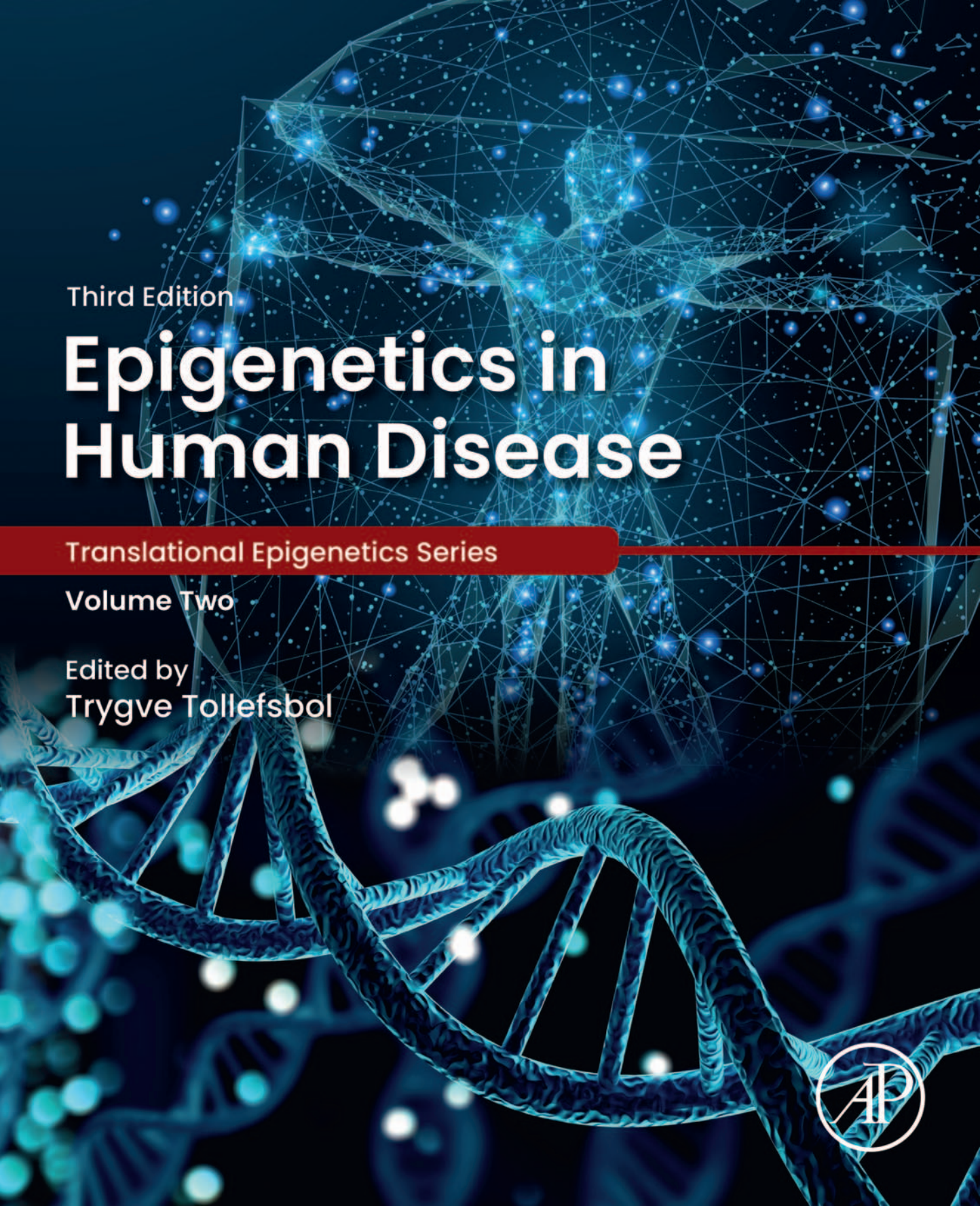
Third Edition
Epigenetics in Human Disease
Translational Epigenetics Series
Volume Two
Edited by
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Epigenetics in Human Disease

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Translational Epigenetics

Epigenetics in Human Disease

Third Edition

Volume 2

Edited by

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ACADEMIC PRESS
An imprint of Elsevier

Academic Press is an imprint of Elsevier
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525 B Street, Suite 1650, San Diego, CA 92101, United States
50 Hampshire Street, 5th Floor, Cambridge, MA 02139, United States
The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, United Kingdom

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ISBN: 978-0-443-21811-8 (Set)
ISBN: 978-0-443-21863-7

For information on all Academic Press publications visit our website at https://www.elsevier.com/books-and-journals

Publisher: Stacy Masucci
Acquisitions Editor: Ali Afzhal-Khan
Editorial Project Manager: Matthew Mapes
Production Project Manager: Swapna Srinivasan
Cover Designer: Christian Bilbow

Typeset by TNQ Technologies



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Preface

The field of epigenetics has moved to the forefront of biological sciences and this area has expanded significantly, especially with respect to applications to human diseases and disorders. This third edition of *Epigenetics in Human Disease* highlights translational aspects of this burgeoning field of science and is intended to provide the most recent, pertinent, and comprehensive information pertaining to the role of epigenetics in human disease. Epigenetic diseases consist of the increasing number of human diseases that have at least part of their basis in aberrations of epigenetic processes such as DNA methylation, histone modifications, or noncoding RNAs. Unlike most genetic defects as a cause for human disease, epigenetic alterations are potentially reversible. This is perhaps the most important aspect of epigenetic diseases because their reversibility makes these diseases amenable to pharmacologic and other modalities of prevention and treatment. The goal of this volume is to highlight those diseases or conditions for which we have considerable epigenetic knowledge such as cancer, autoimmune disorders, and aging, as well as those that are yielding exciting breakthroughs in epigenetics such as diabetes, neurological disorders, obesity, reproductive organ diseases, and many others.

The numerous chapters that comprise this book have been updated and revised and methodological protocols or case studies have been incorporated into many of the chapters. In addition, I was able to recruit numerous authors from the previous editions to contribute to this volume thereby maintaining the standards of those volumes and to recruit other leaders in the field to contribute novel chapters to this volume. A third edition of this volume also conferred the advantage of incorporating exciting extant topics on the epigenetics of human disease which includes advances in noncoding RNA aberrations in diseases such as cancer and the role of nutrients in preventing or treating epigenetic-based maladies. Advanced epigenomic and bioinformatics techniques based on next-generation sequencing are important aspects of this new edition as well as coverage of methods for profiling histone modifications and chromatin-modifying proteins.

For each of the diseases/disorders that are addressed, efforts were made to delineate the basic mechanistic role of epigenetics in these diseases and to present novel epigenetic approaches for the treatment of these diseases. Where possible, each featured human disease is first described in terms of the underlying role of epigenetics in the disease followed by a chapter describing the most recent advances in epigenetic approaches for treating the disease. This approach allows basic scientists to readily view how their efforts are currently being translated to the clinic and it also allows clinicians to review in side-by-side chapters the epigenetic basis of the diseases they are treating. Although aging is not considered to be a human disease *per se*, there are many age-associated diseases. Moreover, since epigenetics plays a major role in the aging process, advances in the epigenetics of aging are highly relevant to many human diseases. This updated and expanded volume consists of chapters on aging epigenetics and breakthroughs that have been made to delay the aging process through epigenetic approaches. There is also an increasing need to understand the relatively recent merging between the fields of epigenetics and epidemiology.

This edition of *Epigenetics in Human Disease* is intended to be of use to the many students, scientists, physicians, and others who are interested in the mechanisms of human diseases and/or novel means to treat human diseases that are caused by reversible epigenetic processes. Those with interests ranging from basic molecular biology to clinical therapy will find a wealth of new information pertaining to the rapidly advancing knowledge that has driven the highly evolving and exponentially growing field of epigenetic-based diseases and disorders.

Trygve O. Tollefsbol

PART

Other disorders/ diseases

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CHAPTER

Epigenetic processes—driven disorders and therapeutics

16

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16.1 What is epigenetics?

Epigenetics comprises of the stable and heritable (or potentially heritable) changes in gene expression that do not involve a change in the base sequence of DNA [1]. This cellular machinery is responsible for remotely regulating the expression of genes. As mutations give rise to an abnormal gene functioning and its pathological effect, epimutations can give rise to epigenetic phenotypes, obvious in loss-of-imprinting disorders. Technological advancements have given a boost to the increasing interest in the epigenetic regulation of the "blueprint of life." Knowledge of genetic changes alone will not give us a complete solution to understand the enigma of human disease; epigenomic regulation directed by the intrinsic and extrinsic environment of the cell will need to be comprehended. Major epigenetic mechanisms include DNA methylation and demethylation reactions, chromatin restructuring, RNA methylation, and regulation of gene expression via noncoding RNAs (ncRNAs) [2]. A know-how of what causes disease is essential not only to arrive at suitable treatments but also to work toward ways of preventing disease in the first place. This chapter deals with various epigenetic phenomenon in play, to maintain normal homeostasis and their variations, which are responsible for disease of different organs in the human system.

Epigenetic study now allows the mapping of epigenetic marks, such as DNA methylation, histone modifications, and nucleosome positioning, which are critical for regulating genes and for ncRNA expression. There are genetic disorders where the chromatin structure and remodeling are affected in *trans* or in *cis*, as well as the expression of both imprinted and nonimprinted genes [3]. The characterization of a human DNA methylome at single-nucleotide resolution, the discovery of the CpG island shores, the finding of new histone variants and modifications, and the unveiling of genome-wide nucleosome-positioning maps highlight the accelerating speed of epigenomic research over the past couple of decades.

Within cells, there are essentially three systems that can interact with each other to silence/affect gene expression: DNA methylation, histone modifications, and RNA-associated silencing.

Epigenetics in Human Disease. https://doi.org/10.1016/B978-0-443-21863-7.00001-9
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16.1.1 DNA methylation

DNA methylation is a crucial epigenetic modification of the genome that is involved in regulating many cellular processes that include embryonic development, transcription, chromatin structuring, X chromosome inactivation, genomic imprinting, maintaining chromosome, and normal cellular homeostasis [4]. Consistent with these important roles, a growing number of human diseases have been found to be associated with aberrant DNA methylation. There are three types of enzymes involved in the basic mechanism of the DNA methylation processes.

(1) Writers: Writers are the enzymes that catalyze the addition of methyl groups onto cytosine residues; they are Dnmt1, Dnmt3a, and Dnmt3b enzymes. Dnmt1 is called the maintenance Dnmt because it maintains the original pattern of DNA methylation in a cell lineage by methylating and repairing activity. Dnmt1 binds to the newly synthesized DNA (hemimethylated DNA) and methylates it to precisely mimic the original methylation pattern present before DNA replication. Dnmt1 has the ability to repair DNA methylation [5].

(2) Erasers: Erasers modify and remove the methyl group. DNA demethylation can occur in both dividing and nondividing cells, but the process requires enzymatic reactions to process the 5 mC to revert it back to a naked cytosine. Demethylation occurs through a series of chemical reactions that further modify 5 mC, by deamination and/or oxidation reactions to a product that is recognized by the base excision repair pathway to replace the modified base with naked cytosine.

(3) Readers: Readers recognize and bind to methyl groups to ultimately influence gene expression. DNA methylation may itself reduce gene expression by impairing the binding of transcriptional activators (Fig. 16.1). DNA methylation is recognized by three separate families of proteins: the

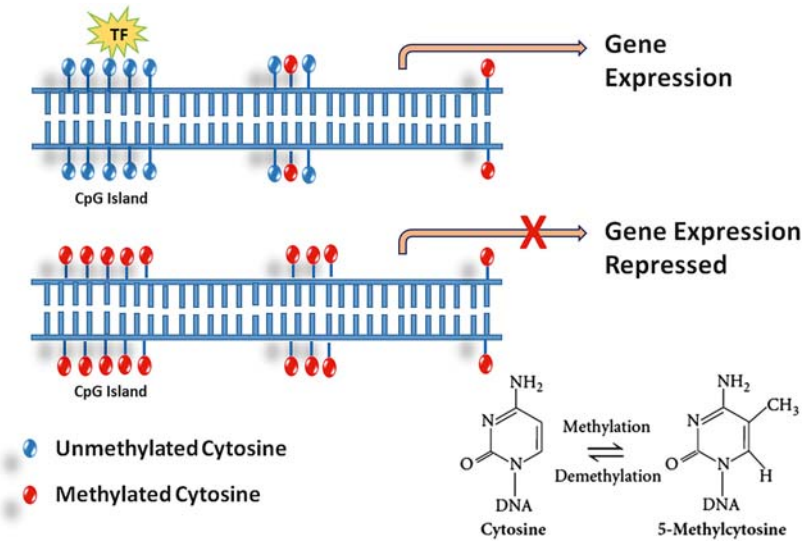

FIGURE 16.1

DNA methylation.

MBD (methyl-CpG-binding domain) proteins, the UHRF (ubiquitin-like, containing PHD and RING finger domain) proteins, including UHRF1 and UHRF2, are multidomain proteins that flip out and bind methylated cytosines via a SET- and RING-associated DNA-binding domain [6,7]. The UHRF protein family first binds to Dnmt1 and then targets it to hemimethylated DNA to maintain DNA methylation, especially during DNA replication [8,9]. UHRF1 appears to interact so closely with Dnmt1 that its deletion, like the deletion of Dnmt1, leads to embryonic lethality [10].

The last family of methyl-binding proteins binds to methylated DNA by a zinc-finger domain and is composed of Kaiso, ZBTB4, and ZBTB38 [11]. Kaiso preferentially binds to two consecutively methylated CpG sites [12]. Zinc finger domain proteins repress transcription in a DNA methylation–dependent manner [11,13–15].

16.1.2 Histone modifications

16.1.2.1 Histone acetylation, histone phosphorylation, histone methylation

For a compact chromatin to be able to express itself by allowing interaction with other molecules, histone modifications play an important role and can be transferred as an epigenetic mark to the daughter cells. Histones are methylated on their lysine or arginine residues by histone MTases, which use S-adenosyl methionine (SAM) as the methyl donor. Depending on the type and position of the residues methylated, they either promote or repress transcription [16]. Each of the residues may be methylated more than once, signaling a different outcome to the chromatin. Acetylation targets lysine residues in the amino terminal tails of core histone proteins. This process does not permit the nucleosome array to fold further and helps in chromatin decondensation. This gives access to the various transcription factors, their cofactors, polymerases, etc. for active transcription from the genes (Fig. 16.2). Elevated glycolysis in cancer is associated with global histone hyperacetylation. Inhibition of glycolysis holds promise for modulating histone acetylation. Therefore, like methylation, acetylation process is another epigenetic mechanism by which gene expression is regulated, and this process can be tissue- and time-specific (regulated during the development process). Acetyl-CoA is a central

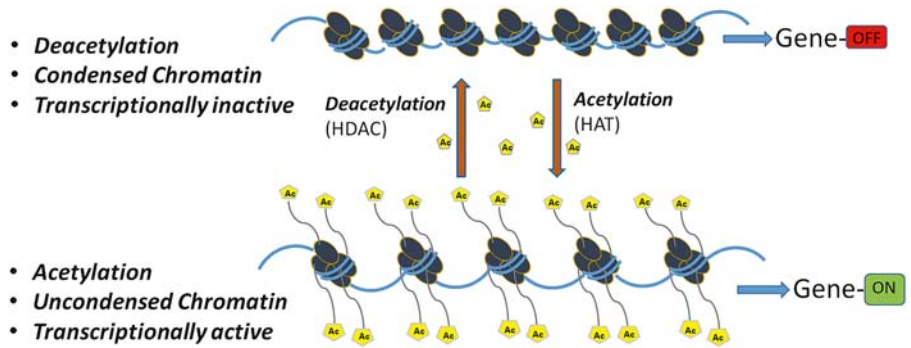

FIGURE 16.2

Gene regulation by Histone acetylation.

metabolic intermediate and the acetyl donor for acetylation modifications. It plays important roles in energy production, lipid metabolism, and epigenetic modifications, consequently positioning it at the cross-roads of metabolism and epigenetic regulation. Cellular glucose availability is linked to acetyl-CoA abundance and histone acetylation. Glucose is the main carbon source of acetyl-CoA in oxygenated cells. Cancer cells shunt glucose-derived carbon to lactate during hypoxia. Subsequent studies reveal that cancer cells can utilize acetate as alternative acetyl-CoA source to support cell survival and proliferation under hypoxia [17].

Histone phosphorylation was identified and reported long before other histone modifications; it has now become a topic of great interest owing to studies describing its association with a variety of cellular processes, including transcriptional regulation, DNA repair, cell cycle progression, apoptosis, chromosome condensation, and developmental gene regulation [18]. The role of histone H3 phosphorylation in transcription has been discussed in detail by Thomson et al. [19]. C-terminal phosphorylation of threonine119 in histone H2A is linked to the regulation of chromatin structure and function, during mitosis, and H3 Serine10 (H3S10) phosphorylation is related to chromatin compaction during mitosis [20]. These histone phosphorylations occur in combination with varying histone alterations giving rise to a multitude of cellular effects. In the recent years, several advances have been made toward understanding the cellular outcome of these post−translational histone modifications.

16.1.3 RNA-associated gene silencing

Transcriptional gene silencing by RNA interference (RNAi) is an important aspect of epigenetic regulatory processes in plants and mammals. Small RNAs modify chromatin folding and regulate gene expression via RNAi. This has a very central role in heterochromatin formation and in the maintenance of pericentromeric DNA repeats via histone H3 lysine 9 (H3K9) methylation. Furthermore, they also play a role in viral resistance, transposon suppression, non−random chromosome segregation and stem cell maintenance [21−23]. The effect of microRNAs (miRNAs) on the mRNA content is also a manner of epigenetic regulation of gene expression. Although it is known that miRNAs help degrade mRNAs and repress translation, they are also known to have a role in determining stem cell fate and differentiation [24]. Recent studies also reveal that chromatin-associated long non−coding RNA scaffolds recruit chromatin-modifying complexes that aid in gene silencing [25]. This kind of a surveillance system detects and helps repress inappropriate transcriptional events, in addition to creating a memory to counter their recurrence, a good example of which is transposon suppression. Synthetic miRNAs have been used to mimic tumor suppressor miRNAs to repress specific oncogenes in cancer. Treatment with 5-Aza-CdR alone was found to be sufficient in reactivating miR148a, miR34b/c and miR-9, a group of miRNAs with metastasis suppression activity [26].

16.1.4 Cross-talk of DNA methylation and other epigenetic mechanisms

DNA methylation works with other epigenetic mechanisms such as histone modifications and miRNA (micro RNA) to regulate transcription (gene expression). As we know in eukaryotes, DNA associates with histone proteins that help in condensation of nuclear material into a compact nucleus. Dnmt1 and Dnmt3b can both bind to histone deacetylases that remove acetylation from histones to make DNA pack more tightly and restrict access for transcription [27,28]. Elevated histone acetylation can trigger

DNA demethylation [29,30]. Tet1 contains a DNA-binding motif similar to Ctf1, suggesting that both proteins target similar sites, in this case CpG islands, to maintain DNA demethylation [31].

Another important epigenetic regulation of gene expression are the miRNAs that are processed by Dicer to generate a 22−23 nucleotide miRNA:miRNA* duplex. Mature miRNAs associate with a miRNA-induced silencing complex (miRISC) that can bind to their target mRNA and repress gene expression by inhibiting translation or inducing RNA degradation [32]. miRNAs can also regulate histone modifications and Dnmt expression, thereby regulating DNA methylation [33,34]. Upstream binding factor (UBF) is important for activation of ribosomal RNA transcription. Histone architecture affected by acetylation or deacetylation controls the output of the rRNA promoter. Methylation of the rRNA promoter at nucleotide position-133 also influences transcription, chiefly by serving to dampen expression by impairing the binding of UBF [35]. In addition to all these, it is known that genic DNA methylation has a positive effect on the expression of associated genes and prevents spurious transcription initiation events as in cancer cells.

16.2 Epigenetics and environment

16.2.1 Nutrition and metabolomics

Barker [36] proposed the fetal origin hypothesis of adult disease, a fundamental idea put forth on the origin of human diseases in utero. Nutrition has a huge role in designing and bringing about epigenetic changes, to maintain human health and contribute to disease—this has been the focus of a great deal of research. Starting from the preconceptional health and nutrition status of the mother, through conception, birth, and up to the age of 2, a woman's diet could affect her child's susceptibility to chronic diseases such as heart disease, obesity, diabetes, and even cancer in his lifetime.

Obesity and overweight are significant burdens on public health due to increased consumption of foods high in calories and sedentary lifestyles. Significant body fat buildup that results in an increased body mass index (BMI) between 25 and 30 kg/m^2. Elevated BMI leads to increased risk of cardiovascular diseases (CVDs), diabetes, musculoskeletal ailments, and various malignancies. It is known that LINE-1 methylation and obesity are related and that these two factors can be used clinically to predict obesity risk and how well people would respond to weight loss regimens. Italian researchers examined the relationship between women's LINE-1 methylation levels and BMI, and obesity [37]. It has been shown that dietary habits, foods, and nutrients can affect methylation levels. In particular, eating foods that support one-carbon metabolism, eating fruits and vegetables, and following healthy dietary guidelines all seemed to be linked to greater LINE-1 methylation levels [38,39]. These results were supported by evidence that weight loss therapies may considerably raise the LINE-1 methylation level in blood samples [40,41].

For instance, insufficiency of vitamin B class and folate leads to lowered homocysteine metabolism. This directly affects the methylation cycle to which methyl groups are donated, from the homocysteine pathway [2,42]. To provide the required methyl groups to the process, generally trimethylglycine and folate are given as supplement. Although there may be a genetic predisposition of the individual to high homocysteine due to mutation in different genes related to this pathway, such as methylenetetrahydrofolate reductase (MTHFR), 5-methyltetrahydrofolate-homocysteine methyltransferase, cystathionine-beta-synthase, etc., dietary care with sufficient folate and B vitamins may take care of this to a certain extent. Inadequately functioning methylation cycle may affect the

antioxidant systems, detoxification, cardiovascular health, neurotransmission, mitochondrial function, and energy output of the individual, thus affecting many organ system functions. For this very reason, it is recommended for a person to be aware of his serum homocysteine, B vitamins, and urine sulfur levels so that dietary supplements can be suitably taken to suit their test findings. Apart from genetic susceptibility, food sensitivities, exposure to environmental toxins and heavy metals, etc. are also responsible for adding stress to the methylation pathway. Anemia, mean corpuscular volume more than 95, can indicate methylation issues. Aging can affect the absorption of vitamins from diet, medications such as methotrexate (used in cancer, autoimmune diseases, etc.) lower vitamin B12, leading to elevated homocysteine and methylation defects [43].

It is now well understood that the blueprint of life, our DNA code, can be regulated by the nutrients we eat and the related epigenetic processes. This process is important all through the life of an individual; however, it is most pronounced at the time of embryonic and fetal development. During this time, any abnormal epigenetic programming can put the fetus and/or the individual at a lifetime's risk of developing chronic ailments, sometimes not even compatible with life.

Although it may be thought that the cellular metabolic profiles might have little impact on the genetic level, it appears that they could play a decisive role in epigenetic regulation of gene expression. Metabolic profile at a given time point in a cell can direct the epigenome and may even be responsible for producing oncogenic signals. When we talk of cancer cells directing their intrinsic pathways to promote malignancy, in addition to metabolizing glucose, they are addicted to glutamine. By means of a process known as glutaminolysis, cancer cells divert a major part of glutamine to replenish the tricarboxylic acid (TCA) cycle [44]. Mutations in KRAS, PIK3CA, PTEN, or AKT have been shown to hyperactivate mTOR-AKT pathway, which stimulates glycolysis via upregulation of glucose transporter 1 (GLUT1). The oncogenic transcription factor MYC mediates the transcription of almost all the genes involved in glycolysis and glutaminolysis. For instance, in gliomas and acute myeloid leukemia metabolic genes, isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2) are known to be driver genes for carcinogenesis. "Metabolic rewiring" is considered as one of the hallmarks of cancer.

In other words, the cellular metabolome could alter the cofactors and the epigenetic-modifying enzymes eventually altering the cellular landscape from a normal to a cancerous state. An excess supply of SAM might contribute to DNA hypermethylation at CpG sites and inappropriate gene silencing. Cancer cells have also been shown to boost SAM availability via promoting one-carbon metabolism [45]. One-carbon metabolism is a network of pathways that donate and regenerate carbon units, involves methionine and folate cycles, and integrates nutrients such as amino acids and vitamins to generate diverse outputs for methylation reactions, maintenance of redox status, and biosynthesis of nucleotides, lipids, and proteins. The carbon units provided by serine and glycine metabolism satisfy many of diverse biological functions. Serine metabolism can contribute to methionine cycle and DNA and RNA methylation through de novo ATP synthesis in cancer cell. Glycine *N*-methyltransferase (GNMT) can catalyze SAM-mediated methylation of glycine to produce sarcosine (Fig. 16.2); it acts as a cellular buffer for maintaining the SAM level [46]. Other dysregulated metabolites that involve in the regulation of acetyl-CoA abundance also play important roles in cancer. Arginine and its metabolic products are crucial to cellular physiology, and their dysregulation is associated with cancer. Acidosis is considered as a hallmark of cancer. Some tumor metabolic peculiarities, including the release of lactate that is a by-product from elevated aerobic glycolysis, account for low pH in tumor microenvironment.

Mutations abrogate the ability of IDH1 and IDH2 to synthesize isocitrate from α-ketoglutarate (α-KG) and aid conversion of α-KG to 2-hydroxyglutarate (2-HG), an oncometabolite. Methylated cytosine (mC) is demethylated in the presence of ten-eleven translocation family of proteins, and 2-HG acts as competitive inhibitor, thus affecting the epigenomic regulation (5-mC is demethylated to 5-hmC and later cytosine as part of DNA methylation process). DNA hypermethylation can promote silencing of tumor suppressors in cancer; multiple tumor suppressors that repress PI3K/AKT/mTOR and HIF-1 signaling are epigenetically silenced by promoter hypermethylation, including PTEN, LKB1, and VHL [47]. However, this hypermethylation induced by 2-HG is reversible and therefore represents a viable therapeutic target in IDH1/2-mutant cancers [48]. Unraveled Zaprinast, a phosphodiesterase 5 inhibitor, is a potent inhibitor of mutant $IDH1^{R132C}$-mediated 2-HG biosynthesis in HT1080 cells, which led to a marked reduction in histone H3K9me2/3 methylation.

16.2.2 During development

Tissue-specific gene expression is acquired during developmental stages and cell differentiation. Epigenetic reprogramming is essential to achieve totipotency after the formation of the zygote [49]. Even before fertilization, the oocyte undergoes global hypomethylation, and then after the fertilization process, there is a loss of methylation of the paternal pronucleus followed by the maternal pronucleus; eventually postimplantation, there is a gain of methylation like the normal somatic levels [50]. Cell differentiation along with de novo methylation starts at the blastocyst stage. Specific transcription factors and epigenetic processes then go hand-in-hand to establish a pluripotent state. A broad range of epigenetic interactions that design the integrated morphological and functional aspects of the human body also bring about several infinite phenotypic variations that we see.

The effects of a ketogenic nutritional intervention for weight loss (based on a very-low-calorie ketogenic diet [VLCKD]) on DNA methylation levels in obese patients were investigated in an epigenome-wide association study (EWAS). The study looked at how a ketogenic diet, specifically a low-fat—low-carbohydrate ketogenic diet, affected the pattern of DNA methylation in people who were obese. This study made use of an array-based platform that interrogated 850,000 specific CpG sites. Following VLCKD intervention, 988 CpG sites showed differential methylation. These CpG sites encoded 786 genes involved in biological processes such as adipose tissue function, neurological function, muscle development, and other metabolic processes. The DMCpG methylation profile after a short period of dieting was similar to that observed in normal weight individuals. Changes in DNA methylation profiles after VLCKD may be related to the beneficial effects of nutritional therapy on body weight and body composition [51].

Epigenetic markers may be detectable in easily accessible samples like peripheral blood instead of inaccessible tissues. Peripheral blood DNA methylation variations can indicate future T2D incidence. In obesity, leukocytes were also used to examine the DNA methylation of the mitochondrial genome's displacement loop and were linked to insulin resistance. It has been demonstrated that the DNA methylation of peripheral blood cells is influenced by factors such as gender, race/ethnicity, dietary factors, and physical activity. Leukocytes mirrored the pattern of DNA methylation associated with T2D observed in adipose tissue and pancreatic islets. These studies suggest that leukocytes/blood may be a better sample type to understand tissue-specific epigenetic signatures and the role they play in the development of diseases such as insulin resistance, T2D, and obesity [52,53].

Epigenetics acts to enable neighbouring organs to grow around a certain space and accommodate themselves appropriately. The masticatory system is a very good example of how the morphological integration takes place, while incorporating the functional processes to suit the organismal needs. The human ENCODE (Encyclopedia of DNA Elements) Project Consortium 2012 has characterized epigenetic marks in 147 different cell types. In addition to this, newer aspects of human disease have come to light after integrating the ENCODE data with the genome-wide association studies [54]. It has been shown here that although nearly 3% of the genome codes for the protein-coding 20,687 genes, it is also true that almost 75% of the genome gets transcribed; this amounts to the rest of the non-coding regions giving rise to gene regulatory sequences that impact gene expression, that too in a tissue-specific manner.

Developmental Origins of Health and Disease (DOHaD) is a theory that suggests that early-life environmental insults can increase a person's risk of developing non-communicable diseases in later life. According to the theory "Developmental Origins of Health and Disease," exposure to specific environmental factors during pivotal stages of development and growth may have a major impact on a person's short- and long-term health [55]. The developing fetus responds to a hostile uterine environment by developing adaptations that not only promote its immediate viability but also its survival, if a similar environment is encountered later in life. These insults include poor nutrition, infections, chemicals, metabolites, or hormonal perturbations [56]. This theory has particular relevance for low- and middle-income countries because these regions continue to experience high rates of infection, malnutrition, and poverty.

It turns out that the degree to which epigenetic processes can turn on or off specific genes is going to have a greater impact on our risk for developing these chronic diseases than does a genetic variant in the gene code we have inherited from our parents. Epigenetic mechanisms contribute to establishing a memory of active and silent genes and thus direct a stem cell fate for further differentiation [57]. They are also responsible for maintaining the cellular homeostasis of cells composing an adult tissue. Using advanced genetic tools, it may be possible soon to decipher the epigenetic code that directs and determines a certain cell lineage. There is quite a possibility that epigenetic mechanisms might have shaped various evolutionary adaptations of living beings to support survival and sustenance.

16.2.3 Microbiome

Methylation is also an important aspect in the regulation and functioning of the microbiome, which is an integral part of the human system and comprises of the normal flora. For example, amino-terminal methylation is found in the bacterial ribosomal proteins, such as L11, L16 of *Escherichia coli* [58]. Disrupting methylation processes of symbiotic organisms may also play a role in contributing to pathology of the human organ systems that host groups of microbes. Gut commensal communities impact several metabolic functions within the human body. In addition to this, the gut microbiome also shapes gene expression in the host. Microbial-derived metabolites can influence the host metabolism by inducing epigenetic alterations of key genes so gut dysbiosis has been associated since long with metabolic diseases such as inflammatory bowel disease, liver disease, obesity, and cancer [59]. Chronic inflammation promoted by microbial inflammatory responses in local tissues may be one of the driving forces for cancerous changes.

16.2.4 Pollutants

Chemical pollutants, dietary components, temperature changes, and other external stresses can have long-lasting effects on development, metabolism, and health, sometimes even in subsequent generations. Several classes of environmental chemicals that modify epigenetic events include metals (cadmium, arsenic, nickel, chromium, methylmercury), peroxisome proliferators (trichlorethylene, dichloroacetic acid, trichloroacetic acid), air pollutants (particulate matter, black carbon, benzene), and endocrine-disrupting/reproductive toxicants (diethylstilbestrol, bisphenol A, persistent organic pollutants, dioxin) [60,61]. Metals increase the production of reactive oxygen species (ROS). Cadmium, for instance, is known to enhance ROS and interferes with the DNA-binding domain of methyltransferase enzyme and reduces methylation [62]. Arsenic is known to induce gene-specific hypermethylation and global DNA hypomethylation [63]. Furthermore, arsenic also altered the expression of specific miRNAs related to one-carbon metabolism. Several heavy metals are found in tobacco smoke, such as Cd, Cr, Pb, and Ni that accumulate in tissues and fluids; inorganic arsenic is also present as the tobacco plant takes up arsenic from soil or from the previously used lead arsenate insecticide. Environmental contaminants such as trichloroethane and trichloroethylene deplete SAM and produce decreased methylation of genes such as c-jun and c-myc. Particulate matter in the air has been known to produce global DNA hypomethylation producing various risks to human health such as cardiovascular disorders, neurological conditions, etc. [64,65]. Exposure to these agents during the developmental stages may especially disrupt various endocrine pathways and produce persistent changes, leading to disease.

16.3 Epigenetics in human diseases

16.3.1 Cardiovascular diseases

CVDs are not only specific to particular regions but a worldwide disease, causing much suffering not only health-wise but also economically because of the costs involved in the treatment of this disease. There are many genetic factors involved in the causation of CVDs. However, epigenetic alterations also play a crucial role and impact the severity in cardiovascular conditions in the present world populations. We can now identify the etiological causes of disease and forecast the best course of action based on epidemiology, molecular biology, physiology, and genetic linkage maps.

Little is known about the DNA methylation and progression of atherosclerosis, a now-common CVD. Research points that estrogen receptors (ERs) are constantly hypermethylated in atherosclerosis; ERs are mainly present on smooth muscle cells and endothelial cells of cardiac muscle and may protect the heart from the disease progression. This finding recommends that hormone replacement therapy could be useful in controlling the disease [66]. Studies have demonstrated that elevated serum *CXCL12* expression is positively correlated with the incidence of CAD. GWAS demonstrate that CXCL12 may emerge as a potential therapeutic target for atherosclerosis and thrombosis. Researchers found that hypomethylation of the *CXCL12* promoter region might lead to the upregulation of gene expression, which was increased during the inflammatory response [67]. *CXCL12* can be crucial in the accumulation of smooth muscle progenitor cells (SPCs), participate in the inflammatory response, and induce endothelial cell differentiation into foam cells, eventually leading to atherosclerosis. Recent advances in knowledge about RNA methylation have revealed a relationship between epigenetic

modifications, and CVDs have brought potential targets for diagnosis and treatment. Studies on RNA methylation show that RNA N6 methyladenosine (m^6A) modification seems to have a role in CVDs such as coronary heart disease, hypertension, cardiac hypertrophy, and heart failure. m^6A can modulate almost every step in mRNA metabolism and dictate distinct fates of mRNAs via differential processing, translation, and procedural decay [68]. miRNAs also play a role in therapy, as they are involved in the expression of genes as negative regulators. Animal studies conducted by Montgomery [69] reported that during heart disease progression, therapeutic inhibition of miR-208a is useful for treating the CVD. Dysregulation of ncRNAs may also contribute to the development of CVD; hence, regulation of ncRNAs might be useful, for instance, in treating the patients with CVD caused by insulin resistance [70].

So much of research has been done on epigenetic regulatory mechanisms and factors that may lead to the worsening of cardiovascular conditions; however, the implementation of epigenetic therapeutics is still a less-approached area. There is much more to understand regarding the role of these epigenetic mechanisms and to substantiate with successful trials that epigenetic therapies can prevent and halt the disease progression. Angiotensin-converting enzyme inhibitors and β blockers of conventional pharmacological treatments have had limited success in controlling the disease progression in CVD. Meanwhile, reminding ourselves of the impact of diet on epigenetic processes, polyphenols such as cocoa have been known to have the ability to control inflammation in CVDs and may modify inflammatory markers involved in the disease progression. In vitro studies show that extracts of cocoa reduced the mRNA levels of DNMTs, MTHFR, and MTRR genes; these cocoa extracts are capable of decreasing low-density lipoproteins oxidation susceptibility and increase the antioxidant capacity, thus favorably influencing cardiac disease risk.

16.3.2 In cancer

Mechanism of epigenetics plays a crucial role in different types of cancers by silencing the tumor suppressor genes and activating oncogenes, for example, by disturbing the methylation of CpGs in the gene promoters [71]. Many of the mutations related to cancer genesis, cell migration, evasion of apoptosis, tumor metastasis, etc. occur in genes related to epigenetic processes. Progenitor genes are disrupted epigenetically at the initial stages of malignancies, even before mutations, and lead to altered differentiation and tumor evolution [72,73]. Research in the area of cancer epigenetics has revealed a number of similarities between cancer genes and stem cell reprogramming genes, such as the widespread mutations in epigenetic regulators, and the role played by chromatin structure and stability in both development and cancer. Different epigenetic mechanisms may be involved in the initiation and progression to malignancy. Mutations early in the genesis of common cancers have also been identified that are likely to be associated with tumor initiation. Mutations in DNA may lead to initiation events in cellular transformation, but induced changes in the transformed cell epigenome would support such events and increase the chance of progression and metastasis [74].

According to some animal studies, it has been shown that aberrant DNA methylation alone can activate cancer. For several years, it has been known that cancer cells have a global hypomethylated genome, whereas in specific sites at the same time, mCs have increased in the genome. Genes with repetitive DNA sequences and coding regions of CpGs are methylated in normal cells; however, moderately repeated sequences of DNA, satellite DNA, and repeats of LINE-1 become unmethylated. Genes with CpG islands at their promoters become hypermethylated, resulting in

the lack of expression of the genes. One of the crucial epigenetic mechanisms contributing to cancer transformation is histone modification. It has been proven that histones H3ac, H3K4me3, and H3K4me2 are bulkily present around the transcription start site, whereas H4ac and H3K4me1 are seen in very low concentrations. The presence of abnormal histone modifications of H3K36me3, H3K4me1, H3K4me2, and H3K4me3 genes has been correlated with the stimulation of genes. In contrast, suppression of genes from expressing or gene silencing was linked to the presence of high levels of H3K79me2, H3K27me3, and moderate levels of H3K9me2 and H3K9me3. The loss of gene expression of H4K20me3 and H4K16ac markers is a trademark of DNA hypermethylation, which is associated with carcinogenesis in normal human cells; this hypermethylation occurs at repetitive sequences of the DNA [75]. In addition, mC forms thymine by a process called deamination, creating an aberration in the DNA sequence that is very difficult to correct as the mechanism of DNA repair cannot simply differentiate as to which is the correct base during mismatch repair.

Hence, it is clear that epigenetic modifications in cancer also affect the stability of the genome, providing a link between the organization of the genome, and its replication and repair. Much of the descriptive work has demonstrated the nature of such alterations; however, the precise cause of the epigenetic switch in cancer may lie with the tissue's exposure to specific internal and external cellular environmental factors. Therefore, epigenetic mutations promoted by either environmental agents or oncometabolites with the help of their cofactors, alter the epigenetic-modifying enzymes that influence methylation and acetylation processes, and this in turn brings about large-scale oncogenomic changes [76,77].

There are many technical challenges encountered while investigating epigenetic changes that are tissue-specific. However, research has helped evolve genetic tools to facilitate the same. Several epigenetic molecules are used as targets to regulate or repress transcription. DNMTs mediate methylation reactions, and then there are the methyl-CpG-binding proteins such as MeCP1, MECP2, etc. that bind to large repressor complexes. These repressors target histone deacetylases and histone methyltransferases onto methylated promoter to eventually induce a transcriptionally inactive chromatin. Histone deacetyltransferase (HDAC) inhibitors are mostly pan inhibitors or those that act on a specific class of HDAC enzymes. DNA methyltransferase inhibitors (DNMTis) are used in more clinically advanced stage of disease. Various epigenetic agents are already approved and in use as drugs such as azacytidine, decitabine (approved by the US FDA in 2004 and 2006, respectively), valproic acid, etc. In case of hematological malignancies, these epigenetic drugs have clinical application. Decitabine is a DNMTi used in myelodysplastic syndrome (MDS).

HDAC inhibitors such as suberoylanilide hydroxamic acid (SAHA, vorinostat, approved by FDA in 2006) and romidepsin (depsipeptide in 2009) are being used for the treatment of patients with progressive, persistent, or recurrent cutaneous T cell lymphomas [78]. Panobinostat in combination with bortezomib and dexamethasone has been approved in 2015 for the treatment of patients with multiple myelomas [79]. Furthermore, small-molecule nonnucleoside compounds such as BML-210 (HDAC inhibitor) and UNC-2170 (methyl-lysine binding protein inhibitor) can be used in therapy for inhibiting epigenetic changes. DZNep is a specific HMTi with promising results in cancer immunotherapy. Lot of these epigenetic therapies may be combined to act synergistically with the chemotherapy modalities and may even aid in reducing adverse events such as drug resistance (Fig. 16.3). Not only this, epigenetic markers can help predict prognosis in many cancers, for instance, EHZ2 enzyme alterations indicate a poor prognosis in breast, prostate, and other types of cancers.

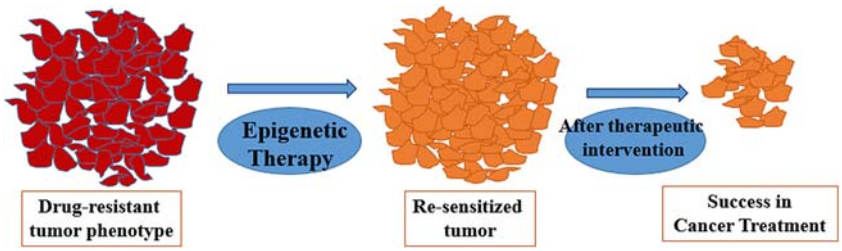

FIGURE 16.3

Combined therapy for cancer.

16.3.2.1 Methylation biomarkers in cancers

In the current-day context of precision medicine, disease-related methylation changes can be used as biomarkers at all stages of clinical management such as risk assessment and predisposition screening through early diagnostics to personalization of patient care and monitoring of the relapse and chronic disease. However, the translation of the research findings in methylation biomarkers field to clinical practice is at the very least not satisfactory. DNA methylation seems to be sufficiently stable epigenetic modification to be utilized as a biomarker with in vitro diagnostic (IVD) settings. Dietary components on the other hand with an epidrug effect may offer more promising venues for clinical use. Lunasin is a 43-amino-acid peptide found in soybean, barley, wheat, and rye and is a competitive inhibitor of HATs. It inhibits histone acetylation and regulates the cell cycle.

Gene-specific methylation has been linked to survival, treatment response, and risk of recurrence when patients received adjuvant or neoadjuvant therapy. Demethylation of the MGMT promoter in tumor tissue was linked to a greater risk of recurrence in individuals who had undergone adjuvant chemotherapy. Those who had MGMT hypermethylation had a lower risk of recurrence [80]. It is conceivable that promoter methylation-suppressed MGMT expression results in a reduction in its DNA repair activity, increasing the efficacy of chemotherapy [81].

16.3.2.2 Risk assessment biomarkers

It is well established that environmental exposure (e.g., smoking) can induce methylation changes, affect gene expression, and lead to adverse health effects. Methylation changes at the CpG site in the AHRR gene were identified involving smoking adults and children of smoking mothers. Considering the magnitude of the evidence, these changes at the CpG site can be proposed as a biomarker of exposure to smoking and adverse smoking-related health effects even in former smokers [82].

Occupational agents such as trichloroethylene (TCE), a volatile, colorless liquid used in some household products such as cleaning wipes, aerosol cleaning products, tool cleaners, paint removers, spray adhesives, carpet cleaners, and spot removers, also affect the epigenetic machinery in our body. The wide use of this substance makes it an occupational toxicant for various working groups. TCE exposure has already been associated with kidney cancer and autoimmune diseases. Researchers identified that the promoter of TRIM68 gene showed hypomethylation with increased exposure to TCE. The functional consequences seemed to link them to pathways important in the development of autoimmune diseases and genes related to cancer development [83,84]. Lead (Pb) exposure has been reported to have adverse effect mainly on the nervous, hematopoietic, and renal systems. Two of the

differently methylated CpG sites within GSTM1 gene identified were identified as causative; other methylation changes were linked to CRIM1 and NINJ2 genes, which are important regulatory genes of nervous system development [85–87].

16.3.2.3 Diagnostic biomarkers

Early detection of the disease significantly increases the chances of cure. Studies in carcinogenesis of multiple cancers showed methylation changes occur early in the disease development.

Colorectal cancer (CRC) is the third most common cancer. Five-year survival rates in this cancer drop drastically from 90% to 10% with increasing stage at diagnosis [88]. FDA-approved Cologuard, Epi proColon, CE-marked real-time mS9 CRC Assay and EarlyTect Colon Cancer are liquid biopsy-based tests targeting methylation changes intended for CRC screening [89]. Hepatocellular carcinoma (HCC) is the sixth most common cancer and the second most frequent cause of cancer death worldwide. The clinical utility of the Epi proColon 2.0 CE test to detect HCC was with a sensitivity of 90.6% (95% CI 81.9–99.2) and specificity of 87.2% (95% CI 80.8–93.7), which is significantly higher than the accuracy of alphafetoprotein (AFP), a widely used serum-diagnostic marker for liver cancer. HCC blood test analyzes methylation status of genes in cfDNA extracted from plasma, using next-generation sequencing (NGS) approach, to confirm the presence of liver cancer [90]. Likewise in lung cancer, Epi proLung is a CE-marked test, intended for diagnosis in patients at increased risk. The test detects methylation of SHOX2 and PTGER4 genes in the cfDNA extracted from plasma using real-time PCR with a fluorescent hydrolysis probe [91].

Bladder CARE test is intended for both BC screening and BC recurrence surveillance. The test targets methylation of three genes: SOX1, IRAK3, and LINE1 by real-time PCR technology-based methylation detection (MSRE-qPCR) [92,93]. Prostate cancer is the most prevalent cancer in men worldwide. ConfirmMDx (MDxHealth, Irvine, CA, United States) was shown to be useful in the assessment of the risk of occult disease in men with negative prostate cancer biopsy. The test targets the methylation status in three genes: GSTP1, RASSF1, and APC in the DNA derived from the prostate core biopsy tissue [94]. Persistent infection with carcinogenic human HPV is necessary for the development of CIN and subsequently cervical cancer; its prevention is based on the detection of the CIN and assessment of the oncogenic subtypes of HPV. GynTect is a methylation biomarker-based CE-marked test dedicated to the diagnosis of CIN or cervical cancer in HPV-positive women. Other CE-marked tests for the detection of cervical cancer are QIAsure Methylation Test (hypermethylation of FAM19A4 and MIR124-2 genes), PAX1 DNA Detection Kit, and ZNF582 DNA Detection Kit, built on real-time PCR technology [95,96].

Glioblastoma multiforme (GBM) is the most common primary malignant brain tumor in adults. Standard chemotherapy in glioblastoma is based on alkylating agents such as temozolomide, and studies have long shown that the effectiveness of temozolomide therapy depends on the methylation status of the MGMT gene. MGMT protein is a key component in the process of repair DNA damage induced by alkylating agents. Thus, tumors with MGMT gene promoter methylation and down-regulation of the MGMT protein respond to the treatment [97,98]. Breast cancer kit (QIAGEN) for prognosis targets methylation changes at three CpG sites within PITX2 gene, utilizing real-time PCR with fluorescent hydrolysis probes and methylation-unspecific primers [99,100]. There is an exhaustive list of these epigenetic biomarkers used for diagnostics and prediction of therapeutic response.

16.3.3 Imprinting disorders

Genomic imprinting is a clear evidence of the influence of epigenetic mechanisms on gene expression or alterations in them with respect to human disease. Imprinting of the human genome is an epigenetic process that marks its DNA in a sex-dependent fashion [89,101]. So far, about 60 genes are added to this list, many of them present in clusters comprising imprinted loci [102]. An epigenetic state is basically coded for, by the configuration of the chromatin. This epigenetic regulation permits hereditary alterations in expression of gene without alterations in the DNA sequence; these regulations include degrading transcripts or blocking the RNA synthesis [103]. Imprinted genes show expression from only one parent member of the gene pair (allele), and their expression is determined by the parent during the production of gametes. Imprinted genes represent only a small subset of mammalian genes that are present but not imprinted in other vertebrates [104,105]. Genomic imprints are erased in both germlines and reset subsequently, thus reversible, depending on the parent of origin and lead to differential expression during the course of development [106,107].

Most of the autosomal genes show biallelic expression, of which a small number of genes are expressed from one of the parents; these genes are important for viability. It is believed that genes from maternal origin show growth-limiting activity, whereas genes from paternal origin show growth-promoting activity. The alleles of imprinted genes are marked epigenetically at discrete elements termed imprinting control regions with their parental origin in gametes through the use of DNA methylation [108,109]. Imprinted gene expression is subsequently maintained using ncRNAs, histone modifications, insulators, and high-order chromatin structure [110]. Imprinted genes evade the genome-wide reprogramming that occurs after fertilization and remain marked with their parental origin. Examples include Prader—Willi syndrome, Albright hereditary osteodystrophy, Beckwith—Wiedemann syndrome (BWS), pseudohypoparathyroidism, and Silver—Russell syndrome (SRS) [109].

DNA methyltransferase 1 (DNMT1) enzyme is important to maintain the stable imprinted cell during differentiation and cell division. DNMT1 enzyme has got two important properties: one, its association with replication of DNA, and the other is its strong preference toward hemimethylated DNA. These two properties of this enzyme take care to see that DNA, once methylated, will be likely to stay methylated, thus maintaining the cell stability. Some animal model studies have shown that the loss or the absence of this enzyme activity leads to the distinct loss of genomic imprinting [107,110,111].

Imprinting disorders, caused by epigenetic mutations, are a group of congenital diseases that affect the metabolism, growth, and development of an individual throughout his/her lifetime. The four molecular mechanisms of imprinting disorders are uniparental disomy (UPD), chromosomal rearrangements, epimutations, and intragenic point mutations within the imprinted gene. These result in the abnormal expression of the imprinted genes.

In UPD, there is an inheritance of both the homologous chromosomes from a single parent of origin. UPDs affect acrocentric chromosomes, and the recurrence of getting UPD risk to another offspring is very less ($>1\%$) [112]. In chromosomal rearrangements such as translocations or deletions, there are chances of gene duplication, thereby leading to overexpression of imprinted genes. Imprinting disorders such as SRS and BWS are well-known examples. In SRS, very often, a hypomethylation of the ICR1 DMR in the imprinted region 11p15 has been reported, whereas in other cases, a maternal UPD for chromosome 7 has been observed. In BWS, ICR2 hypomethylation accounts for about 50% of the cases, and paternal UPD (11p15) is the second most frequent molecular aberration.

Very similar to the manifestation of a UPD is the presence of epimutations. Epimutations are results of abnormal methylation reactions in the genome that may lead to change in the expression of the affected gene; this may be germinal or somatic. There are two types of epimutations: primary epimutation occurs in the absence of any DNA sequence change, giving rise to somatic mosaicism, whereas a secondary epimutation occurs secondary to a DNA mutation in a *trans*- or *cis*-manner. Epimutations in the imprinted genes can involve hypermethylation of the expressed gene (absence of gene product of the imprinted gene) or hypomethylation so that there is an overdose of the gene product due to biallelic expression [113]. First evidence of an epimutation in imprinted gene was seen in biparental complete hydatidiform mole where imprinted genes such as PEG1, PEG3, and SNRPN are normally methylated on the maternal chromosome but were found to be unmethylated on both the parental alleles.

Females show X chromosome inactivation, which triggers the inaction of one of the X chromosomes in each female cell and occurs during early embryogenesis. This results in X-linked gene mosaicism during the expression of genes. The process is highly regulated by various epigenetic mechanisms, such as nuclear rearrangement of X chromosome, ncRNAs, and chromatin remodeling. Genomic imprinting and X chromosome inactivation, when not properly regulated, lead to disease [108]. Both these mechanisms, though not fully understood, involve DNA methylation epigenetics.

16.3.4 Pediatric disorders

Clinical application of epigenetics is a rapidly growing area throwing light on mechanisms regulating gene expression without the involvement of changes in the genotype. The period around childbirth, early stages of growth, first few years of development, and the infections we are exposed to, are important in deciding how our genetic makeup gets modulated as we reach adulthood. The impact of each of these is bound to have heterogenous effects even within a given population of individuals, their genetic susceptibilities apart. Whole-genome approaches have yielded a lot of information on genetic and epigenetic players in pediatric disorders.

16.3.4.1 Exposome concept

In 2005, Wild coined the term "exposome," which he described as the "totality of environmental exposures from conception onward." To better understand the impact of environmental factors on chronic diseases, a set of all environmental elements that a person is exposed to from conception onward has been recommended [114–116]. Wild CP persisted in highlighting the value of the exposome in completing our knowledge of the human genome. The exposome paradigm is responsive to two bodies of evidence that have evolved over the past few decades; first evidence is that which gave way to the early origins of health and disease (DOHaD) [117]. Second body of evidence that supports exposome studies is epigenetics, which is defined as molecular changes in gene expression or phenotype without concurrent changes in DNA sequence. Cellular and tissue differentiation are regulated by epigenetic processes, which cause the expression or inactivation of genes at particular developmental stages [118]. These paradigms are based on the DOHaD paradigm, in which epigenetics is mechanistically grounded. Two paradigms have been proposed to comprehend how early exposures alter reproductive health, and affect health and disease across the lifetime and, presumably, future generations [119,120].

Examples of pediatric disorders caused by well-known epigenetic factors include childhood leukemia, BWS, asthma, allergies, fetal alcohol spectrum disorders, type 2 diabetes mellitus, and childhood obesity. Currently, epigenetically active treatments are being used to treat childhood leukemia. Pediatric high-grade gliomas are marked with epigenetic dysregulation, which can be reversed by epigenetic therapies. An H3K27M mutation leads to the inactivation of the polycomb repressive complex 2 (PRC2) methyltransferase and results in global hypomethylation of H3K27; this leads to transcriptional derepression of potential oncogenes in gliomas. Here, inhibition of H3 demethylation could contribute to therapeutics in H3.3-K27M diffuse pontine gliomas [121]. The epigenetic-based therapies for these diseases focus on methods that either aim to restore the epigenetic homeostasis or skew it to counter the disease into a manageable proportion. Even in the area of dentistry, although there is currently no application, studies have proven that epigenetic therapies may be important to prevent hypodontia, periodontitis, and a range of dental anomalies [122,123].

16.3.4.2 CHARGE syndrome and Kabuki syndrome (KS)

CHARGE is a multiple-organ system disorder with congenital features of coloboma of the eye, heart defects, growth retardation, genital abnormalities, and one of the leading causes of deaf-blindness [124]. This is caused by haploinsufficiency of the CHD7 gene, which encodes an ATP-dependent chromatin remodeling protein involved in the epigenetic regulation of gene expression. KS, which is also a multiple-organ system disorder, includes distinctive facial features, growth delays, varying degrees of intellectual disability, etc. MLL2 (mixed lineage leukemia) mutations cause KS where MLL family of histone methyltransferases is essential in the epigenetic regulation of active chromatin states [125]. Both these syndromes are caused by epigenetic deregulation of important genes involved in embryogenesis and development [126].

16.3.4.3 Overgrowth with intellectual disability

Overgrowth with intellectual disability (OGID) shows different phenotypic appearances such as those involving height and head circumference, along with other abnormal phenotypic characteristics. Overgrowth disorders may be germline or sporadic, running in a family, and this can be due to many mechanisms such as chromosomal abnormalities, mutations in genes, and imprinting interruptions. Epigenetic mechanisms play an important role in OGID; here, epigenetic processes in some of the responsible genes are dysregulated according to one study by Tatton-Brown et al. [127,128].

16.3.5 Immunity

In humans, there are two major types of immune systems: innate and adaptive immunity. Innate immunity comes by birth, whereas adaptive immunity gets established when we get exposed to specific type of pathogens and remains in our body. Monocytes are the immune cells forming a part of innate immunity. They are mainly present in blood circulation and in surrounding tissues, differentiated to macrophages, and fight against pathogenic organisms and dead cells [129].

Many human disorders are characterized by deregulation of inflammatory and immune activities in the body. The epigenetic status of these cells is fine-tuned under physiological conditions; however, its deregulation often causes the development of disease states [127,130]. Some of the environ mental factors influence the function and differentiation of immune cells through different epigenetic alterations. Vertebrate animals have established good host defense mechanisms against potentially

aggressive antigens. These processes mostly involve epithelial cells and the immune system that protects the body surface. Plenty of recent studies have discovered that epigenetic mechanisms in association with signal transduction networks regulate gene expression over the course of differentiation, proliferation, and function of immune and epithelial cells [131]. Commensal microbiota-derived metabolites inhibit histone deacetylases to induce regulatory T cells [132]. Histone methylation is an important epigenetic mechanism in immunity, for example, $CD4^+$ T helper cell differentiation is increased by histone methylation. Some infectious agents may also induce DNA methylation, resulting in the development of cancer. These data imply that epigenetic regulation of host defense cells, which are usually the first to encounter external antigens, is yet another factor implicated in disease development [133].

As we know, the machinery of the condensed chromatin decides which genes to express or not to express to control their function. It is known that in an open chromatin (decondensed chromatin), the nucleosomes are present with sufficient space for the DNA to allow the transcription process, whereas condensed chromatin does not allow the transcription factor to bind to the DNA. During immune responses, it is crucial that cells have to quickly turn on the expression of genes (for example, when exposed to foreign materials such as infectious microorganisms) and then genetically often preserve these states even during the division of cell, immunological memory, and clonal expansion. Innate cells such as monocytes and neutrophils can also have memory, which is trained to respond rapidly to infectious agents, where the epigenome has a huge role to play [134].

Several studies indicate that there is epigenetic control of major immune cell functions, such as hematopoietic lineage choice, antigen receptor rearrangement, allelic exclusion, and immune responses to pathogens. Epigenetic processes govern the differentiation of T helper cells and their lineage stability; exposure of dendritic cells to oxidized phospholipids can change the phenotype of the cells through epigenetic mechanisms, and epigenetic processes control the production of antibodies by B cells. These immunological developmental events that are regulated by epigenetic mechanisms could possibly be altered and promote autoimmunity [132].

Epigenetic factors, from both external sources (e.g., environmental exposures, diet and nutrition, drugs and pharmaceuticals, retroviruses) and internal agents (e.g., aging, gender, and stress and exercise levels), acting on the immune system and/or the target organ, may all contribute to either enhancing the development of autoimmunity or protecting against it. We are unsure as to which of these epigenetic factors and mechanisms could affect the immune system and effector mechanisms in autoimmunity [135].

X-linked genes are typically unmethylated (active) in men, whereas women have one methylated (Xi) and one unmethylated (Xa) gene. It has been found that some genes on Xi are demethylated in females with autoimmune disease (e.g., SLE) [136]. Females can have elevated levels of expression of molecules encoded on the X chromosome, due to gene dosage irregularities. There are observations of elevated levels of CD154 in a multitude of autoimmune disorders, including SLE, autoimmune thyroid disease, T1D, inflammatory bowel disease, psoriasis, multiple sclerosis, and rheumatoid arthritis. Treatment of $CD4^+$ T cells with the DNMTi 5-azacytidine leads to elevated levels of CD40 ligand (CD154—which is encoded on the X chromosome) on T cells from females but not males [137]. Several molecules thought to be of great potential importance for autoimmunity and encoded on the X chromosome include interleukin (IL) 2Rγ chain (also known as the "common γ chain" because it is shared by receptors for IL2, IL7, IL15, and IL21), Foxp3, the master regulator in the development and function of Tregs, tissue inhibitors of metalloproteinases 1−4, which are important in inflammation,

the X-linked inhibitor of apoptosis that regulates T cell function and Toll-like receptor 7 and Toll-like receptor8, which are pattern recognition receptors, both of which are important in recognition of single-stranded RNA [138]. Skewing of X inactivation, so that there is an overrepresentation of the Xa from one parent, has been reported in females with some autoimmune diseases, including autoimmune thyroid disease and scleroderma, but not in SLE or multiple sclerosis. Skewed X inactivation could influence expression of genes on the X chromosome and could lead to inactivation of a gene that protects against autoimmunity, or overexpression of a susceptibility gene, leading to increased autoimmune disease [135]. It will be very interesting to understand how the human system has evolved to use the various epigenetic modifications to chromatin, and understanding them will pave way for us to modulate these including the immune system toward better treatment outcome.

16.3.6 Neuropsychiatric/neurodevelopmental disorders

Epigenetic studies are of increasing relevance to the field neuroscience with related mechanisms being found essential for neuronal differentiation and brain development; in addition, they have also been found important in directing more dynamic processes such as cognition. Studies of neuropsychiatric epidemiology using either blood or target brain tissues each have distinct advantages and methodological challenges. The mental disorders of unknown etiology have complex inheritance patterns connecting many genes and environmental factors, unlike the neuropsychiatric disorders that have simpler inheritance patterns. There are two major categories of mental illness. One includes the idiopathic disorders such as major depressive disorder (MDD), schizophrenia, and bipolar disorder where there are no definitive structural changes in the brain. The other comprises the neuropsychiatric disorders such as Alzheimer's disease and Huntington's disease [139]. In mature, differentiated neurons in the central nervous system (CNS), epigenetic mechanisms play critical roles in encoding memory-related and environmental stimuli into stable, behaviorally meaningful patterns of gene expression [140]. For example, epigenetic changes in mature hippocampal neurons have been implicated in learning and memory and in a variety of neuropsychiatric disorders, including depression. With all the recent research and findings on epigenetic modifications in mature neurons, it is with emphasis that we recollect epigenetic mechanisms were initially described for their important role in promoting differentiation and driving cell fate in embryonic and early postnatal development, including neurogenesis. These discoveries of neurogenesis in the adult brain and the intriguing links among adult hippocampal neurogenesis, hippocampal function, and neuropsychiatric disorders have been complemented with the studies on the role on epigenetic pathways in neuronal development and their function. The process of adult hippocampal neurogenesis is complex, with neural stem cells (NSCs) giving rise to fate-restricted progenitors and eventually mature dentate gyrus granule cells. Notably, neurogenesis occurs within an increasingly well-defined "neurogenic niche," where mature cellular elements such as vasculatures, astrocytes, and neurons release signals that can dynamically regulate neurogenesis [141]. Epigenetics directs the intrinsic changes occurring within NSCs and their progeny that are critical for neurogenesis. A more thorough understanding of these mechanisms of regulation will lead to novel therapeutics for the treatment of neuropsychiatric disorders.

Major depressive disorder (MDD) is a devastating neuropsychiatric disorder encompassing a wide range of cognitive and emotional dysfunctions. The prevalence of MDD is expected to continue its growth to become the second leading cause of disease burden (after HIV) by 2030. Despite an extensive research effort, the exact etiology of MDD remains elusive, and the diagnostics are

uncertain. Moreover, a marked interindividual variability is observed in the vulnerability to develop depression, as well as in the response to antidepressant treatment, for nearly 50% of patients. Although a genetic component accounts for some cases of MDD, it is now clearly established that MDD results from strong gene and environment interactions. Such interactions could be mediated by epigenetic mechanisms, defined as chromatin and DNA modifications that alter gene expression. Some epigenetic mechanisms have recently emerged as particularly relevant molecular substrates, promoting vulnerability or resilience to the development of depressive symptoms. Although the role of brain-derived neurotrophic factor (BDNF) in the pathophysiology of MDD remains unclear, its modulation of the efficacy of antidepressants is clearly established. There are epigenetic mechanisms regulating the expression of BDNF in humans and play a role in individual differences in vulnerability to depression and response to antidepressant drugs.

With respect to the importance of methyltransferases, a significant levels of DNMTs were noticed specifically in postmitotic neurons, suggesting a functional importance of DNMTs in the nervous system. This supports a maintenance role of DNMT to prevent active demethylation in postmitotic neurons. Accumulating evidence showed that DNA methylation changes correlate with certain neuropsychiatric disorders such as schizophrenia, Rett syndrome, and immunodeficiency, centromere instability, and facial anomalies syndrome. Studies of methyl-CpG-binding proteins, DNMT inhibitors, and DNMT knockout mice explored the key role of DNA methylation in neural development, plasticity, learning, and memory. Although an enzyme exhibiting DNA demethylation capability in vertebrates still remains to be identified, DNA methylation status in the CNS appeared to be reversible at certain genomic loci. It is widely accepted that the incorporation of unhealthy habits as part of lifestyle may also speed up the aging process. For instance, tobacco exposure can alter the CpG methylation status of genes associated with cellular homeostasis and development of aging-associated disorders [142]. Likewise, alcohol inhibits the methionine synthase pathway, and consequently the SAM levels, affecting hypomethylation of several genes and accelerating aging.

Importantly, epigenetic dysregulation presently has been marked as a key player in aging and age-related neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease, where it may mediate interactions between genetic and environmental risk factors, or directly interacts with disease-specific pathological factors. Epigenetic mechanisms are involved in the regulation of neural differentiation and in functional processes related to memory consolidation, learning, or cognition during healthy lifespan. More studies are needed to focus on the potential use of epigenetic enzymes as druggable targets to ameliorate neural decline during normal aging and especially in neurological disorders, tapping into the fact that these epigenetic anomalies are reversible.

In case of disorders such as schizophrenia and autism, carnitinoids (inhibit histone deacetylases) have been used as an epigenetic therapeutic approach to reinstate synaptic plasticity and protect from neuronal damage [24]. In autism spectrum disorder patients, it was also seen that deletion or promoter methylation of the oxytocin receptor gene led to loss of this peptide hormone function. The implication of this oxytocin dysfunction in other neuropsychiatric disorders also has paved way for therapeutics involving exogenous oxytocin administration with reports of ameliorated disease severity in cases of mood disorders, autistic features, etc. [143].

Puberty is a critical period of development during which the re-emergence of gonadotropin-releasing hormone secretion from the hypothalamus triggers a cascade of hormone-dependent processes. Maturation of specific brain regions including the prefrontal cortex occurs during this window,

but the complex mechanisms underlying these dynamic changes are not well understood. Particularly, the potential involvement of epigenetics in this programming has been underexamined. The epigenome is known to guide early stages of development, and it is similarly poised to regulate vital pubertal-driven brain maturation. Furthermore, as epigenetic machinery is highly responsive to environment, its involvement may also lend this period of growth to greater vulnerability to external insults, resulting in reprogramming and increased disease risk. Importantly, neuropsychiatric diseases commonly present in individuals during or immediately after puberty, and environmental perturbations including stress may precipitate disease onset by disrupting the normal trajectory of pubertal brain development via epigenetic mechanisms. Epigenetic processes are involved in pubertal brain maturation, and future studies for understanding this dynamic developmental window are essential to gain a better understanding of pubertal-onset psychiatric conditions, such as depression and poor social interaction.

16.3.7 Aging

Aging is an inevitable consequence of living, characterized by progressive decline in organ functions and increased risk for disease and death. Although the molecular basis of aging is currently being investigated in many experimental contexts, there is no consensus theory to fully explain the aging process. Functional studies in humans and animal models specify that epigenetic changes have a strong influence on the aging process. The well-known phenomenon exemplifying epigenetic drift (modification of epigenetic patterns during aging process) is the steady decrease of global DNA methylation. Different tissue types, aging cells, and a variety of human diseases acquire their own distinct DNA methylation profiles, although the functional impact of these is not always understood. These various epigenetic changes take place at different levels, during both aging and replicative senescence of the organism [144,145].

Aging is a natural phenomenon, and the epigenetic processes that get disrupted in the process cause the precipitation of several disorders such as cardiovascular, neurological, and even malignancies, once again providing evidence to the importance of epigenetics in age-related human disease development [146]. Epigenetics, in concert with environmental exposure, can drive age-associated decline in various functions of the body by lowering gene expression, disrupting genomic stability, and reducing the fidelity of maintaining the high-order chromatin architecture. As a consequence of aging, global decrease in CpG methylation has been observed along with locus-specific gain of methylation leading to silencing of what could be genes related to the aging process in addition to other bodily functions [147]. Now, whether this is actually a consequence of the aging process or a cause driving the aging mechanism is a matter of further research.

In other age-related complications also, epigenetic mechanisms are actively involved. With chronic kidney disease (CKD), there is an increase in serum homocysteine levels; this affects methyl transfer reactions by inhibiting DNMTs, thereby promoting DNA hypomethylation in CKD patients. In diabetic islets, an increase in DNA methylation of the PPARγ coactivator 1α gene (*PPARGC1A*) was seen, a factor that plays a key role in regulating mitochondrial genes and modulating diabetes [148]. Type 2 diabetes involves insulin resistance, obesity, environmental factors, nutrition, lifestyle, etc. in addition to hyperglycemia, where each of these risk factors could induce epigenetic changes to the chromatin structure, eventually giving rise to frank diabetic condition. The complex role of nutrition in all these conditions is a matter of interest where emphasis on the role of ncRNAs may provide exponential understanding and open up an array of natural, epigenetic therapeutic applications.

16.3.7.1 Telomere *length and DNA methylation–based epigenetic clock*

Telomeres are essential for a variety of cellular functions because they guard against end-to-end fusions and chromosomal instability [149]. The protective protein complex shelterin binds to the repeated TTAGGG sequences that make up telomeric DNA. The telomere structure is shaped by this complex and proteins involved in chromatin remodeling, safeguarding the ends of chromosomes [150]. The median leukocyte TLs (LTLs) in the blood of healthy neonates ranged from 8.5 to 13.5 kb in the study by Okuda et al. [151] and from 7.0 to 11.6 kb in the study by Factor-Litvak et al. [152]. LTL was observed to shorten with age at an average annual rate of 30–35 bp, reaching roughly 5–6 kb in adults over 60 years old [153]. Telomere length (TL) has many properties that make it suitable for use as an aging biomarker. These chromosomal structures are known to play an important role in the process of cellular senescence [154], and their age-related shortening is widely regarded as an important contributor to organismal aging. As a result, many authors now regard TL as a potent biomarker of aging and aging-related pathological conditions [155].

The comparison of LTL and other aging biomarkers from the same individuals may undoubtedly offer useful information when discussing the applicability of LTL as a trustworthy biomarker of aging. In this context, it appears to be particularly useful to compare LTL with biological age measures like the DNA methylation–based epigenetic clock (DNA methylation age), which is currently thought to be the most accurate way to estimate biological age [156–158].

About 4% of the genomic DNA in mammals contains DNA 5-methylcytosine, mostly at cytosine-guanine dinucleotides. These CpG sites are primarily located in hotspots or CpG islands, which are non-randomly distributed throughout the genome. It is expected that a 5′ or promoter CpG island can be found in around half of all human genes [159–161]. Mammalian telomeres only have TTAGGG repeats and no CpG sites. However, both mouse and human investigations have demonstrated that the subtelomeric region, a section of DNA next to telomeric repeats, is highly methylated. By use of knockout mouse models, TL control was discovered to be dependent on DNA methyltransferases (DNMTs) and other epigenetic enzymes, such as histone methyltransferases (HMTs). This finding emphasizes the critical role that epigenetic mechanisms play in maintaining telomere length homeostasis in mammals [162].

16.4 Conclusion

Although DNA continues to reign as the “blueprint of life,” it is now evident from a whole lot of research work that the environment (external and cellular) works strongly to direct the expression or lack of expression of different parts of the genetic sequence, resulting in normal functioning or functional derailment of specific bodily processes. Epigenetic processes are mitotically and meiotically inheritable, however, without involving any change in the DNA sequence. Diet plays a very significant role in directing the epigenetic machinery to work in a certain manner right from the preconception time to last the lifetime of an individual and affect disease state or well-being. It is being recognized now that because epigenetic variations may be responsible for disease, the same can be used for therapeutic correction of the condition in a patient (Fig. 16.4). In the past few years, using epigenetic targets for personalized treatment of various disorders including cancer has improved owing to increased awareness among the medical practitioners. Caution has to be taken in applying these epidrugs to target abnormal cells; otherwise, they could lead to more normal cells developing the same

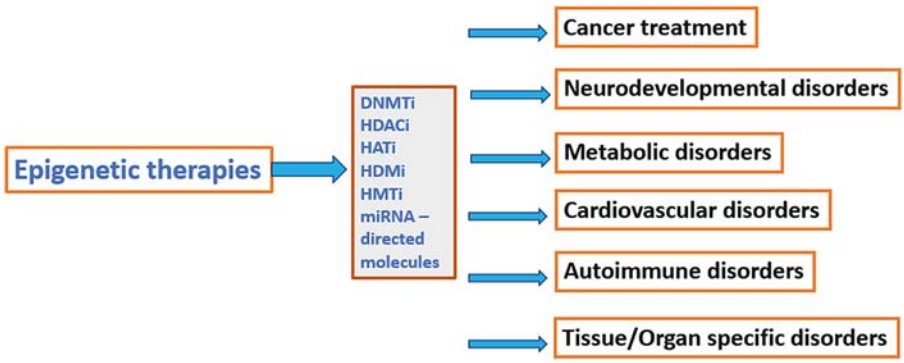

FIGURE 16.4

Potential areas of epigenetic therapies.

condition they are being treated to counter. Epigenetic signatures change over time and course of disease offering best therapeutic options, which when appropriately identified could give us the exact window period for epidrug delivery and maximize benefit for treatment. Epigenetic profiling could be done before choosing the treatment modality, and its use in prediction of outcome may soon become a routine in healthcare.

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CHAPTER

17 Epigenetics of allergic diseases: Allergies, eczema, asthma, and allergic rhinitis

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17.1 Prevalence of allergic diseases

In spite of variations, allergic disease such as asthma, allergic rhinitis, food allergies, and eczema (atopic dermatitis) appeared to increase worldwide in the four decades [1,2], and this increase is too rapid to be due to changes in genetic polymorphisms alone [3]. Worldwide, eczema affects 15%−30% of children and 2%−10% of adults [4]. Similarly, the prevalence of asthma (6%−25%) and rhinitis (up to 30%) is high [5−7]. Natural history investigations have demonstrated close and complex relationships between these allergic disorders [8−13]. Allergic conditions are considered to result from gene−environment interactions, leading to epigenetic changes during critical periods of immune developments [14,15]. However, mechanisms and pathways underlying their development are an area of ongoing scientific dispute and exploration.

17.2 Plasticity of allergic diseases

The "allergic march" concept suggests that allergic disorders develop in a sequential pattern, starting with eczema in early infancy and followed later in childhood and adulthood by asthma and rhinitis [16,17]. However, supporting replicable evidence and consensus is far from complete [18−20]. An opposing concept is the "coexistence" of allergic morbidities [18,21,22]. A report based on the German Multicenter Allergy Study showed that single occurrence of eczema early in life does not result in an allergic march, but the coexistence of eczema and wheezing predicted asthma [22]. Results from the Swedish Children, Allergy, Milieu, Stockholm, Epidemiology (BAMSE) birth cohort revealed that the comorbidity of allergic disorders is frequent during the first 12 years of life [21]. Such observations speak in favor of coexistence of different allergic morbidities instead of a progressive development. The interrelationship between these conditions, the plasticity of their progression, and the risk factors that predispose individuals to develop multiple allergic disorders are an ongoing field of research.

Epigenetics in Human Disease. https://doi.org/10.1016/B978-0-443-21863-7.00002-0
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17.3 Time order and the concept of the developmental origins of health and disease

To understand the role of epigenetic changes, we need to consider the time order of exposures, epigenetic changes, and allergic diseases [23]. Since genetic polymorphisms (the DNA) do not change with time, there was no need to address this challenge in past genetic studies. However, epigenetic markers, e.g., DNA methylation (DNAm), can change over time, both, as a consequence of exposures, diseases, or their treatments. Up to now, most epigenetic studies include concurrent assessments of disease status and epigenetics (cross-sectional studies). Hence, we cannot exclude that allergy, eczema, or asthma themselves lead to subsequent changes of DNAm as part of the body's response to cope with the disease or as part of the medical treatment (reverse causation: disease → change in DNAm; treatment → changes in DNAm). Hence, we need to evaluate the information provided by studies with concurrent assessment of epigenetic markers and disease [24–28] with a lot of hesitation, since the identification of epigenetic markers may be driven by reverse causation.

There are several approaches in epidemiological studies to address the time order of epigenetic markers and disease:

(1) One attempt is to start (1) with an exposure that is linked with an allergic manifestation. With this focus we can (2) identify epigenetic markers related to the exposure. Assuming that the exposure (but not the disease) changed the level of the identified epigenetic marker, we consequently can (3) link the identified epigenetic marker with the disease. Such approaches were conducted linking oral contraceptive → DNAm → asthma [29] or linking tetanus vaccination → DNAm → asthma [30]. Also unlikely, we cannot rule out the possibility that the disease outcome had an effect on the exposure (reverse causation).

(2) A better attempt is first to establish an epigenetic baseline and then to challenge study participants with and without allergic disorders with an allergen exposure. For instance, studies had been conducted for airborne [31] and food-born allergens [32]. Those epigenetic markers that respond to the allergen exposure are likely to represent factors that can trigger a worse disease status. Such an approach is possible, since the expression of allergic disease can vary individually, over time, and related to exposures (plasticity of the allergic diseases). However, such tests can only be conducted for exposures related to long-term responses.

(3) The best design is to measure epigenetic markers in nonaffected individuals excluding all early signs of allergy and then to follow them and determine their disease status (follow-up studies). In this setting, epigenetic markers are predictors of the disease and not their consequences. For instance, a recent study by DeVries and colleagues has used DNAm measured in cord blood monocular cells (at birth) and assessed its role for asthma development by 9 years of age [33]. A similar design was used by Martino et al. for food allergies [32]. However, it is still possible that maternal asthma status during pregnancy affects the cord blood DNAm [34], establishing epigenetic changes as an intervening variable between maternal susceptibility (disease status) and offspring asthma. Moreover, since genetic polymorphisms predispose to disease risks and might also modulate DNAm, it is preferable to also control for genetic polymorphism to determine the net epigenetic effect.

There are only a few "perfect" assessments of epigenetic effects and allergic diseases. However, the Developmental Origin of Health and Disease (DOHaD) hypothesis (also called the Barker hypothesis [35]) suggests that early life conditions influence the susceptibility for diseases later in life [36] and offers a setting for improved study results. Epigenetic markers may constitute an archive of a variety of early developmental exposures. This assumption combines both exposure-oriented assessments and follow-up studies (attempts (1) and (3), mentioned before). For example, in utero exposure to cigarette smoke has been associated with several adverse health conditions in offspring including asthma, wheezing, eczema, rhinitis, and poor pulmonary function [37–40]. Moreover, increased risk of developing allergic sensitization, a common thread that links allergic diseases, was associated with exposure to gestational smoking [41]. The developmental plasticity theory, an extension of the DOHaD hypothesis, suggests that environmental conditions surrounding critical developmental periods, such as prenatal and neonatal periods, can lead to differential physiological and morphological states across genetically identical organisms [42]. Hence, epigenetic modifications in critical developmental periods constitute biologically mechanisms that could explain such observations. Furthermore, for such environmentally induced epigenetic modifications, a transgenerational inheritance has been demonstrated, which no longer requires a direct exposure of the offspring [42a,42b]. A possible environmental epigenetic transgenerational inheritance is exemplified by an observed increased risk of asthma in children whose maternal grandmother had smoked during the fetal period of their mothers given that the child was not directly exposed to gestational smoking [43].

The focus on exposures combined with follow-up studies (attempt (1) and (3)), however, should not be restricted to the neonatal starting points (DOHaD). Also other time windows such as puberty, pregnancy, or menopause are linked to new onset of allergic diseases [44] and can provide changes in exposures that can be linked with epigenetic changes [45].

17.4 Allergy and atopy

Immune responses have been divided into the innate and the adaptive immune system [46,47] and two type of reactions, humoral and cell-mediated. Innate immune responses are not very specific, but they provide an immediate defense against pathogens, whereas the adaptive immune system reacts to specific antigens and this response takes time to evolve after contact with the pathogen [48]. Adaptive immune responses are mediated by T and B lymphocytes. B cells and their antibodies (immunoglobulins) constitute humoral immunity; T cells provide cell-mediated immunity [47,49]. T cells represent a subset of T helper (CD4+) cells, which have been subdivided into Th1-, Th2-, Th17-, and regulatory T cells (Treg). The Th1 response is characterized by proinflammatory cytokines, whereas the Th2 response is antiinflammatory [46,47]. The Th17 pathway plays an important role inducing inflammation [50,51].

Immunoglobulins (Igs, or antibodies) are Y-shaped proteins that consist of an antigen recognition part, which binds with high specificity to an antigen, and an effector part, which mediates complement activation and receptor binding. Five different classes (isotypes) of antibodies (IgA, IgD, IgE, IgG, IgM) are found in mammals. The immunoglobulins are synthesized by B lymphocytes and plasma cells; they can be integrated in their cell membrane, and they are secreted by these cells to exert their function throughout the body. IgE binds with their effector part to Fc-receptors, which are found on different white blood cells, notable high-affinity receptors on mast cells. When an antigen is binding to

these cell-associated IgE molecules, it will trigger the release of various mediators. This then induces within seconds a strong inflammation. Antigens that trigger such a response are termed allergens. IgE antibodies are found in plasma and in secretory fluids such as mucus, milk, and sweat. However, compared with other antibodies, IgE antibodies have a low concentration. For instance, in blood serum, IgE concentrations in a nonatopic individual constitute only 0.05% of the total antibody concentration compared with 75% for the IgGs in 10 mg/mL serum [52]. Generally, adult IgE levels are achieved at 5−7 years of age. Between the ages of 10 and 14 years, IgE levels may be higher than those in adults. After age 70 years, IgE levels have been reported to decline [52]. IgEs are capable of triggering very powerful inflammatory reactions. Although the original function of IgEs is the defense of parasites, these antibodies also recognize environmental and food antigens in a specific process and trigger the release of substances leading to allergic inflammation. Several immune-modulating agents are involved in allergic inflammation. For instance, small proteins such as cytokines are involved in cell signaling.

The European Academy of Allergy and Clinical Immunology (EAACI) defined atopy as tendency to produce IgE antibodies in response to low doses of allergens and to develop typical symptoms such as asthma, allergic rhinitis, or eczema [53]. Nevertheless, some patients do not show IgE-related atopy, but nonallergic bronchial hyperreactivity when having asthma or a disturbed skin barrier function when developing eczema. Atopy is considered to be heritable and has been shown that the risk of a 7-year-old child to develop and IgE-mediated allergy is 40%−60% if both parents were atopic and 5%−10% if neither parents were affected [54]. Similar findings have been reported for asthma, eczema, and allergic rhinitis, modified by the sex of the child [55]. Nevertheless, no single genetic marker for atopy has been identified as yet. Rather a variety of genetic variants have been described, suggesting that a network of genes, environmental conditions, and epigenetic effects are involved.

The EAACI has defined allergic hypersensitivity as "objectively reproducible symptoms or signs, initiated by exposure to a defined stimulus at a dose tolerated by normal subjects" [53]. In addition, EAACI allows the term "nonallergic hypersensitivity" for condition, when an immunologic mechanisms is not detectable, for instance, hypersensitivity to aspirin [56]. According to EAACI, "allergy is defined as a hypersensitivity initiated by immunologic mechanisms" both, cellular or antibody-mediated [53]. While this broad definition covers many types of immune reactions, allergy is commonly used to describe responses that are mediated by IgE and mast cells. To determine allergic sensitization, skin prick tests (SPTs) and total and specific IgE serum levels are used. Regarding SPTs, having a positive SPT to at least one allergen with mean wheal diameter of 3 mm greater than the negative control is considered as a marker of allergic sensitization. Regarding IgE, blood tests are usually considered positive if the allergen-specific IgE level is greater than 0.35 kU/L [57]. Since allergic sensitization is a dynamic phenotype, sensitization and related risks can change over time and with age.

17.5 Eczema

Eczema, also referred to as atopic dermatitis, is a chronic inflammatory skin disease characterized by intense itching, chronically relapsing, and recurrent lesions of inflammatory skin [58,59]. The main hallmarks of eczema are skin barrier defects and immunologic dysregulation. Following a position paper from the EAACI nomenclature task force [53], the term "atopic eczema/dermatitis syndrome"

should be used as the "umbrella" term to cover different subtypes of atopic dermatitis [60]. Eczema is then subdivided into an allergic, IgE-associated form, and a nonallergic-type skin disease. Hence, eczema is not one single disease, but rather an aggregation of several diseases with similar symptoms. Patients with nonallergic eczema, who also have no bronchial asthma or allergic rhinitis, show normal serum IgE levels, and negative skin-prick tests (see Section 17.2 Plasticity of allergic diseases). Following this distinction, it has been shown that the risk of an "atopic march" is lower in children with a non−IgE-associated type [60]. The prevalence of nonallergic eczema varies between 16% and 45% in different countries [61]. Although eczema most often starts in infancy and affects 20% of the children, it is also highly prevalent in adults [62]. It is the leading nonfatal health burden attributable to skin diseases, inflicts a substantial psychosocial burden on patients and their relatives, and increases the risk of food allergy, asthma, allergic rhinitis, other immune-mediated inflammatory diseases, and mental health disorders [58]. There is a need to improve our understanding of the heterogeneity of eczema and its subtypes, the role of atopy and autoimmunity, the mechanisms behind disease-associated itch, and the comparative effectiveness and safety of therapies. These variations may be explained by differences of epigenetic, genetic, or environmental influences.

In addition to immune-related characterizations of this skin disease, defects of the epidermal barrier play a central role [58]. Filaggrin (filament-aggregating protein) is a key structural protein for the formation of functional skin barrier that inhibits the penetration of allergens, microbes, and irritants and that limits transepidermal water loss [63]. The filaggrin gene (*FLG*) encodes a large insoluble polyprotein, profilaggrin, which is cleaved by dephosphorylation to produce multiple filaggrin peptides [64]. The liberated filaggrin monomers undergo further processing to form a functional/intact skin barrier that protects the host organism from external insults [65]. In 2006, several loss-of-function variants in the *FLG* gene were discovered and subsequently emerged as the strongest and most replicated genetic risk factors for eczema [66−68]. However, *FLG* variants only explain less than 10% of the heritability of eczema [69].

17.6 Asthma

Asthma is the most common inflammatory chronic disorder of the lungs that affects children and adults [70]. In children, the prevalence of wheezing ranges from 1% to 25% in different countries of the world [71]. In the United States, the prevalence of asthma in children is about 10% and in adults about 8% [72]. In adults, the prevalence of doctor diagnosed asthma is about 4%, but varies as much as 20-fold among 70 countries [73]. There is a complex heterogeneity of the clinical manifestations of asthma, which includes recurrent airway inflammation, variable airway obstruction, and bronchial hyperresponsiveness [74,75]. A complex interplay between environmental, metabolomic, genetic, and immunological factors has been speculated to underlie the pathogenesis of asthma [76−79]. However, the mechanisms leading to asthma development and its recurrence (exacerbations) are not well understood. The recognition of chronic inflammation and structural remodeling of the lungs, even in the absence of overt clinical symptoms, is a breakthrough in understanding the pathophysiology of asthma [80,81]. More recently, the role of the bronchial epithelial barrier in asthma development has emerged as an essential component of the inflammatory cascade found in asthmatic patients: a compromised respiratory epithelium facilitates the penetration of airborne allergens that initiate the inflammatory process [82,83], further highlighting the importance of gene−environment interactions in asthma.

Typically, shortness of breath and wheezing or whistling in the chest are first indicators of asthma. To this end, the International Study of Asthma and Allergies in Childhood (ISAAC) provided a validated and internationally used questionnaire [84]. Finally, asthma needs to be determined by lung function tests or spirometry. Spirometry is the most accurate test as it measures the overall lung function and capacity. In addition, peak flow measurement can be used for monitoring of the airway obstruction. Peak flow meters measure the maximum (or peak) speed at which a patient can blow air out. This provides information on how narrow (obstructive) or responsive the airways are. Comparable with eczema (see before), asthma can be classified as allergic and nonallergic asthma [85]. Hence, also asthma presents a heterogenous collection of respiratory diseases with differing phenotypes [86]. Immune mechanisms seem to distinguish airway inflammation among different subtype of the disease [87]. In addition to the frequent Th2 type inflammation asthma, chronic asthma also exists with an increase in eosinophils in peripheral blood, bronchoalveolar lavage fluid, and sputum (eosinophil asthma), which constitute a more severe phenotype [88]. Similarly, smoking-related asthma may also represent a unique phenotype with neutrophilic predominance and Th1 inflammation [87].

17.7 Food allergies

In a sensitized individual, ingestion of food protein allergen can give rise to pathological immune reactions often mediated by IgE, causing clinical symptoms that range in their severity from mild itching to severe anaphylaxis, which could be fatal. Food allergy has been on the rise in developed countries [89,90]. Between 1997 and 2007, the prevalence of food allergy has increased from 3.9% to approximately 5.0% in the United States [91]. In 2016, a cross-sectional study of 333,200 children up to 5 years of age reported food allergy prevalence to be 6.7% [92]. Diagnostic testing for food allergy remains a major challenge. To diagnose food allergies, there is a need to demonstrate a response to allergen exposure. Hence, food allergies need to be dissected from food intolerance, lactose intolerance, or gluten-induced diseases.

17.8 Allergic rhinits

Allergic rhinitis is a health problem with sneezing, or a runny or a blocked nose without a cold or specifically influenza infections [9]. It represents an inflammatory disease affecting the nasal tissue and is mainly driven by IgE-mediated reactions often to airborne allergens that affect a large proportion (up to 30%) of the general population [7]. Common symptoms of allergic rhinitis are nasal congestion and itching, sneezing, and rhinorrhea, which has high financial costs and negatively impacts the quality of life [7,93].

17.9 Epigenetics

On the one hand, epigenetic changes are influenced by genetic polymorphisms also called methylation quantitative trait loci (methQTL) [94]. On the other hand, environmental stressors can affect susceptible polymorphisms, leading to variations in DNAm [95]. The exposures include nutritional, behavioral, chemical, and physical factors [96–99]. However, the manner of the interplay between the environment and the genome that changes the epigenome remains unknown [98]. In the 1940s, Conrad

Waddington used the term epigenetics to describe how the genotype manifests itself as a phenotype [100]. In 1958, David Nanney borrowed the term to describe inherited phenomena that could not be explained by conventional genetics [101]. Epigenetics has been defined concisely by Mark Ptashne in 2007 by three criteria: (1) a change in the activity of a gene that does not involve a mutation, (2) that is initiated by a signal, and (3) that is inherited (mitotically or meiotically) in the absence of the signal that initiated the change [102]. Classically, four epigenetic mechanisms have been identified: (1) DNAm, (2) histone modification, (3) chromatin remodeling, and (4) small (21- to 26-nt) and non-coding RNAs. There is ample evidence that DNAm fulfills all three criteria required to be considered as an epigenetic mechanism [103−105]. Histone modifications satisfies some of the criteria for being epigenetic mechanisms in that they can result from exogenous signals such as cigarette smoke and alter gene activity [106−108]. However, meiotic inheritance of DNAm has only been demonstrated in *Caenorhabditis elegans* [109]. DNAm usually works hand-in-hand with histone modifications to activate or silence genes by influencing chromatin structure and its accessibility by transcription factors [110]. So, it is possible that DNAm constitutes a mechanism of mitotic inheritance for some histone modifications. Given the complex and ever-changing structure of chromatin, there is little information on chromatin remodeling regarding initiation, alteration of gene activity, and inheritance [111−113]. MicroRNAs (miRNAs) also have been shown to be controlled by exogenous factors and to alter gene activity by either inhibiting translation or degrading messenger RNAs (mRNAs) [114,115]. For instance, in humans, miRNAs have been demonstrated to be differentially expressed in current and never smokers and to be related to particulate matter exposure [106,116]. Currently, there is little evidence that environmentally induced miRNAs expression patterns can be inherited [117]. However, since miRNAs are part of the genetic code, it is possible that DNAm affects the activity of miRNAs and thus facilitates inheritance. Hence, in the following, we will focus on the truly and well-established epigenetic mechanism of DNAm.

DNAm in vertebrates is characterized by the addition of a methyl or hydroxymethyl group to the C5 position of cytosine, occurring in the sequence of CG dinucleotides, which are called cytosine−phosphate−guanine dinucleotides or CpG. Additional non-CpG methylation in a CHH and CHG context (where H = A, C, or T) exists in embryonic stem cells [118]. DNAm is believed to be involved in gene regulation/expression; however, such a role highly depends on the context in which methylation occurs in our genome. For instance, prior studies in cancer research had mainly focused on methylation of promoter regions, which were speculated to be the main modulators of gene expression (higher methylation suggested to suppress gene expression vs. lower methylation suggested to promote gene expression) [119]. However, more recent studies have demonstrated that methylation occurring at different genomic regions (e.g., gene body, intergenetic) can also influence gene expression [105].

When analyzing multiple investigations for various phenotypes of allergy (atopy, IgE levels, eczema, food allergy, asthma, and allergic rhinitis), we emphasize DNAm sites on identical genes that were discovered and replicated in at least two different populations. Our assessment requires that differential methylation sites on identical genes were at least detected twice as statistically significant. We did not demand that these sites are in the same genetical position for two reasons: (1) Although having identical effects, variations of genetical polymorphisms between genetically different populations can influence the specific location of the methylation. (2) Different approaches of epigenetic measurements may address identical methylation sites/regions, however, focusing on different parts of a methylated region.

17.10 Epigenetic changes and changes in cell composition

In the burgeoning field of epigenetics, epigenome-wide association studies (EWASs) seek to identify epigenetic alterations, which are associated with disease predisposition, the disease itself, or are predictive of future developments, but are not explained by other factors such as the cell composition of the tissue of interest. However, DNAm is cell-type specific. Hence, the question for inflammatory diseases such as allergies is whether the DNAm or the number of cells carrying the DNAm of interest is altered. Both settings can result is similar DNAm assessments in whole tissue samples, but have a different meaning: for instance, either the number of inflammatory cells is increased, or the DNAm of specific inflammatory cells is different. Easily collectable samples such as blood, saliva, or nasal epithelial samples, or cervical smears represent a composition of different cell types or contamination with cell infiltrates [120]. For instance, whole blood is composed of at least eight main cell subtypes (neutrophils, eosinophils, basophils, monocytes, CD4+ T cells, CD8+ T cells, B cells, and natural killer cells). In a British study, a large part of the associations between IgE levels and DNAm seems to be explained by a higher count of eosinophilic granulocytes [24]. Then the dilemma is that associations of specific CpGs with allergy become nonsignificant after adjusting for eosinophilic counts, while at the same time, there are associations of these CpGs within eosinophil cells. There are two "solutions" for this dilemma. First, we may only investigate specific cells such as eosinophils or bronchial cells, but then we may miss changes in other important cell types. Second, we can adjust for cell composition in the whole cell sample. To this end, multiple approaches are available [120,121]. Since such adjustments are overall and do not consider specific networks, the extent of specific DNAm changes will be reduced, often to a few percentages. For instance, if methylation differs in one CpG of eosinophils between allergic and nonallergic individuals by 50%, but eosinophils only account for 5% of all cells, adjusted for all cell types, we only see a 2.5% difference in methylation in the respective CpG between allergic and nonallergic individuals.

17.11 Epigenetics related to atopy and IgE levels

GWASs have identified single-nucleotide polymorphisms (SNPs) linked with total immunoglobulin E levels, but their joint estimated effect accounts for a very small proportion or the trait's heritability [122–125]. Other researchers estimate that 36%–78% of the variance in total IgE is explained by multiple single genetic polymorphism [126–128]. Regarding DNAm, most studies did not investigate atopy or increased IgE levels as a single independent marker but analyzed them as part of clinical features of asthma, eczema, food allergies, or allergic rhinitis (Table 17.1). On the one hand, for different CpGs, there are candidate gene approaches (advances that are based on one or a few aspirant genes), often based on prior knowledge of specific associations. On the other hand, there are epigenome-wide approaches, which do not require specific prior knowledge but attempt to identify novel epigenetic markers (CpGs or differently methylated regions). To this end, statistical tools such as Bonferroni corrections or false discovery rates (FDRs) are used to diminish the chance of false-positive findings. Bonferroni correction reduces the probability of false-positive results by using a conservative significance level for each test determined by the total number of all tests. FDR controls the proportion of wrongly rejected null hypotheses among those that are rejected, instead of among all tests. However, epigenome-wide studies need replications to distinguish chance findings from novel reproducible findings. In addition, few studies underwent metaanalyses, statistically comparing the strength of

Table 17.1 Atopy, increased immunoglobulin levels, and related DNA methylation.

Allergic phenotype	Gene and methylation	First author, sample sizes, and main comparisons
Atopy status	Methylation before and after development of a Th2 linage: after Th2 differentiation demethylation of ***IL4*** and ***IL13*** genes	Santangelo et al. (sample size not provided, nonatopic healthy male volunteers) [129]
	STAT6 gene expression ↑ with higher Stat6 methylation	Kim et al. (n = 41 cases with asthma and n = 50 cases with rheumatoid arthritis, unknown number of healthy donors) [130]
	Higher ***FOXP3*** methylation*:* *Asthma* ↑ *Atopy* ↑ A higher count of Treg cells was protective against asthma Lower ***FOXP3*** methylation was found in the Treg ($CD4^{+}CD25^{high}FOXP31^{+}$) cells	Swamy et al. (n = 30 participants, 5 years or older were randomized). Twenty subjects received either timothy grass or dust mite dual sublingual immunotherapy; 10 subjects were in the placebo group [131] Lluis et al. (n = 298 children, atopic sensitization, asthma, Treg cells) [132] Paparo et al. (n = 40 children with and without cow milk allergy) [133]
	CRIP1*: Asthma* ↑ *Atopy* ↓ ***STAT5A****: Asthma* ↑ *Atopy* ↓	Stefanowicz et al. (n = 69 children, comparison of airway epithelial cells in nonatopic and atopic asthmatics) [135]
Increased immunogloblin E (IgE)	*COL15A1, IL5RA, ITGA2B, KLF1, L2HGDH, LPCAT2, PIK3CB, PRG2, SLC17A4, SLMAP, TMEM86B, ZNF22*	Liang et al. (metaanalysis of three studies: Medical Research Council (MRCA): n = 355, mean age: 28 years; Poblogaeth Asthma Prifysgol Albertawe (PAPA): n = 149, mean age 21 years, Saguenay-Lac-Saint -Jean (SLSJ): n = 160, mean age: 29) [24]
	FARP1	Martino et al. (11−15 months old children who underwent skin prick tests to egg white, peanut, cow's milk or shrimp, and objective oral food challenge tests) [32]
	ACOT7, *FARP1*, *PRG2*, *ZFPM1*	Everson et al. (18-year-old adolescents, n = 245 girls, and n = 122 boys, adjustment for predicted cell proportions, replication with the Swedish BAMSE cohort) [28]
	ACOT7, *ZFPM1*	Chen et al. (metaanalysis if two studies: Puerto Rico Genetics of asthma and lifestyle study (PR-GOAL), n = 306, and Genes-environments and Admixture in Latino Americans (GALA II), N = 573, age 6−22 years; adjustment for cell proportions, FDR adjustment) [139]

Continued

Table 17.1 Atopy, increased immunoglobulin levels, and related DNA methylation.—cont'd

Allergic phenotype	Gene and methylation	First author, sample sizes, and main comparisons
Increased immunogloblin E (IgE)	*COL15A1, IL5RA, ITGA2B, KLF1, L2HGDH, LPCAT2, PIK3CB, SLC17A4, SLMAP, TMEM86B, ZNF22*	Ek et al. (Northern Swedish Population Health Study (NSPHS), n = 728, age 14−94) [140]
Increased immunogloblin E (IgE, food, environmental allergens)	No statistically significant CpG sites detected for allergic sensitization applying a false discovery rate of <0.05. Regarding CpGs with differences in methylation between birth and 7−10 years and cross-sectional associations with IgE, one gene overlaps with findings by Acevedo et al.: *ANKRD11*	Peng et al. (396 mother−child pairs from Project Viva (Boston, the United States) and 343 mother−offspring pairs from the Generation R Study (Rotterdam, the Netherlands) [141]. DNA methylation measured at birth and around 7−10 years (midchildhood); IgE measured in midchildhood [141]
Increased immunogloblin E (IgE, food, and aeroallergens)	Associated with IgE sensitization to food allergens: *ALOX12P2, CRIP2, RUFY1, S100A13, TRIM27, TSKS, HNRNPKP3, SEC14L4, ZNF503-AS2, NNAT, SPATA19, RPTOR, HIST1H2BB, C17orf104, CACNA1D, GSTM3, C10orf11, UTS2B, GDF7, CD40LG, ABAT, FKBP11, PRKCZ, ACY3, TNK2* Associated with IgE sensitization to aeroallergens: *CCDC124, KIAA0247, TGIF1, ROR1, GNB5, PRDM15, ANKRD11, FAM100A, APOLD1*	Acevedo et al. (discovery: 288 PBMC samples collected at birth from 74 mother/child pairs with IgE levels at 5 years from the Assessment of Lifestyle and Allergic Disease During Infancy/ ALADDIN birth cohort, replication: 256 children, IgE measured at 4 years linked to DNA methylation from offspring whole blood samples at birth, Children, Allergy, Milieu, Stockholm, Epidemiology/BAMSE cohort [138]
Increased immunogloblin E (IgE)	*CACNA2D1, ADARB2, PCDH17, OR8B8, CTNS, BMI1, PTK2B, ATP6V1C2, SHQ1, DDN, MYO15B, LOXL1, FBXO6, GRM1, FLT3*	Han et al. (discovery: n = 299 from the Taiwanese maternal and infant birth cohort, replication: n = 64 from the Isle of Wight (IoW) birth cohort [137]

associations found in separate studies of multiple populations. The studies presented in Table 17.1 identified some hundred CpGs on genes or intergenic region related to atopy or increased IgE concentrations. However, to focus on a limited number of CpGs and genes, we required that in epigenome-wide studies that epigenetic markers were identified in at least two independent studies.

Using a candidate gene approach, it has been suggested that *IL4*, *IL13*, and *STAT6* genes are differently methylated in atopic and nonatopic patients (Table 17.1) [129,130]. At least three investigations demonstrated that *FOXP3* gene methylation is involved in atopy [131−133]. In addition, Hinz et al. used specific DNAm in T-regulatory (Treg, CD4+CD25^{hi}) cells to estimate the number of Treg cells in 346 cord blood samples. The authors demonstrated that Treg cells in cord blood were associated with atopy development [134]. Also, CRIP1 and STAT5a methylation differences in airway epithelial were found to be associated with atopy (Table 17.1), but this has not been replicated as yet [135].

Regarding epigenome-wide studies, 28 genes identified by Kim et al. [136] comparing DNAm in bronchial mucosa cells of 10 adult atopic asthmatics and seven adult nonatopic asthmatics have not

been replicated by other investigations as yet. However, such cross-sectional studies cannot distinguish "what comes first—atopy or DNAm?" [23]. Also, the study of Peng et al., when linking midchildhood DNAm with midchildhood allergic sensitization, suffers from a clear time order. Nevertheless, this study belongs to more recent publications (Table 17.1) such as Acevedo et al. and Han et al. using two different cohorts to check whether the result of the first study could be replicated in the second [137,138].

In Table 17.2, we describe 16 genes identified in eight studies (Table 17.1) for which differential DNAm was identified related to increased IgE levels in at least two independent publications.

Table 17.2 Atopy or increased IgE levels: list and functions of differentially methylated genes identified in at least two independent epigenome-wide studies.

Gene symbol	Full name	Function
COL15A1	Collagen Type XV alpha 1 chain	Encodes the alpha chain of type XV collagen, which adhere basement membranes to underlying connective tissue stroma maintaining the structure and biological integrity of epithelial barrier [144].
IL5RA	Interleukin 5 receptor alpha	The receptor selectively stimulates eosinophil production and activation [145]; hence, diseases associated with *IL5RA* include atopy and asthma [146].
ITGA2B	Integrin, alpha 2b	The receptor coded by the gene *ITGA2B* (Integrin, alpha 2b) plays a crucial role in the blood coagulation system by mediating platelet aggregation (www.genecards.org/cgi-bin/carddisp.pl?gene=ITGA2B).
LPCAT2	Lysophosphatidylcholine acyltransferase 2	It encodes lyso-platelet-activating factor (PAF) acetyltransferase, which is essential to induce formation of PAF, a proinflammatory lipid mediator [147,148], associated with atopy and asthma [149].
KLF1	Kruppel-like factor 1	A critical transcription factor for erythropoiesis [150].
L2HGDH	L-2-Hydroxyglutarate Dehydrogenas2	The gene is considered to be related to mitochondrial suppression of apoptosis in activated eosinophils [24].
PIK3CB	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta	It has been shown to be part of the activation pathway in eosinophils and neutrophils, which have bound immune complexes at sites of injury or infection [151,152].
PRG2	Proteoglycan 2	Its gene expression is upregulated in response to dust mite exposure in mite-sensitized patients [153].
SLC17A4	Solute Family 17 Member 4	The gene encodes a membrane that acts as potential-dependent organic anion transporter. It may recognize hydrophilic anionic drugs such as aspirin, salicylate, and ibuprofen as substrates (http://www.genecards.org/cgi-bin/carddisp.pl?gene=SLC17A4&keywords=SLC17A4).
SLMAP	Sarcolemma associated protein	It encodes a component of a conserved striatin-interacting phosphatase and kinase complex. Striatin family complexes participate in cellular signaling, cell cycle control, cell migration, Golgi assembly, and apoptosis.

Continued

Table 17.2 Atopy or increased IgE levels: list and functions of differentially methylated genes identified in at least two independent epigenome-wide studies.—cont'd

Gene symbol	Full name	Function
ZNF22	Zinc Protein 22	The gene seems to be involved in governing transcription by RNA polymerase II.
FARP1	FERM, RhoGEF (ARHGEF), and pleckstrin Protein 1	Exact function is not known or validated.
ACOT7	Acyl-CoA thioesterase 7	The gene has been recognized to play a role in inflammation through the production of arachidonic acid and is involved in increased prostaglandin production. [154] Prostaglandins play a key role in inflammatory responses [155].
TMEM86B	Transmembrane protein 86B	It may control levels of plasmalogens and lysoplasmalogens in cells and modulate cell membrane properties (http://www.genecards.org/cgi-bin/carddisp.pl?gene=TMEM86B&keywords=TMEM86B).
ZFPM1	Zinc Protein, FOG Family Member 1	*ZFPM1* is involved in the production of hematopoietic stem cells, their proliferation, maturation, and terminal differentiation [156].
ANKRD11	Ankyrin Repeat Containing 11	Mutations in *ANKRD11* have been associated with distinctive craniofacial features, short stature, skeletal anomalies, global developmental delay, seizures, and intellectual disability (https://www.genecards.org/cgi-bin/carddisp.pl?gene=ANKRD11&keywords=ANKRD11). The gene was also found to be differentially methylated related to long-term ambient air pollution exposure [157] and related to isocyanates (respiratory and skin sensitizers) [158].

Surprisingly, when investigating IgE levels in childhood, three recent studies using an epigenome-wide approach with replications based on two samples each (Table 17.1, Peng et al.; Acevedo et al.; Han et al.) [137,138,141] did not identify methylation of any identical genes. It is likely that IgE levels vary so much in children with age and season of the year [142,143] that no stable epigenetic association can be identified across studies. In addition, it is also possible that air pollution, not controlled for a most studies, is responsible for some associations (e.g., *ANKRD11*, Table 17.2). Hence, there is need to identify more stable IgE trajectories including specific trajectories (e.g., house dust mites) in childhood, before further epigenetic associations controlling for air pollution should be researched.

In summary, regarding atopy and increased IgE levels, differential methylation of specific CpGs located on a variety of genes involving blood cell production, in particular of Treg cells, cell membrane function, cell barrier, inflammatory mediators, and mitochondrial function may be involved in the epigenetic regulation of atopy and IgE concentrations.

17.12 Epigenetics of eczema

The interplay of epidermal barrier and the innate and adaptive immune systems [159,160] in the etiology of eczema remains not well understood even after decades of research in this field [159]. Until recently, two concepts were discussed for the pathogenesis of atopic dermatitis: the outside−in concept (leaky epithelial barrier allows entry of allergens leading to immunological imbalance) and the inside−out concept (systemic immune defect triggers epithelial barrier dysfunction) [161]. Accumulating evidence has demonstrated that these two mechanisms may be interdependent [162]. Disruption of the epidermal barrier function may stimulate Th2 cytokine-driven inflammatory mechanisms that further compromise barrier function, thus creating the outside−inside−outside vicious cycle [163]. Studies have shown eczema to be heritable [164−166], yet classical genetics fails to explain the risk of eczema [160], paving the way for investigating environmental effects mediated by epigenetic processes such as DNAm [167]. However, several genome-wide analyses have produced the same genetic polymorphisms associated with eczema across populations [159,160], strengthening causal associations. In contrast, studies related to eczema and DNAm are still in their exploratory phase and lack coherence with each other, often due to the lack of replication studies. Owing to the scarcity of epigenome-wide methylation studies for eczema, our review focused on studies using candidate gene approach and investigations exploring the effects of environmental exposures on eczema that are mediated through alterations in the DNAm.

Peripheral blood methylation of CpG cg07548383 of the filaggrin (*FLG*) gene, one of the major components of the epidermal barrier, has been shown to modify the effect of *FLG* loss of function mutation on eczema [168]. A study by Tan et al. found no association between buccal cell methylation of other *FLG* CpGs and eczema [169]. However, Lee et al. found higher *FLG* methylation levels measured in lesional epidermis than those in matched nonlesional epidermis of subjects with severe eczema [170]. The methylation status of the β-defensin-1 (*HBD-1*) gene, involved in the skin barrier functioning, has been shown to be associated with eczema, with higher methylation levels observed in lesional samples than in nonlesional skin samples and normal skin samples [171].

Follow-up studies have explored links between various prenatal exposures, methylation of immunity-related genes and eczema [134,169,172−174]. In one study, regulatory T (Treg) cells were identified to play an important role in maintaining tolerance, preventing autoimmunity and controlling allergic responses [175]. The *FOXP3* gene, located on X-chromosome, has a Treg-specific demethylated region (TSDR). Demethylation of TSDR served as a biomarker to quantify Treg cells in several studies. Prospective studies have shown maternal smoking, family history of atopy, and higher expression of microRNA miR-223 to be associated with low number of Treg cells in cord blood (measured by *FOXP3* TSDR demethylation), which, in turn, is associated with a high risk of eczema later in life [134,172]. In addition to these findings, a case−control study reported higher peripheral blood count of Treg cells in adult atopic dermatitis (AD) patients and a positive association with disease severity [173] (Table 17.3).

The role of DNAm of *TSLP* (thymic stromal lymphopoietin) gene has also been explored [169]. Luo et al. demonstrated an association between promoter demethylation of *TSLP* and its overexpression in skin lesions of eczema patients [169]. Wang et al. in a Taiwanese cohort showed prenatal smoking exposure to be associated with methylation of 5′ CpG island of the *TSLP* gene in cord blood. In this prospective study, they found the methylation at this region to be associated with a risk of atopic

Table 17.3 Two genes *FOXP3* and *TSLP* found in more than one studies linking epigenetics and eczema.

Genes	Description	First author and sample size
FOXP3 TSDR	Male gender, maternal hay fever, paternal asthma, maternal smoking during pregnancy, maternal cytokines (IL-13, IL-17E, and IFN-c) ↓ Lower Treg numbers in cord blood (measured by higher methylation of TSDR of *FOXP3*) ↓ Higher risk of atopic dermatitis and food allergen sensitization in the first year of life	Hinz et al. N = 346 mother−child pairs [134]
	High miR-223 expression ↕ Lower Treg cell numbers in maternal and cord blood (measured by higher methylation of TSDR of *FOXP3*) ↓ Lower Treg cell numbers at birth had a higher risk of atopic dermatitis during the first 3 years of life	Herberth et al. n = 441 [172]
	Higher Treg frequencies in adult whole blood (measured by *FOXP3* TSDR methylation) ↕ Higher disease severity	Roesner et al. 30 AD patients Controls: 20 suffering from systemic lupus erythematosus (SLE) and 20 healthy volunteers [173]
TSLP	Increased expression of TSLP mRNA and decreased methylation of TSLP promoter ↕ In skin lesions (keratinocytes) from patients AD	Luo et al. 10 children with AD and 10 healthy controls [169]
	Prenatal smoke exposure ↓ } Screening Lower methylation of 5′ CGI of *TLSP* in cord blood ↓ } Follow-up Higher risk of AD	Wang et al. N = 14 for screening (7 each for low and high smoke exposure group) N = 150 for follow-up study [174]

The arrow ↓ *indicates prospective time order, and the arrow* ↕ *indicates concurrent findings.*

dermatitis at 2 years of age [174] (Table 17.3). Another study detected differentially methylated CpGs of *VKORC1* and, between *PCED1A* and *TMEM239* (intergenic) linked to autumn and winter season of birth, respectively, and with eczema at age 18 years [176]. Epidemiologic evidence, although conflicting, suggest an inverse association between vitamin D levels and eczema risk and severity. As such, lower vitamin D levels have been implicated in the development of eczema and its severity [177–179]. Strengthening a potential causal association, birth cohort studies have shown inverse associations between maternal and cord blood vitamin D levels with eczema during childhood [178,180–182]. Cho et al. suggested that the increased eczema risk that is related to vitamin D deficiency could be mediated by lower methylation of the microtubule associated monooxygenase, calponin, and LIM domain containing 3 (*MICAL3*) gene [183]. Moreover, Yang et al. showed that placental DNAm modified the association between particulate matter 2.5 μm ($PM_{2.5}$) exposure during the first trimester of pregnancy and early-onset persistent eczema [184].

Regarding eczema, *FOXP3* and *TSLP* were the genes carrying differentially methylated CpGs, which were identified in at least two independent studies. Table 17.4 briefly describes the function of these genes.

Atopic dermatitis may not always be associated with elevated serum IgE levels (intrinsic type); nevertheless, elevated IgE levels and allergic reactions may aggravate disease severity as seen in the extrinsic type of eczema [61,185] (see before). A study reported decreased expression of the DNA methyltransferase-1 (*DNMT-1*) gene in peripheral blood mononuclear blood cells of atopic dermatitis patients with high (≥5000 IU/mL) compared with those with low serum IgE levels (<5000 IU/mL) [186]. As the name suggests, the enzyme DNMT-1 is responsible for gene methylation by transferring methyl groups to the DNA [187], lower expression of which may lead to reduced methylation. Interestingly, reduced methylation in B cells is linked to IL4-driven IgE production [186]. Thus, assessing

Table 17.4 Eczema: list and functions of differentially methylated genes identified in at least two independent epigenome-wide studies.

Gene symbol	Full name	Function
FOXP3	Forkhead Box P3	This gene is a transcription factor of the forkhead/winged-helix family and plays an immunosuppressive role. It suppresses NF-κB and IL2 genes and activates other genes like CD2S and is the master regulator for Treg cell lineage [197,198].
TLSP	Thymic stromal lymphopoietin	The gene *TSLP* codes a cytokine that promotes B cell and dendritic cell development and Th2 responses. Stimulation of cells by TSLP induces STAT phosphorylation visa JAK1 activation. TSLP mitigates TH1/TH17 responses and is involved in allergic disorders of airway and skin, autoimmunity, and cancer [199].

the role of methylation and IgE production may provide a new insight toward the etiology of eczema. A case–control study showed that increased methylation of the *FCERI* (the high-affinity IgE receptor) promoter in monocytes of atopic dermatitis patients compared with controls [188]. Monocytes bearing this surface receptor may differentiate into inflammatory epidermal dendritic cells and stimulate T cells, thus exacerbating atopic dermatitis [188]. The cutaneous lymphocyte antigen (CLA) expressed by T cells is known to mediate the inflammatory responses found in eczema. Hence, Acevedo et al. investigated the role of DNAm in subsets of $CD4^+$ T cells and observed DNAm differences in $CD4^+CLA^+$ T cells between eczema patients and controls in 40 genes [189]. Many of the differentially methylated genes are involved in inflammatory responses and cytokine signaling pathways. For instance, lower methylation in the upstream region of the *IL-13* gene was observed among eczema patients compared with controls, and lower methylation was correlated with increased IL13 mRNA expression [189]. A study by Thürmann et al. investigated the role of methylation of nucleotide-binding oligomerization and pyrin domain–containing receptors (NLRPs), known to modulate the innate immune response, in eczema development [190]. This study showed that lower methylation of the promoter region of *NLRP2* gene was associated with an increased risk for early-onset eczema.

Assessing the correct time order of exposure and event and tissue specificity of DNAm in predicting diseases remains an unresolved issue in epigenetic studies (see Section 17.3 Time order and the concept of the developmental origins of health and disease). Rodriguez et al. found significant differences in the methylation profile of peripheral blood compared with epidermal shaves of atopic dermatitis patients in their epigenome-wide analysis [191]. The 127 differentially methylated sites between lesional atopic dermatitis and controls belonged to the functional pathways related to plasma membrane, signal transducers, and ion channels. Several of these sites located within the epidermal differentiation complex and belonging to the keratin encoding genes were also correlated with their mRNA transcripts, reinforcing the functional role of DNAm. However, these differential methylation sites may be the result of eczema, but not its "cause." In an epigenome-wide analysis by Quraishi et al., 83 differentially methylated CpGs showed a concurrent association with concurrent eczema using peripheral blood at age 18 years, of which two CpGs significantly were replicated in cord blood predicting eczema in infancy [192]. The enriched set of genes were related to biological pathways of epithelial barrier integrity and cellular adhesion [192]. More recently, Chen et al. conducted epigenome-wide methylation analysis using peripheral blood of 24 eczema patients and 24 control subjects [193]. This study identified 16,840 differentially methylated CpG regions between eczema patients and control subjects, with >97% of the identified CpG loci showing lower methylation in eczema patients as compared with controls. Authors identified two CpG clusters within the golli-mbp locus of the *MBP* gene. However, no replication was conducted.

Whereas environmental exposures may mediate their effect through DNAm, interaction between genetic polymorphisms (single nucleotide polymorphisms, SNPs) and DNAm of CpG sites may also influence the risk of allergic diseases. The genes of the cytochrome oxidase (CYP) enzymes have been found to be overexpressed in keratinocytes treated with coal tar [194], and genetic variants of these genes are also associated with AD and high levels of serum IgE [195]. Thus, it may be important to investigate the combined role of genetic polymorphisms and DNAm in the etiology of eczema. In line with this concept, Stevens et al. demonstrated that the minor alleles of *KIF3A* SNPs rs11740584 and rs2299007, which have been previously shown to be associated with eczema, were associated with CpG sites that resulted in increased methylation and decreased expression of *KIF3A* [196]. Subsequently, the authors showed that this increased methylation is associated with increased transepidermal

water loss (TEWL) in those carrying the risk alleles of the aforementioned *KIF3A* SNPs. This evidence further supports necessity to incorporate genetic polymorphisms in DNAm studies.

Epigenetic studies related to eczema until now have produced varying results due to small sample sizes, use of different arrays to obtain DNAm (27 vs. 450K vs. 850k arrays) and preprocessing techniques, and different tissue types (peripheral or cord blood or skin samples) apart from the ethnicities and age of the participants. Facilitating more investigations, replicating and validating results, and performing metaanalyses with several cohorts are the most pressing needs to unravel underlying mechanism that could lead to eczema.

17.13 Epigenetics of food allergies

Table 17.5 lists studies of DNAm related to food allergies, which focused on cow's milk allergy and peanut allergy. Interesting features of most food allergy studies are the use of clinical trials to treat food allergies [200] and thus the related small sample sizes of these investigations. As for other allergic manifestations (Tables 17.1 and 17.3), methylation of *FOXP3* also plays a role for food allergy (a description is provided in Table 17.4).

Table 17.6 records gene names identified in two independent studies. In addition to *IL4*, a gene known to be involved in allergies [201,202], *IL10* and *RPS6KA2* were identified to be associated with food allergy.

Looking at the potential epigenetic candidate genes, time order raises a challenge. Methylation of some genes, particularly *IL4* and *IL10*, may only have been changed since these genes reacted to an existing food allergy. Etiologic research in the epigenetics of food allergy will benefit from birth cohort studies, that, given their clear time order of processes, can distinguish which DNAm can be considered as cause and that should be considered as effect [23]. Hence, clinical trials or challenge test approaches should be nested in birth cohort studies or exploit existing blood spots collected at birth. Currently, there are no reliable epigenetic biomarkers [215]. In addition, epigenetic factors in food allergy vary between different studies and collaborative approaches are necessary to improve our understanding, in particular the analyses of different cohort studies to distinguish between etiologic epigenetic factors and epigenetic responses to existing food allergy.

17.14 Epigenetics of asthma

The high heritability of asthma, estimates varying between 35% and 95%, and clustering within families demonstrate the importance of genetic or epigenetic predisposition in disease pathogenesis [216]. Since the completion of the Human Genome Project in 2003, much of the etiologic research in asthma focused on identifying genetic factors, in addition to environmental determinants, that predict asthma onset and the role of life-course changes.

GWAS, a hypothesis-free approach, have identified multiple genetic loci that predispose to asthma risk [217–220]. For instance, Moffatt and colleagues, using a GWAS approach, were the first to show that multiple markers on chromosome 17q21 strongly predispose to the onset of childhood asthma [221]. This study demonstrated that gene expression of *ORMDL3* is regulated by genetic variants within the 17q21 locus. Subsequent studies have confirmed the role of this locus on asthma risk [122] and further showed that genetic variants at the 17q21 locus influence expression of *ORMDL3*

Table 17.5 Food allergy and related DNA methylation.

Gene and methylation	First author, sample sizes, and main comparisons
Candidate gene approaches	
Promoter methylation of *IL4* and *IL5* genes was lower in children with cow's milk allergy (CMA), and the methylation of the promoter of the *IL10* and *IFN-γ* genes was higher in children with CMA.	Canani et al. (40 children, 10 with IgE-mediated CMA, 20 children who acquired tolerance to CMA, 10 controls; assessment of the DNA-methylation of promoter region of *IL4*, *IL5*, and *IL10*, and *IFN-γ* genes of peripheral blood mononuclear cells; no adjustment for cell composition of the blood cells) [203].
FOXP3 methylation was lower in children who acquired tolerance against cow's milk.	Paparo et al. (10 children with IgE-mediated CMA; 20 children who outgrew CMA, 10 controls; assessment of the methylation of *FOXP3* gene) [133].
FOXP3 methylation was lower after induction.	Begin et al. (unknown number of healthy adult blood bank donors, naïve T cells) [204].
The genes *BDNF*, *SERPINE1*, *FLG*, *IL12B*, *IL2, CXCL12, IL10*, *IKZF2*, *CCL5*, *IL21*, *CCR9*, and *IL-33* were differentially methylated.	Zhou et al. assessed targeted DNA methylation of 70 immune-related genes in 10 children with peanut allergy (PA) and 10 nonallergic participants, aged 5−10 years old (both twins and nontwins) [205].
Methylation of the *HLA-G* promoter region and some CpGs of *HLA-DRB1* were different in samples of food allergic participants.	In a cross-sectional study, Kostara et al. recruited 64 orally challenged food allergic children/adolescences and 44 controls. Targeted pyrosequencing analysis of *FOXP3*, *HLA-G, HLA-DRB1*, and *HLA-DQB1* were applied to identify DNA methylation and genetic variations [206].
Epigenome-wide approaches	
Significantly differential methylation of 49 genes; *RPS6KA2*.	Martino et al. (12 children with IgE-mediated food allergy, 12 nonallergic controls; comparison of DNA-methylation of CD4+ T cells at birth and at 12 months) [32].
Significantly differential methylation of 13 genes: (*HOXC4/HOXC5*, *NBPF1*, *KLHL17*, *IGR*, *OR8U8/OR5M8*, *GABPB2*, *C10orf58*, *PARD3*, *ZNF281*, *CCR3*, *THAP3*, *NAV3*, *C7orf50*).	Petrus et al. (20 children with CMA, 23 controls, 10 tolerant boys; assessment of DNAm from peripheral blood mononuclear cells; adjustment for cell composition of the blood cells) [207].
FOXP3 CpG sites in antigen-induced	Syed et al. (23 participants undergoing oral immunotherapy against peanut allergy, 7 developed immune tolerance, 13 remained nontolerant, 20 age-

Table 17.5 Food allergy and related DNA methylation.—cont'd

Gene and methylation	First author, sample sizes, and main comparisons
regulatory T cells were significantly different between patients with immune tolerance and those who remained nontolerant.	matched controls (age not provided), assessment of DNA methylation from peripheral blood mononuclear cells; no adjustment for cell composition of the blood cells; assessment of DNA methylation in Treg cells) [208].
IL4, RPS6KA2.	Hong et al. (106 children with cow's milk allergy (CMA) and 76 nonatopic controls; assessment of DNA methylation in whole blood; adjustment for cell composition [209].
The genes *ZBED2* and *BCL11B* were identified a differently methylated at baseline and significantly changed in follow-up (persistent vs. transient allergy).	Martino et al. included participants of the HealthNuts cohort study recruited through community immunezation clinics in Melbourne, Australia. 44 allergic subjects were selected for this sub-study (mono-sensitized and clinically reactive to egg at 12 months of age); age-matched controls (n = 21) were non-atopic to any of the allergens tested [210].
Differential methylation of the genes *PHACTR1* and *ZNF121* in CD4+ cells of baseline samples detected with the Illumina Infinium HumanMethylation450 array.	In an epigenome-wide approach, Do et al. studied a discovery cohort with 21 peanut-allergic children who reacted to peanut challenge during a randomized, double-blind, placebo-controlled food challenge. The replication cohort had 19 independent peanut-allergic children identified with the same protocol as the replication cohort [211].

(ORMDL Sphingolipid Biosynthesis Regulator 3) and *GSDMA* (Gasdermin A) genes and regulate the secretion of interleukin (IL)-17 in cord blood cells [222]. Hence, these genetic variants link asthma-associated loci with inflammatory processes that characterize the clinical symptoms of asthma. Moreover, a large GWAS using severe asthma cases supported the association of asthma with *ORMDL3* and *GSDMA* and added strong evidence of associations between the *IL1RL1/IL18R1* loci on chromosome 2q12 with severe asthma [223].

However, genetic investigations have not been successful at identifying genetic variants that explain the observed high heritability, a setting known as the "missing heritability" [224,225]. Not accounting for gene−environment and gene−gene interactions when trying to explain the heritability could be one explanation for the "missing heritability" phenomenon [226]. Nevertheless, heritable genetic regulatory elements, other than DNA sequence variants, may offer a complementary explanation for the unexplained genetic effect. DNAm, addressing heritable genetic regulatory elements that alter gene activity (e.g., expression) without changing the underlying nucleotide sequence of the DNA [227,228], has been speculated as plausible predisposing factors in the development of asthma and might also account for the unexplained heritability [95,229,230].

In this context, given the rapid emergence of exciting literature supporting a pivotal role for epigenetics in the developmental origins of asthma [33,231,232] and given that epigenetic markers may constitute an archive of a variety of early developmental exposures, etiologic research has recently been focused on identifying epigenetic markers for asthma.

Table 17.6 Food allergy: list and functions of differentially methylated genes identified in at least two independent epigenome-wide studies.

Gene symbol	Full name	Function
IL4	Interleukin 4	The cytokine IL-4 triggers the differentiation of naive helper T cells to Th2 cells, stimulates B cell proliferation, and switches B cells to IgE production [212].
IL10	Interleukin 10	This cytokine is primarily produced by monocytes and to a lesser extent by lymphocytes. It plays multiple roles in immunoregulation and inflammation (pleiotrop) [213].
RPS6KA2	Ribosomal Protein S6 Kinase A2	The activity of then kinase may be involved in controlling cell growth and differentiation. It has been shown to be associate with inflammatory bowel disease [214].

Candidate−gene association studies, a set of hypothesis-driven approaches, have contributed to advancing our knowledge and understanding of the complex relationship between genetic, environmental, and epigenetic factors in the development of asthma. Studies have investigated the methylation of genes in the pathways known to be involved in asthma and allergy such as receptor tyrosine kinases, glucocorticoid receptors, DNA repair and senescence, and specific cytokine-related pathways [233−239]. These studies have identified differential methylation of key genes such as *AXL* [236], *GLCCI1* [238], *FOXO3* [237], *TP53* [237], *IL13* [239], and *CCL26* [239] to be associated with asthma. A recent cross-sectional study focused on coronavirus-related candidate genes has identified 40 CpGs linked to asthma in 242 Caucasian participants aged 26 years, of which six CpGs showed a sex-specific effect [240]. Candidate−gene association studies also demonstrate the importance of integrating genetic information in epigenetic studies. For instance, interactions between DNAm and genetic variants within the leptin gene (*LEP*) in the association with decreased lung function and increased asthma risk have been described [235]. Another study showed an interplay between DNAm and genetic variants in Th2 pathway genes to contribute to asthma risk [234]. Moreover, it has been shown that DNAm modulates the risk of asthma related to genetic variants in the *IL4R* gene [233]. Results of the studies provide strong evidence for an interplay between genetic variants and DNAm and provide a novel model on how a joint effect of genetic variants and DNAm can explain occurrence of asthma.

Epigenetic candidate−gene association studies provided essential insights and paved the path for EWASs to discover novel epigenetic loci that predispose to the development of asthma. The rest of this

section will focus on recent and novel findings reported by epigenome-wide investigations in relation to asthma and asthma-related phenotypes. Specifically, we review studies reporting genome-wide investigations of human DNAm because the role of other epigenetic mechanisms (e.g., histone modifications) has not been systematically investigated in asthma research. Detailed information about the studies we reviewed (study population, sample size, tissue used to measure DNAm, exposures, studies outcome, and summary of findings) is presented in Table 17.7.

Over the past few years, several epigenome-wide association analyses have been conducted using DNAm from various tissue types. Studies with bronchial biopsies and bronchial epithelia provide a close insight toward the methylation dynamics in the airway. A study by Vermeulen et al. [241] compared bronchial biopsy tissues from 26 subjects with persistent asthma, 39 subjects in remission of asthma in the past 3 years without medication, and 70 healthy controls. They identified four differently methylated CpGs and 42 differentially methylated regions (DMRs) between persistent versus remission of asthma. Of note are two CpGs on the atypical chemokine receptor 2 (ACKR2) and diacylglycerol kinase theta (*DGKQ*) genes that had lower methylation and consequently lower gene expression in subjects with remission compared with those with asthma. The *ACKR2* gene encodes a chemokine receptor and is involved in abatement of inflammation [242]. The *DGK* family of genes are known to be expressed in immune cells and are required for signal transduction in inflammatory processes [243]. They also identified a CpG on the tenascin XB (*TNXB*) gene, an extracellular matrix glycoprotein that although not associated to *TNXB* gene expression has been linked to asthma in prior studies [244]. A DMR that is located on chromosome 19 was also associated with gene expression of the ribosomal protein L13a (RPL13A) *implicated in inflammation repression*. The authors also identified 1163 CpGs and 328 DMRs with around one-third of CpGs and 20% of DMRs being more methylated in remission compared with controls. Cell-type composition was found to be significantly different between the comparison groups and impacted the findings. In a study by Clifford et al. [245], differential DNAm between asthmatics (n = 17) and nonasthmatics (n = 16) in airway epithelial cells was identified to be different based on cell isolation technique. The top significant genes identified were dual specificity phosphatase 22 (*DUSP22*) involved in cell proliferation and Wnt signaling pathway−related Wnt Family Member 7B (*WNT7B*) that have earlier been implicated in asthma [246,247]. In another study by the same group [248], methylation of airway and parenchymal lung fibroblasts was compared between donors with and without asthma. They identified 17 and 112 DMRs linked to asthma in airway and parenchymal lung fibroblasts, respectively, without any overlap between the two tissues indicating the importance of considering the different cell types to understand asthma pathogenesis. Perry et al. compared epigenome-wide methylation levels between healthy donors (n = 5) and donors with nonsevere asthma (n = 5) and severe asthma (n = 5) in airway smooth muscle cell obtained from bronchial biopsies [249]. They detected 15 "hub" genes with differential methylation and gene expression patterns correlated with severity of asthma on genes of the innate immunity and cellular proliferation pathways [249]. Several CpGs of these genes were also associated with differential miRNA expression [249].

Nasal epithelium cells are easier to access without invasive procedures, and a large body of evidence links DNAm in the nasal epithelia to be linked to asthma in the past 5 years. In a study by Forno et al., 483 nasal epithelium cells were obtained from Puerto Rican children aged 9−20 years [250]. They identified 7104 differentially methylated CpGs between participants with atopic asthma (n = 169) and nonatopic controls (n = 171). The results showed a high degree of overlap with that of atopy and associated methylated genes were implicated in epithelial barrier formation and Th1/Th2

Table 17.7 Summary of recent epigenome-wide studies assessing associations of DNA methylation with asthma and asthma-related phenotypes.

Author and year	Study population and sample size	Sampled tissue	Results of epigenome wide association	Exposure or effect modifier	Outcome	Summary of findings
Vermeulen et al. [241]	26 subjects with persistent asthma, 39 remission subjects, and 70 healthy controls	Bronchial biopsies	4 CpGs and 42 DM regions were identified between persistent versus remitting asthma, whereas 1163 CpGs and 328 DM regions were identified between remitting versus healthy groups.	None	Asthma	DM CpGs were correlated with the expression of genes involved in inflammation namely, atypical chemokine receptor 2 (*ACKR2*) and diacylglycerol kinase (*DGKQ*) and those expressed in ciliated epithelium.
Clifford et al. [245]	17 asthmatic participants and 16 healthy controls	Airway epithelial cells	A total of 864 differentially methylated regions were identified.	None	Asthma	Two top genes with maximum differences in methylation were DUSP22 and WNT7B known to be involved in asthma pathogenesis.
Clifford et al. [248]	17 asthmatic participants and 17 healthy controls	Airway and parenchymal fibroblasts	17 differentially methylated regions airway fibroblasts and 112 regions in parenchymal fibroblast.	None	Asthma	Genes from the HOXA and HNF1A/HNF1A-AS1 gene family had 20% difference in DNA methylation between asthmatics and nonasthmatics.
Perry et al. [249]	Five healthy subjects, five nonsevere asthma patients and five severe asthma patients	Airway smooth muscle cells	12,353 CpGs were identified in an hierarchical clustering approach, 15 hub genes had varying expression between the asthma phenotypes.	None	Asthma	CpGs linked to nonsevere asthma belonged to cell proliferation and apoptosis and axon guidance. Genes linked to severe asthma associated with calcium signaling, proliferation, and endocytosis.

Forno et al. [250]	483 Puerto Rican participants aged 9–20 years	Nasal epithelium	7104 DM sites identified. Top 30 replicated in an African American (n = 72) and European cohort (n = 432) to have statistically significant associations in the same direction.	None	Atopic asthma	Identified DM genes involved in epithelial barrier process and Th1/Th2 immune responses.
Kim et al. [251]	Puerto Rican subjects (aged 9–20 years) with (n = 219) and without (n = 236) asthma	Nasal epithelium	16,867 significant methylation–gene expression pairs were identified in a genome wide expression quantitative trait methylation (eQTM) analysis.	None	Asthma	Top 500 eQTM sites were located on immune regulation and epithelial barrier–related genes.
Qi et al. [252]	455 children at the 16-year follow-up in PIAMA cohort	Nasal epithelium	1 CpG linked to asthma in the discovery cohort and validated in a metanalyses of three cohorts.	None	Asthma	The CpG belongs to gene PDE6A epithelial cell function and extracellular matrix remodeling [291].
Qi et al. [253]	Whole blood (n = 72) and nasal brushings (n = 97)	Whole blood and Nasal brushing samples	1 out of 7 CpG sites associated with clinical remission and 8 out of 129 complete remission associated CpG sites in whole blood samples showed significant associations ($P < .05$) in the same direction in nasal brushes.	None	Children with clinical remission (i.e., no symptoms in the past 12 months) and complete remission of asthma.	Identified genes belong to the peroxisome proliferation and Wnt signaling pathway.
Zhang et al. [254]	54 asthmatic and 54 nonasthmatic African American siblings with ages between 5 and 18 years	Nasal epithelium	Three CpGs linked to asthma replicated in independent cohorts. Two of the CpGs associated with traffic-related air pollution.	Traffic-related air pollution	Asthma	CpGs belonged to the genes TET1, OR2B11, and NLRP3 that are involved in inflammation, olfactory reception, and innate immune system.

Continued

Table 17.7 Summary of recent epigenome-wide studies assessing associations of DNA methylation with asthma and asthma-related phenotypes.—cont'd

Author and year	Study population and sample size	Sampled tissue	Results of epigenome wide association	Exposure or effect modifier	Outcome	Summary of findings
Zhu et al. [255]	33 no severe and 22 severe asthmatic African American children	Nasal epithelial cell	816 differentially methylated CpGs and 10 DMRs associated with asthma severity. 6 CpGs linked to asthma and associated allergic markers such as IgE and FenO in independent cohort. 27 CpGs also linked to traffic-related air pollution and second-hand smoke.	None	Asthma severity	The identified genes were enriched to cell signaling linked with asthma such as Rho Family GTPases and Eicosanoid signaling.
Yang et al. [257]	12 patients with allergic asthma and 12 healthy controls	Nasal epithelial cell	Differential expression of *HLA-DMB, IL4, HLA-DPB1*, and *CD40LG*, were related to the occurrence of asthma. Expression of *HLA-DMB* was associated with its methylations at one CpG.	None	Asthma	DNA methylation and gene expression of Major Complex, Class II, DM Beta was identified to be linked to asthma.
Cardenas et al. [258]	547 children (mean age 12.9 years)	Nasal epithelial cell	285 asthma-related CpGs were detected	None	Asthma, FeNO, IgE	Genes involved in Th2 pathway and eosinophilia.
Shi et al. [256]	72 atopic asthmatic subject samples and 69 healthy samples	Nasal epithelium	146 differentially expressed genes with methylation changes were identified.	None	Asthma	The tumor necrosis factor and major histocompatibility complex, class II, DP alpha 1 were the main immune-related markers to be identified.

Jiang et al. [259]	1136 Latino participants aged 6−20 years	Whole blood	2 DM sites were identified at an FDR *P*-value cut of off 0.1, whereas 193 DM sites were identified with an FDR *P*-value cutoff of 0.2.	None	Asthma	Top two DM sites were located on inflammation-related genes, and TGF-beta pathway was identified as most relevant for asthma.
Patel et al. [263]	n = 124 females, 151 males with methylation and asthma information at age 10 and 18 years	Whole blood	535 CpGs linked to asthma of which 13 show changes in DNAm associated with asthma acquisition in a sex-specific manner.	None	Asthma	Gender-related switch in asthma acquisition is related linked to changes in DNAm.
Rathod et al. [264]	102 females and 113 males	Whole blood	205 CpGs showed associations with asthma acquisition at FDR = 0.05 in IOWBC, of which 112 showed consistent associations in the ALSPAC.	None	Asthma	Genes that the identified CpGs were mapped to, e.g., AKAP1 and ENO1, have been shown to be associated with the risk of asthma.
Hoang et al. [260]	Subjects having atopy without asthma (n = 185), nonatopic asthma (n = 673), atopic asthma (n = 271), and a reference group of neither atopy nor asthma (n = 1157)	Whole blood	A number of CpGs identified with FDR *P*-value $\leq$ 0.05 were as follows: none for atopy without asthma, 524 in nonatopic asthma, and 1086 in atopic asthma.	None	Asthma	35% of the DM CpGs in non-atopic asthma and 91% in atopic asthma were replicated in other tissue types. The identified genes were implicated in inflammation and nervous system−related processes.

Continued

Table 17.7 Summary of recent epigenome-wide studies assessing associations of DNA methylation with asthma and asthma-related phenotypes.—cont'd

Author and year	Study population and sample size	Sampled tissue	Results of epigenome wide association	Exposure or effect modifier	Outcome	Summary of findings
Song et al. [261]	20 patients with difficult-to-control asthma	Whole blood	Epigenetic and metabolic profile linked to asthma were identified.	None	Asthma	A DM CpG of the aldehyde dehydrogenase 3 family member A1 (ALDH3A1) and two related metabolic pathways were identified as potential markers for diagnostic classification of difficult-to-control asthma.
Everson et al. [262]	N = 370 in discovery cohort IOW and n = 720 in replication ALSAPC cohort	Whole blood	10 CpG sites at age 18 years associated with adolescent asthma at a 5% false discovery rate, five of which showed similar associations in the replication ALSAPC cohort.	None	Asthma	One of the CpGs on HK1 gene showed strong associations in both discovery and replication cohorts and was linked to cord blood gene expression in a smaller subset.
Dekker et al. [266]	1688 children from five cohorts	Cord blood	59 DMRs associated with childhood lung function, of which 18 were associated with childhood asthma and nine with COPD in adulthood.	None	Lung function, asthma, and COPD	Genes of the top 10 identified DMRs were HOXA5, PAOX, LINC00602, ABCA7, PER3, CLCA1, VENTX, NUDT12, PTPRN2, and TCL1A involved in respiratory developmental pathways.

Liang Li et al. [265]	796 participants with newborn blood and asthma information at age 10, 18, and 26 years	Heel prick blood	Four CpGs linked to asthma acquisition in adolescence and four CpGs to asthma acquisition in adulthood. All eight replicated in an independent cohort. All genes were biologically linked to asthma.	None	Asthma	The EWAS study identifies eight candidate epigenetic markers at birth linked to asthma later in life.
DeVries et al. [33]	Children aged 9 years (18 subjects developed and 18 did not develop asthma by age 9 years)	Cord blood monocular cells	Identified 589 DMR between asthmatic and nonasthmatic children.	Maternal asthma	Asthma	SMAD3 promoter was DM between asthmatic and nonasthmatic children of asthmatic mothers in three independent studies.
Janjanam et al. [30]	Participants aged 18 years (n = 370) had their epigenome profiled and asthma status assessed	Whole blood	Tetanus vaccination was associated with 40 DM CpG sites. Of these 40 candidate CpGs, 2 were associated with increased asthma risk.	Tetanus vaccination	Asthma	Tetanus vaccination associated with DM of CpG site within the KIAA1549L gene and another intergenic region between PSMG3 and TFAMP1 genes, which in turn were associated with asthma risk.
Yang et al. [257]	228 children at age 3 years N = 57 with asthma and n = 171 without asthma	Whole blood	Urinary BPA glucuronide level was inversely correlated with *MAPK1* promoter methylation, which in turn was linked to asthma.	Bisphenol A	Asthma	Mediation analysis suggested that MAPK1 methylation may act as a mediation variable between BPA exposure and asthma providing a mechanistic insight on the exposure effects.

Continued

Table 17.7 Summary of recent epigenome-wide studies assessing associations of DNA methylation with asthma and asthma-related phenotypes.—cont'd

Author and year	Study population and sample size	Sampled tissue	Results of epigenome wide association	Exposure or effect modifier	Outcome	Summary of findings
Yan et al. [271]	487 Puerto Ricans aged 9–20 years	Nasal epithelium	In a metaanalysis of three cohorts (EVA-PR, PIAMA, Project Viva), which included the top CpGs associated with stress/violence measures in EVA-PR, 12 CpGs were linked to atopic asthma at FDR-$p < 0.05$.	Maternal perceived stress or gun violence	Atopic asthma	The genes belonged to STARD3NL, SLC35F4, TSR3, CDC42SE2, KLHL25, PLCB1, BUD13, OR2B3, GALR1, TMEM196, TEAD4, and ANAPC13 genes of which PLCB1 and TMEM196 have been implicated in asthma.

DM, *differentially methylated;* DMRs, *differentially methylated regions.*

signaling. To determine the best predictive CpGs, a top 30 CpG panel was selected that could classify participants in the discovery Puerto Rico cohort with 88% accuracy and an area under the curve ranging from 0·93 to 0·94. Similar results were obtained upon replication in African American children (n = 72), and in a European cohort (n = 432) [250]. In another genome-wide expression study, quantitative trait methylation assessments were conducted by the same group using nasal epithelia (Table 17.7). The authors identified 5934 paths where DNAm-affected atopic asthma via gene expression of nonnearby cis genes localized in the enhancer region [251]. A study by Qi et al. conducted among 16-year-old participants (n = 37 with asthma, n = 418 controls) of the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) cohort has identified 16 DMRs and 95 CpGs at a less conservative *P*-value cut off 0.0001 [252]. A metaanalysis was then conducted using data from the discovery cohort and two replication cohorts after which only identified one CpG cg08844313 (*PDE6A*) as statistically significant [252]. The same group also studied differences in whole blood and nasal brushing methylation between clinical remission, complete remission, and persistent asthma [253]. In whole blood, 7 and 129 CpGs were linked to clinical remission and complete remission, respectively. Among these, one CpG linked to clinical remission and eight CpGs linked to complete remission were also identified with DNAm from nasal brushing showing the same direction of effects at nominal statistical significance (*P*-value $\leq$0.05) [253]. An interesting study with nasal epithelium cells of 29 sibling pairs discordant for asthma identified six differentially methylated sites linked to asthma of which three were successfully replicated in independent cohorts [254]. Two of the three sites, also associated with traffic related air pollution, were in transcription factor−binding sites [254]. The same group detected that 10 DMRs and 816 CpGs were associated with asthma severity in African American children (n = 22 severe asthma; n = 33 nonsevere asthma). The 398 genes linked to 816 CpGs were enriched in the signaling pathways of Rho family GTPases, and cardiac β-adrenergic signaling, CREB signaling in meurons, eicosanoid signaling, and G protein signaling. 39 of these CpGs were correlated to their mRNA levels [255], and six were replicated in independent cohort and were associated with asthma, allergic asthma, total IgE, environment IgE, and fractional exhaled nitric oxide (FeNO) levels [255]. Shi et al. found 146 differential methylation and gene expression changes linked to asthma involved in immune response processes [256]. Yang et al. identified 169 differentially methylated genes in nasal epithelia methylation linked to allergic asthma in African American patients (n = 24) that explained 9.81% of the variation in gene expression [257]. In an EWAS with 850K CpGs in the Project Viva cohort, differential methylation of genes involved in allergic asthma, eosinophilia, and Th2 immune pathways was detected [258]. In addition, they identified that nasal epigenetic age is accelerated by asthma and positively correlated with allergic biomarkers such as IgE levels and fractional exhaled nitric oxide [258].

Whole blood methylation provides a window to systemic processes involved in asthma (Table 17.7). Recently, a metanalyses in 1136 Latino children with whole blood DNAm discovered two CpGs: *CAMK1D* (calcium/calmodulin-dependent protein kinase ID) and *TIGIT* (T cell immunoreceptor with Ig and ITIM domains). Both are involved in inflammatory processes [259]. In addition, 25 DMRs were found that belong to transforming growth factor beta (*TGF-β*) pathway [259]. In a large adult asthma case−control study with 2286 participants, Hoang et al. identified 524 and 1086 differentially methylated CpGs between nonatopic and atopic asthma, respectively, compared with the control group after adjusting for multiple testing [260]. Upon comparison with other studies, they found that the highest overlap of the identified CpGs was linked to DNAm in nonatopic and atopic asthma measured in nasal epithelium (29%; 81%), followed by whole blood (13%; 27%), eosinophils

(9%; 25%), and airway epithelium (0%; 5%) [260]. A small study followed 20 difficult-to-control asthma patients (age range of 38−65 years) using a multi-omics approach [261]. They identified epigenetic and metabolic profiles in whole blood including the aldehyde dehydrogenase 3 family member A1 (*ALDH3A1*) to be a marker that can be used for diagnostic classification. A study using data from the Isle of Wight birth cohort used a recursive random forest technique and identified five newborn CpGs from heel prick blood-linked adolescent asthma that were replicated in the Avon Longitudinal Study of Parents and Children (ALSPAC) [262]. Cord blood methylation of one of these CpGs cg16658191 in the *HK1* gene was additionally linked to its mRNA expression levels and wheezing in infancy. While these studies provide novel and insightful knowledge, a limitation is their inability to account for time order of event. Concurrent associations between DNAm and disease status cannot distinguish between differential DNAm, leading to asthma or DNAm resulting from asthma. However, other studies with whole blood and newborn blood investigated prospective associations, which provided a time order of exposure and events. In a study based on the Isle of Wight birth cohort, Patel et al. identified sex-specific changes in whole blood DNAm of 13 CpGs with asthma acquisition from preadolescence to postadolescence [263]. One of these CpGs belonging to IFRD1 gene showed a significant association in another Caucasian birth cohort, ALSPAC. Nine additional CpGs also showed same direction of association but did not reach statistical significance in the replication analyses [263]. The same group also performed a prospective study to identified whole blood CpGs at age 10 and 18 years linked to asthma acquisition from 10 to 18 years and 18 to 26 years of age, respectively [264]. Of note the CpGs *that were also linked to asthma acquisition in another cohort* mapped to the genes AKAP1 and ENO1 *known to be linked to asthma* [264].

Studies using cord blood DNAm with later-onset asthma provide a prospective setting to identify CpGs that predict asthma. Li et al. using DNAm measured in heel prick blood of the Isle of Wight birth cohort identified eight CpGs on eight genes that were linked to asthma acquisition in adolescence and adulthood in females that were successfully replicated in ALSPAC [265]. All the eight genes were known to be biologically linked to asthma [265]. Another large metaanalysis study, with cord blood of 1688 children from five cohorts identified 59 DMRs linked to childhood lung function among which 18 were associated with childhood asthma located on genes associated with respiratory developmental or pathogenic pathways [266]. A noteworthy study by DeVries et al. evaluated DNAm in cord blood and its association with asthma development by age 9 years (Table 17.7) [267]. Results of this study showed that 589 differentially methylated regions distinguished children who developed and did not develop asthma by age 9 years. Network analysis showed that differentially DNAm was evident and clustered in immunoregulatory and proinflammatory pathways. Specifically, the transcription factor *SMAD3*, an established high-risk gene for asthma, was the most connected node in the network and pathway analysis [33,267]. Investigators showed that methylation of *SMAD3*, in three independent studies, to be increased in asthmatic children born to asthmatic mothers and was associated with asthma risk. Hence, indicating the methylation of *SMAD3* could be causally related to asthma development only among children of asthmatic mothers. Such an observation supports the parent-of-origin concept of asthma [55,268].

Environmental exposures, such as smoking, air pollution, endocrine disrupting chemicals such as bisphenol A, have shown consistent associations with DNAm [99,269,270]. An EWAS-reported tetanus vaccination is associated with differential methylation of two CpG sites in whole blood; one located on the KIAA1549L gene and the other between *PSMG3* and *TFAMP1* genes [30]. Subsequently, DNAm of these two CpG sites showed association with asthma risk. In a metaanalysis of three

cohorts, namely, PIAMA, Project Viva, and PIAMA, 12 CpGs in nasal epithelium linked to chronic stress were also linked to asthma [271].

Table 17.8 shows 35 genes that are associated with asthma and that were reported to carry differentially methylated CpG sites in at least two independent epigenome-wide studies that we reviewed. Briefly, most genes that were associated with asthma in at least two EWASs belong to pathways involved in immune regulation, extracellular matrix development, signal transduction, and lung development. The genes *PF4* [272], *CYR61* [273], and *PCSK6* [274] are involved in extracellular matrix related adhesion process [272–274] and that is known to be involved in airway remodeling and asthma pathogenesis [275]. Several of the identified genes function in immune and inflammatory processes including *PF4* [274], *SPOCK2* [276], *CD9* [276], *KCNH2* [140], *FGR* [277], and *IFITM3* [278]. Genes implicated in lung development and implicated in lung fibrosis are *FGR* [279]), *HOXA5* [280], *ANO1* [281], *PLEC* [282], *CYR61* [283], and *PTPRN2* [284]. Notably, the gene KCNH2 (potassium voltage-gated channel subfamily H member 2) that codes for a potassium voltage-gated ion channel, which was found as an epigenetic marker linked to asthma in two studies, is a known target for several approved medications including antiarrhythmic agents and β-adrenergic receptor antagonists [285]. The genes *PRRT1* and *PCSK6* were identified in three independent epigenome-wide studies. The protein encoded by the gene *PRRT1* (proline-rich transmembrane protein 1) aids to maintain a pool of extra synaptic glutamate receptors required for synapse development [286] that are known to have role in airway hyperresponsiveness [287]. *PCSK6* (proprotein convertase subtilisin/kexin type 6) is a protease that is known to mediate mediator of TGF-β signaling [288] and is highly expressed in the lung parenchyma in idiopathic pulmonary fibrosis [289].

In summary, a variety of methylation markers have been identified in bronchial epithelium, nasal epithelium, and whole blood. Among the different tissue types, assessing methylation from nasal epithelial cells has some advantages. First, nasal epithelium is easier to collect in a noninvasive manner. Second, it serves as an interface between the external environment and may reflect better the impact of air pollution, inhaled allergens, and virus infections [290]. Third, it is less affected by a mixture of immune cell types that must be considered while assessing whole blood methylation. Future studies should assess both nasal epithelium and whole blood to understand the effect of external stimuli and its systemic effect on the immune system in the pathogenesis of asthma.

17.15 Epigenetics related to allergic rhinitis

Both genetic and environmental factors and their interplay have been shown to have a role in the development and natural course of allergic rhinitis [292,293]. When compared with asthma, eczema, and allergic sensitization, there are a very limited number of well-designed genetic and epigenetic studies for allergic rhinitis.

GWASs have identified some susceptibility loci for allergic rhinitis, of which variants in *MRPL4*, *BCAP*, *C11orf30/LRRC32*, and *FERD3L* genes appear to be strongly associated with allergic rhinitis [294]. These genes are involved in inflammatory and immune regulation, which further support their role in the pathogenesis of allergic rhinitis [294].

Regarding epigenetic association studies, we identified four investigations that related differential DNAm to varying phenotypes of allergic rhinitis [31,295–297]. All studies had small sample sizes. Nestor et al. [295] measured genome-wide DNAm of $CD4^+$ T cells from seasonal allergic rhinitis

Table 17.8 List and functions of differentially methylated genes identified in at least two independent epigenome-wide association analyses.

Gene	Gene name	Gene function
ANO1	Anoctamin 1	Enables calcium activated cation channel activity and involved in anion and cation transport.
C7orf50	Chromosome 7 open reading frame 50	Enables RNA-binding activity.
CAPN14	Calcium-activated neutral proteinase 14	These are cytosolic calcium-activated cysteine proteases involved in integrin−cytoskeletal interactions, apoptosis, and cell division.
CD9	CD9 molecule	A member of the transmembrane 4 superfamily. The functions of the gene include adhesion and signal transduction.
CLIC6	Chloride intracellular channel 6	A member of the chloride intracellular channel family of proteins.
CYP2E1	Cytochrome P450 family 2 Subfamily E member 1	These synthesize monooxygenases that catalyze drug metabolism and synthesis of cholesterol, steroids, and other lipids.
CYR61	Cellular communication Network factor 1	Plays a role in adhesion, cell proliferation, differentiation, angiogenesis, apoptosis, and extracellular matrix formation.
DGKQ	Diacylglycerol Kinase Theta	Aids in the formation of phosphatidylinositol (PI) from diacylglycerol during cell signal transduction.
FGR	FGR protooncogene, Src family Tyrosine Kinase	Functions as a negative regulator of cell migration and adhesion triggered by the beta-2 integrin signal transduction pathway.
GDNF	Glial cell−derived factor	These are ligands that bind various TGF-beta receptors, leading to recruitment and activation of SMAD family transcription factors.
HOXA5	Homeobox A5	A transcription factor that regulates gene expression, morphogenesis, and differentiation.
IFITM3	Interferon-induced transmembrane protein 3	These are antiviral proteins induced by interferon.
KCNH2	Potassium voltage-gated channel subfamily H member 2	Voltage-activated potassium channel found in cardiac muscle, nerve cells, and microglia.

Table 17.8 List and functions of differentially methylated genes identified in at least two independent epigenome-wide association analyses.—cont'd

Gene	Gene name	Gene function
LITAF	Lipopolysaccharide-induced TNFF	Stimulator of monocytes and macrophages, causing secretion inflammatory mediators such as tumor necrosis factor-alpha.
LRRFIP1	LRR-binding FLII-interacting protein 1	Represses transcription by RNA polymerase II through a DNA-binding activity.
NRF1	Nuclear respiratory factor 1	Transcription factor that encodes for genes required for growth, differentiation, and respiration.
OLIG1	Oligodendrocyte transcription factor 1	Involved in neuron differentiation and regulation of regulates transcription by RNA polymerase II.
OTX1	Orthodenticle homeobox 1	Transcription factor and may play a role in brain and sensory organ development.
PAX6	Paired box 6	DNA-binding activity and regulation of gene transcription.
PCSK6	Proprotein convertase subtilisin/Type 6	Includes proteases that process precursors of peptides and proteins.
PDLIM4	PDZ And LIM domain 4	Involved in bone development.
PF4	Platelet factor 4	It is a chemokine involved in platelet aggregation, inhibition of hematopoiesis, and T cell function.
PLEC	Plectin	Maintaining cell and tissue integrity and orchestrating dynamic changes in cytoarchitecture and cell shape.
PODN	Podocan	It inhibits smooth muscle cell proliferation and migration.
PRR5	Proline-rich 5	A tumor suppressor gene regulates platelet-derived growth factor receptor beta expression.
PRRT1	Proline-rich transmembrane protein 1	Protein-binding activity involved in synaptic depressions and protein localization to cell surface.
PTPRN2	Protein tyrosine phosphatase Type N2	Plays a role in vesicle-mediated secretory processes.
PVRL4	Nectin cell adhesion molecule 4	Involved in cell adhesion through transhomophilic and transheterophilic interactions.
RBMS1	RNA-binding single-stranded protein 1	This protein has diverse functions such as DNA replication, transcription, and cell cycle regulation.

Continued

Table 17.8 List and functions of differentially methylated genes identified in at least two independent epigenome-wide association analyses.—cont'd

Gene	Gene name	Gene function
SH2D4B	SH2 containing 4B	Involved in decreased TP53 expression and associated with decreased virus infection.
SIRPD	Signal regulatory protein delta	Active in cell plasma membrane and located in extracellular region.
SLC9A3	Solute family 9 Member A3	Plays a role in ion transport across the cells and signal transduction.
SPOCK2	SPARC, Cwcv And Kazal-Like domains proteoglycan 2	Binds to glycosaminoglycans to form part of the extracellular matrix.
STARD3NL	STARD3 N-Like	Endosomal cholesterol transport and involved in immunodeficiency.
TMEM151B	Transmembrane protein 151B	Is an integral component of membrane.

Information on gene functions were retrieved from GeneCards database (http://www.genecards.org/).

patients (n = 8) and healthy controls (n = 8). They demonstrated that DNAm profiles robustly distinguished seasonal allergic rhinitis patients from healthy controls. However, their study did not report the gene names that showed differential methylation across cases and controls. In another study, Morin and colleagues [297], using an epigenome-wide approach, showed that DNAm of caudal type homeobox 1 (*CDX1*) gene to be differentially methylated between allergic rhinitis cases (n = 30, mean age: 28 years) and healthy controls (n = 31, mean age: 29 years). However, the mechanisms underlying the observed association need further elucidation. Although *CDX1* has not been implicated previously in the development of allergic rhinitis, prior studies showed that genetic variants within *CDX1* to be associated with asthma and eczema [298,299], suggesting the possibility that the methylation changes in the gene *CDX1* may be linked to allergic rhinitis.

In a small study (n = 40) integrating genetic and epigenetic effects, one CpG on the *MTNR1A* gene was found to mediate the effect of a paternally inherited allele on comorbidity of asthma and allergic rhinitis [300]. *MTNR1A* codes for melatonin receptor that regulates melatonin levels, which is known to have an immunomodulatory effect and be involved in TH2 signaling pathways [300] relevant for the pathogenesis of allergy. In another case–control study comparing children aged 8–10 years with allergic rhinitis (n = 105) to controls (n = 90), the effect of particulate matter $(PM)_{2.5}$ was identified to be mediated through epigenetic modification of *IFN-γ* promoter region [301].

In an experimental investigation, cases of allergic rhinitis (n = 38, age range: 22–58 years) and nonallergic controls (n = 8, age range: 21–61 years) were exposed, in a controlled environment, to grass pollen for 3 h on two consecutive days [31]. Epigenome-wide DNAm pre- and postexposure was assessed in peripheral blood mononuclear cells. At baseline, DNAm of allergic rhinitis cases and nonallergic controls did not differ. However, allergic rhinitis cases experienced changes in their DNAm profiles after exposure to grass pollen for 3 hours on two consecutive days. The nonallergic controls did not show a pre- and postexposure difference. Among the allergic rhinitis cases, changes in DNAm

of tryptase gamma 1 (*TPSG1*), schlafen 12 (*SLFN12*), and mucin 4 (*MUC4*) genes in response to grass pollen exposure were identified by the epigenome-wide survey and further validated by pyrosequencing. Moreover, DNAm levels of *SLFN12* gene were associated with severity of allergic rhinitis symptoms. The discovered genes are considered to be functionally involved in immune modulation and allergic inflammation [31].

Taking a candidate−gene association approach, Li and colleagues [296] investigated the role of DNAm upstream of *IL13*, a gene that has been consistently and strongly associated with allergic disorders, with allergic rhinitis that is characterized by house dust mite (HDM) sensitization. The study analyzed data of 60 patients with HDM-sensitized allergic rhinitis subjects and 65 controls. Results of this study showed that a lower methylation at *IL13* gene is associated with increased risk of allergic rhinitis that is accompanied by HDM sensitization [296].

In summary, results of epigenetic studies have implicated genes that regulate immune responses and inflammation in the pathogenesis of allergic rhinitis. Due to the limited number of investigations, future studies are necessary to identify both genetic and epigenetic markers that predispose to the development of allergic rhinitis.

17.16 Putting it all together

There is a large body of evidence that DNAm is related to allergic diseases and that DNAm may change with the status of the disorder, for instance, food allergies. It is also obvious that exposures, in particular early-life exposure, can result in DNAm changes (an archive of a variety of early developmental exposures), which in turn are risk factors for asthma and eczema. Given these results, there is an enormous potential to identify intervention strategies (diet, lifestyle, medication, etc.) that can prevent adverse changes in the methylation of specific genes and consequently prevent allergic diseases. However, to achieve this, we need to improve our scientific work. The reason is that in the current research structure, we may need too long (another 50−100 years) to identify epigenetic approaches to prevent allergic diseases.

First, a number of investigations did not involve replications, comparisons with studies, or collaborations to determine if other investigations have identical findings. We cannot request that single investigators take on this burden, but we need to develop a scientific structure to support collaboration and cross-study comparisons. One example is the Pregnancy and Childhood Epigenetics (PACE) group at the National Institute of Environmental Health Sciences. This group includes researchers around the world in the field of epigenetics who are interested in studying the early-life environmental impacts on human disease (https://www.niehs.nih.gov/research/atniehs/labs/epi/pi/genetics/pace/index.cfm).

Second, in epigenetics, a widely unused concept is the idea of Mendelian randomization. This concept can help to overcome confounding of associations between factor X (DNAm) with outcome Y (e.g., allergy) and thus provide an assessment of causality. It is assumed that genetic polymorphisms are randomly allocated at birth and thus are not or less affected by confounding. Thus, given that a genetic polymorphism (instrument Z) can influence DNAm (factor X), but associations of genetic polymorphisms are not or less disturbed by confounding, genetic polymorphisms that affect DNAm can help us to investigate whether DNAm is a causal factor [302].

Third, some journals and their reviewers pose a challenge. A number of studies with methodological limitations such as a lack of time order of epigenetics and diseases are published in high-impact

journals. This hinders the scientific progress, since other studies need to explain why they do not find associations, which were simply identified due to reverse causation (disease → DNAm). Hence, it is imperative that investigators need first to understand and second to address their study limitations. In addition, it is the responsibility of peer reviewers and editors to ensure that the study findings are reported and interpreted in light of the study strengths and limitations. To improve such settings, collaborative groups of epigenetic researchers to be established in Europe, Asia, Arabic countries, or the United States can help to separate which approaches and results are useful and which are not.

List of abbreviations

CpG	cytosine−phosphate−guanine dinucleotides
DMRs	differentially methylated regions
DNAm	DNA methylation
DOHaD	Developmental Origin of Health and Disease Hypothesis
EAACI	European Academy of Allergy and Clinical Immunology
Fc-receptor	Protein found on the surface of certain cells, known as the fragment, crystallizable region
HDM	house dust mite
IgA, IgD, IgE, IgG, IgM	immunoglobulin A, D, E, G, M
methQTL	methylation quantitative trait locus
miRNA	microRNA, noncoding RNAs that play a role in regulating gene expression
mRNA	messenger RNA, a single-stranded RNA sequenced from the DNA template, which provides the information for protein synthesis
SNP	single-nucleotide polymorphism, each SNP represents a DNA building block called a nucleotide, which can vary among different individuals
SPT	skin prick test: an allergen is introduced under the skin's surface, and results, such as swelling or redness, are seen after 15
T cells	Lymphocytes (T helper cells) that orchestrate the immune response
Th1 cell	Generate responses against intracellular parasites such as bacteria and viruses
Th2 cell	Produce immune responses against helminths, other extracellular parasites, and allergens
Treg	regulatory T cells
TSDR	Treg-specific demethylated region

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CHAPTER

Nutrient intake, epigenetics, and asthma

18

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18.1 Introduction

Asthma is described as a complex chronic inflammatory airway disorder of variable severity and intensity, inflicting over 300 million people worldwide of all ages, residing in both developed and undeveloped countries [1,2]. It has been estimated that an additional 100 million people will suffer from asthma by the year 2025 [1]. There are wide geographical variations in asthma prevalence and severity, with prevalence dominating in high-income countries and asthma-related mortality in low- to middle-income countries [1]. Secular trends show that asthma incidence and prevalence are higher in children, up to 20% in English-fluent countries versus (vs.) 4% in adults [3,4]. Despite advances in pharmacotherapy, asthma control is suboptimal in children and associated with a higher rate of exacerbations, hospital admissions, pediatric emergency visits, school absenteeism, poor academic performance, and an impaired quality of life [5,6]. Asthma causes considerable morbidity along with an economic burden for the individual, family, and healthcare system due to increased medical costs, work absenteeism, and loss of productivity days [6]. Therefore, it is of utmost global public health importance that the etiology of asthma induction and progression is fully understood.

Unequivocally, asthma is caused by the complex interaction of genetic and environmental exposures, including atmospheric pollution, cigarette smoke, upper respiratory infections, pet hair, cold weather, high humidity, dampness, obesity, and diet [7], which trigger immunological and inflammatory mechanisms, subsequently leading to symptoms of wheeze, cough, shortness of breath, and chest tightness [2]. Multiple immune cells are involved in the inception of asthma including T cells, macrophages, dendritic cells, eosinophils, neutrophils, mast cells, and basophils [8]. More specifically, macrophage (M1) polarization stimulates the expression of CD80, CD86, TLR4 (Toll-receptor 4), and inducible nitric oxide synthase (iNOS), further producing T-helper 1 (Th1)-associated cytokines (TNF-α, IL-1β, IL-2, IL-6, and IL-12), Th17-associated cytokines (IL-23 and IL-27), monocyte chemotactic protein- (MCP-) 1, reactive oxygen species (ROS), and chemokines (CXCL9, CXCL10, CXCL11, CXCL16, CCL2, CCL5, and CCL8) [8]. Combined with the imbalance of T cells (Th1/Th2) and skewing toward the Th2 immune response, this results in the secretion of Th2-derived proinflammatory cytokines (IL-4, IL-5, IL-9, and IL-13), the subsequent activation of B cells and

Epigenetics in Human Disease. https://doi.org/10.1016/B978-0-443-21863-7.00003-2
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production of IgE, mucus secretion, hyperplasia of goblet cells, and airway hyperresponsive. Altogether, this contributes to asthma progression and the manifestation of symptoms [8].

The growing awareness that genetics can only partially explain the rapid rise in allergic disease and the fact that asthma has a strong environmental component, as illustrated by country variations in asthma prevalence as well as the critical role of early-life exposures as determinants of asthma in later life, has stemmed great interest in epigenetic processes and respiratory disorders, including asthma. Epigenetics, a budding science, is defined as the study of heritable changes of a phenotype (involving gene expression and chromatin organization) without causing structural alterations to the underlying DNA sequence (genetic code) [9]. Investigating epigenetic alterations by using -omics analysis may uncover asthma etiology, noninvasive biomarkers, and molecular pathways involved in disease pathogenesis.

The purpose of this narrative review is to provide an overview of the latest scientific evidence on (1) the role of epigenetics in asthma inception, (2) dietary influences on gene expression/DNA methylation in relation to asthma etiology, and (3) uncover potential epigenetic traits and possible molecular targets for the development of future primary preventive strategies and personalized treatment of this disease. Understanding how these mechanisms impact asthma will allow for better asthma care in patients, reduce the burden of asthma, improve the quality of life, and decrease individual and societal costs.

18.1.1 Epigenetic mechanisms

External insults can stimulate epigenetic changes via three main mechanisms: DNA methylation, histone modification (posttranscriptional histone modification, ATP-dependent chromatin remodeling complexes), and small and noncoding RNAs, which could play a critical role in T cell differentiation and novel aspects of asthma progression [10].

DNA methylation refers to the process mediated by DNA methyltransferase (DNMT) enzymes that catalyze the addition of methyl groups at the 5-carbon (C^5) of cytosines into (cytosine−phosphate−guanine) CpG dinucleotides and in clusters enriched in CpG sites known as CpG islands, which are found in locus control regions, promoter regions, and the first exons of many genes [10]. Cytosine methylation involves the transfer of a methyl group from S-adenosyl-L-methionine (SAM), a methyl precursor, to the cytosines in CpG dinucleotides [10]. This event facilitates histone deacetylation and, with DNA methylation, results in a condensed chromatin structure with reduced accessibility, leading to suppression or downregulation of gene expression [10]. In addition, higher methylation of CpG islands by DNMT inhibits the assembly of transcription factors and leads to transcriptional gene silencing and gene inactivation [10]. Promoter methylation of CpG islands (hypermethylation) may contribute to gene silencing (as is the case in cancer), whereas an unmethylated promoter (hypomethylated) inhibits expression [11]. Therefore the outcome of DNA methylation is the downregulation of gene expression. However, methylation of CpG sites within gene bodies is frequently associated with increased gene transcription and alternative splicing [12]. In mouse models, Jones et al. demonstrated that CpGs affect transcription of the IFNγ gene and that de novo methylation of -53 CpG by DNA methyltransferase (Dnmt3a) suppresses IFN-γ transcription in activating Th2 polarization within the CD4+ T cell by directly inhibiting transcription factor binding [13]. Therefore, DNA methylation associated with gene repression might be related to the onset of disease. In contrast, demethylases belonging to the 10−11 translocation (TET) family of DNA dioxygenases (TET 1, 2, 3) remove the methyl groups from the cytosine bases favoring an open chromatin state enhancing active gene expression [14].

Histones are proteins that organize chromatin and histone modifications are important in transcriptional regulation, DNA repair and replication, alternative splicing, and chromosome condensation [15]. Histone modifications, including acetylation, methylation, and phosphorylation reactions, are posttranslational modifications of the N-terminal tail of histone proteins creating a code that is decoded by other chromatin remodeling complexes [15,16]. These modifications are catalyzed by histone methyltransferases (HMTs), histone demethylases (HDMs), histone acetyltransferases (HATs), and HDACs. HMTs act by adding methyl groups to lysine/and or arginine residues in histones, while HDMs remove methyl groups [15,16]. Likewise, histone acetylation involves the acetylation (or addition) of lysine residues on histone tails via HATs, and HDACs function by decreasing the number of acetyl groups in histone tails [15,16]. The effect of histone methylation on gene expression is dependent on the specific amino acid position. Lysine 4 methylation is related to gene activation [17] and lysine 9 methylation with gene silencing [18], whereas arginine methylation upregulates gene transcription [15]. Comparably, histone hyperacetylation leads to increased DNA transcription and consequently gene activation, whereas hypoacetylation in gene silencing [15]. Harb et al., in a birth cohort study, examined the role of placental histone acetylation in the development of allergic sensitization in offspring [19]. According to the investigators, higher placental histone H3 acetylation levels at IFNG and SH2B3 promoter regions and H4 acetylation in HDAC4 were associated with a decreased risk of food allergen sensitization in childhood. Sex differences were noted in acetylated placental genes. This study highlighted the link between allergen sensitization early in life with modifications in histone acetylation of immunoregulatory genes in the human placenta. Regarding asthma, previous studies have reported the activity of HATs and the reduction of HDAC activity in asthma patients [20,21].

Therefore, given that histone modification can lead to hypermethylation of N-terminal histone tails, opening of chromatin structure, and increased gene transcription [22], then it is possible that these modifications could contribute to increased expression of genes involved in inflammatory responses characteristic to asthma.

In the same line, small noncoding single-stranded RNA molecules of 19−24 nucleotides microRNAs (miRNAs) are key regulators of gene expression and DNA stability [23]. MiRNAs target 3′ untranslated mRNA regions causing degradation of protein products or translational silencing by the formation of heterochromatin [23]. Studies examining the composition of circulating miRNAs in relation to childhood asthma differentiated 122 circulating miRNAs in asthmatic children as compared with nonasthmatic counterparts [24] including correlations to lung function parameters (forced expiratory volume in 1 s (FEV_1), forced vital capacity (FVC), and the ratio of FEV_1/FVC) [25].

In relevance to allergic inflammation, the epigenetic control of inflammatory cytokine gene expression is critical for immune homeostasis. More specifically, differentiation and synthesis of cytokines by T cells along with transcription factors are driven by epigenetic mechanisms [26,27]. For example, during Th1 maturation, loci at TBX21 and IFNG undergo DNA demethylation, whereas loci at IL4, IL13, RORC, and 1L17A essential features of the Th2 and Th17 response remain methylated. Notably, DNA methylation of IFNG and RORC is maintained in Th2 and Th17 cells [28−31]. Contrastingly, IL4 and IL13 loci are hypomethylated in Th2 cells, and FOXP3 undergoes demethylation in developing T-regulatory (Treg) cells [32,33]. Concerning Th17 cells, the inverse is observed with demethylation of RORC, preserved methylation of the FOXP3 locus, and demethylation of ILI7A and TBX21 [26,34]. Furthermore, histone acetylation or methylation contributes to Th1 lineage differentiation. The IFNG locus in Th1 cells is characterized by high H4ac, H3K4m2/3 (permissive

mark), and H3K9me2 (repressive mark), while low H3K27me2/3 levels. As for Th2 cells, high H3K4me2 and H3K9me2 were observed [28,35]. Additionally, high levels of H3K4me3 were annotated at IL4 in Th2; IL17A, IL17F, and RORC in Th17, including FOXP3 in Treg cells [36].

18.1.2 Epigenetics mechanisms and asthma-emerging evidence

18.1.2.1 Fetal programming: Tracking childhood asthma from intrauterine life to midchildhood

The surge in allergic disease over the past 30 years can only be partially explained by genetic variations. Environmental changes, including exposure to risk factors (such as atmospheric pollution, cigarette smoke, poor diet, and respiratory viral infections) interacting with genes, could activate a series of events initiating and orchestrating allergy induction [7,37]. Conceptually, the perinatal period offers a window of opportunity for the influence of these environmental factors. During early life, the plasticity in T cell development and adaptation to environmental insults skews toward a Th2 inflammatory phenotype and, as a result, the manifestation of allergy symptoms [37].

Epigenetic regulation could provide a link between genes and early-life exposures during fetal development that impact susceptibility to asthma [10,38,39]. According to the Barker theory, environmental events (including nutrition) during the first 1000 days of life (ranging from preconception up to 2 years of age) have a major influence on the development of chronic diseases, including cardiovascular and allergic disease in adulthood [40]. It has been postulated that in utero epigenetic programming as a result of external insults during pregnancy induces specific genes and pathways that control fetal development and disease risk [40]. Therefore, maternal health and epigenetic vulnerability to the intrauterine environment shape fetal maturation, and the genome, which determines disease progression.

Accumulating evidence suggests that prenatal exposures alter epigenetic profiles and subsequent immune function in exposed offspring. Moreover, perturbations in epigenetic patterns might contribute to the pathogenesis of diseases that manifest during childhood [41]. The concept of intrauterine programming as the origin of disease has been extended to the respiratory system through a range of cohort studies [38,42,43]. These studies have revealed that one of the main mechanisms through which nutrition can influence long-term health is via the modulation of epigenetic programming. DNA methylation appears to play a pivotal role during intrauterine development. Previous birth cohorts have examined DNA methylation as a proxy for associations between parental exposures and asthma risk in offspring [38,44]. Reese et al. within the context of the PACE (Pregnancy and Childhood Epigenetics) consortium, using data from 17 cohorts, examined prospective (8 cohorts, $n = 668$) and cross-sectional associations (9 cohorts, $n = 631$) between CpG methylation regions (from blood, respiratory epithelium, and blood eosinophils) and asthma in schoolchildren [44]. Metaanalysis of asthma and neonate methylation identified 9 CpGs and 35 regions that were differentially methylated in relation to asthma development, with 7 of the 9 showing greater methylation in asthmatic children than in noncases. Furthermore, 179 CpGs and 36 regions were identified from cross-sectional analyses of data from children aged 7−17 years and found to be annotated to genes associated with asthma *(IL5RA),* which is the target for pharmacotherapy in severe asthma patients [44]. Pathway analyses revealed enrichment related to immunological and inflammatory processes (including endothelial nitric oxide synthase (eNOS) signaling, the inflammasome, and nuclear factor κB (NF-κB) signaling along with organ development [44]. Overlapping in pathways was observed between neonates and children. Annotated

genes included IL5RA, RUNX1, and KCNH2, as well as transcription marks that could be valuable targets for potential drug therapy. Remarkably, in children, asthma-related differential methylation in blood was replicated in eosinophils and respiratory epithelium [44].

Den Dekker et al. investigated the epigenome data of cord blood from 1688 children participating in five cohorts to identify differentiated methylated regions (DMRs) correlated to lung function and asthma at ages 7−13 years [38]. Overall, 59 DMRs were associated with childhood lung function, of which 18 were associated with childhood asthma and 9 with COPD in adulthood. Among the top annotated genes (HOXA5, PAOX, LINC00602, ABCA7, PER3, CLCA1, VENTX, NUDT12, PTPRN2, and TCL1A), DMRs located in the homeobox A5 (*HOXA5*) gene promoter involved in fetal lung development were found to be related to FEV_1 in children [38]. The possibility that DNA methylation at birth could reflect fetal exposures and mechanisms involved in asthma inception is fascinating.

In contrast, De Vries et al. surveyed DNA methylation in cord blood mononuclear cells from children (asthmatic vs. nonasthmatic) participating in three cohort studies [42]. According to the investigators, hypermethylation of the promoter of the SMAD3 (small mother against decapentaplegic 3) gene during neonatal life predicted childhood asthma risk at 9 years in subjects born to asthmatic mothers. Furthermore, positive associations were found between SMAD3 methylation in neonates with maternal asthma and neonatal production of IL-1β, an innate inflammatory mediator [42]. Wu et al., in a birth cohort of 1629 neonates, showed that paternal tobacco smoking stimulated hypermethylation of inflammatory genes (LMO2) and interleukin 10 (IL-10) that were correlated with asthma development at age 6 years [43]. Interestingly, the higher the paternal smoking exposure, the higher the CG methylation levels. Overall, higher CG methylation levels of LMO2_E148, IL10_P325, and GSTM1_P266 corresponded to the highest asthma risk [43].

Altogether, these findings consolidate evidence that parental transgenerational effects can disrupt biological processes in offspring via DNA methylation modifications [45]. Parental exposure to toxins, diet, and other external factors could precipitate a series of epigenetic events that are transferred through gametes to offspring and subsequent generations [45]. Then, one could speculate that the inheritance of asthma from parents to offspring could be attributed to epigenetic mechanisms. The concept that fetal epigenetic programming may influence epigenetic-sensitive pathways involving the inheritance of respiratory disease is indeed remarkable. Methylome profiling in neonates could potentially be a useful clinical tool to identify subjects with high asthma risk and targets to inhibit childhood asthma development. Therefore, this novel science offers opportunities for primary prevention before the onset of disease. One could conclude that the trajectory to childhood asthma originates at birth and involves epigenetic modifications in immunoregulatory and proinflammatory pathways that influence disease development across the lifespan. In addition, there appears to be a strong influence between maternal asthma and epigenetic mechanisms that contribute to asthma induction.

Regarding the obese−asthma phenotype, Rastogi et al. examined DNA methylation patterns in pediatric obese−asthma patients [46]. Peripheral blood mononuclear cells collected from 32 children in preadolescence ($n = 8$ obese asthmatics, 8 obese only, 8 asthma only, and 8 healthy controls) revealed that among obese asthmatics, there was decreased promoter methylation of CCL5, IL2RA, and TBX21 genes, which encode proteins involved in Th1 polarization, innate immune and nonatopic inflammation; whereas increased promoter methylation of FCER2 (a low-infinity IgE receptor) and TGFB1 (T-helper cell activation inhibitor) was observed. Overall, the annotated methylation profiles in

obese asthmatics indicate hypomethylation in regions linked to T cell differentiation and macrophage activation [47]. These observations suggest that dysregulation in DNA methylation is associated with nonatopic airway inflammation, features characteristic of the obese−asthma phenotype [47].

In another study, Cardenas et al. measured DNA methylation from nasal epithelial cells of 547 adolescents (11−15 years) participating in the Project Viva Cohort Study [48]. Compared with non-asthmatics, associations were found between elevated airway inflammatory biomarkers, namely fractional exhaled nitric oxide (FeNO), total IgE, and methylated regions that were annotated to genes involved in the structure and function of epithelial cells, oxidative stress, and mucin production enzymes in asthmatic peers. According to the investigators, hypomethylation at the eosinophil peroxidase (EPX) gene promoter has been implicated in driving Th2 and eosinophilic responses. Of note, no statistically significant associations were observed between DNA methylation and lung function [48]. Nevertheless, NO is a mediator involved in chronic inflammatory diseases and Th2-mediated immune responses [49].

Liang et al. explored epigenetic associations between serum IgE concentrations (a mediatory of atopic inflammation) and methylation at loci concentrated in CpG islands in 95 nuclear families (355 subjects aged 2−61 years), using DNA from peripheral blood leukocytes [50]. Positive results were observed for IgE and low methylation at 36 loci encoding for eosinophil products and genes (IL5RA) encoding a receptor that stimulates eosinophil production and activation, eosinophil eotaxin receptor (CCR3), eosinophil-activating cytokine (IL33), and eosinophil transcription factor (GATA1) along with phospholipid inflammatory mediators and mitochondrial proteins. Furthermore, methylation at these loci differed significantly in eosinophils isolated from asthmatic versus nonasthmatic subjects, with higher IgE levels noted in the asthmatic group. Overall, this study identified novel therapeutic targets for discriminating allergic diseases and biomarkers for patient stratification.

In another study, Seumois et al. investigated whether histone modulation regulated cytokine production in 12 asthmatics versus 12 nonasthmatic subjects [51]. Genome mapping of histone modifications in CD4+ memory, Th1 and Th2 cells revealed differential enrichment of histone H3 lysine 4 dimethylated in Th2 enhancers associated with asthma susceptibility transcription binding sites involved in T cell differentiation (such as GATA3, TBX21, and RUNX3) as well as genes involved in chemokine and Toll-like receptor signaling pathways [51].

With respect to miRNAs, circulating miRNAs are attractive biomarkers for predicting disease outcomes. Kho et al. reported strong associations between serum-derived miR-206 and exacerbation risk in 153 asthmatic children (5−12 years) participating in the Childhood Asthma Management Program (CAMP) [52]. Maes et al. demonstrated associations between MiRNA expression (miR-629-3p, miR-223-3p, and miR-142-3p) and neutrophilic asthma in sputum collected from 9 severe adult asthma patients as compared to 10 healthy and 17 mild-moderate asthma patients [53]. In light of the aforementioned findings and their significance in the clinical setting, information from circulating serum miRNAs might improve the accuracy of predicting asthma exacerbations in children, including their utility as putative biomarkers for the neutrophilic severe asthma phenotype in adult populations.

From a different perspective, the beneficial health effects of maternal human milk are indisputable. Breast milk is recommended as the ideal source of nutrition for the first 6 months of an infant's life, promising optimal growth, development, and maturation of the infant's immune system [54]. Most importantly, it provides protection against allergies and life-threatening conditions, including necrotizing enterocolitis in premature infants [55]. Human milk is rich in immunological nutrients and bioactive components, including a spectrum of exosome-derived miRNAs: miRNA-148a,

miRNA-30a, miRNA-146b, miRNA-200a, miRNA-21, miRNA-200c, miRNA-26a, let-7f, let-7i, and miRNA-146a [55]. These small molecules (miRNA) can bind and regulate gene expression, affecting the immune system via modification of T and B cell development, and activation of inflammatory cytokines [55]. It is believed that it is the action of these breast milk miRNAs that provide the allergy-protective effect during infancy. Therefore, breast milk is not only a food for the developing infant but also an immunomodulating agent for early-life programming mediated by miRNAs.

Interestingly, miRNAs are not exclusive to human milk but have been identified in cow's milk, an important substitute for maternal milk [56]. Up to 52 differential mRNAs species have been isolated from processed cow's milk and 25 from raw milk [56]. Candidate genes targeted by miRNAs are signal transducers and activators of transcription 3 (STAT3), which transduces signals from receptors involved in innate immunity, contributes to T helper cell differentiation, and mediates the inhibitory effects of the proinflammatory cytokine IL-6 on dendritic cells [56]. Other key players involved in allergic inflammation are prostaglandin-endoperoxide synthase 2S (PTGS2), IL13, IL6, and IFN-γ, which are involved in the synthesis of proinflammatory eicosanoids [56]. Prior research examining the effect of raw milk intake in relation to pediatric asthma has reported that in children residing in rural areas, raw milk consumption was associated with 40% lower odds of asthma, 26% lower odds of atopy, 23% lower odds of respiratory infections, and 46% lower odds of hay fever, independent of other farm exposures [57,58]. Hence, cow's milk consumption by children could potentially explain the protective effect of farm life and of asthma inception. Although the promising data, consumption of unpasteurized raw cow's milk should not be encouraged, due to the high risk of foodborne illness or "food-poisoning," which can be serious in infants, children, pregnant mothers, and in the immune-compromised [59]. Further investigations are warranted to uncover the components of cow's milk, which confer prophylaxis against asthma as well as epigenetic processes.

18.1.3 Asthma and diet

18.1.3.1 Maternal nutrition

Prenatal exposures can cause long-term alterations in the epigenetic profiles of offspring. During fetal development, epigenetic processes such as DNA methylation, histone modification, and mRNA expression are dynamic (undergoing continual configuration), reversible, and sensitive to external cues, thus affecting genes and pathways that are critical for early fetal development and later-life physiological functions.

In this context, maternal diet and prenatal exposure during pregnancy play a pivotal role in fetal growth and development. Increased nutritional requirements (both macronutrients and micronutrients) are needed to support the metabolic demands of embryonic growth and maturation (including nutrient transport, cell motility, and biosynthetic pathways) along with physiological changes that occur in the mother including placental growth [60]. There is convincing evidence from studies focusing on the fetal origins of health and disease linking early-life nutrition to disease [40]. Maternal nutrition can alter DNA methylation patterns, which are associated with changes in gene expression and phenotype in offspring mediated by fetal programming [40,61]. Most importantly, epidemiological studies indicate that undernutrition during intrauterine growth may have adverse consequences in later life and can be carried to future generations [40,60]. In an early study, Bygren et al. demonstrated that food deprivation in female fetuses for 7−12 weeks was related to increased prevalence of metabolic syndrome in adult life, in their children, and in their grandchildren [62]. A plausible mechanism leading to

transgenerational effects of food restriction might be via alterations in DNA methylation and histone modifications that result in developmental adaptations that permanently alter the structure, physiology, and metabolism of offspring [45,60], hence predisposing individuals to metabolic, endocrine, and cardiovascular disease in adulthood [60]. Although the concept of fetal programming has been replicated in animal models [63–65], causal relationships in human populations have yet to be established, and there is a great need for well-designed clinical trials to validate this theory.

18.1.3.2 Methyl donors, DNA methylation, and asthma

Molecular studies indicate that some dietary components can actively contribute to epigenetic mechanisms and modify gene expression. Perturbations in the methyl donor activity of DNA methyltransferase enzymes and increased methylation of specific CpG sites, along with associated changes in chromatin structure, can silence gene transcription, leading to alterations in cellular activities [10]. One potential mechanism by which dietary intake might affect respiratory health in children is through epigenetic modulation of immunoregulatory and inflammatory cytokines mediated by hypo- or hyper-DNA methylation [10]. Current evidence suggests that DNA methylation is dependent on the presence of specific nutrients derived from the diet such as folate, betaine, choline, methionine, vitamins B_{12} and B_6, as well as magnesium, zinc, and sulfur [66]. These nutrients act as substrates and cofactors that are crucial for DNA methylation and regulation of the one-carbon cycle [66]. Therefore, dietary sufficiency of these nutrients via consumption of a healthy diet, especially during the prenatal and perinatal time periods as well as throughout the life course, could help to support DNA methylation and inhibit expression of certain genes associated with asthma onset.

18.1.3.3 Folate and asthma

Prior research has shown that diet is an important determinant in the manifestation of the future disease [67–70] and constitutes one of the major environmental factors that exert a profound effect on the development and functioning of the immune system [71]. During intrauterine life, the developing fetus is nourished and oxygenated via the amniotic fluid. However, whether allergens and other external exposures can be transferred to the fetus via the amniotic fluid is unclear [72]. In consideration of the impact of the early environment on asthma induction, it is essential to consider how environmental changes could trigger immune dysfunction and Th2 responses. Given that maternal exposures are in direct contact with the intrauterine environment, any factors that induce inflammation in utero could potentially affect DNA integrity and ultimately gene expression giving rise to allergic disease. An example illustrating the impact of prenatal nutrition and disease in offspring is maternal folate supplementation during pregnancy, which effectively reduces the risk of neural tube defects in offspring [73]. On the other hand, there is considerable concern about maternal folate supplementation as a risk factor for respiratory disease. The infamous murine study conducted by Hollingsworth et al. demonstrated that maternal folic acid supplementation was associated with allergic airway disease in mouse offspring [74]. A most likely explanation is epigenetic alterations in DNA methylation that caused perturbations in immune gene expression [74]. Comparably, in a human prospective birth cohort ($n = 557$), folic acid supplementation in late pregnancy was associated with increased risk of childhood asthma at 3.5 years [75]. Veeranki et al., in a retrospective cohort study of 104,428 children, reported that folic acid supplementation during the first trimester only or later than the first trimester increased asthma odds by 10%–30% in children at 4.5–6 years as compared with children from mothers with no folic acid supplementation exposure [76]. Roy et al. studied 858 mother–child dyads

and found that the relative odds of current wheeze at 3 years were 33% lower odds in children born to mothers with folate levels ≥20 ng/mL compared with those with folate-deficient mothers [77]. No significant associations were noted between third-trimester folate and asthma outcome. Miyashita et al., in a Japanese study of 6651 mother−child pairs, documented a 27% increase in odds of childhood asthma at 2 years in children from mothers with high folate levels versus low folate, although null associations were observed at 1, 4, and 7 years of age [78]. Interestingly, no associations were found between DNA methylation and maternal folate levels at all ages. Latest data from a Chinese case−control study on childhood asthma that included 1364 school children (548 cases and 816 controls) reported that folic acid supplementation before gestation was associated with a 31% increased risk of childhood asthma, 9% in the first month of gestation, 90% beyond the first month of gestation, and 75% for >6 months duration [79]. Overall, periconceptional folic acid supplementation was associated with a 28% increased risk of childhood asthma. A plausible explanation for these findings could be that in utero exposure to methyl donors, mediated by maternal folic acid supplementation, might influence T-helper cell development, favoring the proliferation of the Th2 phenotype and increasing susceptibility to asthma [74]. In terms of fetal growth and maturation, T cell development commences at 12 weeks of gestation, and IgE receptor activity increases at 16−20 weeks of gestation [80,81]. This might clarify why stronger associations were observed with folate supplementation beyond 6 months of gestation. In contrast, a metaanalysis of cohort and case−control studies reported no associations between maternal folic acid supplementation before pregnancy, during the first trimester and childhood asthma at 8 years in the offspring [82]. Clearly, it seems that the timing of exposure to folate supplementation in utero may be critical to childhood asthma disease development. In light of the mechanism by which maternal folate promotes asthma induction in offspring, Harb et al., using a miniaturized chromatin immunoprecipitation (ChIP) assay to identify epigenetic marks, demonstrated that high maternal folate exposure during fetal life was associated with increased H3 and H4 histone acetylation at *GATA3* and/or *IL9* promoter regions of CD4+ T cells (Th2-associated genes) in cord blood of neonates [83]. These findings support the notion that folate, a methyl donor from the maternal diet during pregnancy, has the potential to epigenetically modify gene expression in prenatal life and influence asthma progression.

On the other hand, studies investigating the effect of folate status during midchildhood on airway function have documented mixed results. Lin et al., in a prospective cohort study of 150 children (5−17 years old), documented no differences in the airway function parameter (FEV_1/FVC) according to folate level, although low folate levels were associated with low FeNO and low-moderate levels with increased inflammation [84]. Similarly, Nicholson et al., in a case−control study of 884 Peruvian children 9−19 years old (n = 413 with asthma), found that a 10-ng/mL decrease in plasma folate level was associated with a 0.28 decrease in FEV_1/FVC [85]. Along the same line, we conducted a cross-sectional analysis of 64 mild-asthmatic Greek children (5−12 years) to investigate the role of folate and airway function in pediatric patients [86]. We found that girls in the lowest folate quartile had an 8.64% lower FVC, 10.35% FEV_1, and an 18.72% lower PEF than those in the highest folate quartile. No associations were observed in asthmatic boys. Regarding asthma exacerbations, Basanti et al. demonstrated in 45 Egyptian schoolchildren (6−12 years) that folate concentrations were inversely correlated with asthma exacerbation risk and severity [87].

It is worth noting that discrepancies between studies could be due to study design, age and population differences, genetic variability, dietary habits, timing/season of folate assessment, thresholds used for folate levels, asthma severity, or differences in lung function parameters measured.

Nevertheless, overall, the findings of these studies postulate that there might be a window of exposure at which folate confers protective effects against asthma induction in children [88]. This has important future implications because intervening during childhood as opposed to during pregnancy by modifying folate intake could be an effective strategy for preventing childhood asthma. Therefore, one might speculate that, in strategies targeting folate deficiency in pediatric asthma patients, increased consumption of fruits and vegetables could benefit lung function. The potential of dietary folate as a therapeutic adjunct to conventional asthma care remains to be clarified in future studies.

The proposed mechanism by which folate influences asthma progression is by acting as a methyl donor, promoting DNA methylation, and thereby suppressing the expression of key genes involved in T cell differentiation and regulation [27]. It is possible that normal serum folate levels could prevent the Th2 proallergic immune response that leads to airway and systemic inflammation, and as a result, improve lung function. Alternatively, folate deficiency could trigger a Th2 inflammatory response and proliferation of inflammatory cytokines, characteristics of asthma pathogenesis. Furthermore, a deficiency of methyl groups impairs one-carbon metabolism, resulting in DNA instability along with the accumulation of homocysteine, which could consequently lead to increased production of reactive oxygen species that favor the expression of proinflammatory genes and vascular inflammation [89]. It is this state of chronic oxidative stress that activates the Th2 immune response associated with asthma [90]. Feasibly, sufficient folate concentrations could prevent inflammation by reducing oxidative stress in the airways [90].

18.1.3.4 Folate, B_{12}, methionine cycle

Folate or vitamins B_9 and B_{12} are two members of the vitamin B family that are critical for human health. Both micronutrients are key players in the one-carbon metabolism and are essential for DNA methylation, synthesis of nucleotides, and protein, especially methionine. Folic acid can normalize high homocysteine levels by remethylating homocysteine to methionine via methionine synthetase, utilizing B_{12} as a cofactor [91]. This is important because hyperhomocysteinemia is a cardiovascular risk factor [92].

Inadequate maternal nutrition during early-life development can create permanent alterations in offspring, leading to poor health outcomes. A large epigenomic-wide association study of folate and vitamin B_{12} was conducted on the DNA of 5841 subjects derived from 10 cohorts [93]. Folate and B_{12} intakes were estimated from food-frequency questionnaires. According to the investigators, novel epigenetic loci were associated with folate and B_{12} intake. Six differentially methylated positions (DMPs) were negatively associated with folate intake. Genes involved in centrosome localization, cell proliferation, and tumorigenesis were annotated and included FAM64A, WRAP73, FRMD8, CUX1, and LCN8. Of the 74 folate-associated differentially methylated regions (DMRs), 73 were negatively associated with folate intake, with a 400-base pair spanning region annotated to the LGALS3BP gene. Regarding vitamin B_{12} intake, 29 DMRs were annotated to 48 genes in a 1100-base pair spanning region that corresponded to the calcium-binding tyrosine phosphorylation-regulating gene (CABYR) [93].

Therefore, folate and B_{12} intake during pregnancy affect the establishment of DNA methylation marks and subsequent alterations in epigenetic mechanisms. In the case of folate deficiency, studies have shown that global DNA methylation decreased, while the inverse was observed with folate supplementation [61,94].

Folate is a water-soluble B vitamin that is naturally found in leafy vegetables (such as spinach, beans, asparagus, and broccoli), legumes, egg yolks, liver, yeast, whole-wheat bread, some citrus fruits

(oranges), and fortified products (cereals, dairy) [91]. It is essential for normal cell growth, replication, erythropoiesis, DNA synthesis, and repair, including brain development, and therefore critically important during periods of rapid growth from childhood to adolescence [95,96]. Folate participates in a variety of metabolic pathways, including the histidine cycle, thymidylate cycle, purine cycle, and serine/glycine cycle. Then, folate depletion in the body would undermine the turnover of all cycles and lead to adverse health effects. Folic acid refers to folate added to foods and dietary supplements [91].

In the one-carbon folate cycle, folate in foods is reduced by the enzyme dihydrofolate reductase (DHFR) to form dihydrofolate (DHF), while further reduction by the action of the same enzyme generates tetrahydrofolate (THF), the active coenzyme [66] (Fig. 18.1). Then, THF is converted to 5,10-methylene tetrahydrofolate (5,10-MTHF) (the biologically active form of folate) by serine hydroxymethyltransferase (SHMT) via the donation of a methyl and hydroxy group from serine, which results in the production of the amino acid glycine in the process. 5,10-MTHF is synthesized to 5-methylene tetrahydrofolate (5-MTHF) by methylene tetrahydrofolate reductase (MTHFR). In addition, 5-MTHF can be converted back to THF by methionine synthase (MS), which also generates methionine from homocysteine in the presence of vitamin B_{12}. In addition, methionine is converted to S-adenosyl methionine (SAM) by methionine adenosyltransferase (MAT). The methyl group of SAM can be donated to form a variety of vital methylated compounds, including creatine, epinephrine, sarcosine, methylated DNA and RNA along with proteins. It is worth noting that the utilization of 5-methyltetrahydrofolate as a methyl donor by MS links B_{12} metabolism with folate one-carbon metabolism, which, apart from the synthesis of methionine and SAM, is required for de novo purine synthesis and production of deoxythymidine monophosphate (dTMP), an important cellular energy source, through the reduction of NADP + to NADPH [66]. Under conditions of folate deficiency, methionine is not produced, which leads to low production of glutathione (an antioxidant) and the amino acids taurine and cysteine, which are involved in the elimination of toxins from the body and are important for cardiovascular health [91]. Moreover, low methionine levels lead to hepatic steatosis, including deficits in creatine and collagen production [91].

As for 5,10 MTHF, this is a crucial intermediate since it can be recycled back to DHF by thymidylate synthase (TS), undergo further conversion by methylene tetrahydrofolate dehydrogenase (MTHFD) to 5,10-methenyl THF and to 10-formyl THF, a precursor for nucleic acid synthesis by the action of methenyl THF-cyclohydrolase [66]. In humans, the major source of one-carbon units is serine, which, in the presence of THF, is reversibly metabolized to glycine and 5,10-MTHF by the enzyme serine hydroxymethyltransferase (SHMT) [66]. It has been speculated that the folate cycle may commence with the demethylation of serine. In the case of folate deficiency, serine is not produced from glycine, which leads to many health problems such as dysfunction of the brain and central nervous system along with the impaired function of RNA, DNA, fatty acid metabolism, and muscle formation [91].

With respect to vitamins B_6 and B_{12}, these two vitamins are important in controlling blood levels of homocysteine [91,92]. In addition, both B_6 and B_{12} are cofactors involved in the regulation of the catalytic activity of enzymes from the folate cycle, and indirectly, determine SAM bioavailability [66]. Therefore, supplementing diets with vitamins B_6 and B_{12} could contribute to the maintenance or establishment of DNA methyl markers [97]. Most importantly, using B_{12} as a cofactor, folate can normalize high homocysteine levels by remethylating homocysteine to methionine via methionine synthetase. It is worth noting that high alcohol consumption inhibits folate metabolism, reduces the availability of vitamins B_6 and B_{12} [98], and interferes with the activity of methionine synthase,



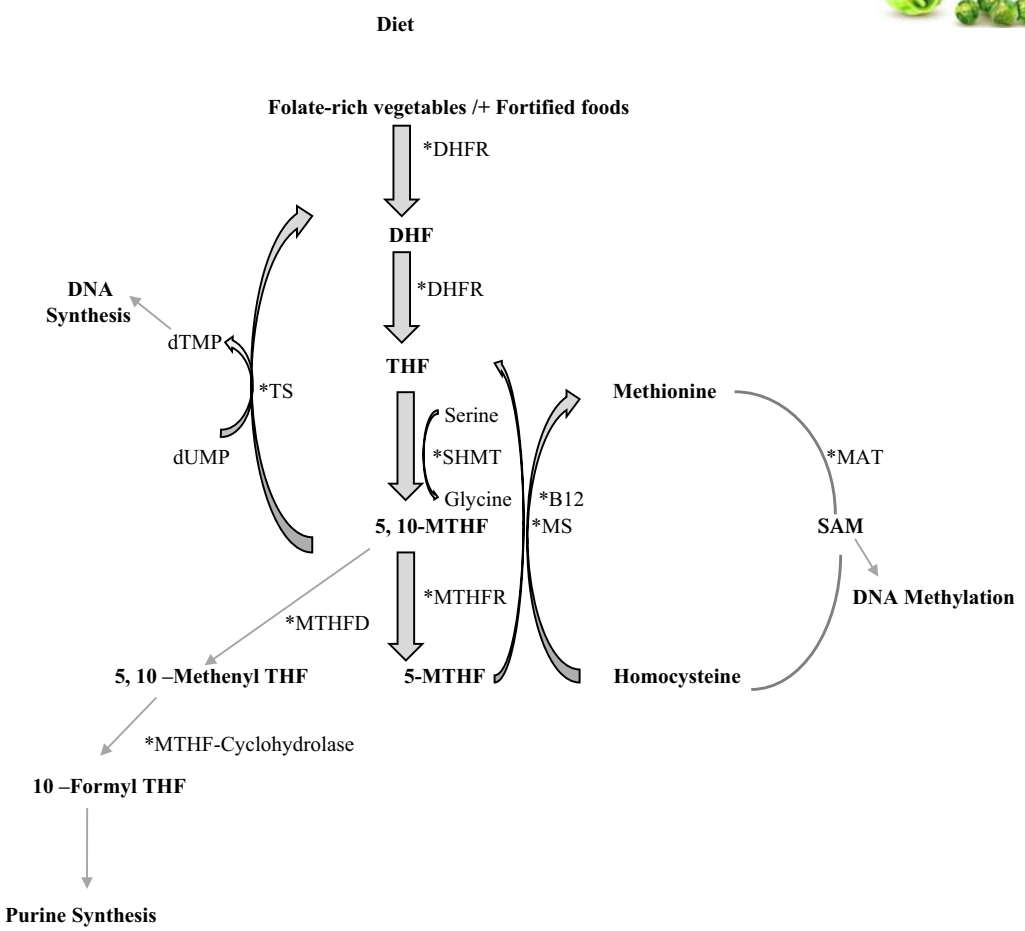

FIGURE 18.1

One-carbon folate and methionine metabolism.
5-*MTHF*, 5-methyl tetrahydrofolate; *DHF*, dihydrofolate; *dTMP*, deoxythymidine monophosphate; *dUMP*, deoxyuridine monophosphate; *MAT*, methionine adenosyltransferase; *MS*, methionine synthase; MTHF-cyclohydrolase, methenyl THF-vyclohydrolase; *MTHFD*, methylene tetrahydrofolate dehydrogenase; *MTHFR*, methylenetetrahydrofolate reductase; *SAM*, S-adenosylmethionine; *SHMT*, serine hydroxymethyltransferase; *THF*, tetrahydrofolate.

S-adenosyl homocysteine hydrolase, methionine adenosyltransferase, and MTHFR, enzymes vital for methyl donor synthesis [98]. Alcohol also induces the betaine homocysteine methyltransferase pathway, which perpetuates a decrease in SAM and SAH production [98]. With regard to choline, it is utilized for methylation reactions via betaine and is further converted to SAM and SAH. While phosphatidylcholine is an essential constituent of cell membranes and as phosphoplipase D important for cell signaling [98].

Vitamin B_{12} or cobalamin is not produced in human but is abundant in animal products such as milk, fish, meat, liver, kidneys, egg yolk, and fortified breakfast cereals and is synthesized by bacteria [91]. Both folate and vitamin B_{12} status have been linked to diverse diseases, including cardiovascular disease, megaloblastic anemia, and severe neurological abnormalities [91]. It has been speculated that DNA methylation is the underlying mechanism bridging folate/B_{12} deficiency with increased disease risk. Specific alterations in methylation patterns could serve as biomarkers for nutrient-driven disease risk. Therefore, more insight is urgently required to elucidate the mechanisms underlying the effects of folate and B_{12} on health and disease throughout the life span.

From an epigenetic point of view, lack of folate/methionine pathway intermediates or modification in methionine or folate intake could cause alterations in DNA methylations involving hypomethylation in regions that would normally be methylated, a decrease in histone methylation, regulatory miRNAs, and changes in gene transcription [66, 97–99]. Therefore, folate as the limiting substrate would lead to a decrease in DNA precursors and ultimately cause a decrease in cell division and intrauterine growth restriction [98]. Emerging evidence has established that to maximize the use of often limited substrates and cofactors, the one-carbon metabolic processes undergo allosteric regulation by SAM and adenosylhomocysteine [66]. High levels of SAM result in allosteric inhibition of MTHFR and MAT along with simultaneous activation of cystathionine β synthase, which inhibits SAM production [66]. Through this mechanism, excess homocysteine is released from the cycle. On the other hand, increased concentrations of adenosylhomocysteine activate MTHFR, while inhibiting methyltransferases [66]. Furthermore, the flow of single carbon units to each of these pathways is based on cellular needs [66]. To sum up, folates as a source of SAM, the main cellular methyl donor that modulates gene methylation, could regulate gene expression. In addition, B_{12}, methionine, and folate pathways are interlinked, each exerting a regulatory effect via allosteric control [66]. Therefore, deficiency of either methyl donor in the individual's or maternal diet will influence the turnover rate of the one-carbon cycle, and inevitably, DNA methylation and gene expression [97]. It has been proposed that it is through this process that folate influences fetal programming [41].

18.1.4 Other nutritional factors

A range of other nutritional factors, including vitamin D, antioxidants (selenium, zinc, vitamins A, C, and E), omega-3 fatty acids, and short-chain fatty acids, appear to modulate gene expression through epigenetic effects. Moreover, diet-induced alterations in the microbiome can also induce epigenetic modification in genes related to the immune response and in disease pathobiology.

18.1.4.1 Vitamin D, immune regulation, and asthma

Vitamin D status is influenced by sunlight exposure along with vitamin D–rich food sources such as fatty fish, egg yolk, liver, fortified foods (milk, margarine), and oral supplements [100]. The detrimental effects of vitamin D deficiency as defined by serum 25-hydroxyvitamin D [25(OH) D]

levels < 20 ng/mL extend beyond bone health. Observational studies have consistently found that suboptimal vitamin D levels (25(OH) D < 30 ng/mL) are common among asthmatic children [101−103] and are correlated to poor asthma control [101], increased hospitalization [104], acute asthma exacerbations [104], asthma severity [101,105], airway inflammation [106], and a decline in lung function [107]. It is well recognized that vitamin D plays a key role in bone metabolism and calcium maintenance. The identification of vitamin D receptors (VDRs) in many other cells including the immune (T and B lymphocytes, monocytes) and lung cells [108] provides the rationale that vitamin D could modulate immune system regulatory functions.

During allergen exposure, CD4+ Th2 cells orchestrate the immune and inflammatory response by activating the production of IL-4 and IL-13, which enhances IgE synthesis from B cells; IL-5, in eosinophil maturation and IL-13 in the regulation of airway hyperresponsiveness and mucus hyperplasia [108]. In contrast, IL-10 is a potent antiinflammatory cytokine that is able to inhibit antigen-presenting cell function, cytokine production by macrophages and dendritic cells, inhibition of Th1 cell−mediated immunity, as well as downregulation of cytokine production from Th2 cells, mast, and eosinophil function [108]. Vitamin D deficiency influences Th1 and Th2 cytokine production, thus contributing to the development of allergies, including asthma. Vasiliou et al., in a murine study, showed that a vitamin D−replete diet administered to mothers in utero from day 16 of gestation to 3 weeks postnatal had increased levels of CD3+, CD4+, and TIST2+ cells along with reduced CD4+IL-10+ regulatory cells, signifying a Th2 skewing toward the development of allergic airway disease [109]. The same pattern was observed in the offspring of vitamin D−insufficient mothers fed the vitamin D−deficient diet: high levels of lung eosinophilia and Th2 cells with low CD4+IL-10+ regulatory cells. At weaning, introduction of vitamin D into the diet of deficient offspring led to significant reductions in serum IgE, lung eosinophilia, and peribronchiolar collagen deposition, characteristics of airway remodeling [109]. In a human study, Junge et al., using data from six neonates participating in the LINA (Lifestyle and environmental factors and their Influence on Newborns Allergy risk) mother−child cohort, demonstrated that children with high 25(OH)D levels in umbilical cord blood at birth had low DNA methylation and loss of repressive histone marks in the TSLP enhancer region, which resulted in increased TSLP mRNA expression [110]. More specifically, elevated vitamin D concentrations were associated with decreased repressive histone tail modifications H3K9me3 and H3K27me3 at the TSLP promoter and enhancer regions. Additionally, increased TSLP expression was correlated with an increased incidence of wheezing at 3 years of age in neonates with high 25(OH)D levels in cord blood than in those with low vitamin D levels at birth [110]. In the allergic response, TSLP induces proinflammatory Th2 proliferation via dendritic cell maturation [111]. On the other hand, prior studies document that TSLP mRNA and protein levels are correlated with asthma severity [112]. These findings provide evidence that epigenetic deregulation of TSLP could be involved in the vitamin D−related programming for asthma induction.

Zosky et al., in a cohort study of 2834 mothers, documented impaired lung function and increased odds of childhood asthma in children whose mothers were vitamin D deficient prenatally [113]. According to the investigators, lung function in 6-year-old children (as reflected by FEV_1 and FVC) was positively associated with maternal serum 25(OH)D concentrations at 16−20 weeks gestation, and asthma at 6 years of age was three times more likely in boys whose mothers were vitamin D deficient [113]. This study suggests that maternal vitamin D status is a crucial factor for fetal lung development and maturation, including asthma induction.

Sundar et al. reported that VDR deficiency is common in chronic obstructive pulmonary disease (COPD) patients and evokes airway inflammation and changes in lung mechanics [114]. In addition, rodent studies showed that VDR-deficient mice were prone to develop COPD phenotype, implying the role of VDR in the progression of chronic lung disease [114]. More specifically, increased infiltration of inflammatory cells, phosphoacetylation of nuclear factor-kappa B (NF-κB) associated with increased proinflammatory mediators, and upregulation of matrix metalloproteinases (MMPs) were observed in VDR knockout mice as compared to wild-type mice. These events were associated with lung function decline and emphysema with lymphoid aggregate formation [114]. Hence, understanding the molecular/epigenetic mechanisms of vitamin D for the treatment of airway disease and exacerbations is an emerging area of interest. More well-designed clinical trials conducted in humans are greatly needed to uncover the molecular regulatory mechanisms of vitamin D and VDR in asthma disease.

Inhaled glucocorticoids are the cornerstone of asthma management and symptom control [2]. Glucocorticoids act by inhibiting Th2 synthesis and enhancing the production of IL-10 by human airway cells in asthmatic patients [115]. Therefore, the induction of IL-10 synthesis may contribute to the efficacy of glucocorticoids in asthma. Xystrakis et al., in a human study, demonstrated that vitamin D_3 supplementation in severe glucocorticoid-resistant asthmatic children enhanced response to asthma therapy via activation of IL-10 secretory Treg cells and inhibition of IL-5, Il-13, and IFN-γ production [115]. In clinical practice, analysis of IL-10 synthesis could be a useful tool in determining glucocorticoid therapy responders. Likewise, Zhang et al. demonstrated vitamin D antiinflammatory and corticosteroid-enhancing effects in monocytes of 11 steroid-resistant and eight steroid-sensitive adults with asthma [116]. Vitamin D (1,25 OH-D) preincubation of peripheral blood mononuclear cells increased dexamethasone-induced H4 acetylation, providing antiinflammatory effects [116]. In the same line, Banerjee et al. reported that in TNFα-treated human airway smooth muscle cells, $1,25(OH)_2D$ inhibited expression of chemokines RANTES and interferon-inducible protein 10 (IP-10) secretion as well as reduced expression of the gene coding for steroid resistance *(FKN)* [117]. RANTES is a chemokine that attracts monocytes, eosinophils, and T cells during inflammation and the immune response, while IP-10 is a potent chemokine for activated T cells, NK cells, and mast cells [117].

A plausible explanation for these beneficial effects is that vitamin D increases the expression of VDRs and *CYP241A1* (an important gene in vitamin D metabolism) in airway smooth muscle cells [117]. Amounting evidence indicates that airway smooth muscle cells secrete chemokines in response to allergen-induced inflammation, which results in the recruitment of inflammatory cells and the production of proinflammatory markers [117]. The concept that vitamin D supplementation could control the expression of chemokines by airway smooth muscle cells may provide novel therapeutic targets that decrease cell migration, infiltration, inflammation, and airway remodeling and effectively reverse these processes. Alternatively, vitamin D supplementation could enhance the clinical efficacy of pharmacotherapy in glucocorticoid-resistant patients, thereby restoring lung function.

18.1.4.2 Vitamin D and the immune response

Vitamin D is referred to as a pleiotropic hormone, possessing multiple biological effects. The active form of vitamin D ($1,25(OH)_2D$) contributes to innate immunity by binding to VDRs and coactivating the retinoid X receptor (RXR), which promotes vitamin D−regulated gene transcription [108], Treg secretion, and the antiinflammatory cytokine (IL-10) and suppresses IL-4 mediated expression of proinflammatory cytokines IL-13 and IL-17 as well as IgE synthesis [108]. Most importantly,

$1,25(OH)_2D$ acts on T cells, causing a shift from the Th1 phenotype to the less inflammatory Th2 [108]. With respect to airway remodeling, a feature of asthma, $1,25(OH)_2D$ is able to inhibit fibroblast proliferation and expression of proinflammatory chemokines (TNF-α, TGF-β) from smooth muscle cells, including suppression of matrix metalloproteinases, which are involved in the digestion of the extracellular matrix [108]. Furthermore, vitamin D enhances the expression of genes coding for cathelicidin (camp) and defensin (defβ2), antimicrobial peptides that are important in protecting against respiratory infections, which are the most common factors triggering wheezing and asthma in children [108]. To summarize, based on the aforementioned evidence, it appears that vitamin D is able to attenuate airway inflammation, hyperresponsiveness, and remodeling associated with asthma pathology.

18.1.4.3 Epigenetic modifications and the vitamin D receptor

As aforementioned, it is well established that specific nutrients such as folate can influence DNA methylation equilibrium. However, the impact of vitamin D on epigenetic processes is a matter of controversy. Epigenetic effects of 1, $25(OH)_2$ D are mainly observed via histone acetylation and DNA methylation [118,119]. Vitamin D regulates vital pathways of cellular metabolism and differentiation through its nuclear receptor (VDR), which can interact with chromatin [119]. VDR possesses DNA-binding sites that facilitate binding to DNA sequences in promoter regions of target genes, after which nuclear hormone receptors recruit various transcription factors and regulatory cofactor complexes on promoters to activate or suppress gene expression [119]. In this context, it is possible that epigenetic chromatin remodeling could regulate vitamin D—mediated gene expression. Transcriptional activation is induced by the interaction of the VDR/(retinoid X receptor complex) RXR dimer with HATs as well as HDACs [118]. HATs introduce acetyl groups into the nucleosomes, opening the chromatin and thereby increasing accessibility to transcription factors [118]. Binding of the VDR/RXR dimer to vitamin D response elements, recruitment of transcriptional corepressors (namely NCOR1), and silencing of the mediator for retinoic acid and thyroid hormone receptors (SMRT) result in histone deacetylation and transcriptional inactivation [118]. Zhu et al. reported that subjects with severe vitamin D deficiency had reduced synthesis and increased catabolism of vitamin D [120]. The authors observed that severe vitamin D deficiency was associated with methylation modifications in leukocyte DNA [120].

18.1.5 Long-chain omega-3 fatty acids

18.1.5.1 The impact of omega-3 long-chain polyunsaturated fatty acids in childhood asthma

Essential omega-3 fatty acids, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are well known for their nutritional value and health benefits due to their antiinflammatory properties [121]. The modern diet is low in fish intake, a dietary source rich in omega-3 fatty acids, and high in margarines and vegetable oils, sources of omega-6 fatty acids [122]. Current data indicate that ω-6:ω-3 ratio in our diets ranges from 8:1 to 17:1, the optimal being 1:1 [123]. It is believed that this imbalance in the omega-6: omega-3 fatty acid ratio is responsible for the rise in chronic diseases, especially those with an inflammatory component such as asthma [123].

The fetal programming theory hypothesizes that maternal nutritional status, hormonal balance, and the immediate intrauterine environment affect gene expression and metabolism in offspring. Recent

research supports omega-3 long-chain polyunsaturated fatty acid (ω-3 LCPUFA) supplementation in the mother's diet during gestation and in infant formula due to the importance of the omega-3 fatty acid DHA and the omega-6 fatty acid arachidonic acid (ARA) in brain development, cortical visual acuity, and cognition [124]. On the other hand, there are limited studies focusing attention on the potential benefits of omega-3 fatty acids in preventing asthma risk in neonates [125]. It is unclear how omega-3 fatty acid supplementation during gestation can affect asthma development in infants and during childhood [126], and the findings of meta-analyses are inconclusive [125−127].

Given the dearth in the literature, we conducted a metaanalysis of observational studies to investigate the role of fish (a dietary source rich in omega-3 fatty acids) in childhood asthma [128]. Pooled analysis of data from 163,744 children up to 15 years showed that early introduction of fish (6−9 months of age) and regular consumption (≥once/week) improved asthma symptoms and reduced risk in children <15 years versus no fish intake. Overall, there were 25% decreased odds of "current asthma" and 38% decreased odds of "current wheezing" in children younger than 4.5 years, while there was a 65% protective effect of fatty fish in older children (8−14 years) for "current asthma" as compared to "no fish" intake [128].

Fetal life is a critical stage for airway and immune development, during which dietary exposures could have irreversible long-term influences on the development of asthma. Omega-3 fatty acids in fish can be transferred across the placenta and exert a protective effect via involvement in immune regulatory pathways [129]. Clinical trials have documented that maternal fish oil supplementation significantly increased ω-3 levels in neonatal erythrocyte membranes and was associated with lower IL-13 plasma levels [129], mononuclear cell PGE_2 [130], and neutrophil LTB_4 production in neonates [131]. Furthermore, fish oil supplementation during gestation altered cytokine production in neonates by suppressing mRNA coding for IL-4, IL-13, and Th2 cells in umbilical cord blood [129,132]. Alternatively, animal and in vitro studies have shown that omega-3 fatty acids can alter the expression of inflammatory genes by modifying transcription factor activation, inhibiting T cell signaling modulated by changes in lipid rafts in cell membranes, decreasing the production of proinflammatory eicosanoids, cytokines, and reactive oxygen species, and giving rise to inflammatory-resolving bioactive molecules [121]. Nonetheless, the effect of fish intake during gestation is unclear. Stratakis et al. conducted a systematic review of the literature to explore the effect of fish intake during pregnancy on asthma risk in children from infancy to midchildhood [133]. Pooled analysis of data from 60,774 mother−child pairs participating in 18 European and US birth cohorts revealed no protective associations between maternal fish intake and wheezing or asthma in children 0−8 years [133]. In contrast, a contemporary systematic review reported that maternal fish intake during pregnancy was associated with a 3% reduced risk of wheezing in offspring but not for asthma [134]. Kachroo et al. showed that fish oil supplementation (EPA/DHA) in pregnant women at 10−18 weeks of gestation (first trimester) reduced wheezing in offspring at age 3 years [135]. High levels of DHA in mothers during the first trimester were associated with a 57% reduced risk of asthma and current wheezing in offspring. Along the same line, Bisgaard et al., using cohort data from the Copenhagen Prospective Studies in Asthma in Childhood (COPSAC $_{2010}$) study, found that fish oil supplementation of pregnant women at 24 weeks of gestation (third trimester) was associated with a 30.7% reduction in persistent wheezing or asthma in offspring at 5 years of age [136]. The protective effect was most prominent in children of mothers with low preintervention blood EPA/DHA concentrations.

Heterogeneity among studies may arise due to diversity in study designs, age of populations, asthma definition, exposure assessment, fish intake assessment methods, geographic location, dietary

habits, and adjustment for confounding factors. Another source of bias is that fish are often exposed to pollutants such as polychlorinated biphenyls and dioxins [137]. There is substantial evidence that intrauterine exposure to these chemicals can exert adverse immunomodulatory effects and increase the risk of developing asthma in offspring [138]. It is possible that these pollutants in fish could mask any beneficial effects between fish intake and asthma in children, thus yielding null associations. Furthermore, most studies were cross-sectional, which can only be used to generate hypotheses and not temporal directionality. To our knowledge, only one randomized controlled trial has been undertaken in pediatric asthma, assessing the efficacy of fatty fish intake within the context of a Mediterranean dietary pattern [139]. In this 6-month clinical trial, we found that two meals of fatty fish intake weekly as part of the Mediterranean diet reduced airway inflammation (as represented by FeNO) by 14 ppb in mild-asthmatic Greek schoolchildren. No associations were observed for lung function as measured by spirometry. To address whether the same effects can be produced in other populations, further trials are required to establish the potential of fish intake (Ω3) as a nutritional therapy for airway inflammation in pediatric populations. Apart from the antiinflammatory properties of omega-3 fatty acids and downregulation of the Th2 immune response associated with asthma onset, whether these effects may be explained by epigenetic processes remains to be elucidated.

18.1.5.2 Omega-3 fatty acids and epigenetic regulation

In terms of epigenetic mechanisms, ω3-LCPUFAs can influence DNA methylation, and maternal intake is associated with modifications in DNA methylation in offspring. Niculescu et al. demonstrated that, in mice, maternal high-fat feeding with ω-3 LCPUFA α-linolenic acid increased DNA methylation in the fatty acid desaturase (*FADS*) gene [140]. More specifically, alterations were observed in the FADS2 promoter and intron 1 of the maternal and offspring livers. Hoile et al. demonstrated in pregnant rodents that increasing maternal dietary fat intake throughout pregnancy and lactation by saturated fat (as butter) or ω-3 LCPUFAs (as fish oil) induced hypermethylation of CpG loci in the FADS2 promoter (encoding Δ6 desaturase, rate limiting in PUFA synthesis) and decreased hepatic expression of mRNA in adult offspring [141]. Interestingly, decreases were also noted in docosahexaenoic acid (DHA) and arachidonic acid content of membranes and plasma phospholipids [141]. In the same study, feeding adult female rats fish oil for 9 weeks also induced lower FADS2 mRNA expression and increased methylation of specific CpG loci in the FADS2 promoter [141]. Similar findings were documented in humans [142]. Hoile et al. reported that supplementation of adult renal patients with 4 g of ω-3 LCPUFA for 8 weeks altered the methylation of fatty acid desaturase (FADS)-2CpGs and elongase ELOVL5 CpGs in peripheral mononuclear cells [142]. Hence, it appears that omega-3 fatty acid supplementation during fetal development and adulthood exerts changes in epigenetic regulation of the long-chain PUFA biosynthesis.

At birth, the neonatal immature immune system is characterized by reduced interferon γ (INFγ) production and the Th2 phenotype [143]. T helper type 2 cells give rise to the proinflammatory cytokines IL-4, IL-5, and IL-13, which trigger the initiation and progression of allergic inflammation associated with asthma [37]. As the infant develops, T cells undergo maturation transitioning from a Th2 phenotype to Th1 dominance, which is maintained throughout adult life. However, during this critical phase of postnatal life, differences in T cell function at birth and during maturation appear to pave the way to allergy development. In neonates, activation of protein kinase C (PKC)−dependent mitogen-activated protein kinases (MARKs), which are important in protein kinase C isozyme ζ (PKCζ) expression and regulation of cytokine production from T cells, is downregulated, thus

affecting signaling pathways. PKC isozyme ζ plays a pivotal role in the regulation of T cell division in neonates by promoting a Th1 antiinflammatory phenotype [144]. In neonates from mothers with a family history of allergy, cord blood T cells expressing low PKCζ levels were associated with the development and maturation of cells toward an inflammatory Th2 phenotype [144,145]. Therefore, PKCζ levels in cord blood T cells could predict the development and severity of allergic disease [146]. On the other hand, empirical evidence suggests that ω-3 LCPUFA could mediate changes in neonatal T cell responses via influencing PKCζ, IL-13, and T-Box 21 gene expression [144,147]. Prenatal exposure to fish oil supplementation increased PKCζ levels with a parallel increase in H3 histone acetylation at the PKCζ gene promoter region [144,147]. Recently, Acevedo et al., in a prospective birth cohort study of 173 families, demonstrated that maternal fish oil supplementation during pregnancy was associated with increased H4 histone acetylation at the CD14 promoter loci in the placentas of female offspring only [148]. Therefore, it is feasible that maternal ω-3 LCPUFA supplementation during gestation could have favorable clinical effects in reducing asthma disease in offspring mediated via epigenetic control of PKCζ synthesis. Intrauterine life may be a window of opportunity for epigenetic intervention, thereby modifying T cell signaling and the perpetuation of asthma in progeny.

In the same direction, DHA supplementation during gestation in women who smoked during pregnancy was associated with DNA methylation modifications in genes coding for IFN-γ and IL-13 of cord blood mononuclear cells in neonates [149]. Furthermore, maternal fatty fish intake during the third trimester was correlated with increased DNA methylation levels in genes involved in PUFA synthesis (fatty acid desaturase [FADS1/2] and ELOVL FA elongase 5 [ELOVL5]), with a corresponding decrease in ELOVL5 mRNA [150].

18.1.5.3 Omega-3 fatty acids: Mode of action

The epigenetic effects of omega-3 fatty acids on the allergic immune response might be accounted for by its bioactive components (Fig. 18.2). Omega-3 long-chain polyunsaturated fatty acids, comprising of at least 18−20 carbons, are classified into two main families according to the position of the first double bond from the methyl end group: ω6 and ω3 [121]. The main ω-3 LCPUFAs are α-linolenic acid (ALA) (C18:3), derived from plants, nuts, and seeds, eicosapentaenoic acid (EPA) (C20:5), docosapentaenoic acid (DPA) (C22:5), and docosahexaenoic acid (DHA) (C22:6), which are common in seafood [121]. The ω-6 LCPUFA family includes linoleic acid (LA) (C18:2), the most abundant PUFA in our diet derived from plants, and arachidonic acid (ARA) (C20:4). DHA is the biologically active end product of ALA. Humans do not possess the enzymes to synthesize ALA and LA; therefore, they are referred to as essential fatty acids that are exclusively obtained from the diet [121]. ALA is the precursor of ω-3 LCPUFAs, while LA gives rise to ω-6 LCPUFAs. ALA is converted into ω-3 LCPUFAs by a series of alternating desaturation and elongation reactions. The first step in ALA metabolism is desaturation, catalyzed by delta-6-desaturase, which is considered to be rate-limiting [121]. In humans, the efficiency of the conversion of ALA to DHA and EPA is low and limited; therefore, dietary sources are essential to achieve the adequate provision of DHA [121]. Both EPA and DHA are obtained primarily from fish oil and fatty fish such as sardines, herring, mackerel, salmon, trout, and tuna, as well as ω-3 fed poultry and their eggs. EPA is a precursor of DHA and is metabolized to DHA in the liver [121]. The content of ω-3 PUFAs varies among fish (wild and farmed) and is dependent on fat composition and type of feeding [151]. Typically, one meal of fatty fish can provide 1.5−3.0 g of EPA and DHA as compared with 0.2−0.3 in lean fish, while 1 g of fish oil supplement provides 0.3 g of EPA and DHA combined [121]. EPA and DHA influence inflammatory pathways via

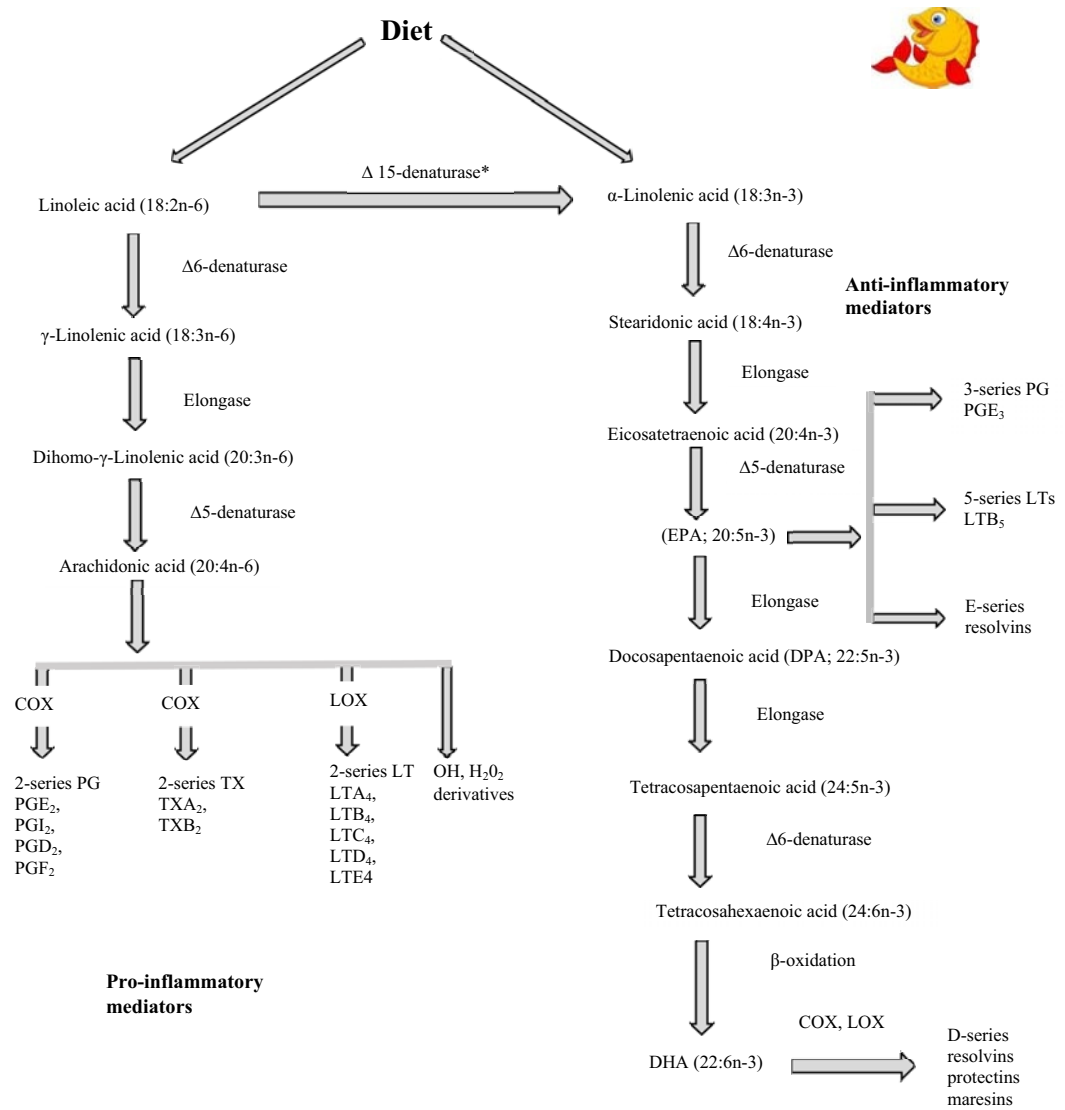

FIGURE 18.2

Omega-6 and omega-3 fatty acids metabolism, and eicosanoid derivatives produced from arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA).
COX, cyclooxygenase; *LOX*, lipoxygenase; *LT*, leukotriene; *PG*, prostaglandin; *TX*, thromboxane.

bioactive products, namely eicosanoids (prostaglandins [PGs], leukotrienes [LTs], and thromboxanes [TXs]). Proinflammatory eicosanoids are derived from ω-6 fatty acid ARA (2-series PGs and TXs, 4-series LTs), whereas EPA and DHA give rise to less biologically potent antiinflammatory eicosanoids (3-series PGs and TXs, 5-series LTs), and inflammatory-resolving bioactive molecules protectins,

maresins, and resolvins (via cyclooxygenase and lipoxygenase pathways), thereby controlling eicosanoid synthesis [121]. The fatty acid composition of cell membranes depends on the ratio of ω6: ω3 provided in the diet. Omega-3 fatty acids actively compete with omega-6 fatty acids in cell membranes, so an increase in EPA in cell membranes via intake will reduce PGE2 and LTB4 production by replacing AA as the substrate and by inhibiting the action of enzymes responsible for eicosanoid production (cyclooxygenase and lipoxygenase) [121]. Therefore, EPA and DHA by influencing membrane fluidity, cell signaling, eicosanoid production [121], and other mechanisms can potentially affect the immune response and development of allergic responses.

In light of the aforementioned, animal and in utero studies in neonates suggest that omega-3 fatty acids can influence epigenetic mechanisms involving DNA methylation and histone acetylation in the FADS, PKCζ, IL-13, and T-box 21 gene promoter regions [140−142,144,147,149], thus modulating the immune and inflammatory response. The potential of omega-3 fatty acids to modify epigenetic markers in utero could be an effective therapy for reducing asthma prevalence and burden in children. Future clinical studies will provide a comprehensive proof of concept.

18.1.6 Gut microbiota

According to the hygiene theory, lack of exposure to microbes during in utero and early life could trigger Th2 immune activity [152] and predispose children to allergic disease [153]. Even though many studies have demonstrated the dynamics of human microbiota early in life, little is known about the influence of microbiota on epigenetic mechanisms and immunological consequences. Forsberg et al. documented that maternal supplementation with *Lactobacillus reuteri* during gestation caused alterations in DNA methylation patterns in CD4+ T cells of neonates [154]. Interestingly, hypomethylation was observed in neonatal DNA, reflecting transcriptional accessibility in the probiotic group [154]. Annotated genes appear to participate in immune pathways involving chemotaxis, P13K-Akt, MAPK, and TGF-β signaling [154]. In regard to rural versus urban life, Conrad et al. documented that intranasal administration of *Acinetobacter iwoffii*, a farm bacterium, to pregnant mice protected offspring against the development of eosinophilic asthma [155]. Maternal exposure to *A. iwoffii* F78 caused a transient increase in lung proinflammatory cytokines (IL-6, IL-1β, TNF, IL-12, IL-4, IL-10, INF-γ) and upregulation of lung Toll-like receptor (TLR) mRNA expression in offspring [155]. TLR signaling is associated with protection against asthma pathobiology [155]. The prenatal asthma protection in offspring mediated by this microbe was attributed to CD4+ histone H4 acetylation at the IFNγ promoter of T cells [156]. Hence, this data supports the hygiene concept and indicates that microbes function via epigenetic mechanisms, with the possibility that maternal microbial exposure in utero could confer asthma-protective effects in offspring.

18.1.6.1 Microbial fermentation by-products short-chain fatty acids

Secular trends show that today's diet is characterized by low-fiber foods high in saturated fats, refined grains, sugar, and salt and less fruit, vegetables, and fish [157]. Adherence to the Western-type dietary pattern, a risk factor for asthma [7], is associated with perturbations in gut microbiome diversity. Accumulating evidence documents that dysbiosis of the gut and lung microbiome is common in asthma [158]. This is important because disturbances in gut microbiota support the activation of inflammatory pathways that contribute to bronchoconstriction and hyperresponsiveness [158]. Therefore, daily consumption of fermented foods may be important for maintaining a healthy, balanced, and

diverse microbial composition resulting in symbiosis, gut integrity, and regulating immune and inflammatory responses, thus reducing asthma prevalence.

Dietary fiber (poly- or oligosaccharides) is fermented by gut microbes to short-chain fatty acids (SCFAs) acetate, propionate, and butyrate, which exhibit antiinflammatory properties [159], promote gut homeostasis [160] and gut integrity [161], and regulate the function of T regulatory cells [162]. There appears to be cross-talk between microbiota in the gut and lung. Interestingly, stool samples of neonates analyzed shortly after birth identified SCFAs [163]. SCFAs are primarily produced in the large intestine and used as an energy source by enterocytes. These fatty acids are crucial in maintaining the balance between pathogenic and nonpathogenic microbes by activating free fatty acid receptors (FFAR2/GRPR43, FFAR3/GPR41) on intestinal epithelial cells, which lead to mitogen-activated protein kinase signaling and rapid production of chemokines and cytokines involved in protective immunity [164]. Furthermore, SCFAs are able to act on GPR43 or HCAR2/GPR109A receptors to mediate oral tolerance as well as inhibit histone deacetylases (HDACs) and modify gene expression by enhancing histone acetylation [164,165], especially in FOXP3 (a gene encoding a transcription factor for Tregs development), stimulating its expression and production in tissues [162,166].

In a murine study, Nakajima et al. showed that a high-fiber diet in mothers increased SCFA and Treg levels in offspring as compared with no-fiber diet−fed mice [167]. A recent study documented that dietary fiber fermentation and propionate production dampened allergic airway disease in mice via changes in the composition of gut and lung microbiota [168]. It was reported that gut microbiota metabolized fiber increased circulatory SCFAs and protected against allergic inflammation in the lung, while a low-fiber diet decreased SCFAs levels and increased allergic airway disease (as reflected by increases in IL-4, IL-5, Il-13, and IL-17, IgE in lung tissue) [168]. Of note, treatment of mice with propionate caused changes in bone marrow hematopoiesis that lead to activation of macrophage and dendritic cell precursors and infiltration of the lungs by these cells, although there was impairment in promoting Th2 cell effector function [168]. These results showed that dietary fiber and SCFAs can shape the immunological environment of the airways and influence the severity of allergic inflammation.

Thorburn et al. reported that mice fed a high-fiber diet developed a selective environment conducive to acetate-producing microbiota [169]. The high-fiber or acetate diet suppressed airway inflammation in mothers as well as in offspring (as evident by a reduction of eosinophils in bronchoalveolar lavage fluid and blood, Th2 cytokines and IFN-γ, IgE, mucus-secreting cells, and airway hyperresponsiveness) suggesting a transgenerational epigenetic effect brought about by the transfer of maternal microbiota at birth to offspring [169]. More specifically, high concentrations of acetate resulted in increased activity of Treg cells by enhancing histone acetylation of the FOXP3 promoter mediated through HDAC9 inhibition [169]. Folkerts et al. showed that treatment of human and mouse mast cells with butyrate and propionate caused a reduction in activation and degranulation of mast cells, facilitated by HDAC inhibition and peroxisome proliferator−activated receptor (PPAR) pathways [170]. In addition, butyrate inhibited human mast cell activation by downregulating genes involved with tyrosine kinases BTK (Bruton tyrosine kinase), SYK (spleen-associated tyrosine kinase), and LAT (essential for T cell activation), but induced upregulation in global histone H3 lysing 27 (H3K27) acetylation, as well as decreased locus-specific H3K27 acetylation at the promoter regions of genes coding for BTK, SYK, and LAT [170]. Similarly, Wu et al., in a mouse study, reported that commensal bacteria stimulated HDAC3 activity through phytate fermentation and production of inositol-1,4,5-triphosphate [171]. Intestinal exposure to inositol-triphosphate and phytate activity

promoted epithelial repair in the gut [171]. This study exhibited that inositol-triphosphate is a microbiota-derived metabolite that activates histone deacetylase to promote epithelial repair in mammals [171]. Comparably, folate synthesized by *Lactobacillus* and *Bifidobacterium* species increased DNA methylation and gut mRNA N6-methyladenosine, which is essential for normal intestinal development. While via butyrate production, Clostridial cluster, *Anaerostipes*, and *Eubacterium* enhanced histone acylation modification, which promotes gut development and immune balance [172]. Therefore, dietary nutrients are pivotal in shaping gut microbes, and the microbial metabolism of these components regulates epigenetic functions that influence host, maternal, and offspring health or disease.

In light of the aforementioned, much research has been undertaken in mice on the epigenetic capacity of SCFAs in Treg induction, but there is a lack of evidence from human studies. To our knowledge, one contemporary study conducted by Hu et al. used SCFA to induce human regulatory T cell (iTregs) synthesis in vitro [173]. In this study, naïve non-Treg CD4 cells were isolated from the peripheral blood of 13 healthy adults and the cord blood of 12 newborns. Cells were induced to differentiate into iTregs using a transforming growth factor β based-protocol with SCFA (acetate, butyrate, or propionate) or without SCFA. Results showed that SCFA enrichment resulted in the in vitro differentiation of human naïve CD4+ non-Tregs to iTregs and inhibited the suppressive capacity of iTregs. Differential activation patterns were observed in the generation of human iTregs in adult and newborn cord blood, independent of SCFAs. In addition, histone H4 acetylation at Treg loci was highest in cells treated with butyrate or propionate. Comparably, the expression of markers CD39, GITR, ICOS, and PD-LI was higher in butyrate-treated iTregs. This innovative study will set the foundation for Nutrigenomics as a potential tool for targeted, personalized nutrition therapies aiming to generate and enhance Treg cell activity using the SCFA approach. The utility of Tregs induction in the prevention or attenuation of allergic symptoms is to be elucidated in future clinical trials.

18.1.7 Dietary patterns

In real-life situations, during meals, individuals consume a variety of nutrients and food groups. Therefore, dietary pattern analysis may provide a more comprehensive picture of an individual's overall intake and add further insight into the relationship between diet and chronic disease risk [174]. Intervention studies manipulating dietary patterns have been more effective at improving asthma outcomes than individual nutrient supplements [175]. The Mediterranean diet is one of the most researched dietary patterns that has been repeatedly shown to have a prophylactic effect against the development of chronic disease (including asthma), promising overall good health, well-being, and longevity [176]. The traditional Mediterranean diet can be described as a healthy dietary pattern and way of life customary to countries surrounding the Mediterranean Sea during the 1950s [177]. Common to all of these Mediterranean populations is the elevated intake of seasonal, locally grown fruits, vegetables, wild salad greens, wholegrain cereals, legumes, olives, and virgin olive oil as the principal source of fat; the moderate intake of dairy products (mainly fermented products such as yogurt, sour milk and feta cheese); eggs and wine usually with meals; and the limited intake of meat and poultry. Fish is consumed by populations dwelling close to coastal regions [177]. The health benefits of the Mediterranean dietary pattern are attributed to the high intake of fiber, vitamins (A, C, E and D), minerals (magnesium, calcium, and potassium), methyl donors (folate, betaine) and antioxidants (selenium, glutathione peroxidase, superoxide dismutase, polyphenols, carotenoids, anthocyanins,

lycopene, resveratrol and phytochemicals); monounsaturated fat (oleic acid), probiotics, omega-3 fatty acids (ALA, EPA and DHA), bioactive molecules, and the optimum ratio of omega-6 to omega-3 fatty acids, which collectively interact synergistically conveying desirable prophylactic effects [123,178,179]. The main active components of olive oil oleic acid, phenolic derivatives (hydroxytyrosol, tyrosol, oleuropein, and ligstroside), and squalene all have potent antioxidant activity [180] and prevent oxidative DNA damage [181]. Notably, allergen-induced oxidative stress in the airways triggers asthma onset [182].

Despite the fact that the Mediterranean diet is acknowledged as a sustainable, ecologically friendly dietary pattern promising health benefits [183], it is being abandoned by the younger generation [184] in exchange for a Western-type diet characterized by a high intake of saturated fats (from junk food), refined grains, sugar (in the form of sweets and sugar-sweetened beverages), and salt with reduced consumption of fresh fruit, vegetables, and fish [157]. Adherence to an unhealthy dietary pattern consisting of fast food, burgers, and butter has been implicated as one of the agents contributing to the global rise in childhood asthma prevalence over the past three decades [7,185,186].

In contrast, accruing evidence from systematic reviews and metaanalyses suggests that adherence to the Mediterranean dietary pattern is associated with decreased wheezing in infants [187] and asthma in children [187−189]. We conducted a systematic search of the literature to evaluate the effect of the Mediterranean diet in childhood asthma. Data from 15 studies, 103,248 children/adolescents up to 18 years revealed an inverse association between Mediterranean diet adherence and childhood asthma [189]. As explained before, a plausible explanation for these asthma beneficial effects could be attributed to the high intake of antioxidants, probiotics, monounsaturated fatty acids, and omega-3 fatty acids, whereby these bioactive molecules, when combined in the context of a dietary pattern, might act synergistically providing respiratory-protective effects, optimum lung function and strengthening of the immune system [174,190,191]. Nevertheless, given the high heterogeneity among study methodologies and the fact that the vast majority of studies were of a cross-sectional design, there is a need for more randomized controlled trials to provide concrete evidence on the causal relationship between Mediterranean diet adherence and pediatric asthma.

With regard to maternal adherence, contemporary data from 14,541 pregnant women participating in the large population-based birth cohort, the Avon Longitudinal Study of Parents and Children (ALSPAC), documented that maternal adherence to the Mediterranean diet was associated with increases in FEF 25%−75%, biomarker of small airway caliber in children at 7−9 years [192]. However, no associations were observed for asthma or allergy outcomes. Comparatively, we recently demonstrated that adherence to a Mediterranean diet enriched with fatty fish for 6 months reduced airway inflammation by 14 ppb in mild-asthmatic children [139]. Amazouz et al., using data from the PARIS population-based study, reported that high adherence to the Mediterranean diet was associated with lower asthma risk and higher FEV_1 and FVC (measures of central airway function) in 975 French children at 8 years [193].

Papadopoulou et al., using cohort data from 2023 Greek children (9−10-year-olds) participating in Phase 2 of the International Study of Asthma and Allergies in Children (ISAAC), reported that adherence to a proantioxidant diet (consisting of vegetables, fruits, fresh juice and fish) was inversely associated with asthma as compared with those consuming a nonantioxidant diet (meat, burgers) [194]. Thanopoulou et al. documented that adherence to the Mediterranean diet (as measured by the KIDMED score) was associated with lower levels of proinflammatory cytokine (IL-17) in 34 Greek asthmatic children [195].

In the same direction, Douros et al. found that the KIDMED score was inversely associated with proinflammatory biomarkers (IL-4, IL-17) and positively associated with IL-33 in Greek asthmatic children versus healthy peers [196]. IL-33 levels are increased in asthmatics and are associated with hyperresponsiveness, airway remodeling, and disease severity [197]. Th2-driven IL-33/ST2 activation amplified IgE synthesis from mast cells, eotaxin production, histamine release, and increased tryptase activity in the airways and lung parenchyma [197]. These findings imply that Mediterranean diet adherence was associated with reductions in airway inflammation but promoted disease severity in pediatric patients. The negative association between the Mediterranean diet and IL-4 indicates that this dietary pattern can inhibit the production of IL-4, which is responsible for Th2 synthesis and IgE production from B cells [198], thus preventing airway inflammation. Correspondingly, the negative correlation between the KIDMED score and IL-17 suggests that the Mediterranean diet exerts antiinflammatory effects by reducing IL-17 levels. This is significant because IL-17 activates IL-33-induced airway hyperresponsiveness by enhancing neutrophilic asthma [199]. Regarding the positive association between IL-33 and the KIDMED score, mouse models reveal that IL-33 responses of mast cells were not associated with the development of airway hyperresponsiveness [199].

Convincing evidence suggests that olive oil is capable of acting at an epigenetic level even during the intrauterine stage of life [148]. It is well established that the maternal diet mediated by the placenta influences gene expression and epigenetic programming in the fetus. Acevedo et al. reported that mothers' olive oil consumption during pregnancy increased H3 histone acetylation at FOXP3, IL10RA, and IL7RA promoter regions of placentas, genes associated with immune regulation [148]. As mentioned earlier, gene expression is regulated by alterations in chromatin arrangement, activating/or deactivating genes associated with both physiological and pathological processes. Molecular studies have demonstrated that olive oil polyphenols oleuropein and hydroxytyrosol increased H3 and H4 histone acetylation by downregulating histone deacetylase (HDAC) inhibitors HDAC2, HDAC6, and the lysine-specific histone demethylase 1 (LSD1) [200]. The concept that olive oil consumption during pregnancy could prime the fetal immune system toward an antiinflammatory Th1 phenotype protecting against allergic diseases, including childhood asthma, is of great interest and a hypothesis worth investigating in future randomized trials.

In another Mediterranean study, Cazzoletti et al., using data collected from 871 Italian adults (20−84 years) participating in the population-based GEIRD Study (Environment Interactions in Respiratory Disease), showed that high dietary intake of monounsaturated oleic acid and olive oil was associated with lower risk of current asthma [201].

Comparable data were reported by Castro-Rodriguez et al., in a Spanish cohort study evaluating the effect of a Mediterranean diet enriched with olive oil during pregnancy and wheezing in the first year of life in offspring [202]. In the univariate analysis, maternal adherence to a Mediterranean diet plus olive oil was significantly associated with less wheezing in offspring at 1 year. However, in the multivariate analysis, only maternal olive oil intake during pregnancy significantly reduced wheezing in offspring during the study period [202]. In a school setting, Kim et al. showed in a cross-sectional study of 1014 Swedish children (5−14 years) that olive oil consumption was associated with less doctor-diagnosed asthma, in fact, 53% lower odds [203].

In reference to eosinophilic inflammation, Agra et al. demonstrated in ovalbumin-induced mice that uvaol treatment (a triterpene present in olives and virgin olive oil) decreased accumulation of eosinophils and mucus production in airways along with IL-5 levels in pleural effluent and bronchoalveolar fluid [204]. IL-5 plays a key role in eosinophil infiltration in atopic inflammation. These

findings indicate the antiinflammatory activity of uvaol in inhibiting eosinophil influx and IL-5 production in ovalbumin-induced allergy in mice.

Despite positive associations documented in epidemiologic data, there is limited robust evidence to support the use of antioxidant supplementation in asthma. Up till now, most studies have used isolated antioxidants with disappointing results [205,206]. This has led to the use of dietary patterns to investigate diet-asthma associations. Indeed, the notion that the Mediterranean dietary pattern could be used as nutrition therapy for pediatric asthma is remarkable. Future longitudinal studies are warranted to test the efficacy of the Mediterranean diet in improving and maintaining asthma control as compared with conventional pharmacotherapy. Given that the available evidence is derived from studies conducted in Mediterranean regions, more intervention studies are needed to investigate if the promising protective effects of the Mediterranean diet and olive oil in asthma can be translated to non-Mediterranean populations.

Oxidative stress and inflammation in airways play a central role in asthma pathophysiology contributing to airway smooth muscle contraction, mucus hypersecretion, epithelial damage (epithelial shedding, vascular exudation), and induction of airway hyperresponsiveness [182]. A plausible explanation for the prophylactic effect of the Mediterranean dietary pattern in childhood asthma might be mediated by the high antioxidant content of fruits, vegetables, legumes, cereals, and virgin olive oil, which can attenuate airway epithelial damage caused by free radicals [175,180,194,196]. Existing evidence from biological studies has documented that antioxidants are capable of downregulating airway inflammatory responses by reducing reactive oxygen and nitrogen species, thereby inhibiting lipid peroxidation and subsequent onset of asthma symptoms [191,194]. Furthermore, the high content of omega-3 fatty acids found in fatty fish activates the synthesis of EPA-derived eicosanoids and bioactive molecules (resolvins, maresins, and protectins), which have antiinflammatory and inflammatory-resolving effects [207], thus improving lung function, decreasing airway inflammation, and preventing asthma exacerbations [128]. An omega-6 to omega-3 ratio of 5:1 was found to be beneficial in patients with asthma [179]. In addition, the immunomodulatory effects of vitamin D obtained from fatty fish consumption and ample sun exposure in Mediterranean countries would optimize lung function [108].

From an epigenetic perspective, as stated earlier, foods typical of the Mediterranean diet are rich in omega-3 fatty acids, vitamin D, folate, SCFA, nonpathogenic gut microbiota, and antioxidants that can influence DNA methylation and histone modifications directly affecting gene expression by activating or suppressing mediators involved in PUFA synthesis including the immune and inflammatory cascade [66,89,118,119,140–142,144,147,149,159,166,169,170,172,175,194,196]. Previous epigenetic studies undertaken in cancer have reported that phytochemicals, active components of fruit, cruciferous vegetables, and legumes (epigallocatechin gallate, resveratrol, quercetin, genistein, lycopene sulforaphane, and isothiocyanates) can modulate inflammatory and immunological mechanisms by interrupting enzymatic activities of DNMT, HDAC, HAT, and HDAC sirtuins [208–214] and miRNA regulation [215].

More specifically, in renal patients, supplementation of virgin olive oil and long-chain omega-3 fatty acids for 8 weeks induced alterations in methylation patterns at CpG loci involved in PUFA biosynthesis in peripheral blood mononuclear cells [142]. Moreover, production of SCFAs from fiber by intestinal microbes suppressed colorectal cancer cell proliferation by affecting DNA methylation [216,217]. Butyrate treatment of colorectal carcinoma cells activated reexpression of apoptosis-associated speck-like protein (a proapoptotic signaling factor), which induced apoptosis of colorectal carcinoma cells, cell cycle arrest, and DNA damage, most likely by promoting transcriptional

activation and reduced catalytic activity of DNMT 1 [216,217]. In vivo, polyphenols derived from virgin oil inhibited hydrogen peroxide-induced DNA damage in human peripheral mononuclear cells and promyelocytic leukemia cells (HL 60) [181]. In animal studies, rats fed selenium-rich diets induced DNA hypomethylation in the liver and colon. With respect to humans, selenium treatment caused promoter DNA demethylation and reexpression of GSTP1, decreased histone deacetylase activity as well as increased acetylation of H3K9 and decreased methylation [218]. Interestingly, selenium deficiency has been linked to global hypomethylation and promoter methylation of tumor suppressor genes [219].

Even though the exact mechanisms have yet to be defined, these findings suggest that the components of the Mediterranean diet can influence the epigenetic profile of an organism. Future studies are urgently needed to confirm whether the protective anticarcinogenic properties of antioxidants can be translated to asthma.

18.2 Discussion

Given the global epidemic rise in asthma over the past 40 years, there is an urgent need to uncover critical molecular pathways that lead to disease development and progression. The identification of putative signatures that can be used as prenatal biomarkers to predict asthma in infants, before the onset of symptoms, has great significance in reducing asthma burden in pediatric patients. During intrauterine and early life, there is a high level of plasticity in T cell maturation and development. The perinatal period is skewed toward Th2 adaptation favoring asthma induction. Therefore, normal immune development depends on switching to a less toxic Th1 response. The concept that nutritional factors could trigger epigenetic mechanisms that perpetuate toward a Th1 response and maintenance of Th1−Th2 balance could prevent asthma progression and reduce risk susceptibility is indeed fascinating. This review illustrated the potential of nutrients that could be utilized as a part of an epigenetic antiasthma-diet implemented preconception, during early childhood, and beyond. Nutrigenomics could be a promising remedy for a condition that inflicts populations of all ages, especially children. Compared with the adverse effects of pharmacotherapy, modification of diet in a real-life family setting is practical, nonevasive, cheap, and of low-patient burden. Currently, there are no biological biomarkers of predictive value, including IgE [220] and cytokine levels, which are reliable markers for asthma onset [220]. Epigenetics, the study of gene−environment interaction, offers an alternative, attractive approach for diseases with an inflammatory background, namely asthma (Fig. 18.3).

18.2.1 Strengths/limitations

With advances in the discipline of -omics and novel pathogenic mechanisms, the identification of putative biomarkers relevant to asthma has given more insight on the biochemical pathways underlying asthma pathobiology and most importantly, the transition from the predisease to the active state [221]. Epigenetics has important future therapeutic implications since identification of asthma-specific biomarkers and molecular mechanisms provides direction for effective treatment, thus paving the way to personalized, nutritional, and pharmacotherapies. The identification of potential targets for high-risk patients and severe-asthma phenotypes in whom current standard treatment is not always successful in maintaining adequate asthma control will improve symptom management of these patients.

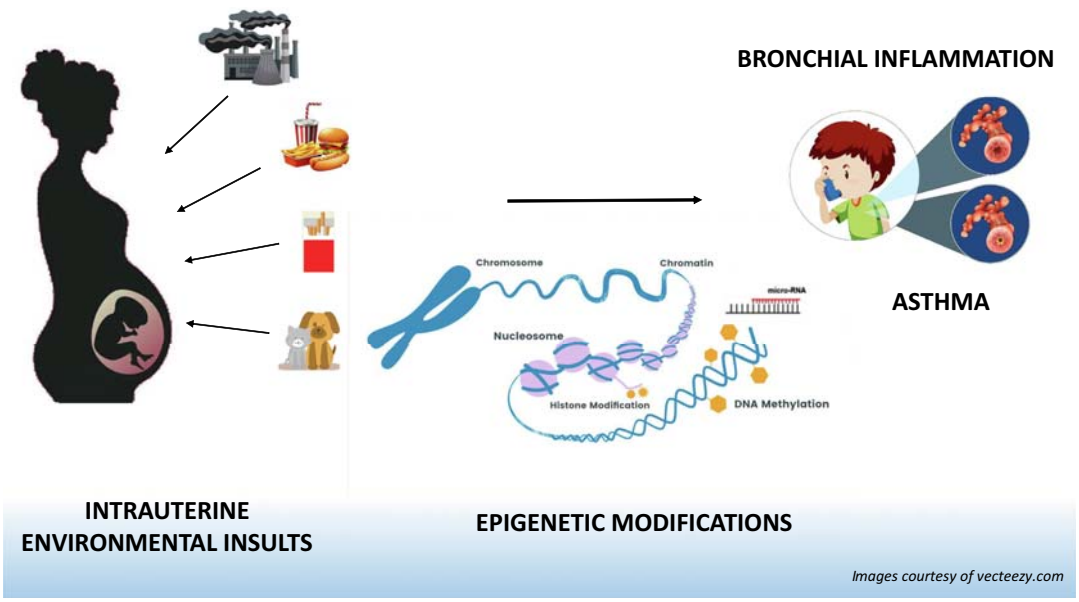

FIGURE 18.3

Epigenetic mechanisms and asthma development in childhood.

Contemporary evidence supports that the "first 1000 days of life" is a critical window during which detrimental external insults may affect epigenetic regulation shaping fetal developmental and health throughout the life span. Over the past few years, despite the growing attention in the research community, to our knowledge, no strategies or policies have been developed or implemented based on the fetal origins of disease theory. A possible downfall is that it is unclear whether aberrant DNA methylation features found in cord blood cells reflect methylation in lung tissue or diseased lungs. Therefore, these findings may not be accurate in predicting asthma risk in patients. Furthermore, DNA methylation patterns and gene expression are dynamic and vary throughout the life cycle. Nonetheless, the current evidence offers insight from an etiological point of view. One other source of bias, DNA methylation marks are sex [222] and age [223] dependent, implying sex- and age-specific epigenetic responses to prenatal insults might serve as biomarkers of disease propensity. Exploration of molecular pathways using the epigenetic model approach, could potentially clarify the mechanistic bridge between exposures, epigenetic variability, and respiratory disease pathogenesis.

In light of future implications, establishing epigenetic traits correlated with gene expression activating eosinophils in asthma would add further knowledge in the molecular pathways leading to the progression of asthma symptomology. Most of the promising data published are based on in vitro, animal, and observational studies. Clearly, more multiomics studies are necessary to deepen our understanding of asthma development during childhood. However, before this promising science is applicable on a universal scale, gaps exist in our knowledge of the human metabolome, the absence of standardized methods for network prediction validation, including lack of randomized trials investigating the clinical utility of epigenetic signatures in human asthma research.

18.3 Conclusion

The global rise in asthma prevalence is a growing public health issue. Dietary intake is a modifiable environmental exposure that could partially explain the current burden of asthma in all ages. Epigenetics, a rapid evolving science, has emerged as an attractive mechanistic link between environmental insults and asthma pathogenesis. Dietary modification via personalized nutrition therapy targeting nutrients involved in DNA modifications may be a practical and feasible public health initiative reducing asthma burden on a world scale. Future studies are warranted to confirm whether nutrients that act as methyl donors can optimize respiratory health and prevent asthma development and onset of symptoms.

Highlights

- The global rise in asthma prevalence is a growing public health issue.
- Dietary intake is a modifiable environmental exposure that could partially explain the current burden of asthma in all ages.
- Epigenetics, a rapid evolving science has emerged as an attractive mechanistic link between environmental exposures and asthma pathogenesis.
- Future studies are warranted to confirm whether nutrients that act as methyl donors optimize respiratory health and prevent asthma development and onset of symptoms in children.
- Dietary modification via personalized nutrition therapy targeting nutrients involved in DNA methylation may be a practical and feasible public health initiative in reducing asthma burden on a world scale.

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CHAPTER

The role of epigenetics in cardiovascular disease

19

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19.1 Introduction

Cardiovascular diseases (CVDs) are the leading cause of death and disability worldwide [1], as they are responsible for 31% of global deaths (https://www.who.int/en/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)). Over the past decade, despite the progress in diagnosis, prevention, and treatment, the number of people diagnosed with CVDs remained very high [2]. This fact indicates that not all cases of CVD depend on classical risk factors, such as hypertension, diabetes, or increased blood lipids [3]. Furthermore, CVDs might also be linked to genetic variations which differs from genetic mutations, such as those observed in conditions such as familial hypercholesterolemia [4] and genetic cardiomyopathies [5] among which are single-nucleotide polymorphisms (SNPs), whose association with cardiovascular pathologies is very complex and must be integrated with specific risk factors. Nonetheless, all this integrated information does not sufficiently explain the deep penetrance of cardiovascular-associated diseases in the global population, calling for other elements that might explain this global picture. This missing link was first theorized in the middle of the 20th century by Conrad Waddington, a British developmental biologist. Although he did not have any mechanistic ideas, Waddington coined the new term "epigenetic landscape" [6], proposing that on top of genetic elements were biological processes that modulated cell fate [7].

The development of organs is a complex process in which fundamental is the tight and coordinated regulation of gene expression activation and silencing, at both the time and spatial levels in cells that share the same DNA sequence [8]. These events depend on the extreme level of chromatin folding needed to fit the DNA into the nucleus and on its modulation necessary for genes to be either accessible to regulator elements and, thus, be expressed (euchromatin), or tightly packed and inactive

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Epigenetics in Human Disease. https://doi.org/10.1016/B978-0-443-21863-7.00004-4
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(heterochromatin) [9]. The transition between these two chromatin states mediates gene regulation, defining what we mean today by "epigenetics," a term that encompasses heritable changes that, independently from the DNA sequence, modulate how chromatin structure affects gene expression. This phenomenon is fundamentally based on chemical modification of DNA mainly on cytosine and histones, but very recently has grown to include many processes mediated by noncoding RNAs, now included in the wider meaning of epigenetics [10,11].

Currently, our understanding of the epigenetic mechanisms involved in cardiovascular-associated diseases is still limited. However, it is evolving at great speed because of massive improvements in sequencing capacity, so much so that translation to innovative therapeutic programs for cardiovascular-associated diseases may be not too far off [12,13]. Indeed, an increasing number of studies are highlighting the central role of epigenetics in human disease etiology, providing strong evidence on how epigenetics is linked to environmental factors and modulates the onset as well as the progression of pathologies [14]. The study of mechanistic roles in disease development has allowed better identification in individuals of variables that impact gene expression and, hence, pathological susceptibility, giving physicians and researchers of different scientific fields, including the cardiovascular one, further tools [15].

In this chapter, we give an overview of the role of epigenetics as the main nongenetic component of CVD risk, highlighting the role of chemical alteration of DNA and histones and the activity of noncoding RNAs in the development of these diseases.

19.2 Etiology and pathobiology of cardiovascular diseases

Cardiovascular-associated diseases comprise a wide variety of conditions affecting the heart and blood vessels, such as heart failure (HF), atherosclerosis, restenosis, and aneurysm, among others [16]. Vascular disease onset and progression depend on transcriptional alterations occurring in vessel-resident cells endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and fibroblasts as well as modification of the communication of these cells with inflammatory cells that migrate into the injured vessel. Continued exposure to stress stimuli triggers vascular remodeling, with the induction of a disease-prone phenotype and altered intra- and intercellular responses [12]. For instance, EC deregulation leads to endothelial dysfunction [17], and VSMCs undergo massive changes that transition them to a synthetic, migratory, noncontractile phenotype and increase their deposition of extracellular matrix (ECM) [18]. Furthermore, immune cells (in particular, monocytes/macrophages) within the vascular lesions present with pathological differentiation, activation, and polarization states, nurturing progression of the disease [19].

To further complicate matters, these vascular pathologies can, in turn, lead to further problems, such as HF, myocardial infarction (MI), and ventricular hypertrophy. HF is a complex syndrome in which the myocardium cannot meet the circulatory demands of the body. Its progression can be accelerated by comorbidities such as diabetes and other metabolic disorders [20]. Indeed, studies have described an increase in HF prevalence, severity, and mortality with such conditions [21,22]. Moreover, structural remodeling is a major feature of HF, with failing hearts having dilated, thin-walled ventricles. This is caused by spatial rearrangement of myocytes within the ventricular walls, with alterations in cellular dimensions [23]. As a consequence, there is an impairment of contractility, a function also negatively impacted by the development of dysregulated calcium metabolism [24].

Here, we will discuss the role of epigenetics in two specific pathological conditions: atherosclerosis and HF.

19.2.1 Atherosclerosis

Atherosclerosis is a slow, chronic inflammatory disorder of the arteries, with largely unknown causes. It represents the most prominent CVD, responsible for the most common cardiovascular complications, such as MI and stroke [25]. Atherosclerotic lesions are characterized by a persistent accumulation and transformation of lipids, inflammatory cells, VSMCs, and necrotic cellular debris in the intima, located beneath the EC monolayer, which lines the vessel's inner wall and interfaces with blood flow. The lesions (called plaques) primarily occur where branching or bifurcations of the arterial tree are present, as these are areas exposed to turbulent blood flow, which exerts a low shear stress on the vascular wall [26]. Here, low-density lipoproteins (LDLs) accumulate and become oxidized (oxLDLs), triggering disease initiation [27]. In this context, ECs undergo pathological transition, and this drives plaque formation. VSMCs then begin to proliferate, and leukocytes are recruited into the vessel via the expression of adhesion molecules and chemotactic proteins [28]. Furthermore, VSMCs start to migrate, losing their contractile properties and producing crucial chemoattractant mediators (i.e., CCL2 and CCL5), reinforcing the recruitment of monocytes to the lesion site [29]. Multiple inflammatory signaling pathways are also activated, leading to the formation of fatty streaks, which are characterized by cholesterol deposits that accumulate in the cytoplasm of VSMCs and macrophages and under the form of crystals in the ECM [30].

Of importance for clinical practice, plaques are classified and graded on the basis of their heterogeneity in terms of cellular composition. Lesions are considered rupture-prone or vulnerable if they display one or more of the following characteristics: large lipid cores, endothelial denudation, a thin fibrous cap, neo-angiogenesis, intra-plaque hemorrhage, and extensive immune infiltrate [31]. Stable plaques have a thick collagen-rich fibrous cap enriched in VSMCs rather than macrophages, and macro-calcified deposits [32–34].

Epigenetic changes are reversible modifications related to imprinting, lifestyle, and environmental cues occurring throughout lifespan. It is becoming increasingly clear how epigenetic mechanisms might impact on CVD pathogenesis and risk [35]. Specifically, the cell-specific transcriptional profile is finely regulated by epigenetics, and atherosclerosis—like other human pathologies—is associated with the perturbation of the epigenetic landscape, aberrant gene transcription, and, consequentially, abnormal biological functions.

Currently, there are no treatments that revert atherosclerosis progression. Protective therapies aimed at lifestyle modifications and cholesterol reduction are the only ones that may slow down advancement the disease. In advanced cases in which the patients experience symptomatic occlusions, surgical intervention via coronary angioplasty or bypass and carotid endarterectomy is mandatory. However, the effectiveness of this solution is only temporary, as stenosis/restenosis—the formation of obstructions within stents—is prevalent (32%–55%) in the first 6 months after surgery [36]. At the cellular level, restenotic obstructions are mainly reliant on EC activation and VSMC reactivation/dedifferentiation, which are then prone to migrate toward the lesion site, causing rapid neointimal hyperplasia [37]. Thus, better comprehension of epigenetic mechanisms leading to the onset and progression of CVDs such as atherosclerosis could play a major role in ameliorating patient

management protocols through the discovery of epigenetic drugs or the development of innovative strategies.

19.2.2 Heart failure

CVD leading to HF and sudden death is the leading cause of morbidity and mortality worldwide. Many pathological conditions have been shown to contribute to the development of HF, including cardiomyopathies, hypertension, MI, and aortic stenosis, to name a few [38]. In brief, upon disease onset, the heart undergoes pathological remodeling, a phenomenon characterized by cardiomyocyte hypertrophy, fibrosis, and inflammation.

Cardiac remodeling is initiated by extracellular cues leading to an inflammatory response, which in turn results in significant changes to ECM composition and deposition and to the secretion of proinflammatory markers (IL-6, TNFα, CRP) [39]. Prolonged exposure to these inflammatory stimuli results in the development of hypertrophy and fibrosis, leading to impairment of cardiac function as a result of ECM deposition causing tissue stiffening and reduction of cardiomyocyte contractility [40,41]. Development of fibrosis is considered the starting point of several pathologies: initiated by an aberrant inflammatory or stress response, it results in structural and functional deterioration of many organs [42]. The process of myocardial remodeling is characterized by increased protein synthesis and cell size, which in turn compromises the sarcomeric structure of cardiomyocytes, and leads to mitochondrial and metabolic dysfunction [43]. At the molecular level, remodeling is generally characterized by a shift from adult gene expression patterns to one mimicking that of the fetal stage (e.g., increased expression of *Actin1* [*Acta1*], *myosin heavy chain 7* [*Myh7*], *natriuretic peptide A* and *B* [*NppA, NppB*]), resulting in perturbation of the homeostatic state of cardiomyocytes and cardiac tissue [40].

Over the past several decades, progress has been made on multidrug treatments including angiotensin II inhibition, diuretics, and beta-blockers. Such therapies, however, are not always curative and do not allow for the recovery of the damaged myocardium [44]. The limitations of these therapeutic strategies are a result of the still-limited knowledge of the underlying cellular and molecular perturbations that occur during heart disease and HF and, importantly, to the limited ability of the adult heart to renew itself and regenerate [45]. Understanding the basal transcriptional programs that underlie correct cardiomyocyte and cardiac function could potentially be exploited in therapeutic strategies aimed at the reversal of disease-associated transcription profiles.

The transcriptome of a cell determined by the epigenome, i.e., the genome-wide landscape of chemical modifications of DNA and histones and the complex network of mechanisms mediated by noncoding RNAs [46]. Epigenetic dysregulation has been correlated with the onset of CVDs and HF through aberrant changes in DNA methylation and hydroxymethylation, chromatin remodeling, histone modifications, and dysregulation of noncoding RNAs [47]. Dysregulation of epigenetic mechanisms in a cell- and tissue-specific manner has been directly linked to various risk factors associated with the development of CVD and HF, including hypertension, diabetes, atherosclerosis, stroke, MI, and obesity [48,49].

19.3 DNA methylation and cardiovascular diseases

The nucleic DNA of eukaryotic cells is wrapped around histone proteins (designated as H1, H2A, H2B, H3, and H4), forming a complex known as chromatin. The accurate and fine-tuned control and

regulation of gene expression occurs through altering the state of the chromatin: it can be compacted (a form called heterochromatin) to hinder transcription, or only lightly packed (euchromatin) to aid access by the elements the transcriptional machinery, such as transcription factors (TFs). This transition is influenced by multiple factors, including DNA methylation (DNAm). DNAm is the most studied epigenetic modification: it is a dynamic, heritable, and reversible process that is commonly associated with gene repression [50]. This chemical modification of the DNA is regulated by enzymes called methyltransferases (DNMTs) and demethylases [51]. DNAm is a ubiquitous mechanism that happens in specific regions named CpG islands [52], loci where a methyl group derived from S-adenosyl-methionine is bound to the 5-position of the cytosine ring, forming 5-methylcytosine (5mC) [53].

DNMT family members include DNMT1, 2, 3A, 3B, and 3L. DNMT1 is responsible for DNAm maintenance, especially during cell division in a processive manner [51]. Within this process, ubiquitin-like with PHD and ring finger domains 1 (UHRF1) is crucial in the recognition of hemi-methylated DNA regions via a SET and RING-associated (SRA) domain and in the guidance of DNMT1 toward these sites [54]. DNMT2 lacks methyltransferase activity and primarily catalyzes RNA methylation as a transfer RNA (tRNA) methyltransferase [55]. DNMT3A and DNMT3B are involved in de novo methylation in undifferentiated cells, contributing to the formation and subsequent maintenance of DNAm marks [56] (Fig. 19.1).

By contrast, DNA demethylation (DNAdm) is mostly related to gene transcription and expression and can occur via two different processes: active or passive demethylation [57]. The former occurs in either dividing or nondividing cells and requires 5mC reversion via enzymatic activity [58]. The latter, instead, only occurs during the replication of dividing cells and depends on the proliferation rate and the lack of DNMT1 activity [59].

Active DNAdm requires the involvement of a crucial class of enzymes: ten-eleven translocation (TET) methylcytosine dioxygenases are Fe(II) and 2-oxoglutarate-dependent, and oxidize the methyl group of a 5mC to 5-hydroxymethylcytosine (5hmC). Once 5hmC is produced, two separate pathways can lead to complete DNA demethylation: the first includes 5hmC deamination by activation-induced deaminase (AID)/apolipoprotein B mRNA-editing enzyme complex (APOBEC) to form 5hm-uracil (5hmU), which is then repaired by either base excision repair (BER) or mismatch repair (MMR) into naked cytosine [60,61]; the second includes two further 5hmC oxidation reactions that produce 5-formylcytosine (5fC) and 5-carboxylcytosine(5caC), by TET family members, in an enzymatic activity-dependent manner [62]. This is followed by thymine−DNA glycosylase (TDG) activity, which recognizes and excises either 5caC or 5fC from DNA [60] (Fig. 19.1). The DNA repair machinery (e.g., BER or MMR) can recognize these derivatives and further restore cytosines, completing the DNAdm process [63].

DNAm plays a crucial role in the regulation of physiological processes, and aberrant DNAm patterns are now known to lead to multiple human pathologies, including cancer [64]. Furthermore, aberrant DNAm distribution and uncontrolled expression of DNMTs have also been closely linked to the development of CVDs [65,66].

19.3.1 Atherosclerosis and DNA methylation

A growing body of knowledge generated through epigenome-wide association studies (EWASs) is linking atherosclerosis to aberrant epigenetic marks. Several reports indicated how impaired DNAm might induce cardiovascular events. Genome-wide DNA hypomethylation is reported to cause

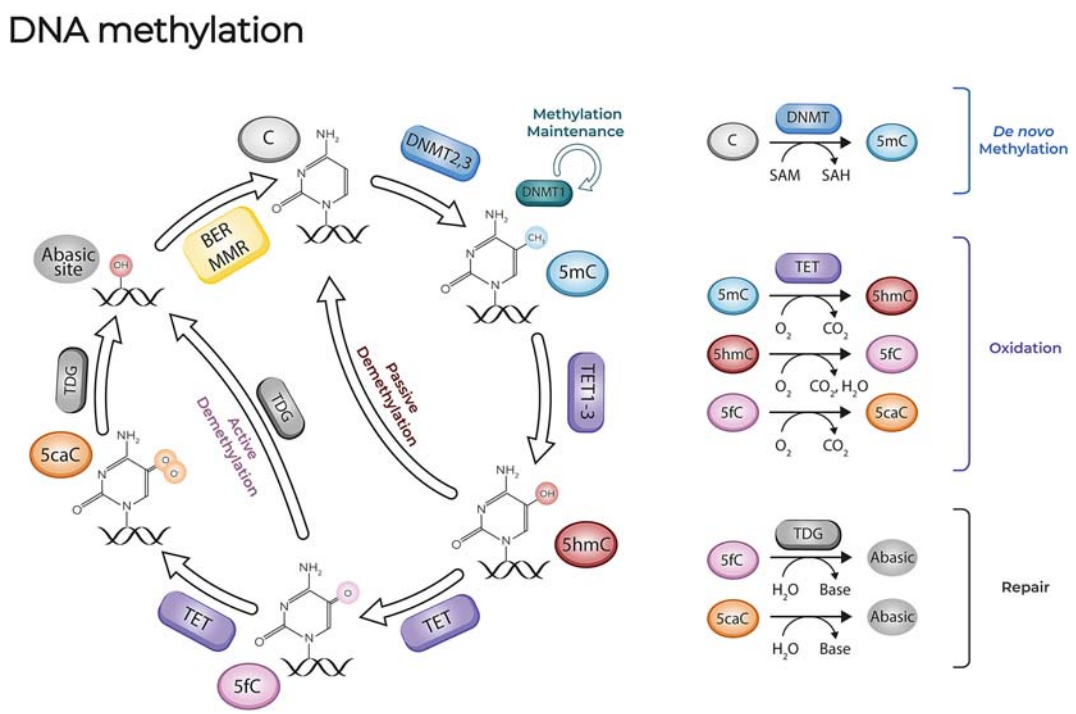

FIGURE 19.1 Illustration of DNA Methylation Effects on Gene Expression

Mechanisms of DNA methylation and demethylation. During differentiation, DNA is subjected to several types of chemical modification. The enzymes DNMT3A and DNMT3B mediate de novo methylation. Upon DNA replication, the newly synthesized DNA strand lacks methylated bases, so DNMT1 is rapidly activated to restore the proper methylation pattern. Adult patterns of methylation are erased by epigenetic mechanisms involving TET enzymes.

genomic instability and structural changes, leading to the expression of pro-atherogenic genes that are meant to be silent [67]. On the other hand, DNA hypermethylation inhibits the transcription of disease suppressor and immune-response association pathway genes, leading to the abrogation of signaling and pro-resolutive responses [68]. Aberrant hypo and hyper-methylation can both affect transcriptional regulation pathways and might lead to atherosclerosis development and CAD. Of note, DNA methylation studies have gained in importance on account of the high variability of methylation levels not only within a given genome but also within different cell types (Fig. 19.2). For instance, an EWAS analysis led to the identification the DNA methylation role in circulating TNF-α concentration, linking these loci to CAD [69]. These information open further innovative opportunities for therapeutic applications.

As described before, endothelial damage can be considered the initial pathological trigger for atherosclerosis development, as oxidative stress, lipid accumulation, and pro-inflammatory-mediator transcription cover critical roles [28]. It is well known how disturbed blood flow induces DNMT1 activity in the intimal and medial layers, in ECs and VSMCs in vitro and in vivo, via the regulation of

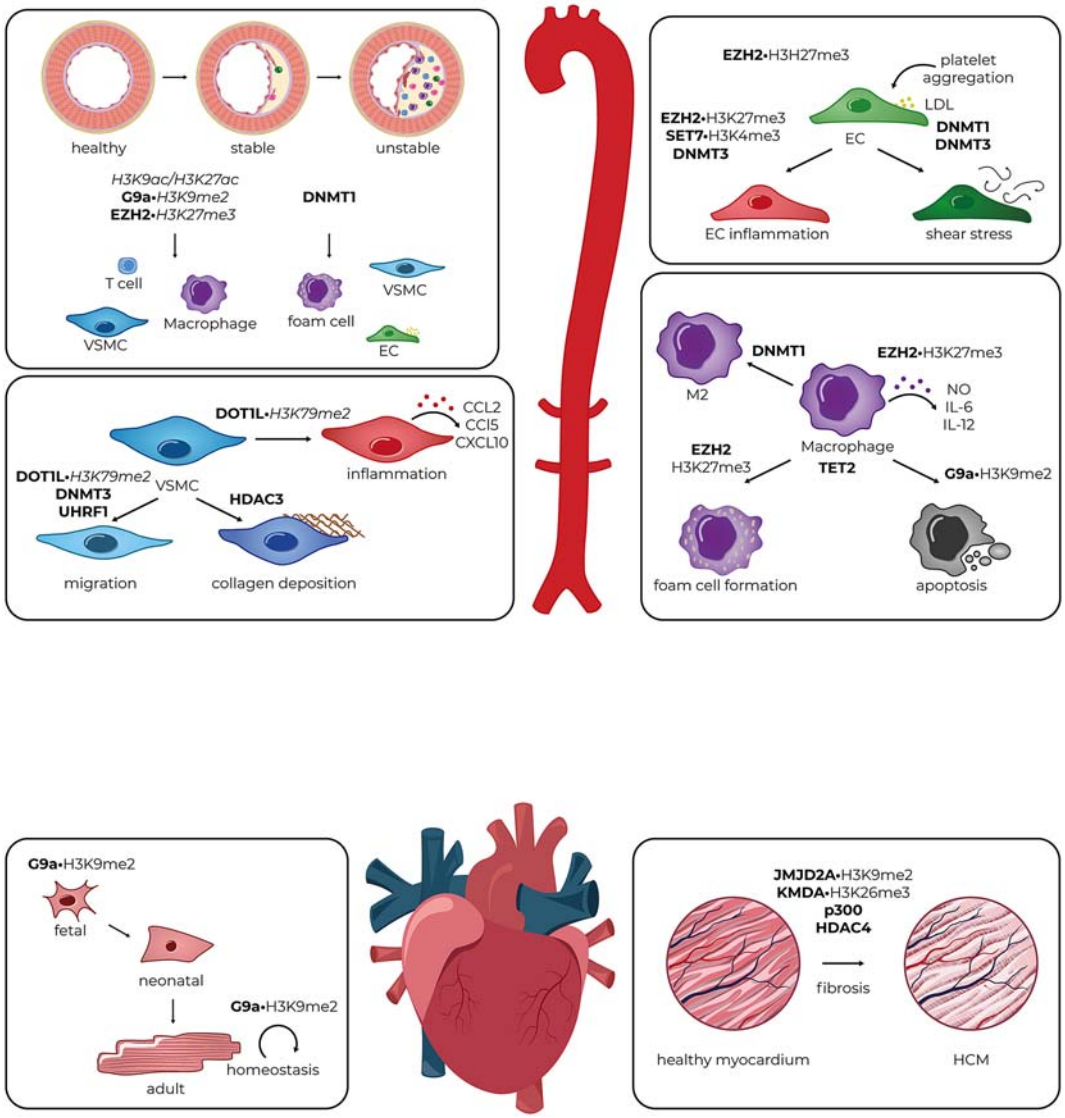

FIGURE 19.2 Illustration Showing Epigenetic Modifications in CVD

Representation of the principal players responsible of DNA methylation and histone modifications involved in vascular and cardiac cell functions, during CVD development.

the maethylation status of the *HoxA5* promoter, thus affecting its expression [70]. In particular, Krüppel-like factor 2 (KLF2) and 4 (KLF4) belong to the zinc finger transcription factor family and play a crucial role in cellular proliferation and differentiation [71]. These two TFs control vasculature integrity and are responsible for endothelial identity. In the context of atherosclerosis, they have been

reported to have an anti-inflammatory role, and experimental data show how DNMT1 could direct target KLF2 methylation status in human umbilical vein endothelial cells (HUVECs) upon oxLDL treatment [72]. Furthermore, KLF4 is a direct DNMT3B target and was reported to affect both inflammatory EC status and endothelial dysfunction during shear stress, in vitro and in vivo [73]. Of note, DNMT inhibition, via 5-aza-2′-deoxycytidine (5-Aza) treatment, was reported to revert endothelial damage and restore aberrant hyper-methylation [72,73].

Oxidative stress refers to accumulation of reactive oxygen species (ROS) in the intracellular compartment. In addition, nutrients and metabolism are crucial keys in CVDs. Methionine is one of the most common residues that can undergo oxidation. Environmental cues and aging are reported to affect metabolites balance, in addition to poor lifestyle and nutritional habits. Plasmatic homocysteine (HCy) is reported to be strongly increased in atherosclerosis patients and is also correlated to the development of unstable plaques [74,75]. Hyper-homocysteinemia (HHCy) can affect either folate or one-carbon metabolisms [76], which are crucial in providing carbon substrates for S-adenosylmethione (SAM) [77]. Therefore, HCy accounts for its high oxidative power, inducing ROS and other superoxide anion release while inhibiting nitrogen monoxide (NO) production [78]. Src homology 2 domain-containing protein (p66Shc) has been reported to promote oxidative stress, leading to endothelial dysfunction and promotion of apoptosis. In particular, increased HCy was correlated to the impairment of methylation level of the *p66Shc* promoter, leading to aortic endothelial dysfunction and atherosclerosis promotion in ApoE$^{-/-}$ mice [79]. This effect was completely reversed by the administration of either a DNMT1 inhibitor or a microRNA targeting DNMT3B mRNA [79,80].

Moreover, HHcy leads to sustained methylation status at the estrogen receptor-α (*ER*α) promoter, which is crucial for vascular homeostasis, oxidative stress, neoangiogenesis, endothelial dysfunction, and osteoblastic differentiation of VSMCs [81–84].

Global DNA hypomethylation was reported to play a critical role also in VSMC phenotypic switch. Balloon denudation of rabbit aortas, as well as advanced patient AS lesions, where intimal hyperplasia was observed, displayed global DNA hypomethylation, suggesting a clear link with VSMC proliferative and migratory properties [85].

Platelet-derived growth factor (PDGF) is one of the growth factors responsible for VSMC phenotypic switch and triggering of cellular proliferation and migration, via the induction of matrix metalloproteinases (MMPs) expression. It has been reported how DNMT3B inhibition, as a direct miR-29b target, induced an impaired methylation status on *Mmp-2/Mmp-9*, leading to aberrant expression and promoting VSMC migration [86].

Similarly, DNA demethylation by TET2 can reduce endothelial dysfunction, reverse the phenotype of VSMCs, affect clonal hematopoiesis, and promote the pro-inflammatory macrophage phenotype within the plaque [87,88]. Reduced methylation was found at the promoters of myocardin (MYOCD), serum response factor (SRF), KLF4, but also genes related to autophagy processes [89]. Due to its ability to impact numerous cell types and biological pathways, TET2 could be a potential innovative drug target for the treatment of atherosclerosis.

On the other hand, DNA methylation was reported to affect pro-atherogenic pathways. As an example, extracellular superoxide dismutase (EC-SOD) is a crucial enzyme for the protection of ECM elements against oxidative stress and damage. It was reported that SOD abrogation via increased DNAm levels induced a pro-atherogenic phenotype and increased inflammatory status [90,91]. Also in this case, DNMT1 inhibition upon 5-aza treatment was effective in reverting this pathological phenotype [91].

Another crucial mechanism responsible for intimal hyperplasia is mediated by UHRF1. This protein bridges DNMT1-driven DNA methylation and chromatin modifications. It may have a crucial role in regulating AS and aberrant proliferation of VSMCs in vitro and in vivo, as a downstream target of PDGF-BB treatment [92].

Inflammatory-driven recruitment of leukocytes within the plaque is a crucial process, leading to plaque instability and poor outcome in atherosclerosis patients. Immune cell types display different DNA methylation patterns and affect the progression of many human diseases via the aberrant expression of target genes. An increased rate of hypomethylated sites was found at promoters of genes involved in the immune response, such as cytokine−cytokine receptors, ATP-binding proteins, and the MAPK signaling pathway [93,94].

Moreover, the abrogation of DNMT1-mediated pro-inflammatory cytokine production in macrophages via the activation of peroxisome proliferator-activated receptor-gamma (PPARγ), one of the most important immune regulators, able to induce macrophage M2 polarization, was able to revert atherosclerosis development in vivo [95].

Overall, extensive evidence in the literature has uncovered multiple mechanisms of action and genes directly modulated by DNA methylation. There are currently no conclusive therapies, but it is becoming increasingly clear that synergistic effects and drug combinations, as well as alternative administration paths, are needed in order to eventually provide a cure for this disease.

19.3.2 Heart failure and DNA methylation

As mentioned earlier, alterations in DNAm has been shown to be key for the pathogenesis of CVDs. Several studies have shown that DNAm patterns are altered in several disease-inducing pathways and can result in various phenotypic alterations (Fig. 19.2); however, its role in HF still remains unclear.

In this context, EWASs have identified additional targets for therapy, providing additional insights into the mechanisms of CVD and HF. An EWAS analysis of the CATHGEN cohort identified six novel methylation sites directly associated with all-cause mortality. Among those, the methylation of the castor zinc finger 1 *(CASZ1)* gene was indicated as central regulatory element associated with mortality in CVD patients [96].

Mouse models of cardiac hypertrophy and HF, such as that generated by transverse aortic constriction (TAC), have been paramount in the study of DNA methylation and its impact on changes in gene expression patterns associated with HF. In general, such studies have highlighted altered DNAm patterns in cardiac hypertrophy and HF; however, the mechanisms still remain unclear. Indeed, one such study showed that the DNA methylation pattern of cardiomyocytes after 3 weeks of TAC shifts to a pattern resembling that of the fetal stage; however, the associated changes to gene expression remain unclear [97]. This shift toward a more fetal-like gene expression pattern has also been observed in the DNAm patterns of the aged heart, although little is known about the consequences in the context of age-related HF [98].

There are conflicting reports on the consequences of altered DNAm patterns in different contexts in HF. For example, one study showed that CpG islands in certain promoter regions were correlated with increased transcription of genes involved in end-stage cardiomyopathies [99]. Other studies have shown that aberrant DNAm leads to repression of key genes associated with maladaptive pathways in HF resulting from dilated cardiomyopathy [100]. Interestingly, one study revealed that prenatal exposure to tobacco severely alters the methylation pattern of the DNA

repetitive element *Alu* Yb8, increasing the risk of development of CVD later in life [101]. In addition, differential DNAm patterns in patients with end-stage HF were correlated to differential expression of angiogenic proteins, such as platelet and endothelial cell adhesion molecule 1 (PECAM1) [99], and one study reported that aberrant methylation can result in a decrease of the heart's capacity to respond to stress [102].

As previously mentioned, DNAm in mammals is catalyzed by the DNMT family of enzymes: DNMT1 is necessary for the maintenance of methylation during DNA replication, whereas DNMT3A and DNMT3B are required for de novo methylation during embryonic development [103]. Conditional knockout of DNMT3B in mouse cardiomyocytes resulted in impaired cardiac function and a phenotype with more severe HF after stress [104]. Interestingly, mice with constitutive knockout of DNMT3A and DNMT3B in their cardiomyocytes did not present with any cardiac phenotype [105]. In contrast, inhibition of DNMTs with the well-known pharmacological inhibitors 5-aza-2-deoxycytidine, 5-azacytidine, or RG108 impeded the onset of HF in mice [106].

DNA hydroxymethylation—a process resulting from the synergism of DNMTs and TET family of proteins—has also gained importance as an epigenetic regulator of transcription. DNA hydroxymethylation is the product of hydroxylation of 5-mC (5-methylcytosine) catalyzed by TET enzymes. High levels of 5-hmC (5-hydroxymethylcytosine) in promoter and enhancer regions have been linked with high levels of transcription [107]. Interestingly, the TET family of proteins do not change in murine models of pressure overload; however, it was reported that upon onset of cardiac hypertrophy, there was redistribution of hydroxymethylated DNA on gene bodies and enhancer regions, resulting in an upregulation of fetal cardiac genes (*NppA, NppB*, *Myh7*) [107].

RNA methylation has also emerged as an important epigenetic mechanism in the control of cellular homeostasis and onset of disease. Just like DNAm, RNA methylation is dynamic and has been largely studied in the context of m6A (N6-methyladenosine), which is the most abundant modification on eukaryotic mRNAs. Two important factors in the regulation of m6A in the context of the heart are methyltransferase 3, N6-adenosine-methyltransferase complex catalytic subunit (METTL3—m6A writer), and fat mass and obesity-associated protein (FTO—m6A eraser). Interestingly, inhibition of METTL3 blocks the hypertrophic response, whereas FTO expression is decreased in failing hearts, reducing cardiomyocyte contractility [108]. The interplay between such epigenetic enzymes in the control of RNA methylation will be an important direction in the understanding of epigenetic mechanisms involved in HF.

It is evident from the literature that there is still much that is unknown with respect to the role of DNAm in the development of HF. The conflicting results from various studies suggest that DNA methylation alone may not be sufficient to cause the changes in gene expression that are responsible for the pathophysiology of HF. Thus, in the future, it will be necessary to consider changes in DNAm patterns along with other epigenetic marks to fully understand the mechanism of aberrant gene expression.

19.4 The histone code and cardiovascular diseases

As described earlier, chromatin consists of a DNA−histone complex. Histone proteins facilitate DNA packing within the nucleus, since two H2A−H2B dimers and two H3−H4 dimers form the core octamer element, also known as a nucleosome, around which exactly 147 DNA base pairs are wrapped [109]. Histones H2, H3, and H4 share a common structure, distinguished by a globular domain from

which protrude flexible domains known as amino(N)- and carbon(C)-terminal tails, depending on their position. In addition, H1 (also known as a linker or H5) participates in nucleosome fiber formation and sealing, and leads to the development of the oligonucleosome [110].

Protruding tails can be targeted by peculiar classes of enzymes that add reversible covalent marks, known as histone posttranslational modifications (HPTMs). HPTMs were originally described in the 1960s, but not until the advent of chromatin immunoprecipitation (ChIP) techniques, and DNA sequencing has become possible to describe them entirely. These include acetylation [111], methylation [112], phosphorylation [113], adenylation [114], ubiquitination [115], and ribosylation of adenosine diphosphate [116].

HPTMs influence chromatin accessibility and, consequently, transcriptional regulation. As previously mentioned, chromatin is characterized by two different states, depending on its degree of compactness: it is defined as euchromatin when its structure is looser and more prone to the action of transcription factors and the transcriptional machinery; heterochromatin, on the other hand, shows a compact assembly, difficult for TFs, and other transcriptional enzymes to reach; therefore, transcription is prevented.

Depending on the amino acid substrate, it is possible to obtain different HPTMs: Arginine (R) can be either deiminated (converted to citrulline) or (mono-, di-, and tri-) methylated [117,118] functions and clinical relevance of arginine methylation. Serine (S) and threonine (T) can undergo phosphorylation or glycosylation (when a carbohydrate is added to a glycosyl acceptor) [119,120]. Tyrosine (Y) can be found phosphorylated, as well [121] (Fig. 19.3). One of the most studied amino acids is lysine (K): it was the first to be found acetylated or methylated [122], but countless other modifications were later discovered, including acylation, biotinylation, crotonylation, formylation malonylation, sumoylation, and ubiquitination, discovered to collectively regulate conversions on this amino acid [123].

19.4.1 Histone acetylation

Histone acetylation is one of the most common HPTMs, and one of the first to be reported. It mainly occurs on K residues of histones H3 and H4. Negatively charged acetyl groups are covalently added by specific histone acetyl transferases (HATs, CBP/p300, MYST, and GNAT) to precise lysine residues on histones H3 and H4. This leads to a reduction in the electrostatic affinity between histone proteins and DNA, resulting in increased chromatin accessibility and in induced gene transcription. Moreover, this chromatin state is accessible to the reader bromodomain-containing protein 4 (BRD4), which plays a crucial role in the regulation of several transcription-mediated biological processes [124,125]. In accordance with its biological role, histone acetylation was preferentially found in specific regions within the promoter and at the 5′ strand of protein-coding genes. As an example, K9 or K27 acetylation on H3 (H3K9ac and H3K27ac) at enhancers or promoters is generally associated with increased gene activity [126,127].

On the other hand, histone deacetylation is conducted by histone deacetylases (HDACs), which induce a more compact nucleosome structure and lead to impaired accessibility and abrogated transcriptional properties [128]. Fine-tuning of HATs and HDACs is crucial for homeostasis, while their impaired function leads to development of diseases, including hypertension [129], MI [130], ventricular hypertrophy [131], and atherosclerosis [132]. Therefore, small molecular compounds targeting HATs, HDACs, or BRDs might provide innovative and resolutive insight into the treatment of CVDs.

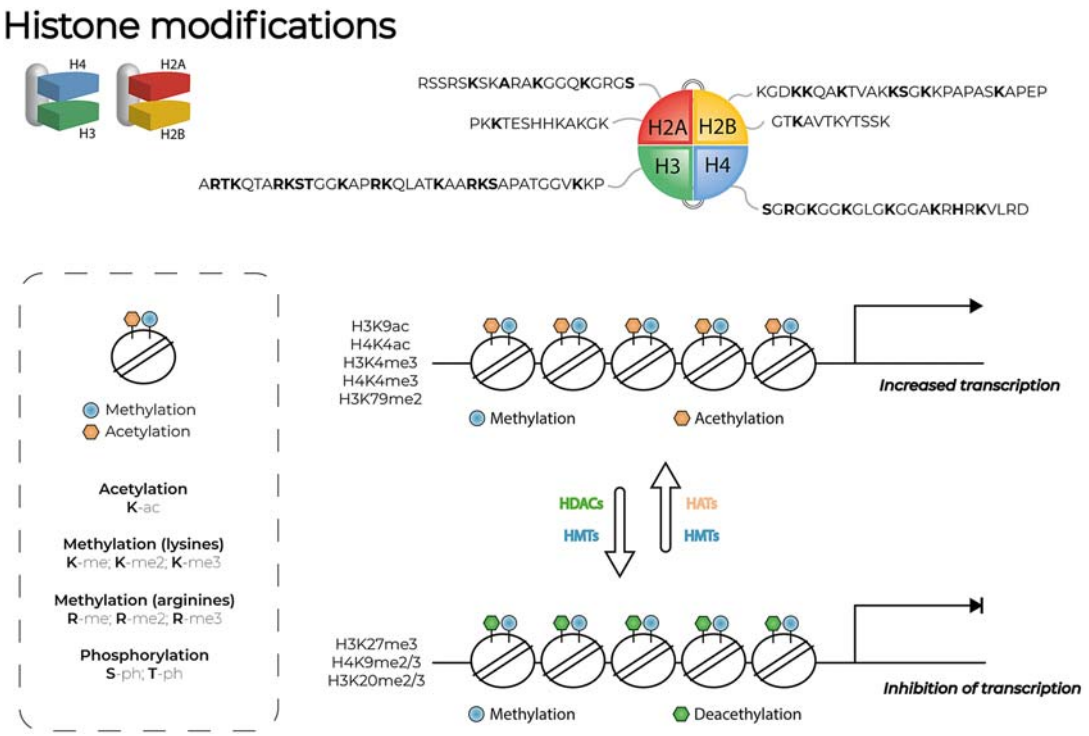

FIGURE 19.3 Illustration of Histone Modification Effects on Gene Expression

The nucleosome is a complex of double-stranded DNA wrapped around a histone protein core. In this context, chemical modifications such as lysine acetylation and methylation on core histones are mutually exclusive. Lysine acetylation is always associated with increased gene expression; lysine methylation indicates gene activation when found at H3K4 or H4K40, but is associated with gene silencing at H3K27, H3K9, or H4K20.

A specific aim is to manipulate their activity to restore abnormal histone acetylation levels. Of note, several HDAC inhibitors have been reported to be beneficial in several CVDs, such as MI [130], atrial fibrillation [133], and cardiac hypertrophy [134]. Nonetheless, there is inconclusive data for atherosclerosis, since HDAC inhibitors are reported to have ambiguous roles in vascular diseases: they were reported to be both beneficial in atherosclerosis development and progression in vivo [135], and detrimental, as HDAC6 inhibition was reported to trigger aortic valve calcification [136].

19.4.2 Histone methylation

Histone methylation, as mentioned earlier, refers to a process in which methyl groups are transferred to specific amino acid residues on histone proteins. In contrast to acetylation, methylation status does not uniquely refer to a specific transcriptional status, with the methylation level on K and R residues inducing different transcriptional programs. Moreover, in terms of genomic location and degree or pattern, methylation can cause different effects on gene activity [137].

H3 is the most extensive histone methylation site, although additional histone cores have been found to be methylated in different loci. Like acetylation, methylation is mediated by crucial enzymes: histone methyltransferases (HMTs) are known as writers; chromodomains (CRDs) or bromodomains (BRDs) represent methylation readers; while histone demethylases (HDMs) are methylation erasers [138]. Their interplay can affect gene transcription and mediate several physiological and pathological processes.

K- and protein R-MTs (KMTs and PRMTs, respectively) accept a methyl group from SAM to produce three different outcomes: namely, mono-, di-, and trimethylation. Among them, di- and trimethylations at H3K4, H3K36, and H3K79 are commonly referred to as transcriptional inducers: H3K4me3 is a promoter-related mark [139], whereas methylations at H3K36 and H3K79 occur within the gene body [140−142].

In addition, monomethylation at H3K4 is an activating mark preferentially occurring at enhancers [142]. Methylation marks present at H3K9 and H3K27 are associated with gene repression [143]. In particular, H3K9me2 mainly occurs at little-expressed genes within euchromatin, while H3K9me3 is associated with heterochromatin [112,144]. On the other hand, H3K27me3 is crucial during development and is considered a reversible and plastic mark, which can act as a switch to finely regulate gene transcription in a time-dependent manner [145].

Methylation erasers (HDMs) remove methyl groups on K and R residues, with the involvement of FAD-dependent amine oxidases [146] and α-KG-dependent dioxygenases [147].

19.4.3 Atherosclerosis and histone posttranslational modifications

Aberrant activities of HPTMs and histone proteins have been reported to play a pivotal role in atherosclerosis development. A large number of genes and target molecules have been studied in the context of histone modification with the aim to develop drugs that reverse or delay CVD progression and, in particular, impact atherosclerosis (Fig. 19.2).

Increased H3K9ac and H3K27ac with a subsequent impairment H3K9 and H3K27 methylation status both affect VSMC and inflammatory cells and were correlated with advanced lesions. Moreover, H3K4 methylation was reported to correlate with atherosclerosis progression on account of the specific activity of crucial HATs and HMTs, such as lysine acetyltransferase 2B (LAT2B), lysine acetyltransferase 8 (KAT8), and lysine methyltransferase 2 and 4 (MLL2/4) [148,149].

Similarly, HHCy was reported to affect enhancer zeste homolog 2 (EZH2), the catalytic subunit of the polycomb repressive complex 2 (PRC2) in vivo, leading to increased H3K23me3 [150].

Contrasting results have been reported in patients, in whom reduced H3K23me3, but without any alteration in EZH2 expression, seemed to be a clear indication of the presence of advanced plaques [151]. Moreover, reduced H3K27me3 triggered an inflammatory EC status via the activation of nuclear factor kappa B (NF-κB) pathways, leading to aberrant adhesion molecules and cytokine production [152]. NF-κB-dependent oxidative and inflammatory signaling was found to be affected by H3K4me in human aortic endothelial cells (HAECs), via the histone methyltransferase Set7 [153].

In addition, LDL was observed as an EZH2 trigger, reducing KLF2 activity and platelet aggregation on ECs [72]. H3K27 methylation was also reported to stimulate foam cell formation in ApoE$^{-/-}$ mice, ameliorating the phenotype [154]. Finally, genetic EZH2 deletion in macrophages has reduced atherosclerotic lesion size in vivo and specifically impaired nitric oxide (NO), interleukin-6 (IL-6), and IL-12 production [155].

H3K9 is a direct target of the euchromatic histone-lysine N-methyltransferase 2 (G9a) writer, which mono- and dimethylates H3K9, and recruits DNMT3A and 3B, which in turn catalyze de novo DNA methylation at the promoter of target genes [156]. HHCy ApoE$^{-/-}$ mice displayed reduced G9a activity and, consequently, H3K9me2 deposition, inducing macrophage apoptosis and leading to plaque instability [157].

Disruptor of telomeric silencing 1 (DOT1L) is the only HMT that mono-, di-, or tri-methylates the nucleosomal histone H3K79 in a nonprocessive manner. It was reported to play an important role in the regulation of cardiac function via the regulation of dystrophin expression [158] and define cardiomyocyte lineage, via its action on genes involved in RNA metabolism, and cardiac specification pathways. Recently, DOT1L abrogation was also reported to critically regulate VSMC phenotype in vitro and in vivo in a model of intima hyperplasia and atherosclerosis [159,160]. In particular, DOT1L, via H3K79me2, regulates NF-κB pathways via direct methylation of inflammatory genes, resulting in leukocyte recruitment to plaques [160].

Moreover, aberrant histone hyperacetylation with consequent reduction of H3K9 and H3K27 methylation marks correlated with atherosclerosis progression and poor prognosis in patients [148].

Additionally, VSMC differentiation was reported to be modulated by HAT activity on serum response factor (SFR) and myocardin (MYOCD) recruitment [161].

Of note, HDAC3 inhibition in myeloid cells promotes collagen deposition by VSMC within the plaque, leading to a more favorable phenotype and promoting plaque stability [162].

Overall, several reports show how pharmacological inhibition of epigenetic enzymes could ameliorate atherosclerosis phenotype, leading to the identification of new therapeutic targets. Among them, HDAC inhibitors can be useful in the regulation of inflammatory status [163], endothelial dysfunction [164], vascular permeability [165], and macrophage polarization [166].

Five HDAC inhibitors (SAHA, LBH589, PXD101, VPA, and romidepsin) are currently FDA-approved for the clinical treatment of cancer [167]. Drug repositioning might pave the way for better management of atherosclerosis patients, lowering the burden of the disease.

19.4.4 Heart failure and histone posttranslational modifications

Several HMTs and HDMs have been described in the context of gene expression regulation in cardiac hypertrophy (Fig. 19.2). One such HDM is lysine demethylase 4A (JMJD2A), which catalyzes the demethylation of H3K9me3 and H3K26me3, and regulates genes mediating the hypertrophic response. Studies on JMJD2A knockout mice reveal that these mice are more resistant to cardiac hypertrophy-inducing pressure overload [168]. Conversely, the HMT G9a (EHMT2) has been shown to play a critical role in cardiomyocyte homeostasis: G9a regulates cardiomyocyte function through deposition of H3K9me2, by interacting with the catalytic subunit of PRC2, the enhancer of EZH2. Interestingly, it was shown that G9a plays an anti-hypertrophic role in healthy cardiomyocytes by repressing the transcriptional activity of myocyte enhancer factor 2C (MEF2C). At basal level, the repression of MEF2C maintains the silencing of fetal genes in the adult heart [40].

Another important post-translational modification of histones is acetylation, which, unlike methylation, is associated with activation of transcription. Histone acetylation has been shown to be an important process governing cardiovascular development and disease, including HF. Key players governing this process are histone HATs and HDACs. The HAT p300 has been widely studied in the context of cardiac development, and it has been shown that deletion of p300 results in embryonic

lethality associated with severe cardiac defects and structural abnormalities. Conversely, p300 overexpression has been associated with cardiac hypertrophy, as it acetylates GATA-binding protein 4 (GATA4) and MEF2C, increasing their binding to promoter regions of fetal genes associated with the hypertrophic response [169]. On the other hand, HDAC4 (a class II HDAC) functions as a repressor of MEF2C activity through its deacetylation and reverts the hypertrophic phenotype [170]. Several other class II HDACs have also been associated with the development of cardiac hypertrophy and HF, such as HDACs 5 and 9, which also negatively regulate the activity of MEF2C, reducing the cardiac hypertrophic response [171].

19.5 ncRNAs and cardiovascular diseases

Although most of the mammalian genome does not encode protein, it does possess a notable regulatory potential. In fact, a great communal scientific effort in the field of noncoding RNAs (ncRNAs) had led to their identification as active players in the gene-regulation machinery [172]. The exponential increase in ncRNAs discovery and characterization due to the new-generation sequencing, novel experimental approaches, and a better understanding of their involvement in CVDs place ncRNAs as good candidates for diagnostic and therapeutic purposes [173–176].

While commonly classified on the basis of size (into small and long ncRNAs) [177,178], alternative ncRNA classifications have been suggested, such as that based on localization (either nuclear or cytoplasmic) at a precise moment of gene transcription [179,180]. Overall, small ncRNAs (sncRNAs; < 200 nucleotides) include regulatory elements, microRNAs (miRNAs, miRs), Piwi RNAs (piRNAs), and YRNAs, as well as steadier expressed housekeeping ncRNAs, such as small nucleolar RNAs (snoRNAs) and transfer RNAs (tRNAs) [181–183]. Generalization and classification are more complex for long ncRNAs (lncRNAs; > 200 nucleotides): these can be grouped and assigned to specific categories according to genomic localization, specific function, or mechanism of biogenesis [184]. Interestingly, circular RNAs (circRNAs) have been identified as a specific group of lncRNAs that derive from back-splicing of a precursor mRNA [185–189]. Moreover, ncRNAs have been found to actively participate in gene regulation by modulating chromatin structure, further increasing the complexity of the epigenetic landscape in vessel biology [190–192].

Several studies have demonstrated the centrality of ncRNAs in a wide variety of pathological scenarios, including those of the cardiovascular system [16,182]. While current knowledge on miRNAs is extensive, further investigation is required to elucidate the contribution of lncRNAs to cardiovascular pathophysiology [172,193].

miRNAs, which are sncRNAs $\sim$ 20 nucleotides (nt) long that regulate gene expression at the post-transcriptional level, have a central role in the regulation of gene expression of many important pathways and biological processes, such as development, differentiation, apoptosis, and cell survival [194]. Thus, it might not be surprising to find that dysregulation of miRNA expression has been directly linked to many diseases, including those of the cardiovascular system [195,196].

miRNAs are first transcribed in the nucleus into a hairpin primary miRNA (pri-miRNA) structure by RNA polymerase (Pol) II. The pri-miRNA is specifically recognized and cleaved by the microprocessor complex DGCR8 microprocessor complex subunit/Drosha ribonuclease III (DGCR8/Drosha), generating a precursor miRNA (pre-miRNAs), which is exported by the exportin 5 (XPO5)/RanGTP complex to the cytoplasm. Once in the cytoplasm, pre-miRNAs are further processed by Dicer, which removes the terminal loop to produce an intermediate miRNA duplex. Remarkably, the

name of the mature miRNA form is determined by the directionality of the strands: the 5p strand comes from the 5′ end of the pre-miRNA hairpin, while the 3p one originates from the 3′ end. The duplex is then further processed by Argonaute (AGO) proteins. Although both strands can be loaded onto AGO proteins, usually the strand with lower 5′ stability or 5′ uracil is preferentially used and is considered the guide strand. The other strand, named the passenger strand, is removed from the guide strand and is eventually degraded. Afterward, the guide strand is loaded onto the RNA-inducing silencing complex (RISC), allowing miRNA:mRNA interaction and promoting recruitment of poly(A)-binding proteins, which shorten the poly(A) tail of mRNAs [196] (Fig. 19.4). mRNA target recognition occurs in a sequence-specific manner, inhibiting gene expression by blocking the translational mechanism or activating nucleolytic mRNA degradation mainly through targeting the 3′-untranslated region (3′UTR) of the target gene [177]. The specific 3′UTR sequence recognized from the mRNA target by the miRNA is known as the miRNA recognition element (MRE) and contains a small region of ∼ 8 nt at the 5′ end called the seed sequence [177,197,198] (Fig. 19.4).

LncRNAs are a miscellaneous group of transcripts with a wide range of actions and that differ for biogenesis, genomic origin, and biological role [174,184]. Mostly, lncRNA synthesis relies on spliceosome-mediated processes via Pol II and Pol III, and produce mature transcripts presenting a 5′-cap as well as 3′ polyadenylated (polyA) tail [199,200]. Hence, lncRNAs are commonly subject to splicing events, being presumably transcribed and processed similarly to mRNAs [199], from which they differ mainly by the absence of translational coding potential.

The resulting mature lncRNAs are low-copy number transcripts with specific subcellular localizations [186,201]. It is worth mentioning that this is a generalization, and several exceptions have been reported. For instance, lncRNAs of intergenic origin differ from the mRNA-like ones due to the lack of a polyA tail. Additionally, a few lncRNAs have been shown to contain functional cryptic open reading frames (ORFs) that may give rise to small peptides [202].

Most lncRNA sequences are poorly conserved among species, yet they present highly conserved structural properties, a fact supporting the possibility of preserved interspecies functionality [203]. Of note, their expression is finely modulated at different stages of development, presenting tissue and cell specificity, indicative of their contribution to gene regulation [178,204]. In addition, lncRNA transcripts can be classified based on their function via *cis*- or *trans*-regulatory circuits [185,205]. By contrast, they can be classified based on their direction with respect to the coding gene, and grouped as sense, antisense, exonic, or intronic. Additional lncRNA classes include a group with bidirectional promoters, the enhancer RNAs (eRNAs), the intergenic RNA (lincRNAs), and the circular RNAs (circRNAs) [206,207] (Fig. 19.5). Commenting on circRNAs, these are generally transcribed by Pol II as a pre-mRNA, like the majority of RNAs [208], but they differ from linear transcripts because of an alternative splicing event (called back-splicing) that promotes the formation of RNA loops: tail-to-head folding uses canonical splicing sites and goes from an upstream 5′ splice site (acceptor) to a downstream 3′ one (donor) [188,189]. Consequently, covalently closed RNAs with neither 5−3′ polarity nor a poly(A) tail are produced. In addition, this structure confers higher stability compared with other lncRNAs, increasing half-life and resistance to exoribonucleases [207,209]. In addition, the RNA loops can be secreted within exosome vesicles in response to specific stimuli and, as a consequence, are detected in body fluids (serum and saliva) [185]. Thus, also circRNAs are potential candidates for human disease prognosis, prevention, and treatment.

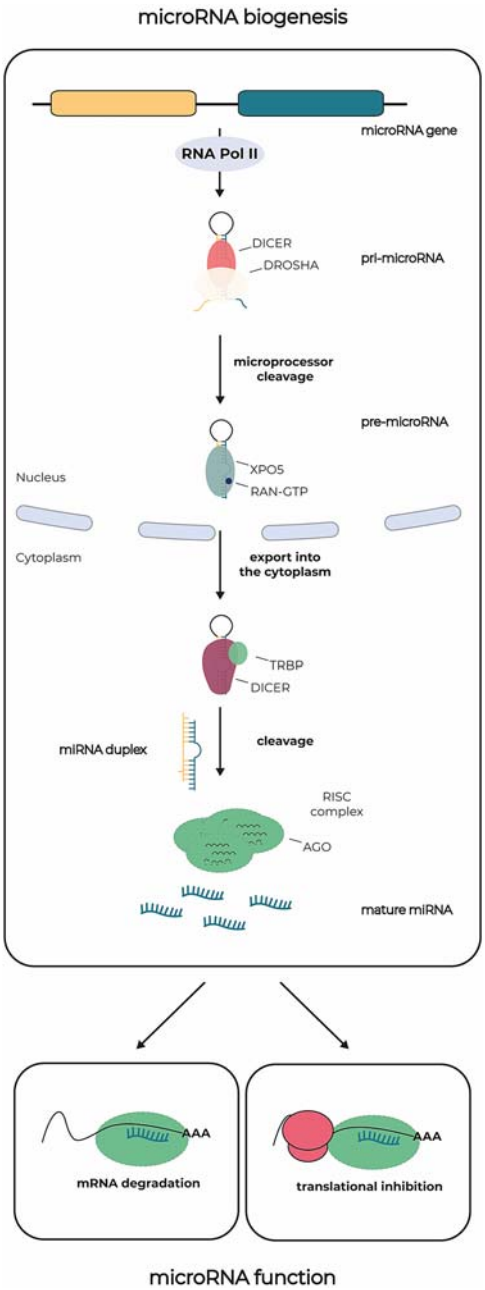

FIGURE 19.4 MicroRNA Biogenesis

A microRNA is transcribed as a pri-microRNA, which is processed first to a pre-microRNA, and then to a mature microRNA, which binds the 3′UTR of target genes, inhibiting gene expression.

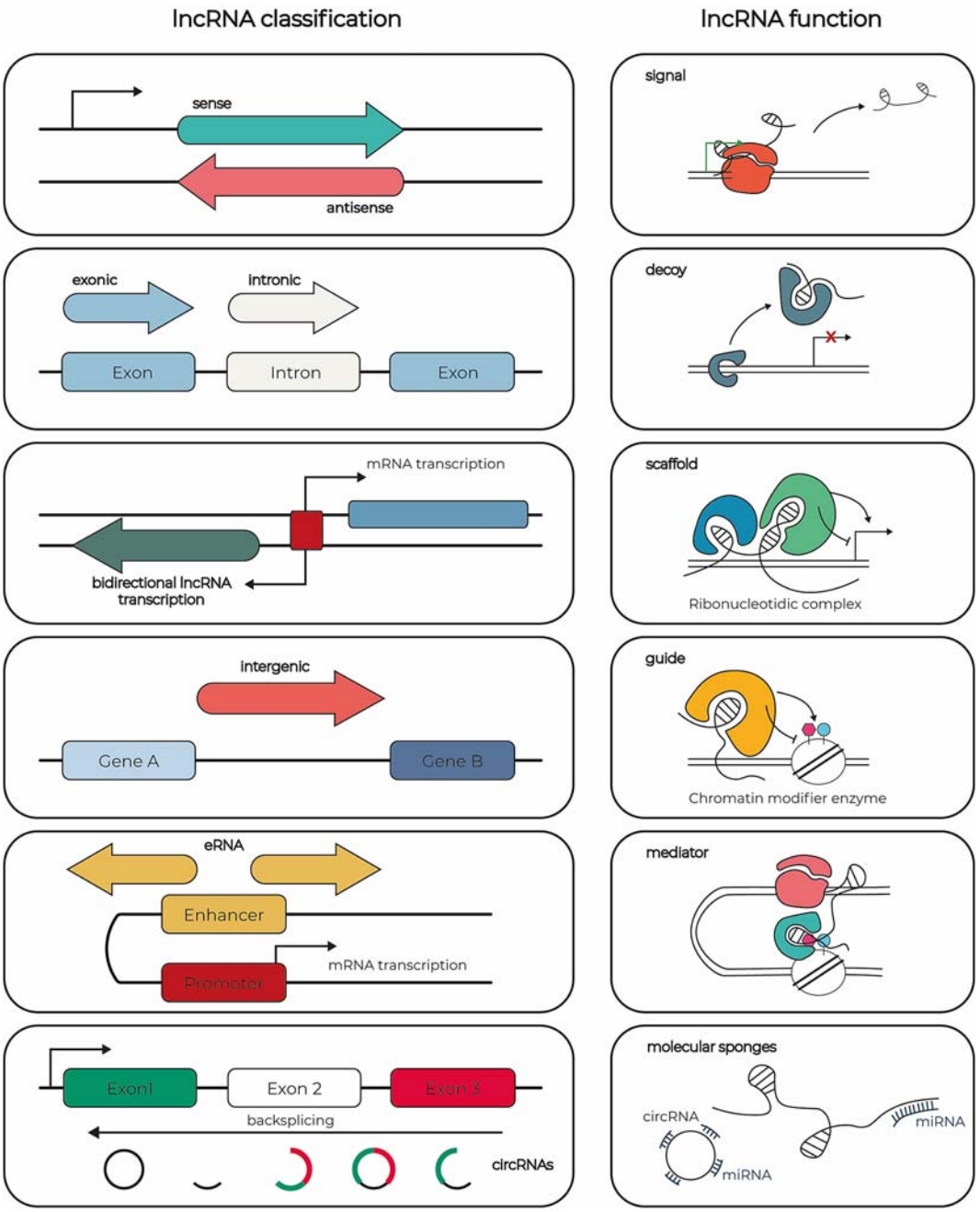

FIGURE 19.5 Schematic Classification of the Different Types and Functions of lncRNAs

LncRNAs classified based on their genomic position: Exons are colored in green, orange, white, and light red, enhancers in yellow, promoters in dark red, and introns in gray. Arrows indicate transcription start sites. Right: LncRNAs modulate gene expression through different mechanisms: in the nucleus, they might guide TFs to promoter regions (1) Or sequester TFs, inhibiting their function (2) They can also modulate chromatin structure by acting as a scaffold (3) Or guide (4).

Our understanding of the importance of lncRNAs in biological systems is increasing relentlessly. Functions found so far include (1) Signaling in reaction to environmental cues at the intra- and/or extracellular level; (2) Decoy, capture, and stalling biologically active molecules to alter chromatin accessibility; (3) Scaffolding to alter biological processes by acting on specific multiriboprotein complexes; (4) Guiding to modulate chromatin-modifying complexes; (5) Mediating to participate in 3D nuclear organization; and (6) Molecular sponging, sequestering active miRNA molecules to inhibit their function and fine-tune the expression of miRNA-modulated targets [184,186] (Fig. 19.5).

This category of ncRNA is now acknowledged as a critical player for the regulation of gene expression at different levels in health and disease, with important impacts on the incidence of human diseases [16,98]. Therefore, a deeper understanding of the potential roles of lncRNAs in vascular health and disease deserves further investigation [172,210].

In conclusion, seminal work in the fields of epigenetics and cardiovascular medicine have shown that ncRNAs such as miRNAs, lncRNAs, and circRNAs could be exploited and harnessed for the development of novel RNA-based therapeutic options. Further studies are needed to overcome the existing limitations. Nevertheless, ncRNA-based strategies represent a valuable tool for the generation of innovative approaches in vascular disease.

19.5.1 Atherosclerosis and miRNAs

Atherosclerosis is a pathology in which there are functional and structural alterations of the vascular wall. The main cause is high levels of circulating cholesterol (hypercholesterolemia), which plays a central role in the pathological activation of vascular cells and the immune system [211,212]. Several other conditions, such as diabetes mellitus, hypertension, aging, and obesity, can accelerate development of the disease process [213]. In most cases, the progression of atherosclerosis leads to carotid and coronary artery disease, which are the primary cause of stroke and heart attack, respectively.

Several miRNAs have been found to be involved during different biological stages of atherosclerosis [214] (Fig. 19.6), including VSMC biology (i.e., contraction, migration) such as miR-21-5p, -34a-5p, -146a-5p, -146b-5p, 210−3p, and -4487, which have been found to be increased in atherosclerotic plaques from the aortic, carotid, and femoral arteries [215,216]. Moreover, specific miRNAs have been found to play roles in the individual vascular cell types (ECs, infiltrating immune cells, and VSMCs). For example, miR-34a, -217, and -146a have been associated with senescence in ECs, and miR-200c, -126, -10a, and -181b have roles in regulating oxidative stress and pro-inflammatory factors [213,217]. Disruption of other miRNAs (i.e., miR-126, miR-31, and miR-17−3p) leads to the dysregulation of adhesion molecules, directly triggering endothelium dysfunction [217]. Moreover, extracellular vesicles play an important role in the delivery of miRNAs from one cell type to another [218−220]. Indeed, ECs have been found to secrete microparticles carrying miRNAs, such as miR-19b, which have an effect in other cellular components within the plaque [221].

During atherosclerosis progression, monocytes/macrophages and VSMCs engulf oxLDLs, leading to the formation of foam cells and activated VSMCs. Macrophages play an important role in several stages during plaque development (i.e., cellular adhesion, lipid uptake, and inflammatory responses), and many miRNAs have been found modulated in this context. Indeed, dysregulation of miR-99b, -320a, -152, -125a-5p, and -155 in monocytes/macrophages influenced the accumulation of foam cells [213,217,222]. Other miRNAs, such as miR-10a and -181a2b2, contribute to the pro-inflammatory process by impacting on EC phenotype during atherosclerosis [223,224]. miR-21 and

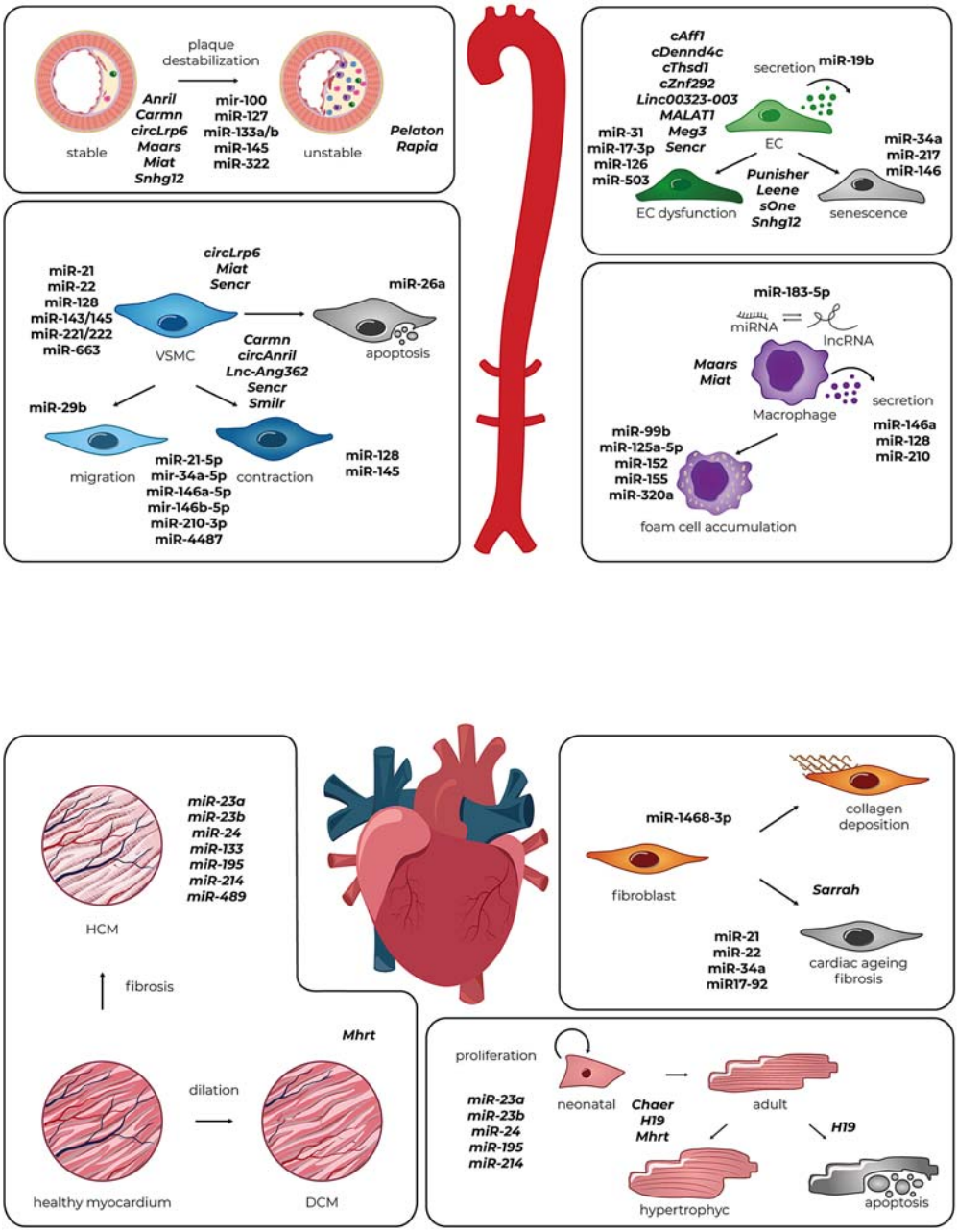

FIGURE 19.6 Illustration Showing ncRNAs in CVD

Representation of the principal ncRNAs involved in vascular and cardiac cell functions, during CVD development.

miR-223 are regulated in monocytes, promoting atherosclerosis progression [225,226]. Furthermore, macrophages secrete extracellular vesicles containing miRNAs (i.e., miR-146a and -128) for transfer to other cell types [226]. Interestingly, macrophages also secrete miRNAs bound to high-density lipoprotein (HDL), such as miR-210, found dysregulated in the plasma of atherosclerotic patients [226].

VSsMC phenotypic switch, which leads to increased migration in the intimal layer, is a critical characteristic of atherosclerotic lesions. Thus, alteration of miRNAs regulating VSMC differentiation has an important impact on disease development [227,228]. A few examples of miRNAs that directly affect atherosclerotic development by changing VSMC phenotype are miR-143/145, -663, -22, -21, -221/222, and -128 [212,213,226,229]; miR-26a, which targets transforming growth factor-β (TGF β), a critical factor for VSMC differentiation and apoptosis, also directly regulates VSMC differentiation [217]; miR-29b targets metalloproteases to affect phenotypical switch by regulating migration; and miR-145 and miR-128 regulate KLF4 to affect contraction [217,230]. Of note, miR-143/145 availability can be modulated by genetic variants, namely rs41291957 (G > A), thus impacting VSMC biology and disease outcome [231]. Finally, increases in inflammatory macrophages and VSMCs lead to plaque destabilization and rupture in the final phase of atherosclerosis. Many miRNAs have been identified to regulate this final step of the diseases: miR-322, -100, -127, -133a/b, and -145 are all significantly more expressed in patients with destabilized plaques [213,226].

19.5.2 Atherosclerosis and lncRNAs

While studies on miRNAs have provided a wealth of information on their role in atherosclerosis, the role of lncRNAs in this disease requires additional investigation. Based on a handful of key studies published up to date, lncRNAs have the potential to affect different stages of atherogenesis by acting on a wide variety of processes, orchestrating the transcriptional programs of all three main cell components of atherosclerotic plaques [204,232] (Fig. 19.6). Many lncRNAs, such as, *AGAP2 antisense RNA 1 (Punisher)*, *long intergenic nonprotein coding RNA 520 (Leene)* and *sOne*, among others, have been found to participate in EC differentiation, homeostasis, and function [233–235]. Several others have been identified in the atherogenic setting, for instance, *human metastasis–associated lung adenocarcinoma transcript 1* (*Malat1*), *maternally expressed 3* (*Meg3*), *Linc00323-003*, and miR-503, as well as the circRNAs *cZnf292*, *cAff1*, *cDennd4c*, and *cThsd1*, which are differentially expressed upon hypoxic conditioning, a stimulus known to trigger EC dysfunction [185,236,237]. Yet, only a few transcripts have been extensively characterized so far. *Malat1* is highly expressed in vascular endothelium and is deregulated in a number of CVDs. Indeed, *Malat1* downregulation promotes aberrant phenotypic changes leading to vessel growth in vivo [238]. Coherently, this translates also in profound cell cycle dysregulation, as assessed by gene expression profiling [238]. Of note, a model has been proposed in which vessel deterioration is caused by increased accumulation of hematopoietic cells [238,239].

Smooth muscle and endothelial cell enriched migration/differentiation-associated lncRNA (*Sencr*) is a vascular cell-specific lncRNA expressed in human ECs and VSMCs, partaking in EC differentiation and homeostasis [240,241]. Interestingly, *Sencr* may be involved also in endothelial dysfunction as its expression is significantly reduced in ECs isolated from patients with premature CAD, a finding suggestive of it having a central role in atherosclerosis [242]. Finally, *Sencr* contributes to EC biology by maintaining barrier functions and cell permeability [241].

The lncRNA small nucleolar host gene-12 (*Snhg12*) is highly expressed in the vascular endothelium and is negatively regulated during atherosclerotic lesion progression. In vivo, *Snhg12* restoration produced beneficial effects by reducing atherosclerotic burden and promoting DNA damage repair in the intimal layer via its interaction with DNA-depended protein kinases (DNA-PK). While further studies may clarify the *Snhg12*-specific action within the EC compartment, it provides novel insight into additional processes affecting vessel homeostasis beyond lipid content and inflammation [243].

Aberrant VSMC changes (i.e., deregulated phenotypic switch and apoptosis impairment) are central events in atherosclerosis initiation and progression. NcRNA-mediated orchestration of VSMC biology plays a critical role and may involve either the independent action of miRNAs, lncRNAs, and circRNAs or the modulation of their axis. For instance, *circLrp6*/miR-145 network modulation is essential for VSMC homeostasis and may play a role in atherogenesis as well [244]. Additionally, lncRNAs/miRNAs sharing the same hosting gene may play a role in disease, as discussed in the following [245,246].

Lnc-Ang362 is an angiotensinII(AII)-induced lncRNA that affects VSMC by enhancing aberrant cell growth. It shares the same locus with miR-221 and miR-222, well known for their detrimental effect on vessel biology [245]. Considering the aforementioned, the effects of *lnc-Ang362* on proliferation may be due to a cross-talk between the processing of the host gene and the two encoded miRNAs. Of note, as these transcripts are modulated in the same manner, further studies might cement *lnc-Ang362* as a therapeutic target in atherosclerosis [247]. Similarly, the lncRNA smooth muscle—induced lncRNA, enhancer of proliferation (*Smilr)* promotes VSMC aberrant proliferation via centromere protein F (*Cenpf*), concurring in disease development. While its overexpression results in neointimal hyperplasia, its disruption ex vivo promoted adverse vascular remodeling [248]. The aforementioned lncRNA *Sencr* was initially described in human coronary artery VSMCs, where it contributes to cell differentiation processes by acting on cell contraction—related genes such as myocardin (*Myocd*), while reducing the pr-migratory gene signature. Therefore, it can alleviate the action of migratory VSMCs during atherosclerotic lesion formation [240].

The VSMC-enriched cardiac mesoderm enhancer-associated noncoding RNA (*Carmn*) is a lncRNA located in a locus of interest upstream of the miR-143/145 cluster, which plays a central role in VSMC function and differentiation. Interestingly, recent work has postulated a miRNA-independent role for *Carmn* during atherogenesis via cell proliferation regulation. In particular, its depletion exacerbates atherosclerosis in mice [246]. Independent work supports these findings corroborating the role of *Carmn* in maintaining VSMC function and reduced expression in injured vessel, through different murine models (mouse and rat) and analysis of human scRNA-seq data. Mechanistically, *Carmn* might mediate its anti-atherogenic function via MYOCD binding in the nucleus [249]. However, a third study had different findings, with *Carmn* expression promoting VSMC proliferation, migration, and differentiation in vitro, with its knockdown in vivo reducing severity of vessel injury via SRF binding [250]. Further studies may help clarifying these contrasting reports. Nonetheless, one should consider the use of different experimental setups and animal models as well as the complexity of the *Carmn* locus, which presents multiple promoters and transcript isoforms.

Macrophages are also key players in atherosclerotic events, driving inflammation and innate immune responses. Myocardial infarction—associated transcript (*Miat*) is upregulated in vulnerable atherosclerotic plaques in which it furthers persistent and aberrant immune responses. *Miat* ablation eases the burden of inflammation and vessel damage by fostering macrophage clearance capacity (efferocytosis) via the miR-149—5p/CD47 axis [251]. Interestingly, additional work has proposed a

role for *Miat* in aberrant phenotypic switching of VSMCs, via the ERK/ELK1/EGR1 pro-proliferative pathway; such cell program changes lead, in turn, to VSMC trans-differentiation into pro-inflammatory macrophage-like cells [252].

Concordantly, the monocyte- and macrophage-enriched lncRNA *plaque-enriched lncRNA in atherosclerotic and inflammatory bowel macrophage regulation* (*Pelaton)* is upregulated in vulnerable plaques and is localized at inflammation sites. Its knockdown mitigates atherosclerosis progression through promotion of macrophage phagocytosis. *Pelaton* acts in *trans*, and unlike *Miat*, its modulation does not have an impact on efferocytosis. Nonetheless, additional investigation is required to elucidate its mechanism of action and coding potential [253].

The lncRNA associated with the progression and intervention of atherosclerosis (*Rapia*) is highly expressed in advanced stages of atherosclerosis. *Rapia* has a sponge-like activity and modulates miR-183−5p. Additionally, its silencing advances athero-protection by reducing proliferation and triggering the apoptotic program in macrophages via miR-183−5p/integrin beta 1 (ITGB1) network regulation [254].

The macrophage-associated atherosclerosis lncRNA sequence (*Maars*) is a macrophage- and nuclear-specific lncRNA; its expression is markedly induced during atherosclerotic progression in murine disease models by impairing macrophage efferocytosis and cell death. During atherosclerosis progression, *Maars* tethers the RNA-binding protein human antigen R (HuR), stabilizing mRNA translation [255].

The already-mentioned lncRNA *Malat1* is also involved in the innate immunity response and is atheroprotective. Its role in maintaining vascular functionality may work via a network of ncRNA-mediated processes involving *MALAT1-associated small cytoplasmic RNA (mascRNA)* and *nuclear paraspeckle assembly transcript 1* (*NEAT1*). However, further studies are needed to better characterize its mechanism of action [256].

Finally, the lncRNA antisense noncoding RNA in the INK4 locus (*Anril*) seems to be modulated in all the three principal cell components of the plaque, so it is directly associated with lesion progression and disease severity [257,258]. *Anril* is a guide lncRNA that binds to specific subunits of PRC1 and PRC2 and mediates interaction with target promoters [259], fostering H3K27 trimethylation and transcriptional repression of the *Ink4* locus in *cis*. Of note, *Anril* depletion blocks aberrant cell proliferation also in *trans* [259,260]. Additionally, its circularized form (*circAnril*) has an opposite expression pattern and behavior, participating in atheroprotection by modulating apoptosis and proliferation in human vascular cells and tissues independently of its cognate linear transcript. While further studies are required, it is tempting to speculate a role for SNPs or specific environmental signals in linear versus circular *Anril* maturation [261].

Leukocyte recruitment, activation, and lipid metabolism are beyond the focus of this chapter and so they have not been discussed in detail. However, such processes have been recently reviewed elsewhere [232,262].

19.5.3 Heart failure and miRNAs

As previously mentioned, ncRNAs have emerged as critical players in several physiological and pathological contexts. One subclass of ncRNA is miRNA. miRNAs are necessary for cell development, differentiation, and other physiological processes and have also been highly implicated in disease. They been extensively studied over the past decade and have been shown to play a prominent

role in regulating cardiovascular biology and HF development. Alteration of miRNA expression has been shown in animal HF models as well in human patients (Fig. 19.6). Very interestingly, the miRNA profile of the failing heart correlates with that of the fetal heart, consistent with the fact that upon hypertrophic onset, there is re-expression of fetal cardiac genes in the adult heart [263]. More specifically, several in vitro and in vivo studies have demonstrated the crucial role of miRNAs in orchestrating the gene expression changes observed during the onset and progression of HF. In neonatal murine cardiomyocytes, overexpression of miR-23a, miR-23b, miR-24, miR-195, or miR-214 individually promoted cardiac hypertrophy by regulating cell growth [264,265]. Transgenic miR-195 mice were found to develop dilated cardiomyopathy [266]. Overexpression of miR-133 inhibited cardiac hypertrophy, whereas miR-133 deletion promoted cardiac hypertrophy in vivo and in vitro [264,265].

Not only have miRNAs been shown to regulate a myriad of gene expression pathways involved in HF, but several studies have shown their importance in regulating other epigenetic pathways. Of note, the DNA methylation reader methyl-CpG-binding protein 2 (MECP2) was found to be repressed in mouse and human HF by miR-132 [267]. As previously mentioned, the histone methyltransferase G9a plays an important role in the maintenance of cardiomyocyte homeostasis, and its dysregulation is implicated in the development of cardiac hypertrophy and HF. At another level of regulation, it has been shown that after onset of pathological hypertrophy, expression of miR-217 inhibits G9a, resulting in a reduction of H3K9me2, de-repressing fetal cardiac genes and resulting in pathological remodeling of the heart [268].

It is also important to consider aging as an important risk factor for CVDs, including HF. In a recent study, aged hearts presented with increased expression of miR-1468−3p, which promoted cardiac fibrosis through cardiac fibroblast activation via the TGF-β1-p38 signaling pathway. This resulted in an accumulation of ECM and collagen deposition in the myocardium and a consequential progressive decline in cardiac function until the development of HF [269]. The miRNAs miR-21, miR-22, and miR-34a and the miR17-92 cluster have been shown to be significantly altered in cardiac aging. These miRNAs are necessary in the regulation of TFs involved in fibroblast-to-myofibroblast transition, cardiac fibroblast senescence, cardiac contraction, and collagen synthesis, respectively. Dysregulation of these miRNA clusters has been shown to play a key role in fibrosis onset in the aged heart [270−273].

Finally, the use of miRNAs as biomarkers for screening of CVDs such as HF has been increasing over the past several years. Such studies are critical in the development of novel diagnostic tools and the discovery of potential therapeutic targets [274].

19.5.4 Heart failure and lncRNAs

The study of lncRNAs in cardiac hypertrophy and HF is still in an initial stage (Fig. 19.6). High-throughput sequencing has identified thousands of lncRNAs dysregulated in cardiac pathologies, like HF, but the majority of them still remain unstudied. Understanding the diverse mechanisms of regulation of these ncRNAs in cardiac hypertrophy and HF will be paramount for the development of novel therapeutic strategies [275].

One of the first lncRNAs to be studied in the heart is myosin heavy chain−associated RNA transcript *(Mhrt)*, which overlaps with the *Myh7* locus. *Mhrt* directly binds to the chromatin remodeling factor BRG1 and protects against hypertrophic growth. Overexpression of this lncRNA in TAC

mice improved cardiac function by regulating the switch between *Myh6* and *Myh7*, a key mechanism involved in cardiomyocyte hypertrophy [276]. Another very important lncRNA in the context of cardiac hypertrophy is cardiac hypertrophy–associated epigenetic regulator *(Chaer). Chaer* directly interacted with the catalytic subunit of the PRC2 complex, EZH2, and prevented H3K27me3 deposition at the promoter regions of several hypertrophic genes (e.g., *Myh7*, *NppA,* and *Acta1*), promoting cardiac hypertrophy. In a murine model lacking *Chaer*, it was shown that these mice had better cardiac function and less hypertrophy after pressure overload [277]. Another important lncRNA is *H19*, which has been shown to play a role in cardiomyocyte hypertrophy and apoptosis. Studies revealed that *H19* increases in the initial hypertrophic phase of HF, but it is then downregulated in the decompensated heart. *H19* also interacts with PRC2 and is involved in the suppression of the prohypertrophic transcription factor NFAT (nuclear factor of activated T cells). Murine knockouts of *H19* developed a larger hypertrophic heart upon pressure overload compared with wild-type littermates, and overexpression of *H19* improved heart function in a mouse model of afterload-induced HF [176]. Moreover, the lncRNA *Chrf* has been implicated in the control of miR-489: repression of miR-489 promoted maladaptive cardiac hypertrophy [278], providing yet another example of multifactorial epigenetic control.

Finally, in the context of the aged heart, the lncRNA *OXCT1 antisense RNA 1 (Sarrah)* was identified as playing an important role: *Sarrah* was found downregulated during aging, playing an antiapoptotic role in cardiomyocytes, and was required for cardiomyocyte survival [279].

19.6 Conclusions

The findings described in this chapter strongly support the notion that structural modulation of chromatin is directly involved in the pathogenesis of CVDs. This is very important since, in the past decades, the incidence of CVDs has massively increased. Fortunately, research aimed at defining the mechanisms at the basis of CVD development has grown in step, with many studies highlighting the epigenetic processes involved in these pathological processes. It is clear that a better understanding of individual epigenetic landscapes will help identify the causes of these diseases and the development of tailor-made therapeutic approaches. Accelerating technological advances in terms of data generation and analysis will increasingly facilitate the creation of epigenetic maps for CVDs, and eventually endow clinicians with new tools in the search for links between environmental effects and traditional risk factors.

EWAS have, indeed, identified additional targets for therapy, providing additional insights into the mechanisms of CVD. An EWAS approach has identified physiopathological mechanisms and unexplained heritability in many diseases. They have, for instance, established relationships between DNA methylation signatures and several phenotypes, including CVD [96] and CAD [69]. These information open further innovative opportunities for therapeutic applications.

Another important factor to consider is the contribution of aging to the onset of various CVDs, since atherosclerosis, stroke, and myocardial infarction incidence increase in the elderly [49]. The natural aging process has been shown to be accompanied by several functional changes to the heart, including diastolic and systolic dysfunction, arrhythmias, and atrial fibrillation [280]. The high prevalence of CVD in the aged population has been linked to inflammation, oxidative stress, production of ROS, apoptosis, myocardial deterioration, and degeneration [39]. Although epigenetic mechanisms have become a very prevalent avenue for the elucidation of CVD onset, such mechanisms

still remain elusive during the aging process. For example, studies have shown that changes to DNA methylation patterns in aging mimic those of the changes occurring during cardiac hypertrophic remodeling [47]. Dysregulation of many epigenetic factors has been implied in the cardiac aging process; however, the exact mechanisms still remain elusive [98]. Therefore, it is of great importance to determine the role of such factors (HATs, HDACs, HMTs, ncRNAs) in the context the pathophysiology of age-related CVDs, using our current knowledge of the epigenome and disease onset.

A further aspect that is worth to discuss is how these epigenetic mechanisms can be introduced into diagnostics. A biomarker is defined as a measurable elements that indicates a specific pathophysiological state and/or a response to a therapeutic treatment [281]. Thus, a biomarker might provide fundamental information to physicians, helping them to correctly diagnose a patient and define the therapy. Epigenetic elements might be used ad biomarkers as well. Among them, miRNAs are the one with greater potentials and most attractive, since they can be easily detected in sample with limited volumes, are very stable, and can be measured in several biological fluids, including urine, plasma, serum, and saliva. Furthermore, they are very highly conserved in different species, making a comparison between animal models and patients very reliable [282]. Therefore, although all epigenetic mechanisms are deeply investigated, miRNAs are the ones also evaluated for their use as predictive biomarkers.

However, full deciphering of the epigenetic networks of the cardiovascular system is still far from completion. We must clearly comprehend the fine relationship between epigenetic mechanisms and transcriptional programs in specific tissues. Without this information, it will be difficult to develop therapies aimed at reversing cell-specific, disease-generating programs of the cardiovascular system. This approach will, hopefully, avoid the undesirable effects induced by off-target modulation of gene expression. It is foreseeable that in the not-too-distant future, RNA-based therapeutics will be used to target all the types of epigenetic mechanism: silencing RNAs (siRNAs), antisense oligonucleotides (ASOs), RNA aptamers, single guide RNAs (sgRNAs) for CRISPR/Cas9 systems, lnRNAs, miRNAs, etc. Differently from classical drugs, these molecules can target any gene, including those encoding proteins with no enzymatic activity.

Key points

- Epigenetics is a major player in the development of CVDs.
- Innovative therapeutic approaches are arising from the current knowledge on epigenetic mechanisms.
- Next-generation sequencing is a critical methodology for exploring these innovative concepts in new research topics.

List of abbreviations

3′-untranslated region	3′UTR
5-azacytidine	5-aza
5-methyl-cytosine	5mC
cardiovascular disease	CVD
circular RNA	circRNA

coronary artery disease	CAD
DNA methyltransferase	DNMT
endothelial cell	EC
endothelial-to-mesenchymal transition	EndMT
high-density lipoprotein	HDL
long ncRNA	lncRNA
low-density lipoprotein	LDL
microRNA	miRNA, miR
miRNA recognition element	MRE
nucleotide	nt
open reading frame	ORF
precursor miRNA	pre-miRNA
primary miRNA	pri-miRNA
pulmonary artery hypertension	PAH
single-nucleotide polymorphism	SNP
transcription start site	TSS
vascular smooth muscle cell	VSMC
venous-occlusive disease	PVOD

Acknowledgment

This work was supported by grants from the Horizon 2020 Research and Innovation Programme under Grant Agreement (No. 828984), the Italian Ministry of Health (No. GR-2016-02364133), and Italian Ministry of Research (No. 2017HTKLRF) to L.E.

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CHAPTER

Therapeutic potential of epigenetic drugs 20

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20.1 Introduction

According to the 2021 World Health Organization report, cardiovascular diseases (CVDs) remain one of the leading causes of death globally, taking almost 17.9 million lives yearly. Various factors, including genetic mutation, metabolic changes, protein aggregate disease, cardiac hypertrophy, myocardial infarction, viral infections, inflammatory responses, arrhythmia, atherosclerosis, and blockage of blood vessels, can compromise the blood flow in the vessels and lead to the development of the CVDs and heart failure [1−5]. Additionally, environmental factors, pollution, nutrition, stress, and lifestyle change can also act as an additive to the development of CVDs [6]. Due to the severity of the CVDs, progress has been made in understanding the cause of the disease and the therapeutic potential of different drugs. However, the complex nature of CVDs is still not fully understood. Epigenetic changes play a significant role in cellular function through posttranslational modification of DNA without altering the DNA sequence. Posttranslational modifications such as methylation, acetylation, SUMOylation, phosphorylation, and ubiquitination can activate or suppress any gene function to fulfill the cell's metabolic demand. These modifications may eventually lead to the development of disease conditions [5,7−13]. Additionally, noncoding RNAs (ncRNAs) can also alter cellular epigenome and cellular transcription [14,15]. Due to the advancement of genomic technology, it is now evident that epigenetic regulation plays a significant role in the development of several diseases, including CVDs. Furthermore, the recent advancement of diagnostic technology suggests that early diagnosis of epigenetic alteration of a gene can predict the cause of pathological signaling and the development of CVDs [16]. Due to the enormous potential of epigenetics, several molecular and natural products have been in clinical trials to correct epigenome related to CVDs. We discuss the epigenetic changes associated with CVDs and potential therapies (Fig. 20.1).

20.2 Epigenetic regulation in cardiovascular diseases and potential epigenetic drugs

Since epigenetic changes play an active role in the development of different diseases, epidrugs could be useful in reversing pathological epigenetic changes. Several potential drugs and their targets were

Epigenetics in Human Disease. https://doi.org/10.1016/B978-0-443-21863-7.00005-6
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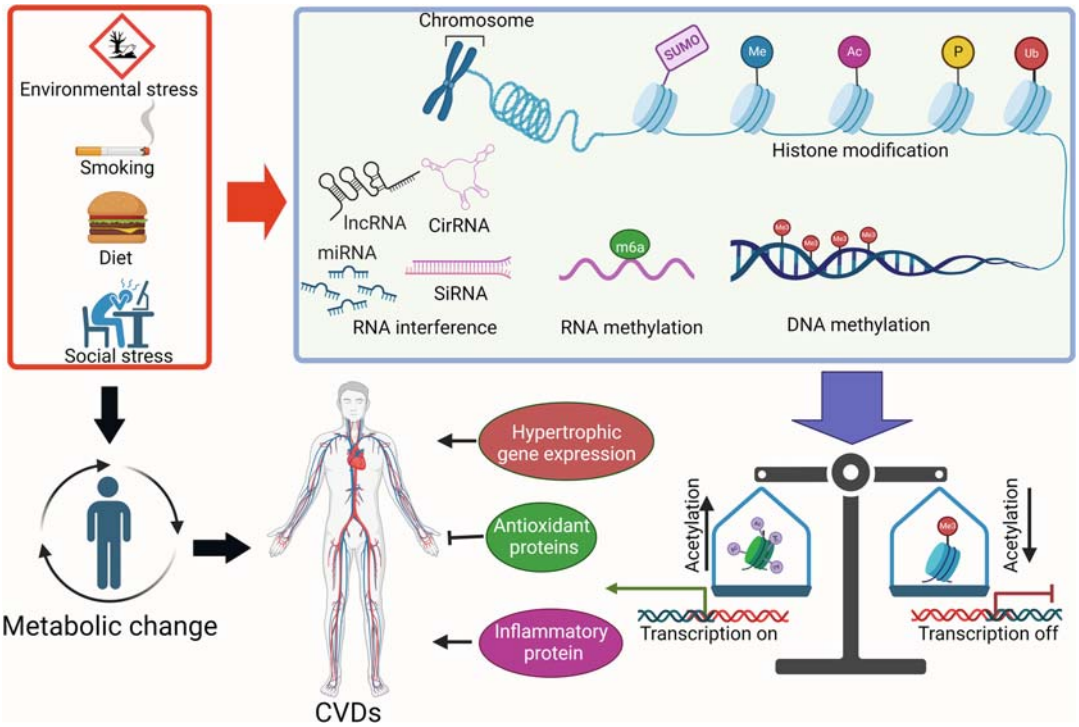

FIGURE 20.1 Schematic Representation of Epigenetic Regulation in the Cell

Ac, acetyl; *CirRNA*, circular RNA; *lncRNA*, long noncoding RNA; *Me*, methyl; *m6a*, m^6-methyladenosine; *miRNA*, microRNA; *ncRNA*, non-coding RNA; *P*, phosphoryl; *Ub*, ubiquitin.
Histone protein in chromosome post-translationally modified by SUMOylation, methylation, acetylation, ubiquitination, and phosphorylation. DNA and RNA modification by methylation regulate cellular gene expression. Noncoding RNAs regulate cellular gene expression through transcription and translation. An optimum epigenetic regulation can cause chromosomes to be in an open configuration (transcriptionally active) or closed configuration (transcriptionally inactive). Gene transcription can express protective or detrimental signaling.

identified to treat pathological epigenetic modification. Recently, therapeutic potential of some epi-drugs was tested in clinical trials, and it showed a beneficial effect. Due to the advancement of epigenetic studies, the scientific community also started analyzing CVDs from a different angle to get answers to the causes of CVDs. However, advanced clinical trials and better delivery methods must be standardized before they are recommended for clinical use. Below, we have discussed the differential regulation of posttranslational modifications in DNA, RNA, and histone during CVDs (Fig. 20.2).

20.2.1 DNA methylation

DNA methylation regulates transcription through the chemical modification of DNA at the C5 position of cytosine to form the 5-methylcytosine [17]. DNA methylation occurs by DNA methyl transferases

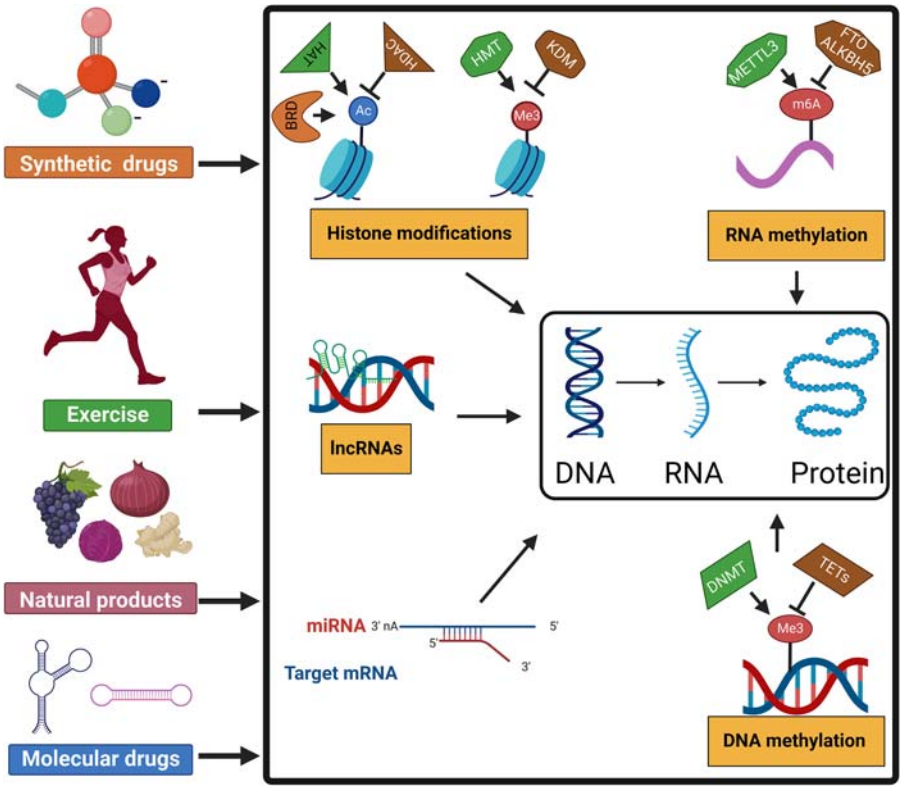

FIGURE 20.2 Epigenetic Mechanism to Improve Cardiovascular Diseases

ALKBH5, alkylated DNA repair protein AlkB homolog 5, an m6A demethylase; *BRD*, bromodomain containing protein; *DNMT*, DNA methyltransferase; *FTO*, fat mass obesity-associated gene (FTO), an RNA demethylase; *HAT*, histone acetylase; *HDAC*, histone deacetylases; *HMT*, histone methyl transferase; *KDM*, histone lysine demethylase; *METTL3*, m6A methyltransferase; *TET*, ten–eleven translocation, a DNA demethylase.

The cellular epigenome can be modified using synthetic drugs against specific enzymes, regular exercise, natural products, or molecular drugs. These tools can regulate cellular epigenetic modification by correcting posttranslational changes or inhibiting the specific chromatin remodeling enzymes. The writer enzymes (HAT, HMT. METTL3, DNMT, etc.) and eraser enzymes (HDAC, KDM FTO, ALKBH5, TETs) regulate the posttranslational modification, whereas the reader (BRD) recognize the posttranslational modifications.

(DNMTs), which add a methyl group to cytosine, followed by guanine (CpG islands). Conversely, DNA demethylation can occur passively or through an active process. In the passive process, the expression of DNMTs level is gradually reduced or unavailable in the cell to methylate the DNA, whereas in the active process, ten–eleven translocation demethylases (TETs) convert 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmc) [18,19]. DNA methylation is believed to be a docking site for chromatin regulatory enzymes. After binding DNMTs with the chromatin, they

cause chromatin remodeling and condensation, inhibiting the binding of RNA polymerase to the gene promoters and suppressing the target gene expression [20]. Recent studies show a unique pattern of DNA methylation associated with the different CVDs, and aberrant methylation is common in cardiac stress [21–23]. A genome-wide study was conducted by Nakotochi et al. using 192 subjects with myocardial infarction (MI) and 192 control subjects to understand the epigenetic changes in the blood DNA. This study concluded that two DNAm sites, cg07786668 in ZFHX3 and cg17218495 in SMARCA4, are independently associated with MI [24]. Similarly, another genome-wide study with a cohort of 4794 participants shows a unique pattern of NLRC5 and PARP9-DTX3L gene DNA methylation associated with the level of circulating TNF-alpha level and incident of coronary heart disease [25]. Another study conducted by Zheng et al. detected 45 unique patterns of blood DNA methylation associated with cardiovascular health. These DNA methylation sites can be used to detect subclinical CVDs [26]. Recently the role of DNMT inhibitor was tested in a pressure overload (PO) rat model generated by transverse aortic constriction (TAC). Interestingly it is found that DNMT inhibitor RG108 can attenuate cardiac hypertrophy [27]. Similarly, another study showed that treating hypertrophied rats with 5-aza-2′-deoxycytidine can improve cardiac hypertrophy [28]. DNMT inhibitor hydralazine also shows a protective role in improving cardiac function through the demethylation of SERCA promoter and increased SERCA2a expression [29]. However, optimum DNA methylation is important for the growth and development of different organs. The impact of DNA methylation on cardiac development was tested in the zebrafish model using DNMT inhibitor 5 azacytidine. It is found that hypomethylation of certain genes causes cardiac abnormality [30].

20.2.2 RNA methylation

Like DNA methylation, RNA can be methylated to adenosis molecules at the N6 position (m6A). Methyltransferase-like 3 (METTL3), in association with other cellular factors, forms the methyltransferase complex to methylate RNA [31]. Different locations of the RNA, such as UTR, near the stop codon, can be methylated [32,33]. Conversely, RNA demethylase enzymes such as FTO and ALKBH5 can remove the m6A modifications [34,35]. The evolving concept of RNA methylation suggests that RNA methylation regulates RNA stability, transport, expression, etc. [36]. RNA methylation levels are significantly induced in CVDs such as cardiac hypertrophy, myocardial infarction, ischemia, and genetic mutation [37]. Similarly, it was noticed that the expression of METTL3 significantly increased during CVDs [4]. Further global METTL3 knockout mice study shows early embryonic lethality at E8.5 [38]. Furthermore, the role of METTL3 in the heart was studied using cardiac-specific knockout mice or transgenic mice. Studies with the mouse model show that overexpression of METTL3 causes compensatory hypertrophy. However, adeno-associated virus 9 (AAV9)–mediated transient expression of METTL3 can protect the heart from hypertrophy [4,39]. Conversely, METTL3 KO mice show cardiac abnormality at an early age, which later progresses to heart failure [4].

20.2.3 Histone posttranslational modifications

DNA wraps around the histone octamer in eukaryotic cells to form a stable nucleosome-based chromatin structure. Histone proteins in the nucleosome can undergo a series of posttranslational modifications according to cellular demand and causes chromatin remodeling. Chromatin-regulating

enzymes writers, erasers, and readers through posttranslational modification of chromatin can lead the chromatin in an open or closed configuration. The role of histone posttranslational modification and its association with CVDs are discussed in the following.

20.2.3.1 Targeting histone methyltransferase

Like DNA methylation, methylation-mediated posttranslational modification of histone can regulate gene expression. Histone methyl transferase (HMT) adds a methyl group to the histone using S-adenosine methionine as a substrate, whereas histone demethylase removes the methyl group from the histone [40]. Histone can be methylated at lysine or arginine molecules of the protein. Different lysine molecules of the protein can be methylated as mono- (me1), di- (me2), or trimethylated (me3) state. However, arginine molecules can be methylated as mono- (me1) or dimethylated state (me2) [40]. The level and position of methylation are important for the epigenetic regulation of gene transcription. For example, H3K4me3 can activate transcription, whereas H3K9me3 and H3K27me3 suppress gene expression [41,42]. Similarly, arginine methylation can promote gene transcription, and demethylation can suppress gene activation [43]. In disease conditions, the amount of methylation or expression of methyl transferase enzyme expression significantly changes and causes aberrant gene silencing or activation. Methylation levels are altered considerably for several CVDs, including atherosclerosis, myocardial infarction, diabetic conditions, and pulmonary hypertension [44]. Consistent with the histone methylation level, the expression of HMTs and demethylase also significantly changed [44]. Over the past several years, it was demonstrated that histone methylase enzymes are actively involved in CVDs [45]. Inhibition of the methylase enzymes can be protective or detrimental, depending on the methylation site and cell types [45]. HMT enzyme EZH2 plays a critical role in the development of atherosclerosis [46,47]. It was demonstrated that overexpression of EZH2 can induce atherosclerosis in apolipoprotein E-deficient (apoE$^{-/-}$) mice [48]. Furthermore, it was shown that methyl transferase inhibitor GSK126 improves CVDs through the reduction of inflammation. Conversely, another study through gain-in and knockdown of the EZH2 gene suggests that EZH2-mediated methylation protects vascular smooth muscle cells (VSMCs) by inhibiting autophagy-mediated cell death. This study was further supported using an EZH2 inhibitor UNC1999 [49]. Another study showed that methylation of H3K36 is essential for neointima formation in VSMC. Further study suggests that a pan inhibitor chaetocin of the Jumonji demethylase superfamily can reduce neointima formation [50]. In addition, it was demonstrated that H3K4m3 and H3K9me3 methyltransferase inhibitors can ameliorate the high salt-induced heart failure in a rat model [51]. In the isoproterenol-induced cardiac hypertrophy model, it was found that the expression of methyl transferase JMJD3 significantly increased. Apparently, inhibition of JMJD3 with GSK-J4 shows a promising effect in the regulation of cardiac hypertrophy [52]. However, GSK-K4 also shows a beneficial effect in the regulation of cardiomyocyte ferroptosis by preserving the H3K27 methylation [53].

20.2.3.2 Targeting histone acetylation

Acetylation regulates chromatin structure through destabilizing chromatin—DNA interaction, which allows the condensed chromatin to an open configuration [20,54]. Histone proteins can acetylate at different lysine residues by histone acetyltransferase (HAT). Conversely, histone can deacetylate by histone deacetylase (HDAC) [20]. It is believed that acetylation of histone causes active histone marks and promotes gene expression, whereas deacetylation causes repressive histone marks and inhibits

gene transcription [55]. However, aberrant acetylation can cause fatal gene expression and the development of several diseases, including CVDs [56]. Additionally, several acetylation-regulating enzymes can translocate to the nucleus and cause changes in chromatin remodeling during cellular stress [57]. In the past, several investigations were performed to detect the level of histone acetylation and expression of acetylation-regulating enzymes in different disease conditions. Changes in histone acetylation and expression of regulating enzymes were prominent in various disease conditions, including cardiac hypertrophy, hypertension ischemia-reperfusion, atherosclerosis, diabetes, and antiretroviral drug treatments [56,58]. Total acetylome studies showed that the acetylation level of histone significantly altered in healthy vs. failing patients [59,60]. Further, it was noticed that the acetylation level of histone correlated with RNA polymerase binding and expression of the transcript [61]. These studies suggest that the acetylation level of histone may help to understand the disease etiology. Some studies found that acetylation by p300 and CBP can upregulate inflammatory gene expression and induce detrimental effects, whereas others report that acetylation of promoter induces protective gene expression [58,62–64]. In the SIRT3 knockout mouse model of dilated cardiomyopathy, histone acetylation levels significantly increased, and concomitantly, expression of inflammatory markers NF-κB, TLR-4, MyD88, and IRAK-4 also increased. However, treating mouse with a HAT blocker C646 can reduce the acetylation and inflammatory protein expression. Further, C646-treated mice show reversed cardiac remodeling with improved cardiac function [65]. Similarly, another study indicates that SIRT6-mediated degradation of HAT enzyme p300 can protect cardiomyocytes from PE-induced hypertrophy [66]. Additionally, it was demonstrated that inhibition of p300 can reduce the aortic valve calcification along with a reduction in histones 3 and 4 and acetylation level [67]. Conversely, it was found that during the ischemic condition, cellular acetylation significantly decreased, and increased acetylation by Nrf2 protein induces the expression of antioxidant factors, and cells get protected by oxidative stress [68,69]. Various studies were conducted to understand the role of HDACs in the cardiovascular system using in vitro cell culture or in vivo animal models. Results support the notion that HDACs are important for cellular homeostasis, and the knockdown of HDACs makes the cells vulnerable to different stress and causes early death in mice [70,71]. Additionally, cardiac-specific HDAC knockout mice show cardiac hypertrophy and dilated cardiomyopathy [70]. However, inhibition of HDACs shows a cardioprotective effect during stress. In cell culture and in vivo rodent models, it was demonstrated that HDAC inhibitor trichostatin A could improve heart function by reducing hypertrophy and fibrosis [63,72–74]. However, the expression of class III HDAC enzyme significantly changes during stress conditions, and it shows differential effects in different cell types. For example, in a hypoxic cell, the expression of eNOS significantly decreased, but SIRT1 expression protects the hypoxic cells through increased transcription of eNOS [75,76]. Additionally, SIRT1 expression can reduce cellular hypertrophy and inflammatory gene expression through the deacetylation of histone [77,78].

20.2.3.3 Other posttranslational modifications of histone

In addition to acetylation and methylation, SUMOylation, phosphorylation, and ubiquitination like posttranslational modifications are also differentially regulated during CVDs.

SUMOylation: SUMOylating regulates various biological processes such as cell division, epigenetic regulation, DNA repair, and neutralization of reactive oxygen species formation, etc., through posttranslational modification of several cellular proteins, including histone [79,80]. Since lysine is the binding site of other posttranslation like ubiquitin and acetylation, a cross-talk happens to

the SUMO protein binding to the substrate [81]. For example, acetylation prefers the binding of the SUMO with the substrate. However, SUMOylation causes transcription suppression after deacetylation of the promoter area of the target gene [80,82]. Moreover, in some instances, SUMOylation can promote ubiquitination and degradation of proteins. For example, SUMOylation promotes the degradation of HDAC4 during I/R, which can protect cells from ROS-induced cellular damage [83].

Ubiquitination: Ubiquitination is the process where a 10 kDa ubiquitin protein attaches to a protein with the help of a cascade of enzymes [84]. Histone ubiquitination participates in DNA repair or regulating target gene expression through chromosomal compactness [85–87]. Histones 2A, 2B, and 3 can be posttranslationally modified at lysine amino acid by binding with the ubiquitin molecule. Posttranslational modification of histone by ubiquitin can also modulate other posttranslational modification. Furthermore, ubiquitinated histone can signal for binding other epigenetic remodeling enzymes and helps in transcriptional regulation. For example, during cardiac development RNF20–RNF40 E3 ligase complex, together with UBE2B, critically regulates the ubiquitination of histone 2b and transcriptional regulation of cilia gene expression. Cilia play a critical role in left–right patterning during cardiac development [88]. Another study shows that RNF20 and RNAF40 can mono-ubiquitinate the H2B at lysine 120. This modification plays an essential role in the transcriptional regulation of cellular genes and cardiomyocyte maturation [89].

Phosphorylation: Multiple studies have shown that the phosphorylation of histone plays a crucial role in the compactness of histone and gene transcription [90–92]. Additionally, cross-talk with other histone modification phosphorylation can regulate chromatin remodeling. For instance, phosphorylation of histone in the promoter area can cause further acetylation and activation of the gene expression. Using in vitro and in vivo mouse models along with clinical samples, Robinson et al. showed that mitogen and stress-activated protein kinase (MSK) mediate phosphorylation of histone 3 at serine 28 in the IEG promoter. This modification enhances the binding of other transcriptional factors and induces chromatin remodeling and hypertrophy [91]. Further, Wang et al., using smooth muscle cells, showed that phosphorylation along with acetylation and methylation of histone 3 at promoter region is important for the IL-1β-mediated regulation of early growth response (EGR)-1 transcription factor expression [92].

20.2.4 Noncoding RNAs and molecular drugs

Recent studies suggest that ncRNAs play a critical role in genetic reprogramming and gene expression. Although ncRNAs do not transcribe protein, they can regulate cellular transcription at the RNA level by interfering the RNA processing or degradation [93,94]. Depending on the size, the ncRNAs can be divided into miRNAs or long noncoding RNAs (lncRNAs). Several studies suggest that the expression of ncRNAs significantly changed during disease conditions, including CVDs, and can be used as a biomarker for the disease [94,95]. In addition, studies suggest that the lncRNAs can regulate the cardiovascular system positively or negatively. Some lncRNAs are important for cardiac regeneration and differentiation, whereas some lncRNA inhibits cardiac regeneration and differentiation by regulating other miRNAs [96,97]. Cells express another ncRNA category called circular RNAs (CirRNAs) [98,99]. CirRNAs are believed to be rich in miRNA-binding sites and act as miRNA sponges. It can facilitate the expression of specific genes through the removal of the inhibitory effects of miRNAs.

Numerous studies showed that miRNAs are conserved in the human genome and can be used as a diagnostic marker for CVDs, as they have been reported in the patient's blood samples [100,101].

Therefore, it is possible to regulate the target gene expression using miRNA at the RNA level. A pathological target gene expression can be knockdown by using siRNAs. However, a critical observation of the miRNA is that a single miRNA can regulate gene expression and modulate a signaling cascade, whereas multiple miRNAs can modulate a single gene expression. So, miRNAs can help target a pathological condition regulated by a set of genes [102]. Preclinical studies show that miRNA has a promising effect in regulating cardiomyocyte generation and treating CVDs [103]. Several pharmaceutical companies are generating different oligonucleotides to control the target gene expression, such as antisense oligonucleotide, siRNA, miRNA sponge, miRNA mimetics, antagomirs, etc. Additionally, recently developed CRISPR/Cas9 gene editing can be used to alter the DNA methylation and regulation of target gene expression [104]. However, these technics are at different stages of clinical trials and need proper evaluation before they can be used to treat CVD patients.

20.2.5 Natural products

Some natural products have been protecting humankind for centuries. However, molecular mechanisms are evolving now. Natural products such as polyphenol (curcumin, resveratrol, quercetin, apigenin, genistein, epigallocatechin-3-gemordin, anthocyanins, ellagic acid, terpenoids, carvacrol, boswellic acids, cocoa, eugenol), isothiocyanates (sulforaphane, allyl-isothiocyanate), and phenylpropanoids (cinnamaldehyde) play a critical role in epigenetic regulation of the cells [105]. The cumulative evidence suggests that natural products play a significant role in the regulation of CVDs through the reduction of inflammation, oxidative stress, or lipotoxicity by epigenetic modification [106].

Curcumin: Curcumin is one of the well-studied natural products found in turmeric. For centuries, turmeric has been integrated into Asian food and cosmetics and has shown several beneficial roles. Published works show that curcumin protects from CVDs, including cardiac hypertrophy, atherosclerosis, IR, etc. [107]. In addition, curcumin has a pleiotropic role in the epigenetic regulation of pathological gene expression. Published works showed that curcumin can prevent cardiac fibrosis and inflammatory gene expression by inhibiting DNA methyl transferase activity and promoting PPAR-mediated protective gene expression [108]. Additionally, curcumin can protect cells by regulating HATs and HDACs activity. The most well-known target of curcumin is p300 [109]. Expression and activity of p300-GATA4 are significantly upregulated in different disease conditions [110,111], and curcumin is known to protect the cardiovascular system by inhibiting p300-GATA4-mediated inflammatory and hypertrophy gene expression [107]. Further, curcumin shows a protective role during ischemia and other CVDs by improving SIRT3 function and induction of the antioxidant activity of cells [107].

Resveratrol: Resveratrol is another well-known natural product found in our regular diets, such as grapes, berries, peanuts, and cocoa. Preclinical studies showed promising effects of resveratrol on CVDs such as ischemia-reperfusion injury, heart failure, atherosclerosis, reduction of blood pressure, and hypertension [112]. Further, resveratrol can ameliorate endotoxin-induced cardiotoxicity through the induction of Nrf2-mediated antioxidant systems [113]. Mechanistically, resveratrol modulates the epigenome through the regulation of DNA methylation and histone acetylation by modulating the function of DNMTs and SIRT1 enzymes, respectively [8].

Quercetin: The phenolic compound quercetin is found naturally in different fruits, vegetables, green tea, etc. [114]. Several studies suggest that quercetin inhibits CVDs by reducing inflammatory

gene expression, ROS production, etc. [115,116]. Quercetin epigenetically silent the inflammatory genes by decreasing the expression of pathological miRNAs. Additionally, it shows an inhibitory effect on the HAT enzyme p300 [117].

Apigenin: Apigenin is found in grapefruits, vegetables, tea, and wheat. Recent studies showed that apigenin protects against cardiometabolic diseases such as hypertension, type 2 diabetes, dyslipidemia, etc. [118]. Apigenin inhibits the expression of an inflammatory gene like NF-κB by downregulating relevant miRNAs. Additionally, apigenin regulates cellular oxidative stress by inhibiting DNMTs and induction of Nrf2 protein expression through regulation of the methylation level of its promoter [119].

Genistein: The phytochemical epigallocatechin is mainly found in green tea. It shows various cardioprotective effects in CVDs, including cardiac hypertrophy, ischemia-reperfusion, myocardial infarction, inflammation, and diabetes. Epigallocatechin shows protection by reducing inflammatory gene expression and inhibiting ROS production [120]. In addition, this phytochemical inhibits inflammatory gene NF-κB expression through regulation of the p300 and HDACs enzyme function [121].

Anthocyanins: Anthocyanins are water-soluble pigments found in several vegetables, flowers, seeds, and berries. Several studies showed the beneficial effect of anthocyanins on CVDs through inhibition of inflammatory gene expression, reduction of ROS production, and upregulation of NO production [122−124]. However, its role in epigenetic regulation during CVD is not clear. Some study suggests that anthocyanins act as an epigenetic regulator through the regulation of mRNAs expression and acetylation of many gene promoters [106,125,126].

Ellagic acid: Ellagic acid shows a beneficial effect on CVD by reducing MI, increasing oxidative enzyme expression, reducing cardiac hypertrophy, and inhibiting fibrosis [127,128]. In a rat model of MI, ellagic acid protects the heart by reducing the expression of HDAC1, collagen 1, collagen III MMP-2 and MMP-9 gene in the fibrotic area [128,129].

Terpenoids: Terpenoids are a significant constituent of essential oils and are found in natural products such as citrus fruit, tea, thyme, cannabis, etc. It protects CVDs through the regulation of vasorelaxation and hypotension of the vascular system, inhibition of MI, etc. [130]. In addition, it shows a protective effect by inhibiting inflammatory gene expression and promotes the expression of the antioxidant protein Nrf2 by regulating the DNMTs and HDACs enzymes [130,131].

Carvacrol: Carvacrol is a phenolic monoterpene found in essential oils of many plants such as thyme, pepperwort, etc. Numerous studies showed that carvacrol has a protective role in CVDs, including atherosclerosis, hyperlipidemia, and hypertension, inhibition of inflammation, reduction of blood pressure and blocking calcium channels, promoting vasorelaxation, etc. [132]. In addition, carvacrol shows epigenetic regulation through the regulation of SIRT-AMPK signaling and miRNA expression [133,134].

Cocoa: Cocoa is a polyphenolic product commonly used in chocolate. Studies have shown that cocoa inhibits various CVDs, including atherosclerosis, through reduced inflammatory gene expression [135,136]. In addition, preclinical studies show that cocoa extracts inhibit the expression of DNMTs [137].

Sulforaphane: The sulforaphane compounds are found in cruciferous family plants such as broccoli, cabbage, etc. Studies showed that sulforaphane has antioxidant function along with anti-inflammatory activity. In addition, preclinical studies showed that it reduces hypertension, cardiac hypertrophy, etc. [138]. Furthermore, the sulforaphane compound promotes cellular antioxidant activity through the regulation of the Nrf2 protein level by inhibiting DNMT-mediated hypermethylation of the Nrf2 promoter [139].

Allyl-isothiocyanate: Allyl-isothiocyanate (AITC) is an aliphatic sulfur-containing compound found in cruciferae plants such as horseradish, mustard, etc. Recent studies show that AITC can reduce doxorubicin-induced cardiotoxicity by reducing cellular inflammation [140]. In addition, AITC shows epigenetic changes in the cell through the regulation of HDACs and HATs activities [141].

Cinnamaldehyde: Cinnamaldehyde is an α,β-unsaturated aromatic aldehyde found in the common spice cinnamon. Several studies showed that cinnamaldehyde attenuates CVDs such as cardiac hypertrophy, atherosclerosis, and vascular dysfunction through modulation of hyperlipidemia, oxidative stress, and inflammation [142]. Based on its beneficial role, it is now advocated by the scientific community for the treatment of CVDs. In addition, cinnamaldehyde inhibits inflammatory gene expression and activation of Nrf2 signaling [143].

Eugenol: Eugenol is found in several common spices such as cinnamon, clove, turmeric, basil, etc. Eugenol shows cardioprotection in several CVDs [144]. It regulates the acetylation of histone proteins and induces cellular antioxidant capacity during stress by transcription factor Nrf2 [145]. Recently, a protective role of eugenol was demonstrated in cardiac ischemia. The authors found that during ischemia, total acetylation of histone is significantly reduced, and eugenol restores the cellular acetylation through the regulation of enzymes such as HMGN1 and PP2A [146].

Short-chain fatty acids: Short-chain fatty acids (SCFAs) are made of less than 6 carbons and play a critical role in the regulation of cardiometabolic disease [147]. SCFAs are produced in the gut through the conversion of natural dietary fibers into monosaccharides by the gut microbiota. Then through different enzymatic processing, SCFAs are converted to short-chain acyl-CoAs. In cell, short-chain acyl-CoAs act as the donor for the posttranslational modification of acetylation to histone and no-histone proteins. Additionally, SCFAs play a critical role in the maintenance acetylation level of histones by regulating the function of p300 and HDACs [148].

20.3 Exercise

Numerous studies suggest that daily exercise has profound health benefits and prevents CVDs. Further, it is now evolving that exercise can regulate the epigenetic modification of DNA or proteins and downstream cardioprotective signaling [149]. For example, prolonged exercise can cause increased expression of PGC-1 α, PPARδ, NRF1, and PDK4 through hypomethylation of the promoter area of these genes. At the same time, exercise causes inhibition of some pathological genes (e.g., RALBP1, GLUT4, $p66^{shc}$, ASC) expression through hypermethylation of these promoters [150]. Exercise also regulates the expression of several miRNAs, which may induce cardiac disease (miR-27a, miR-27b, and miR-143, miR-21, miR-124, miR-144, and miR-145, miR-133 and miR-1) or inhibits cardiac disease (miR-208a and miR-208b, miR-26b-5p, miR-204−5p, and miR-497−3p) [150]. Additionally, exercise shows beneficial effects by inducing the release of several circulating ncRNAs (CirRNAs) within the exosomes from several tissue types. These CirRNAs show cardioprotective function [149].

20.4 Clinical trials of epigenetic drugs and outcomes

Accumulating preclinical data showed that epigenetic drugs could be helpful in the treatment of CVDs such as type 2 diabetes, inflammation, congenital heart defects, hypertension, ischemic stroke, etc. There are three strategies used in the clinical trial: (1) Use of a drug to direct regulation of epigenetic

changes in the histone (e.g., apabetalone); (2) Use of repurposed drugs, which can directly or indirectly modulate the epigenetic changes and protect organs during stress (e.g., statin, metformin, omega-3 polyunsaturated fatty acids [PUFA], etc.); (3) Exercise. Several studies showed that the bromodomain and extra terminal bromodomain (BET) protein induces CVDs through the upregulation of inflammatory gene expression (ClinicalTrials.cov). One of the polyphenolic drugs, apabetalone, completed the phase 1 and phase 2 trials against atherosclerosis, coronary artery disease, and pulmonary artery hypertension and showed an optimistic effect on the suppression of CVDs. However, further study is needed to understand its effect on a diverse population. Another clinical study was performed with menopausal women having prediabetic mellitus (DM). After 14 weeks of exercise, different methylated regions (DMRs) were measured. Interestingly, exercise significantly changed the methylation of certain genes involved in cellular energy metabolism, cellular differentiation, and tumor suppression. This study concluded that exercise could help to control hyperglycemia [151]. Another clinical trial was performed using patients having ST elevation myocardial infarction. These patients were given metformin for 4 months. Interestingly, metformin treatment significantly reduced the phospholipid content in very large high-density lipoproteins [152]. Several clinical trials are going on to understand the efficacy of metformin in the treatment of CVDs. Likewise, several clinical trials were performed to test statin's beneficial role in the control of CVDs. However, US Preventive Services Task Force looking at the different trials, recommended the selective use of statin to prevent CVD in adult patients [153].

20.5 Conclusions

Advancements in cardiovascular research have reached a point where it is more apparent that CVDs are complex and multifactorial diseases. Symptoms of CVDs can vary from the early stage of the onset of illness to the advanced stage of the disease. Again, CVDs can vary with respect to gender, ethnicity, etc. Epigenetic changes also vary accordingly. Integrating bioinformatics data with clinical data can predict the real-time changes in the cellular epigenome and its relationship with the disease. This epigenomic information can be used for the personalized treatment of CVDs. Several epigenetic drugs, including small molecular inhibitor, natural product, and oligonucleotide, are in clinical trial against CVDs. Some of the completed trials showed promising effects in the treatment CVDs. However, some lead molecules need advanced pharmacogenomics and safety validation before getting approved for the treatment of CVDs. Further, it needs better delivery methods to deliver oligonucleotides to modify the epigenetic changes in vital organs such as the heart. Additionally, exercise shows additive benefits in the regulation of cardiometabolic changes by reversing epigenetic modification. The clinical trial suggests that exercise can be used along with other drugs to get better results against CVDs.

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CHAPTER

Epigenetics and human infectious diseases 21

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Certain bacterial and viral infections regularly cause epigenetic alterations in host cells. *Porphyromonas gingivalis* secretes butyric acid, an inhibitor of histone deacetylases, and may thereby elicit increased acetylation of histones H3 and H4 in the cells of the oral cavity. Toxins secreted by *Listeria monocytogenes, Clostridium perfringens*, and *Streptococcus pneumoniae* as well as the phosphothreonine lyase OspF of *Shigella flexneri* induce dephosphorylation of histone H3 at promoters of defense genes, thereby interfering with innate immunity. AnkA, the effector protein of *Anaplasma phagocytophilum*, achieves a similar effect by up-regulating histone deacetylases in granulocytes. DNA-(cytosine C5)-methyltransferases of *Mycoplasma hyorhinis* and *Mycobacterium tuberculosis* directly target cytosines of the infected host cell DNA. Hypermethylation of key cellular promoters at CpG dinucleotides, first observed in human immunodeficiency virus−infected cells, plays an important role in neoplastic development associated with *Helicobacter pylori*, Epstein−Barr virus, Kaposi's sarcoma−associated herpesvirus, human T-cell lymphotropic virus, hepatitis B virus, hepatitis C virus, and the macroparasite trematodes *Opistorchis viverrini* and *Schistosoma haematobium*, whereas the human papillomavirus oncoprotein E7 induces both histone methyltransferase and histone demethylase enzymes. Elucidation of patho-epigenetic consequences of microbe−host interactions may help to design new diagnostic tools and therapeutic strategies.

21.1 Introduction

Patho-epigenetics is a new discipline dealing with the pathological consequences of dysregulated epigenetic processes [1]. In recent years, it became more and more obvious that—perhaps following the lead of cancer research, where epigenetic and genetic theories of neoplastic development complement each other peacefully—epigenetic ideas found their way to virtually all areas of biomedical research. Looking at the exponentially accumulating data one has the impression that epigenetic alterations, induced by certain pathogenic viruses and bacteria in the host cells they are interacting with, play an unexpected but most important role in disease initiation and progression. In this review, we focus mainly on human pathogens eliciting epigenetic changes relevant, or at least potentially relevant, to disease initiation or progression. Although the idea that microbes do induce epigenetic changes in

Epigenetics in Human Disease. https://doi.org/10.1016/B978-0-443-21863-7.00006-8
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host cells is gaining more and more support, so far only a minority of infectious agents was analyzed in this respect. Thus, in this chapter we do not discuss, due to the lack of available data on their potential patho-epigenetic effects, even those pathogens, including protozoan parasites and fungi, that are known to use their own sophisticated epigenetic mechanisms to control the expression of their genomes [2–6]. For the very same reason, we omit alphaherpes viruses in spite of the fact that their latent genomes undergo epigenetic changes in their host cells [7,8], and oncogenic human adenoviruses that induce malignant tumors in experimental animals and elicit spectacular epigenetic alterations in tissue culture [9,10], but lack any association with human neoplasms. As to the bacterial pathogens discussed in this chapter, the epigenetic aspects of *Helicobacter pylori* infection were analyzed most intensively, due to its association with gastric carcinoma. Regarding viruses infecting humans, the epigenotypes of tumor-associated DNA viruses and proviral DNA copies of retroviral genomes were characterized in detail. These data have potential implications as to the therapy of neoplasms and elimination of latent reservoirs of human immunodeficiency virus and human T-cell lymphotropic virus. Deciphering how viral and other microbial proteins and non-translated RNAs alter the host cell epigenome may result in new tools permitting early and stage-specific detection of pathological alterations. Epigenetic studies may contribute to a better understanding of the diseases caused by infectious pathogens, first of all microbes, but also macroparasites, including helminths, fungi, and arthropods. We realize that in mammals, epigenetic alterations regularly occur in parallel with gene expression changes, independently of pathologic processes [11–15]. Understanding how microbe-induced dysregulation affects key epigenetic processes may illuminate, however, distinct aspects of transcriptional control in normal, uninfected cells, too.

It is our impression that within the past 5 years there were significant developments in the field of microbe-induced epigenetic alterations. Further, the application of high throughput sequencing and inventive experimental models significantly contributed to this progress. It is also remarkable, however, that by now, the epigenetic studies extended also to the interactions of microbes with cells of the native immune system ("trained immunity," see Section 21.3.3.2) and cells involved in adaptive immune responses (see Section 21.4.2). Furthermore, there were novel efforts to trace "epigenetic signatures" left by microbes on the host cell epigenotypes after their elimination from the infected cells ("hit and run" tumorigenesis, see Sections 21.3.1.2 and 21.3.5). Due to the recent pandemic caused by SARS-CoV-2, a coronavirus infecting humans, several studies tried to illuminate putative epigenetic mechanisms involved in the pathogenesis of COVID-19. We summarized this initial work in Section 21.3.8.

21.2 Epigenetic modifications elicited in host cells during bacterial infections

Bacteria may affect the epigenetic regulatory mechanisms of their hosts via the metabolic products and toxins released into their environment. In addition, certain pathogens synthesize effector proteins capable of inducing epigenetic alterations. Such effector proteins are either injected into their target cells or enter the host cell nuclei as the products of bacteria that are capable of surviving within the cytoplasm of infected cells. In several cases the exact mechanism of epigenetic changes elicited by bacterial infections remains to be clarified.

21.2.1 Epigenetic alterations induced by bacterial infections in periodontal disease

Pathogenic Gram-negative anaerobic bacteria populating the tooth-associated biofilm may cause an inflammatory reaction (periodontitis). The resulting chronic inflammation, periodontal disease, affects the majority (50%−90%) of the worldwide population [16]. In addition to its local consequences (occasional pain, impaired mastication, loosening and loss of teeth), subgingival infection may have a systemic impact including adverse pregnancy outcomes, atherosclerosis, rheumatoid arthritis, diabetes, and aspiration pneumonia. A critical step in periodontal disease progression is the disruption of the host innate immune system by anaerobic bacteria (reviewed by Ref. [17]). The cells of the innate immune system, including gingival epithelial cells (GECs), recognize pathogen-associated molecular patterns interacting with Toll-like receptors (TLRs) and other receptor classes, and respond by the production of antimicrobial peptides such as human β-defensins and chemokines that activate adaptive immune responses as well [18−20]. In addition to *Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola*, *Prevotella intermedia*, *Fusobacterium nucleatum*, and other pathogenic bacteria causing periodontitis, a series of non-pathogenic bacteria also inhabit the oral cavity of periodontitis patients, forming a microbial community (reviewed by Refs. [17,21]). Similarly, to their pathogenic counterparts, non-pathogenic bacteria may also interact with GECs, modulating native immune responses to invading pathogens.

Porphyromonas gingivalis, one of the major etiological agents of periodontal disease, elicited complex changes when incubated with gingival epithelial cells (GECs): certain gene products were upregulated, whereas others were downregulated in the host cells [22]. As observed by Yin and Chung, *P. gingivalis* suppressed the expression of selected chromatin modifying enzymes, such as histone deacetylase 1 (HDAC1), histone deacetylase 2 (HDAC2), and DNA methyltransferase 1 (DNMT1); these changes could contribute to the stimulation of human β-defensin and CCL20 (CC chemokine ligand 20) expression [22]. In parallel, however, increased DNA methylation, i.e., a repressive chromatin mark was also detected in the promoter region of six genes, including the immune regulator *CD276*, *elastase 2*, *TLR2, IL-12A*, and two putative tumor suppressor genes (TSGs). The level of activating histone modification H3K4me3 decreased in GECs incubated with *Porphyromonas gingivalis,* but not in the presence of another oral anaerobe, *Fusobacterium nucleatum* [21,22].

Benakanakere et al. also observed hypermethylation and inactivation of the TLR2 (Toll-like receptor 2) promoter in gingival epithelial cells in a "chronic in vitro model" of *Porphyromonas gingivalis* infection [23]. This finding was confirmed in the gingiva of mice as well, using an in vivo model of murine periodontitis [23]. Since TLR2 (Toll-like receptor 2) is a key regulator of immune response, de novo methylation-mediated silencing of the TLR2 promoter in the "dysregulated" GECs resulted in the downregulation of TLR2 expression and in a blunted cytokine and antimicrobial peptide response to *P. gingivalis* [23]. Benakanakere et al. speculated that *P. gingivalis*-induced epigenetic dysregulation in host cells may contribute to immune evasion and increased disease susceptibility in patients with chronic periodontitis [23].

Recently, the effect of *P. gingivalis* infection on the barrier function of human gingival epithelial cells was studied by Barros et al. [24]. They found that the transepithelial electrical resistance of *P. gingivalis* infected cells decreased, compared to non-infected controls, in parallel with a decrease of mRNA transcripts expressed from three genes encoding cell-cell junction proteins. This phenomenon

was due to *P. gingivalis* induced epigenetic dysregulation, i.e., DNA methylation-mediated silencing of the genes that specify components of intercellular connections [24]. Thus, epigenetic events initiated by *P. gingivalis* in epithelial cells may compromise the barrier function of gingiva and facilitate the development of periodontitis.

Short-chain fatty acids, the metabolic by-products of *Porphyromonas gingivalis*, are secreted extracellularly and may affect local immune responses, contributing thereby to the development of periodontal disease [25–27]. Butyric acid, the major short-chain fatty acid species produced by pathogenic *Porphyromonas gingivalis* strains, accumulated in periodontal pockets [26–28], and it was suggested that as an HDAC inhibitor [29,30] (reviewed by Ref. [31]), butyric acid may induce acetylation of histone H3 and H4 in neighboring cells. Indeed, supernatants of *Porphyromonas gingivalis* cultures, and butyric acid induced histone acetylation in T-cells and macrophages carrying latent proviral genomes of type 1 human immunodeficiency virus (HIV-1), and caused HIV-1 reactivation [28]. The role of periodontal disease in the progression of acquired immunodeficiency syndrome needs further studies.

We would like to add that recently a kind of epigenetic therapy, using histone mimetics, was suggested to ameliorate the chronic inflammation elicited by *Porphyromonas gingivalis* and other oral bacteria (see Maksylewicz et al. [32], and references therein). Distinct histone mimetics interfere with BET bromodomain proteins that recognize acetylated histone tails and regulate, among others, the expression of inflammatory genes involved in the pathogenesis of periodontitis [32].

Another bacterium involved in periodontal infections, *Campylobacter rectus* may also induce epigenetic alterations in human cells. In experimental mice *Campylobacter rectus* infection down-regulated the expression of the *Igf2* (insulin-like growth factor 2) gene via hypermethylation of the *Igf2* promoter in the murine placenta [33]. This epigenetic change resulted in reduced placental growth and fetal growth restriction, suggesting that a similar mechanism may be involved in preterm births associated with *Campylobacter rectus* infection in humans [34]. The potential epigenetic consequences of *Campylobacter rectus*–gingival epithelial cell interactions remain to be established.

21.2.2 Histone modifications caused by *Listeria monocytogenes*

The Gram-positive bacterium *Listeria monocytogenes* appears to cause only little inflammation during infection. *L. monocytogenes* is associated with foodborne infections, causing usually self-limiting gastroenteritis, but also sepsis and meningitis in immunocompromised patients, perinatal and intrauterine infection, and abortion in pregnant women. It acquired the tools for survival within the cytoplasm of macrophages and endothelial cells (reviewed by Ref. [35]). *L. monocytogenes* appears to use its toxin listeriolysin O (LLO) not only as a pore-forming toxin to escape from the phagosome and enter the host cytoplasm but also in an extracellular form, before entering the host cell, to induce histone modifications in the cells to be infected via an unexplored signaling pathway. Extracellular, pathogenic *Listeria* or purified LLO protein induced, at a subset of genes and within 3 h or even within 20 min, dephosphorylation of histone H3 at serine 10 and deacetylation of histone H4 [36]. In these experiments, the induction of histone H3 dephosphorylation correlated with a decreased expression of the chemoattractant chemokine CXCL2 involved in the recruitment of polymorphonuclear cells during epithelial cell infection. Expression of the phosphatase DUSP4/MKP2 was also down-regulated, similarly to the expression of *IFIT3, a gene coding for the adaptor molecule* interferon induced protein with tetratricopeptide repeats 3; *IFIT3* is known to be regulated by IFN regulatory factor-3 (IRF-3). Furthermore, a decreased expression of the transcription factor EGR1 (early growth response 1) was also observed.

Thus, *L. monocytogenes* and its toxin, LLO, down-regulates key mediators of innate immunity before invading the host cells, by eliciting targeted, local histone modifications at key immune response genes. The situation is complex, however, because transcriptome analysis revealed not only 47 repressed but also 99 induced genes after 20 min of LLO incubation [36].

In addition to the above mentioned, LLO-induced local histone modifications, i.e., dephosphorylation of histone H3 at serine 10 and deacetylation of histone H4, interaction of *L. monocytogenes* with human or mouse cells in vitro also resulted in relocalization of SIRT2, a cellular histone deacetylase, from the cytoplasm to the nucleus [37], SIRT2 (Sirtuin 2 is a nicotinamide-adenine-dinucleotide-dependent deacetylase) underwent dephosphorylation of S25 (serine 25), and is associated with the chromatin [38] (reviewed by Bierne and Hamon [39]) where it repressed a battery of genes by H3K18 deacetylation [38]. Thus, *L. monocytogenes* infection affects the host cell chromatin in a complex manner, inducing histone H3 dephosphorylation and deacetylation as well as histone 4 deacetylation. Furthermore, in epithelial cells, LntA (*Listeria* nuclear targeted protein A), a *Listeria*-encoded effector protein enters the nucleus and modulates the expression of interferon stimulated genes (ISGs) via its interaction with BAHD1, a protein carrying a bromo adjacent homology (BAH) domain (reviewed by Ref. [39]). Thus, LntA belongs to a class of bacterial virulence factors, termed nucleomodulins by Bierne and Cossart, which are targeted to the host cell nucleus ([40]; see also Section 21.2.8). By interacting with the scaffold protein BAHD1, LntA actually activates interferon stimulated host genes [41] (reviewed by Ref. [39]).

Similarly to LLO, pore-forming toxins of other bacteria, belonging to different genera and unrelated to *Listeria monocytogenes*, also induced dephosphorylation of histone H3. LLO and the so-called cholesterol-dependent cytolysines (CDCs) of the LLO family, including perfringolysin (PFO) produced by *Clostridium perfringens* and pneumolysin (PLY) secreted by *Streptococcus pneumoniae*, exerted their histone-modifying effects by the induction of pore-dependent K(+) efflux [42].

Aerolysin, a protein secreted by the Gram-negative enteropathogenic bacterium *Aeromonas hydrophila*, was characterized as a pore forming toxin inducing K^+ efflux [42–45]. Unlike the CDCs produced by Gram-positive bacteria, aerolysin does not utilize cholesterol as a membrane receptor. Incubation of target cells with aerolysin, however, also resulted in dephosphorylation of histone H3 at serine 10 in target cells [42].

21.2.3 Histone dephosphorylation by OspF, the effector protein of *Shigella flexneri*

Shigella flexneri, a Gram-negative bacterium, causes diarrhea that is usually self-limiting. Life-threatening disease may develop, however, in the absence of adequate medical care or in immuno-compromised patients [46]. *Shigella flexneri* abrogated histone H3 phosphorylation at selected cellular promoters by injection of OspF, an effector protein into epithelial cells [47]. OspF entered the nucleus and dephosphorylated the mitogen-activated cellular protein kinases Erk and p38, thereby blocking histone H3 phosphorylation at Ser10 [47]. These events resulted in repression of a narrow set of genes including the NF-κB responsive *IL8* and *CCL20*. By down-regulating IL-8 expression, OspF blocked neutrophil recruitment in vivo in a rabbit model of infection [47]. Li et al. observed that OspF acts as a phosphothreonine lyase, removing phosphate residues from the activation loop of mitogen-activated protein kinases [48].

In addition to histone H3, OspF also alters the phosphorylation of HP1γ (Heterochromatin Protein 1, isoform γ), an HP1 family member which can be modified by phosphorylation at Serine 83 [49]. It was observed that this phosphorylated form of HP1γ is associated, unexpectedly, to euchromatic regions ([49] and references therein). Harouz et al. speculated that OspF interacts with and causes dephosphorylation of HP1γ, practically "using" the targeted HP1γ proteins as beacons to locate and repress immune gene expression [49]. For a recent review of OspF and other nucleomodulins see Bierne and Pourpre, 2020, [50].

21.2.4 Histone and DNA modifications in *Anaplasma phagocytophilum*–infected cells

Anaplasma phagocytophilum, a tick-transmitted rickettsial pathogen, causes an acute febrile disease [51]. It is capable of infecting and surviving in granulocytes by blocking or delaying key antimicrobial mechanisms including oxidative burst, apoptosis, and phagocytosis, and down-regulating defense gene expression in its host cells [52]. AnkA, the effector protein of *Anaplasma phagocytophilum*, is translocated into the nucleus of the host cell where it binds to the host DNA and nuclear proteins and silences the expression of 19 defense genes arranged into three gene clusters. They code for antimicrobial peptides and enzymes or proteins involved in the generation of reactive oxygen intermediates. Silencing correlated with a transient increase of HDAC1 expression and a steady increase of HDAC2 mRNA levels in *Anaplasma phagocytophilum*–infected cells. In addition, at 48 h following infection of the THP-1 acute monocytic leukemia cell line, an increased binding of HDAC1 and a parallel decrease in acetylated histone H3 was observed at most of the defense gene promoters, suggesting that *Anaplasma phagocytophilum* takes over the epigenetic control of host defense gene clusters [52].

The initial data suggested that AnkA affects transcription locally, in *cis,* by the direct binding to AT-rich DNA sequences [52,53]. A reanalysis and reevaluation of gene expression data from *A. phagocytophilum*–infected human peripheral blood neutrophils revealed, however, the presence of both downregulated and upregulated cellular gene-clusters that resembled the differentially expressed genes detected in AnkA expressing HL60 cells [54], based on [53,55]. Thus, Sinclair et al. and Dumler et al. speculated that AnkA, and similar bacterial effectors, called nucleomodulins, may bind to matrix attachment regions (MARs) in the nuclei of infected cells and change the three-dimensional architecture of chromatin loops, eliciting thereby genome-wide changes of cellular gene expression [54,56] (for a recent review see Ref. [50]; see also Section 21.2.8).

A. phagocytophilum infection of human neutrophils resulted in a quick increase of cellular DNA methylation, comprising 16% of all methylated regions in the infected cells [57]. Typically, DNA methylation decreased immediately upstream of the transcription start sites, but increased in the gene bodies. The mechanism of this de novo methylation remains to be established.

We would like to note that in addition to vertebrate cells, *Anaplasma phagocytophilum* infection affects the epigenetic machinery in arthropod cells as well. Cabezas-Cruz et al. gathered transcriptomics and proteomics data of *Anaplasma phagocytophilum* infected tick (*Ixodes scapularis*) tissues and cultured cells [58]. They found that *Anaplasma phagocytophilum* infection increased the levels of distinct histone modifying enzymes (HMEs) Isp300/CBP, IsHDACs, and IsSirtuin in the tick vector [58]. Cabezas-Cruz et al. suggested that these alterations in epigenetic regulation and gene expression may result in the inhibition of host cell apoptosis, facilitating thereby the survival of the pathogen as well as its vector organism [58].

21.2.5 Epigenetic alterations associated with *Helicobacter pylori* infection

Helicobacter pylori (Hp), a Gram-negative spiral bacterium, colonizes more than half of the world's population in early childhood. Primary infection of the stomach regularly triggers an inflammation. Infection can lead to gastric or duodenal ulcer disease and different types of chronic gastritis (atrophic gastritis, enlarged fold gastritis, pangastritis). Chronic inflammation can progress to gastric adenocarcinoma in about 1%−2% of the infected patients through the premalignant stages of gastric atrophy, intestinal metaplasia, and dysplasia. The risk of developing intestinal metaplasia or different types of malignant neoplasms (intestinal type gastric carcinoma (GC), sporadic diffuse type GC, MALT lymphoma) is significantly associated with Hp carrier status [59] (reviewed by Refs. [60,61]). Hp codes for the virulence factors vacuolating cytotoxin VacA and cytotoxicity-associated antigen CagA. CagA is encoded within the bacterial cag-pathogenicity island (cag-PAI). Both proteins are strongly associated with Hp pathogenesis. CagA, a bacterial oncoprotein, can be injected into gastric epithelial cells by a type IV bacterial secretion system and interfere with multiple cellular signal transduction pathways (reviewed by Refs. [62−64]). Through activation of the tyrosine phosphatase SHP-2, CagA may elicit uncontrolled cell proliferation, and induce chromosomal instability by destabilizing microtubules during mitosis [65].

Epigenetic dysregulation is strongly associated with gastric carcinogenesis [66−68]. Both global genomic hypomethylation and CpG island (CGI) hypermethylation correlated with the presence of Hp in gastritis patients, and malignant progression in cancer patients [69−74]. Non-tumorous gastric tissue of Hp-negative GC patients also showed increased CGI hypermethylation [69,70,75]. Concordantly, Hp eradication led to a significant decline of CGI hypermethylation in gastritis patients, and in a small rodent model, but remained still higher than in non-infected individuals [71,76−78] (reviewed by Ref. [79]) or in animals [80]. With the progress of Hp-induced chronic gastritis through the advanced stages of atrophy, metaplasia, dysplasia, and GC, Hp was frequently lost from the gastric mucosa [81]. Thus, the hypermethylation profile of gastric mucosa does not regularly correlate with the Hp-carrier status in the advanced stages [73].

21.2.5.1 *CpG-methylation profiles of* Helicobacter pylori*−associated gastric carcinoma*

TSGs in gastric carcinogenesis are more frequently inactivated by promoter methylation than by mutations [82]. One may wonder, however, whether epigenetic dysregulation is causative for carcinogenesis or a mere epiphenomenon. The study of the E-cadherin (*CDH1*) promoter may help to clarify this question. *CDH1* down-regulation decreases cellular adhesion and therefore increases cellular motility and the disposition of cancer cells for metastasis. *CDH1* germline mutation is regularly observed in families with hereditary diffuse GC. Thus, *CDH1* inactivation clearly predisposes to GC and is therefore co-causal [83]. Somatic *CDH1* mutation is also common in sporadic GC, while transcriptional silencing by promoter CGI hypermethylation represents a second hit for carcinogenesis [84,85]. The following observations support a causative role for epigenetic dysregulation of *CDH1* in gastric carcinogenesis: (1) *CDH1* promoter hypermethylation is especially frequent in enlarged fold gastritis, a high-risk factor for GC [86]; (2) in the gastric mucosa of Hp-infected patients *CDH1* expression is down-regulated [87], due to promoter methylation [69,76,77,88]; (3) epigenetic silencing of *CDH1* occurs early in gastric carcinogenesis [71] (reviewed by Ref. [60]). Overall, hypermethylation of *CDH1* is clearly co-causal in the development of sporadic GC (reviewed by Ref. [89]).

The patterns of hypermethylated CGIs in non-cancerous mucosa from GC patients presented a rather characteristic fingerprint of Hp infection [90,91]. Surprisingly, however, and unlike in other malignancies, DNMTs were not induced by Hp in human gastric mucosa [90], and they were down-regulated when Hp was cocultured with gastric epithelial cells from gerbils [80]. In contrast, another study found an up-regulation of DNMT1 and DNMT3A through coculturing Hp with two human GC cell lines [92]. However, cancer cell lines may react differently from primary cells. Thus, other mechanisms, like altered distribution of DNMTs or inflammation-induced disturbance of factors protecting CGIs against methylation, may explain Hp-associated CGI methylation [80,93,94]. The role of Hp-encoded 5′-CpG-DNMTs remains to be explored [95] .

Ushijima observed a clear association between the methylation levels in the histologically normal gastric mucosae and the risk of gastric cancer development. He suggested that increased CGI-hypermethylation predisposed to carcinogenesis and proposed that the epigenetically altered mucosal tissue corresponded to an *epigenetic field* for cancerization [82]. Methylation was found at the *p16, MLH1, ECAD, DAPK,* and *MTSS1* loci of the healthy mucosa of GC patients with and without Hp-infection [68,72,75,76]. The *MLH1* repair-gene methylation was not seen in non-neoplastic epithelia from healthy persons, but was frequent in non-neoplastic tissue from GC patients [71,75], and it was significantly associated with the intestinal type of GC [96]. The metastasis suppressor gene *MTSS1* is highly expressed in normal gastric mucosa and frequently affected by loss of heterozygosity in GC, suggesting a physiological role for *MTSS1* as a suppressor of gastric carcinogenesis [68].

A series of TSGs and many other genomic loci were methylated at a higher level in Hp-positive than Hp-negative cases [69,71,91,92,97–99]. The degree of methylation at the *CDH1, p16, MLH1, APC,* and *COX2* loci correlated to malignant progression, with *CDH1* methylation as an early, and that of *MLH1* as a late, event. *COX2* hypermethylation was associated with the Hp virulence factor VacA [96], whereas hypermethylation at *p16* and *THBD* (*thrombomodulin*) correlated significantly with inflammatory cell infiltration. Thus, chronic inflammation was potentially involved in methylation induction [72,78]. Hp-eradication led to a decreased methylation at *CDH1, p16, APC, FLNc, THBD, MGMT,* and to the complete disappearance of *COX2* (*cyclooxygenase 2*) methylation [71,78,99]. The specific methylation profiles associated with GC and Hp infection may allow the development of new diagnostic tools to detect precancerous stages, assess the risk of GC development, and estimate its prognosis [91].

21.2.5.2 Epigenetic silencing of miRNA genes in Helicobacter pylori*–associated neoplasms*

Ando et al. found that three putative tumor-suppressor miRNA genes, *miR-124a1*, *miR-124a2*, *miR-124a3*, were unmethylated in normal mucosa, but hypermethylated in tumor cell lines. In biopsies, hypermethylation was strongly associated with a positive Hp carrier status [100]. *miR-34b* and *miR-34c* were highly methylated in GC, but not in normal gastric mucosa from Hp-negative persons. Transfection of these miRNAs suppressed cell growth in vitro, suggesting a tumor-suppressor function [101]. The tumor suppressor *miR-203* was significantly down-regulated by hypermethylation in human low-grade MALT-lymphoma biopsies. Methylation of *miR-203* coincided with the increased expression of its direct target, the non-receptor tyrosine kinase and oncogene *ABL1* [102,103]. Overexpression of miR-203 in ABL1-expressing human lymphoma cells inhibited proliferation. Further, the ABL1 inhibitor imatinib caused tumor regression in a MALT lymphoma mouse model based on

chronic *Helicobacter felis* infection [102]. Hypermethylation of the *miR-124* and *miR-34* groups and of *miR-203* may contribute to the *epigenetic field for cancerization* and may also serve as a predictive marker for GC risk [82,100,101].

21.2.5.3 *The role of polycomb group proteins and histone modifications in* Helicobacter pylori*—associated gastric cancer*

Polycomb group (PcG) proteins play an important role in reversibly repressing transcription factor genes which are involved in development and differentiation in embryonic stem cells. This reversible PcG-mediated repression utilizes trimethylation on lysine 27 of histone 3 (H3K27me3). In cancer cells, but not in normal cells, the H3K27me3 chromatin mark recruited DNMTs, resulting in a permanent CpG-methylation [104]. Thus, in cancer cells, reversible repression was more frequently replaced by methylation and permanent silencing at stem-cell PcG-target promoters than at non-PcG target promoters [105] (reviewed by Ref. [106]). A glutathione peroxidase knockout mouse model for inflammatory bowel disease and intestinal cancer confirmed these observations. Healthy wild-type mice mostly kept the H3K27me3 chromatin mark at PcG target genes, while inflammation in knockout mice led to aberrant methylation mostly of PcG targets which was later also observed in cancer tissue [107].

Changes in global histone modification profiles and HDAC expression patterns correlated with the progression of GC in general and may serve as prognostic markers (reviewed by Ref. [62]). Further, the epigenetic repression through both DNA methylation and histone modification of specific genes involved in chromatin remodeling, cell cycle control and tumor suppression has been linked with GC as well (reviewed by Ref. [62]). Coculture of Hp with gastric epithelial cells caused a global decrease of histone H3 phosphorylation and deacetylation of H3 at lysine 23 [108,109]. Histone dephosphorylation was dependent on the presence of the Hp cag-PAI, but not on the CagA and VacA proteins. As a consequence of Hp-triggered epigenetic dysregulation, the expression of the *c-jun* proto-oncogene increased, and that of *hsp-70,* coding for a heat shock protein, decreased [109]. The epigenetic changes associated with *Helicobacter pylori* infection were reviewed recently by Capparelli and Iannelli [110].

We would like to note that a fraction of GCs is associated with Epstein—Barr virus, a human gammaherpesvirus (see Section 21.3.1).

21.2.6 Uropathogenic *Escherichia coli* infection down-regulates CDKN2A ($p16^{INK4A}$)

Coculture of uropathogenic *Escherichia coli* (UPEC) with human uroepithelial cell lines strongly induced DNMT1 expression in comparison with nonpathogenic strains. In parallel, the tumor suppressor CDKN2A and the DNA repair gene MGMT were downregulated, whereas a set of other genes (CDH1, MLH1, DAPK1, and TLR4) were not affected. Downregulation of CDKN2A correlated with DNA methylation of its promoter. However, the MGMT gene was not methylated. Frequent UPEC infections might increase the risk for bladder cancer through increasing methylation of TSGs [111,112].

Furthermore, UPEC induced EZH2 (enhancer of zeste, part of the repressive polycomb 2 complex) activity which led to the proliferation of cocultured uroepithelial cells through the methyltransferase function of EZH2 at H3K27me3 [113].

Inhibiting apoptosis is another mechanism to increase the risk of cancerogenesis. Actually, uropathogenic *E. coli* have been found to epigenetically silence BIM (a BH3-only bcl2-family member) expression in rat Sertoli cells by decreasing histone 4 acetylation at the BIM promoter [114].

21.2.7 Chlamydophila spp.—encoded histone methyltransferases: putative inducers of epigenetic reprogramming in host cell nuclei

Chlamydophila pneumoniae, an obligatory intracellular bacterium involved in acute respiratory diseases and implicated in chronic inflammatory processes, may modify, in principle, the epigenotype of its host cells. Similarly to *Anaplasma phagocytophilum, C. pneumoniae* introduces a putative bacterial effector, in this case a SET domain protein, into the host cell nucleus. Although histone-like proteins play an important role in the lifecycle of *Chlamydophila pneumoniae,* its putative effector protein, a histone methyltransferase (HMT), preferentially targets histone H3 in murine host cells [115] (Table 21.1).

Analogously, *Chlamydia trachomatis* codes for a SET domain-protein, called "nuclear effector" (NUE), which functions as an HMT. During infection, NUE associates with the host cell chromatin and methylates histones H2B, H3, and H4 [116]. Modification of host-encoded histones may reprogram the

Table 21.1 Epigenetic alterations elicited by bacterial infections in host cells.

Microbe	Effector molecule	Epigenetic alteration	Effect
Porphyromonas gingivalis	Butyric acid	Histone acetylation	HIV-1 reactivation
Listeria monocytogenes	Listeriolysin O InlB	Histone dephosphorylation Histone deacetylation	Blocking the immune response
Clostridium perfringens	Perfringolysin	Histone dephosphorylation	?
Streptococcus pneumoniae	Pneumolysin	Histone dephosphorylation	?
Aeromonas hydrophila	Aerolysin	Histone dephosphorylation	?
Shigella flexneri	OspF, a phosphothreonine lyase	Inhibition of histone phosphorylation and HP1γ phosphorylation	Blocking innate immunity
Anaplasma phagocytophilum	AnkA	Upregulation of HDAC, alteration of chromatin architecture (?) and host DNA methylation	Blocking defense genes ? ?
Helicobacter pylori	Bacterial DNMTs	?	?
	?	Promoter hypermethylation	Gastric carcinogenesis
	?	Histone dephosphorylation Histone deacetylation	?

Table 21.1 Epigenetic alterations elicited by bacterial infections in host cells.—cont'd

Microbe	Effector molecule	Epigenetic alteration	Effect
Campylobacter rectus	?	Methylation of the Igf2 promoter	Preterm births
Uropathogenic E. coli	?	Promoter hypermethylation	CDKN2A silencing
Chlamydophila spp.	Histone methyltransferase	Histone methylation	Gene silencing
Ehrlichia chaffeensis	TRP47 and TRP120; Ank200	Binding to PCGF5 (polycomb group ring finger protein 5); ?	? ?
Bordetella bronchiseptica	BbSET, histone methyltransferase	Histone methylation	?
Burkholderia thailandensis	BtSET, histone methyltransferase	Histone methylation	?
Legionella pneumophila	LegAS4, histone methyltransferase	Histone methylation	Activation of rDNA transcription
Mycoplasma penetrans	M.MpeI, CpG-specific DNA methyltransferase	?	?
Mycoplasma hyorhinis	Mhy1, Mhy2, Mhy3, *DNA-(cytosine C5)-methyltransferases*	Alteration of the host cell methylome	Alteration of gene expression pattern in host cells
Mycobacterium tuberculosis	*Rv2966c* *Rv1988* *?* Rv3423.1	*DNA-(cytosine C5)-methyltransferase* *Protein arginine methyltransferase* *Alteration of HDAC expression* Histone acetyltransferase	Transcriptional repression Repression of host defense genes ? ?
Mycobacterium leprae	*?*	*Alteration of the host cell methylome*	Alteration of gene expression pattern in host cells

?: (1) Microbe-encoded proteins which may cause epigenetic alterations or functional effects have not been identified yet; (2) epigenetic alterations or functional effects have not yet been linked to microbe-encoded proteins; (3) functional effects of microbe-encoded proteins have not been described yet.

infected cell, perhaps by silencing host defense genes, as the *Anaplasma phagocytophilum* effector AnkA happens to do it.

On the basis of putative epigenetic alterations induced by *Chlamydia trachomatis,* Horner et al. recently speculated that in the female reproductive tract such changes may alter the phenotype of epithelial cells, resulting in epithelial-mesenchymal transition (EMT) [117]. Thus, via the induction of EMT, *Chlamydia trachomatis* could possibly be involved in a diverse set of disease pathologies [117].

We would like to add that *Chlamydophila psittaci* that causes a zoonosis called psittacosis or parrot fever in humans, may induce genetic and epigenetic dysregulation as well, because altered DNA methylation was observed in ocular adnexal marginal zone B-cell lymphomas associated with *Chlamydophila psittaci* infection [118–120].

21.2.8 Reprogramming of the host cell transcription by epigenetic regulators of *Ehrlichia chaffeensis*, *Bordetella bronchiseptica*, *Burkholderia thailandensis*, and *Legionella pneumophila*

Ehrlichia chaffeensis, an intracellular rickettsial pathogen, causes human monocytotropic ehrlichiosis, an emerging life-threatening zoonosis [121,122]. *Ehrlichia chaffeensis* is transmitted by ticks and its 1.3 Mb genome encodes several tandem repeat-containing secreted effector proteins that enter the host cell nucleus. The tandem repeat protein TRP47 and TRP120 bind to a component of the cellular epigenetic machinery, PCGF5 (polycomb group ring finger protein 5), but the effect of these interactions on the host cell epigenotype remains to be elucidated (reviewed by Ref. [123]). TRP120 could bind to DNA directly, via a GC-rich motif, and upregulated the expression of chemokines, whereas Ank200 bound to an adenine-rich motif elevating the level of the B-lymphocyte activation marker CD48, the cytokine TNFα and the transcription factor STAT1 (signal transducer and activator of transcription 1) [124,125]. In addition, TRP32, binding to a G-rich motif also affected the expression of a vast array of host genes [126]. Ank200, TRP120 and TRP32 were suggested to function as nucleomodulins that modulate host cell transcription by directly binding to the host chromatin, similarly to AnkA encoded by *Anaplasma phagocytophilum* ([123]; see also Section 21.2.4).

The genomes of the Gram-negative bacteria *Bordetella bronchiseptica*, *Burkholderia thailandensis* and *Legionella pneumophila* encode the histone methyltransferases BbSET, BtSET and LegAS4, respectively. These effector proteins are capable to enter the host cell nucleolus and may upregulate the transcription of the ribosomal DNA (rDNA) [127]. LegAS4 deposited histone H3K4me2, an activating chromatin mark, to the rDNA promoter [127]. *Bordetella bronchiseptica* is a rare cause of bronchitis in humans and *Burkholderia thailandensis* is not known to be associated with human diseases [128,129]. *Legionella pneumophila*, an aerobic, Gram-negative, flagellated bacterium causes, however, a life-threatening pneumonia called Legionnaire's disease in humans (reviewed by Ref. [130]). *Legionella pneumophila* is ubiquitous in natural and technical water systems. It can infect and replicate in free-living protozoa such as *Acanthameba.* Infection of humans via contaminated aerosols is followed by the replication of the facultative intracellular bacterium within alveolar macrophages. Replication starts with the formation of the so-called *Legionella*-containing vacuole (LCV) and the translocation of bacterial effector proteins into the host cell. These effectors create a replication-permissive environment for *Legionella pneumophila* by interfering with a series of physiological processes of the host cell. In addition to LegAS4, another histone methyltransferase called RomA also enters the host cell via the type IV secretion system of the bacterium. The atypical RomA methylase trimethylates histone H3 lysine 14 (H3K14) and represses numerous cellular promoters [131]. Rolando et al. observed that intracellular replication of a RomA-deleted *Legionella pneumophila* strain was reduced in infected *Acanthameba* cells as well as in human cell lines, whereas in liquid medium the growth of the mutant strain, designated *Δlpp1683*, was comparable to that of the wild-type (WT) *Legionella* strain [131]. In human THP-1 macrophages, an increase in H3K14 methylation was observed already 4 h after infection with the WT strain, and 4870 H3K14 methylated promoter regions could be identified. This

genome-wide H3K14 methylation affected the innate immune genes coding for IL-6, TNFα, CXCL1, CXCL2, and the NOD (nucleotide-binding oligomerization domain) -like receptor NALP3, suggesting that RomA-mediated transcriptional repression may lead not only to metabolic downregulation in *Legionella* infected cells, but it may also curb the host's immune response [131]. Using peptide arrays, Schuhmacher et al. investigated the substrate specificity of RomA [132]. They found that in addition to histone H3, RomA methylated a set of nonhistone-proteins as well. Thus, RomA may act as a pleiotropic effector in the infection process [132]. The pathogenesis mechanisms of *Legionella* were reviewed recently by Mondino et al. [133].

21.2.9 Alteration of the host cell methylome by *Mycoplasma hyorhinis*—encoded DNA methyltransferases

In bacterial cells, DNA methyltransferases (DNMTs) are typically expressed together with restriction endonucleases, as components of a defense system protecting against invading foreign DNA. DNA adenine methyltransferases forming 6-methyladenine may play a role in the control of replication, repair and transcription of bacterial genomes [134]. In principle, expression of DNA adenine methyltransferases encoded by intracellular bacteria may modify not only bacterial DNA sequences but also the genome of the host cell and may affect the gene expression patterns of the infected human cells, too. Although in vitro experiments support this idea, a role for bacterial DNA adenine methyltransferases in human pathology remains to be documented [135,136].

Recently, it was suggested that another class of bacterial DNA methyltransferases, methylating the C5 position of cytosine within CpG dinucleotides, may play a role in human diseases. The genome of *Mycoplasma penetrans*, an obligate intracellular pathogen, encodes a CpG-specific DNA methyltransferase called M.MpeI [137]. *Mycoplasma penetrans* contributes to the development of acquired immunodeficiency syndrome (AIDS) in HIV-infected individuals and may cause urethritis and respiratory distress syndrome in patients not infected with human immunodeficiency virus (HIV) (reviewed by Ref. [138]). The effect of M.MpeI on the cellular genome, however, remains to be investigated.

Mycoplasma hyorhinis, another *Mycoplasma* species originally discovered in swine, was associated with human carcinomas, including gastric cancer (reviewed by Refs. [139–141]). Chernov et al. expressed Mhy1, a DNMT encoded by *Mycoplasma hyorhinis,* in various human cells in vitro. They observed that Mhy1 entered the host cell nuclei, and altered the pattern of DNA methylation and gene expression of host cells [142]. Thus, one may speculate that by reprogramming the host epigenome DNA-(cytosine C5)-methyltransferases as well as other bacterial effectors encoded by Mycoplasma species may contribute to the initiation or progression of malignant tumors or to the development of other diseases [137,143,144].

21.2.10 Epigenetic dysregulation elicited by secreted proteins of *Mycobacterium tuberculosis*

Recent whole-genome based phylogenetic studies suggested that *Mycobacterium tuberculosis* (*M. tuberculosis*), the etiologic agent of tuberculosis in humans, emerged in Africa and possibly co-evolved with humans for an extended period of time (reviewed by Ref. [145]). Acquisition of tuberculosis-specific elements by horizontal gene transfer apparently contributed to the adaptation of

M. tuberculosis to its human host [146]. The bacterium is typically transmitted via aerosol droplets and primary pulmonary disease is initiated at lung alveolar surfaces by its interactions with various host cells, including resident macrophages, alveolar epithelial type II pneumocytes and dendritic cells (reviewed by Ref. [147]). *M. tuberculosis* is capable to inhibit the phagosome-lysosome fusion in alveolar macrophages that permits its survival and growth intracellularly [148]. Even before entering the host cells, secreted products of extracellular bacteria may dampen the initial host defense by affecting the gene expression pattern of macrophages (reviewed by Ref. [149]). One of these secreted products is a DNA-(cytosine C5)-methyltransferase encoded by the *M. tuberculosis* genome whereas the other is an unusual histone methyltransferase [150,151]. Both of these enzymes could enter the host cells and were translocated into the host cell nuclei. Unlike human DNMTs that typically methylate cytosines located to CpG dinucleotides, the secreted bacterial DNA-(cytosine C5)-methyltransferase, called Rv2966c, methylated cytosines at non-CpG sites such as CpA and CpT in the host cell genome [150]. These altered methylation patterns caused transcriptional repression of H2AFY2, a gene coding for a variant histone and GRK5, a gene encoding a G-protein-coupled receptor kinase [150]. The situation is more complex, however, because a comparison of differentially methylated genomic regions between M. tuberculosis H37Rv infected THP1-derived macrophages and their uninfected counterparts showed that besides 19,506 hypermethylated regions, 3927 hypomethylated regions were also detected in the infected cells [152]. The distribution of the highly methylated regions was uneven, there were 23 hypermethylation hotspots, distributed on 11 different chromosomes. A series of genes involved in various immunological processes were clustered in a domain on the long arm of chromosome 6 (6q21−6q27) where the highest differentially methylated region (DMR) density was observed in *M. tuberculosis*−infected THP1-derived macrophages [152].

As expected based on the properties of the *M. tuberculosis*−encoded Rv2966c DNA-(cytosine C5)-methyltransferase, most of the hypermethylted cytosines were located in non-CpG dinucleotides. These data suggest that *M. tuberculosis* leaves a typical "footprint" on the host cell epigenome via the expression of an unusual bacterial DNMT. In parallel with the alteration of the epigenome the pattern of gene expression changed as well. As demonstrated by real-time RT-PCR, the transcription of a set of differentially methylated host cell genes was downregulated, whereas another group of genes showed increased expression in *M. tuberculosis*−infected macrophages [152].

The other epigenetic regulator secreted by *M. tuberculosis* was designated as Rv1988. It proved to be a protein arginine methyltransferase targeting an arginine residue at the core region of histone H3 in the host cell chromatin [151]. This enzyme repressed the expression of host defense genes coding for enzymes involved in the generation of reactive oxygen species and nitric oxide. Repressing of antibacterial defense mechanisms may possibly augment the survival of M. tuberculosis in the host organism. In addition, Rv1988 inhibited the expression of TRAF3 (tumor necrosis factor receptor-associated protein 3), an ubiquitin ligase, too [151]. Acting as an adapter protein involved in signaling processes, TRAF3 affects both T lymphocyte and B lymphocyte function [153]. Thus, downregulation of TRAF3 by Rv1988 may influence the adaptive immune response against *M. tuberculosis*.

In vitro experiments revealed a significant difference in the numbers of surviving intracellular bacteria after the infection of THP1-derived macrophages either with wild type *M. tuberculosis* H37Rv or a deletion mutant not expressing Rv1988. Wild-type bacteria expressing the protein arginine methyltransferase Rv1988 survived much better, suggesting that this secreted epigenetic regulator is a virulence factor for *M. tuberculosis* [151].

21.2.11 Alteration of the histone acetylation "landscape" in host cell chromatin by intracellular *Mycobacterium tuberculosis* infection

Chandran et al. observed that the level of HDAC1, a histone deacetylase enzyme, increased in THP-1-derived macrophages 24 h after phagocytosis of live, virulent *M. tuberculosis* H37Rv bacteria. No such increase in HDAC1 protein level occurred after phagocytosis of heat-killed H37Rv bacteria or following phagocytosis of the avirulent *M. tuberculosis* strain, H37Ra [154]. Up-regulation of HDAC1 resulted in histone H3 deacetylation and downregulated the activity of the *IL-12B* promoter. The *IL-12B* gene *codes for* interleukin 12 subunit beta, a regulator of the Th1 response.

In contrast, Moores et al. found, using real-time PCR, that infection of primary human monocyte-derived macrophages by the very same virulent H37Rv strain of *Mycobacterium tuberculosis* decreased the expression of HDAC1, HDAC2, HDAC3, and HDAC8 mRNAs compared with uninfected controls [155]. In parallel, there was an increase in the level of HDAC4 mRNA and protein, whereas the HDAC5 mRNA level did not change. Because HDACs are known to affect the transcription of metalloproteinase genes that are activated by *M. tuberculosis* infection, and because matrix metalloproteinase 1 and 3 (MMP1 and 3) are involved in tissue destruction as well as in the development of cavitating lung lesions, Moores et al. systematically analyzed the effect of various HDAC and histone acetyltransferase (HAT) inhibitors on the expression of MMP1 and 3. They concluded that both HDAC and HAT activity has a role in the induction of MMP1 and MMP3 by *M. tuberculosis* [155].

In addition to altering the expression of various cellular HDACs by an unknown mechanism, *M. tuberculosis* may also affect chromatin acetylation by expressing a bacterial HAT, designated Rv3423.1, in host cells [156]. Rv3423.1 could bind to the host chromatin and generated euchromatic histone marks, acetylated histone H3K9 and K14. Expression of Rv3423.1 in free living, apathogenic *M. smegmatis* apparently did not confer any growth advantage. In contrast, constitutive expression of Rv3423.1 resulted in a higher number of intracellular bacteria in the recombinant *M. smegmatis*–infected macrophages, compared to those infected with wild type *M. smegmatis* [156].

We wish to emphasize that the patho-epigenetic processes revealed so far in connection with *Mycobacterium tuberculosis* infection may help to develop new diagnostic tools and treatments (for review see Gauba et al. [157]). A very recent review of the topic was published by Madden et al. [158].

21.2.12 Induction of epigenetic alterations and epithelial–mesenchymal transition by *Mycobacterium leprae*

Mycobacterium leprae, the causative agent of leprosy, spreads probably by aerosol and damages the peripheral nerves of infected individuals by invading Schwann cells [159]. Schwann cells are glial cells providing trophic support to neurons. They are either involved in the formation of the insulating myelin sheet around axons (myelinating Schwann cells) or do not form myelin (non-myelinating Schwann cells) (reviewed by Ref. [160]). Both myelinating and non-myelinating Schwann cells respond with cell proliferation to the injury of nerve fibers and the subsequent axonal degeneration and demyelinization (reviewed by Ref. [161]). Using an in vitro nerve tissue culture model, Rambukkana et al. observed that myelinating Schwann cells were resistant to *M. leprae* infection, whereas non-myelinating Schwann cells were highly susceptible to bacterial invasion [162]. They also observed that attachment of *M. leprae* to myelinated Schwann cells caused demyelination in the absence of immune

cells, and could also be induced by dead *M. leprae*, bacterial cell wall, or one of the cell wall components, phenolic glycolipid-1 (PGL-1) [162]. Masaki et al. observed that *M. leprae* infection of primary Schwann cells isolated from adult mouse peripheral nerves led to the removal of Sox10 from the nuclei and downregulated the expression of myelin genes [163]. Sox10 is a master regulator indispensable for Schwann cell identity. Accordingly, in the absence of Sox10 the gene expression pattern and phenotype of Schwann cells carrying a high number of intracellular *M. leprae* changed: they were converted to stem cell-like cells. In parallel, the *Sox10* gene became hypermethylated whereas genes involved in mesoderm development and epithelial-mesenchymal transition (EMT) became hypomethylated, and their expression increased [163]. The stem cell-like cells secreted a series of chemokines, cytokines and growth factors that attracted *macrophages* in vitro [164]. How *M. leprae* induces these spectacular changes that may promote the dissemination of infection in vivo remains to be elucidated (reviewed by Refs. [165,166]). Recently, Hess and Rambukkana overviewed the complex interactions of *M. leprae* with the *peripheral nervous system* [167]

Table 21.1 summarizes the epigenetic alterations elicited by bacterial infections in host cells.

21.3 Virus-induced epigenetic alterations

21.3.1 Epigenetic alterations in cells carrying latent gammaherpesvirus genomes

Gammaherpesvirus genomes regularly carry viral oncogenes and are associated, consequently, with malignant tumors. Some of the viral oncoproteins encoded by human gammaherpesviruses turned out to be modifiers of the cellular epigenome.

21.3.1.1 Epigenetic reprogramming in Epstein–Barr virus–associated neoplasms

Epstein–Barr virus (EBV), a human gammaherpesvirus, is associated with a series of malignant neoplasms including lymphomas (Burkitt's lymphoma, Hodgkin's disease, T/NK-cell lymphoma, post-transplant lymphoproliferative disease, AIDS-associated lymphoma, X-linked lymphoproliferative syndrome), carcinomas (nasopharyngeal carcinoma, gastric carcinoma (GC), carcinomas of major salivary glands, thymic carcinoma, possibly mammary carcinoma), and a sarcoma (leiomyosarcoma) (reviewed by Refs. [168,169]). In infected cells, the terminal repeats of the linear double-stranded DNA packaged into the virions fuse with each other. Accordingly, latent EBV genomes persist in the tumor cells as circular episomes. They attach to the nuclear matrix in interphase nuclei and coreplicate with the cellular DNA once per cell cycle using *oriP*, the latent origin of EBV replication.

The expression of latent EBV genes is cell type specific. Epigenetic regulatory mechanisms including DNA methylation and histone modifications, and binding of key cellular regulatory proteins control the activity of the alternative promoters for transcripts encoding the nuclear antigens EBNA1 to 6. Similar mechanisms affect the activity of promoters for transcripts encoding transmembrane proteins (LMP1, LMP2A, LMP2B), too. In addition to the genes transcribed by RNA polymerase II, there are also two RNA pol III transcribed genes in the EBV genome (EBER1, EBER2). The 5′ and internal regulatory sequences of the EBER1 and EBER2 transcription units are invariably unmethylated. The highly abundant EBER1 and EBER2 RNAs are not translated to protein. In addition, microRNAs are also generated by RNase-processing of latent EBV transcripts. EBV-derived

microRNAs may interact both with cellular and viral mRNAs, thereby modulating their post-transcriptional level [170]. Furthermore, EBER-containing exosomes are involved in intercellular signaling thereby influencing immune responses. EBER RNAs and viral microRNAs may play a role in the inflammatory skin reaction observed in patients suffering from systemic lupus erythematosus [171,172].

Based on the cell-type-specific epigenetic marks associated with latent EBV genomes one can distinguish between unique viral epigenotypes [7]. Although containing identical or nearly identical DNA sequences, each viral epigenotype is associated with a different pattern of gene expression. In addition, alternative conformations adopted by the viral episomes in a latency-type-specific manner may also affect the activity of latent EBV promoters [173].

Latent EBV genomes are regularly targeted by epigenetic control mechanisms in different cell types. EBV-encoded oncoproteins may, in turn, affect the activity of a set of cellular promoters. Thus, their interaction with the cellular epigenetic regulatory machinery results in epigenetic "reprogramming" of the host cells. The nuclear antigen EBNA2 transactivates both viral and cellular promoters. EBNA2 interacts with the cellular HATs p300, CBP, and PCAF, whereas the leader protein EBNA-LP (EBNA5) coactivates transcription by displacing histone deacetylase 4 from EBNA2-bound promoter sites [174,175]. At LMP1p, the EBNA2-associated HATs may counteract the silencing effect of histone deacetylases [175]. EBNA2 cooperates with EBNA-LP and several cellular transcription factors, DNA looping factors and chromatin remodeling complexes to derepress and activate cellular promoters and enhancers which are required for cell immortalization and transformation [176].

EBNA3C (EBNA6) was associated both with histone acetylases and deacetylases, although in separate complexes [177,178]. EBNA3C and EBNA3A interact with C terminal binding protein (CtBP) and repress the TSG $p16^{INK4A}$ in lymphoid cells [179]. These interactions contribute to the maintenance of the H3K27me3 repressive chromatin mark at the silent $p16^{INK4A}$ promoter as well as the sustained growth of lymphoblastoid cells immortalized by EBV. EBNA3C and EBNA3A cooperated in repression of *Bim*, a gene coding for the proapoptotic protein Bcl-2-interacting mediator of cell death [180]. In cells carrying latent EBV genomes the *Bim* promoter was enriched in the repressive histone mark H3K27me3 and methylated CpGs [181]. EBNA3C directly targeted the deposition of H3K27me3 through the PRC2 protein complex to the *BIM* promoter [182]. While EBNA3A and EBNA3C in summary act pro-oncogenic, EBNA3B exhibits tumor suppressive properties and balances the effects of the other two members of the EBNA3 family (reviewed by Ref. [183]).

LMP1, a transmembrane oncoprotein, affected both alternative systems of epigenetic memory, DNA methylation, and PcG complexes. In epithelial cells LMP1 up-regulated DNMT1, DNMT3A, and DNMT3B, whereas in Hodgkin lymphoma cells it induced the PcG protein Bmi-1 [184–186]. LMP1 activated the expression of DNMT1 via the c-jun NH(2)-terminal kinase/activator protein-1 (JNK-AP-1) signaling pathway [159]. The NF-κB pathway was involved in Bmi-1 induction [186]. Dutton et al. suggested that by up-regulating Bmi-1 via the NF-κB pathway, LMP1 may induce the loss of B-cell identity in EBV-positive Hodgkin's lymphomas via down-regulating B-cell markers (CD21/MS4A1, BLK, LY9). In addition, Bmi-1 may mark a set of promoters for de novo methylation by DNMTs, thereby silencing TSGs, including *IGSF4* and *ATM*. Other LMP1 target genes are activated by Bmi-1, like *STAT, c-MET,* and *HK*, coding for signaling molecules and hexokinase (reviewed by Ref. [187]). Latent EBV infection was associated with hypermethylation of a set of cellular promoters in LMP1-positive tumors including nasopharyngeal carcinoma (NPC) [188], Hodgkin lymphoma [189–191], and lymphomas developing in AIDS patients [192]. The observation

regarding hypermethylation-mediated silencing of various TSGs in NPC was confirmed in independent studies. Dai et al. reported that aberrant methylation of chromosome 6p21.3 frequently occurred in primary NPC tumors and Li et al. also found that methylated TSGs are a characteristic feature of the NPC methylome [193,194]. Furthermore, using bisulfite amplicon sequencing, Zhao et al. identified 150 hypermethylated CGIs in EBV-associated NPC samples [195]. For recent review see Leong and Lung [196].

LMP1 also activated the expression of poly(ADP-ribose) polymerase (PARP) 1, thereby exerting broad epigenetic effects on the genomes of infected B cells. The increased level of poly-ADP ribosylation through PARP1 led to a dimished overall H3K27me3 level throughout the host genome [197]. Another epigenetic modifier induced by LMP1 was KDM6B (JMJD3), a histone H3K27me3 demethylase which acts in the same overall activating direction as PARP1 [198].

The transmembrane protein LMP2A is also involved in promoter silencing by CpG methylation [199]. LMP2A induces the phosphorylation of STAT3 in GC cell lines resulting in the up-regulation of DNMT1 transcription and methylation of the TSG *PTEN* (phosphatase and tensin homolog). PTEN functions as a phosphatidylinositol-3,4,5-triphosphate (PIP_3) phosphatase causing cell cycle arrest and inhibition of cell migration. PTEN contributes to the maintenance of apical−basal polarity of epithelial cells, too (reviewed by Ref. [200]). Because LMP1 is absent from EBV-associated GCs, Hino et al. suggested that CpG methylation of cellular promoters [201,202], including the *PTEN* promoter, is due to LMP2A expression in these neoplasms [199]. One has to add, however, that in a significant portion of EBV-associated GCs LMP2 expression can't be detected, either [203], suggesting that other latent EBV proteins or RNAs may also contribute to the development of the CGI methylator phenotype (CIMP). In NPC tumor biopsies, LMP2A was overexpressed mainly at the tumor invasive front. In NPC cells, LMP2A overexpression led to an increased number of stem-like cancer cells and cells undergoing epithelial-mesenchymal transition (EMT) [204].

We would like to emphasize that EBV-associated gastric carcinoma (EBVaGC) represents a distinct histologic subtype of gastric neoplasms: it is characterized as an undifferentiated neoplasm with lymphoid stroma. Furthermore, EBVaGC is also distinguished by its unique epigenotype referred to as "extremely high" methylation epigenotype (reviewed by Refs. [205,206]). It is noteworthy that whereas *Helicobacter pylori*−associated GC (see Section 21.2.5) is located typically in the distal stomach (antrum), EBVaGC comprising about 10% of gastric cancer cases usually occurs in the proximal stomach, and has a more favorable prognosis [205,206]. It was noticed that in *Helicobacter pylori*−associated GC aberrant DNA methylation silenced a set of TSGs involved in cell cycle regulation [205,207]. It was suggested, however, that in addition to a battery of TSGs targeted for silencing by polycomb repressor complexes in *Helicobacter pylori*−associated GC cells, other genes are also switched off via hypermethylation in EBVaGCs [205]. The exact mechanism for the induction of the so called CIMP in EBV infected gastric epithelial calls and EBVaGCs remains to be established. A recent study by Okabe et al. proposed a new mechanism based on the interaction of EBV episomes and the host chromatin [208]. A sophisticated analysis of 3D chromatin structure in EBVaGC cells revealed that the unintegrated, latent EBV episomes interacted with distinct regions of the host cell chromatin and induced an alteration of histone marks which led to the activation of silent enhancers and a change in gene expression [208]. Such a phenomenon, called "enhancer infestation" may change the epigenetic landscape, phenotype and behavior of gastric epithelial cells. Further experiments may elucidate how latent EBV episomes, present in multiple copies, interact with the chromatin of distinct target cells. We would like to add that according to recent chromosome conformation capture studies

latent EBV genomes may interact with repressive as well as active regions of interphase chromatin and may even switch their nuclear compartment upon reactivation in lymphoid cells [209–211].

A recent review of epigenetic alterations affecting the EBV genome as well as the host cell genome was published by Zhang et al. ([212]).

21.3.1.2 Epigenetic alterations in neoplasms carrying Kaposi's sarcoma–associated herpesvirus genomes

Human herpesvirus 8 (HHV-8) or Kaposi's sarcoma–associated herpesvirus (KSHV) belongs to the genus rhadinovirus of the human gammaherpesviruses, a subfamily of herpesviruses. HHV8 is causally associated with Kaposi's sarcoma (KS), a tumor of endothelial cell origin. KSHV is also associated with multicentric Castleman's disease (MCD) and primary effusion lymphoma (PEL, also called body cavity-based lymphoma) (reviewed by Ref. [213]). KSHV exists in two replication states, lytic or latent. The key lytic switch and transcriptional transactivator protein Rta is encoded by the viral immediate early gene *ORF50*. During viral latency in B-cells, Rta expression is down-regulated, together with most other viral proteins. The key regulator of viral latency, LANA (latency-associated nuclear antigen encoded by *ORF73*), suppresses both lytic activation and apoptosis (reviewed by Ref. [213]). The KSHV latent origin of DNA replication is located to the terminal repeats (TRs) of the genome where LANA has two binding sites. LANA recruits cellular replication proteins to the TRs. Histone acetylation at the TRs is high, while histone methylation changes throughout the cell cycle [214]. In addition to replication factors, LANA also recruits to the TRs a repressive complex consisting of heterochromatin protein (HP) 1 and the HMT SUV39H1 [215].

Two recent studies analyzed the chromatin structure of entire KSHV genomes in latently infected cells and during the establishment of latency. Immunoprecipitation of methylated DNA and of covalently modified histones in combination with microarrays yielded distinct profiles of epigenetic modifications for latency in tumor cell lines and in newly infected epithelial cells. Characteristic DNA-methylation patterns were found all over the genome. Latency-specific histone modifications were rapidly established upon infection. Silencing of lytic viral promoters was not established by removal of the activating histone marks H3K9ac, H3K14ac, and H3K4me3, but by the deposition of H3K27me3 across the genome. This "bivalent" modification is transcriptionally repressive, but enables rapid activation upon the induction of the lytic cycle [216]. A similar "bivalent" chromatin structure was found at the *IE* genes *ORF50* and *ORF48* in the second study. In addition, Enhancer of Zeste Homolog (EZH) 2, the HMT enzyme of PcG protein complexes colocalized with the H3K27me3 marks during latency, while the reactivation of the lytic cycle led to the dissociation of EZH2 and H3K27me3 from the viral genome and to the expression of the lytic protein cascade [217]. Remarkably, LANA binding sites and the repressive H3K9 methylation chromatin mark were mutually exclusive in the viral genome in infected or transfected cell lines. This effect was due to the association of LANA with histone H3K9me1/2 demethylase KDM3A [218]. More recent studies established the specific epigenetic landscape of KSHV genomes in physiologically relevant clinical material. Upon primary infection of endothelial cells, the viral genome underwent a biphasic transition from euchromatin to heterochromatin, until latency was established, while in oral epithelial cells the viral genome preferentially adopted a transcriptionally active euchromatin [219]. In Kaposi sarcoma tissues active chromatin marks were mainly found at the latency locus, while repressive marks were widespread over the entire genome with an accent on the lytic phase and replication activator RTA [220]. Furthermore, upon primary infection of peripheral blood mononuclear cells, the

KSHV genome acquired uniquely distinct histone modification and gene expression patterns which were temporally coordinated [221].

Binding of the chromatin insulator protein CTCF in association with cohesins to the KSHV episome may also influence viral gene expression by establishing alternative three-dimensional conformations during latency and lytic replication [222].

In addition to PcG proteins, viral microRNAs also contribute to KSHV-latency. A miRNA deletion mutant virus eliminating 10 of the 12 viral miRNAs from the KSHV genome was more lytically active than the wild-type virus. This effect might be explained by the fact that two of the deleted viral miRNAs, miR-K12-5 and miR-K12-4-5p, have a seed sequence targeting and down-regulating transcripts of the viral transactivator *ORF50* and *retinoblastoma-like protein (Rbl) 2*, a repressor of *DNMT3A* and *DNMT3B* transcription, respectively [223].

KSHV infection influences the epigenetic marks of the host genome. In a *LANA*-transduced cell line, the promoters for *CDH13* (H-Cadherin), *CCND2* (cyclin D2), *LDHB* (lactate dehydrogenase B), *FOXG1B_FKHL1* (Forkhead box protein G1B_Forkhead-related protein 1) and *CREG* (cellular repressor of E1A stimulated genes) were strongly down-regulated. LANA was associated with repressed cellular promoters and recruited DNMT3A but also DNMT1 and DNMT3B, resulting in promoter methylation, as demonstrated in the case of the *CDH13* promoter [224]. At the TGF-β type II receptor (*TbetaRII*) promoter, deacetylation of promoter-associated histones was also demonstrated, in addition to promoter methylation and LANA binding to the transcriptional corepressor complex mSIN3A [225].

In PEL cells carrying both KSHV and EBV genomes, transfection of *LANA* decreased the activity of the EBV promoters Qp and Cp [226]. Upon transfection into immortalized epithelial cell lines, LANA was also associated with the repressive HP1 at the cellular chromatin [227]. Accordingly, LANA seems to work as an epigenetic modifier of the viral and host genomes. Transfection of LANA into several tumor cell lines led to a fundamental reorganization of the cellular chromatin. LANA-induced redistribution of DNA staining coincided with redistribution of the methyl CpG binding protein MeCP2. This effect was attributed to the interaction of LANA with the HMT enzyme SUV39H1 [228]. The interaction of LANA with MeCP2 and nucleosomes would enable LANA to direct regulatory complexes to chromosomal sites and thereby stably reprogram cellular and viral gene expression [229]. Accordingly, $p16^{INK4A}$ was frequently silenced through CpG-methylation in PEL cell lines and primary PEL cells [230]. Furthermore, CpG-hypermethylation at the *MGMT* promoter and a loss of the EBV genome in cell culture was observed in a PEL cell subclone derived from a PEL tumor, which was positive for KSHV and EBV, but unmethylated at *MGMT* [231] (Table 21.2). However, a more recent study found that LANA also acted as a transcriptional activator. LANA was associated with the H3K4-methyltransferase protein hSET1. Together with hSET1, LANA was bound at multiple, mostly transcriptionally active viral and cellular promoters, via its consensus binding site, but also via a novel LANA binding site at cellular gene promoters [232]. Furthermore, together with the viral protein vFLIP, LANA activated the repressive epigenetic modifier protein EZH2 in human endothelial cells via the NF-κB pathway, thereby contributing to angiogenesis [233].

We would like to note that in addition to LANA, the KSHV genome encoded vIL-6 (viral interleukin-6) may also contribute to the virus induced epigenetic alterations by upregulating DNMT1 in endothelial cells [234]. A genome-wide study of CpG methylation revealed that a series of cellular promoters and enhancers were hypermethylated in KS biopsy samples compared to normal skin specimens [235]. Journo et al. observed that DNA hypermethylation occurred at an early, plaque stage

Table 21.2 Epigenetic alterations induced by the human dsDNA tumor viruses EBV, KSHV, and HPV in host cells.

Microbe	Effector molecule	Epigenetic alteration	Effect
EBV	EBNA2	Histone acetylation	Switching on promoters
	EBNALP (EBNA5)	HDAC displacement	Switching on promoters
	EBNA3C (EBNA6)	Histone acetylation, histone deacetylation, histone methylation	Modulation of promoter activity
	LMP1	Upregulation of DNMTs	Promoter silencing
		Induction of KDM6B histone H3K27me3 demethylase	Promoter activation
	LMP2A	Upregulation of DNMT1	Promoter silencing
KSHV	LANA	Recruitment of DNMTs to cellular promoters	Promoter silencing
		Association with mSIN3A, recruitment of HDACs	Promoter silencing
		Association with HP1	Promoter silencing
		Interaction with SUV39H1, redistribution of MeCP2	Reorganization of the nucleus
		Upregulation of DNMT1	Promoter silencing
HPV	E7 E7 and E6	Recruitment of DNMTs to cellular promoters Stimulation of DNMT1 activity	Promoter silencing
	E7	Induction of EZH2 expression Upregulation of SUV39H1	Promoter silencing
		Induction of KDM6A and KDM6B histone H3K27me3 demethylases	Promoter activation

of KS development and kept increasing with tumor progression, whereas an increase in hypomethylation of other CpGs was a characteristic feature of a more advanced stage (nodule) [235]. Similar epigenetic alterations, i.e., initial hypermethylation followed by a slow increase in genome-wide hypomethylation, were recorded in a genome-wide methylation analysis of KSHV-associated primary effusion lymphomas (PELs), too [236].

It was observed that oncoviruses may initiate tumorigenesis even if their genome is completely lost from the infected cells, due to the virus-induced heritable genetic or epigenetic alterations affecting the

gene expression pattern and phenotype of host cell (reviewed by Ref. [237]). Such a scenario, called "hit and run-oncogenesis" could potentially explain the coexistence of KSHV-positive and KSHV-negative (LANA negative) spindle cells in certain Kaposi sarcomas [238,239]. Furthermore, "hit and run-oncogenesis" seemed to be a plausible explanation for the unexpected behavior of KSHV-genome transformed murine cells that could formed KS-like tumors not only after inoculation into nude mice, but even after they were removed from the animals, induced to loose the KSHV episome in tissue culture, and inoculated into new recipient mice [239]. Naipauer et al. took advantage of such a multistep in vivo model of KSHV sarcomagenesis to compare the genetic and epigenetic alterations characteristic for the KSHV-positive and KSHV-negative murine KS-like tumors [239]. Sequence analysis of *MBD2* (Methyl-CpG Binding Domain Protein 2) enriched DNA fractions indicated that although some CpG methylation changes attributed to the KSHV-induced malignant transformation were maintained after the clearance of KSHV genome, other components of the cellular CpG DNA "methylation footprint" were lost. According to the results of high throughput RNA sequencing, differential methylation of distinct promoters was correlated with the expression profiles of the relavent cellular genes [239]. In addition, analysis of the mutational landscape showed the presence of putative driver mutations, including an activating mutation of the gene encoding *PDGFRA* (PDGF receptor-alpha) implicated sarcomagenesis, in the KSHV-negative murine KS-like tumors. These data suggested that both epigenetic and genetic alterations could potentially contribute to KSHV-induced "hit and run oncogenesis" [239]. A recent review on the patho-epigenetic changes observed in diseases attributed to KSHV infection was published by Fröhlich and Grundhoff [240].

The epigenetic alterations induced by the human dsDNA tumor viruses EBV and KSHVare summarized in Table 21.2.

21.3.2 Interactions of lytic cycle proteins of human cytomegalovirus with histone deacetylases

Following primary infection, human cytomegalovirus (HCMV), a betaherpesvirus, persists or establishes latency in cells of the myeloid lineage. Although most infections are asymptomatic, life-threatening disease can develop in immunocompromised patients or if infection occurs in utero. Taken together, HCMV is most likely responsible for the largest and most severe overall disease load among all human herpesviruses. Gönczöl et al. observed that HCMV latency and reactivation is connected to the undifferentiated or differentiated nature of the host cell [241]. In undifferentiated cells the major immediate-early (IE) promoter (MIEP) of HCMV was repressed and located to a hypo-acetylated chromatin domain associated with HP1 and Ets-2 repressor factor (ERF), a transcriptional repressor recruiting HDAC1 to MIEP [242,243]. During lytic infection, however, for example when monocytes differentiated to macrophages, MIEP was activated and associated with hypermethylated histones [242]. In addition, IE1 and IE2, the abundant protein products of the IE genes interacted with histone deacetylases, abolishing their repressive effect at the MIEP and the early promoter of the viral polymerase [244,245]. Thus, IE proteins modulate the host epigenetic regulation of viral promoters, thereby facilitating viral replication.

IE1 and IE2 activated cellular promoters either by direct binding to CCAAT box binding factor (CTF1) at TATA-less promoters, or TATA-binding protein (TBP), at promoters with a TATA motif, respectively [246,247]. In human fibroblasts, activation of the human telomerase reverse transcriptase gene (*hTERT*) by IE1 was accompanied by increased binding of the nuclear protein Sp1, acetylation of

histone H3, and a reduction of HDAC binding at the *hTERT* promoter [248]. Since telomerase activation is crucial for immortalization and malignant transformation of cells, these data support the point that HCMV may contribute to tumorigenesis [249].

The interaction of IE1 with host mitotic chromatin has recently been more elucidated. IE1 targets chromatin via its chromatin-tethering domain by docking to the acidic pocket formed by histone H2A and H2B on the surface of the nucleosome. This resembles the action of KSHV's LANA protein during viral latency [250]. Upon primary infection of cultured permissive cells, the viral genome first undergoes a rapid chromatinization. As infection proceeds from early to the late phase, IE1 mediates an extensive redistribution of nucleosomes [251]. Overexpression of IE1 in permissive cells turned gene expression from a STAT3-dependent to a STAT1 dependent pattern of transcription leading to a switch from an IL-6 like to an IFNγ like response. This potentially tempering effect of IE1 on viral replication was mediated by IE1 binding to STAT3 via its acidic domain 2 (AD2) during the course of lytic replication [252].

Also the long-standing suspicion that HCMV infection may accelerate immunosenescence deserves some mention [253,254]. Epigenetic aging is the molecular basis of many diseases, degenerative, cardiovascular, malignant and other. Generally, epigenetic aging of cells is accompanied with an overall hypomethylation of the genome and an overall hypermethylation of CGIs, both occurring in a stochastic manner. The exact mechanisms of epigenetic aging and causality of HCMV therein are still to be established. Fibroblasts infected with HCMV exhibited a significant global hypomethylation which was associated with a relocalization of DNMTs to the cytoplasm [255]. Epigenetic age in two widely separate age cohorts, one nonagenarian and one 21 year-old, significantly correlated with HCMV seropositivity [256]. Also T-cell distribution and function of HCMV−infected individuals resembled T-cell distribution and function of older individuals [257]. Altogether, the development of a vaccine against HCMV would be highly desirable. The importance of HCMV vaccinations was also emphasized in the light of aging, accelerated by virus infections and associated with an increased mortality risk, by Poloni et al. [258].

We would like to add that recently the HCMV genome encoded IE1 protein was implicated in the association of HCMV episomes to mitotic chromosomes, both in myeloid cells and fibroblasts [259]. It remains to be established, whether such interactions occur in interphase cells as well, similarly to the binding of episomal EBV genomes to host cell chromatin (see above in Section 21.3.2).

21.3.3 Epigenetic dysregulation in human retrovirus−infected cells

The RNA genomes of human T-cell lymphotropic virus (HTLV) and human immunodeficiency virus (HIV) replicate via a DNA intermediate that becomes integrated into the host cell genome (provirus). Proviral genomes are frequently silenced by epigenetic mechanisms. In turn, retroviral proteins interacting with the epigenetic regulatory machinery modulate the gene expression pattern of their host cells.

21.3.3.1 CpG island methylator phenotype in adult T-cell leukemia/lymphoma (ATLL): a putative role for the HTLV-1 protein tax in the silencing of key cellular promoters?

The proviral genome of type 1 human T-lymphotropic virus (HTLV-1), a retrovirus associated with ATLL and tropical spastic paraparesis, frequently undergoes epigenetic silencing [260]. Therefore, in

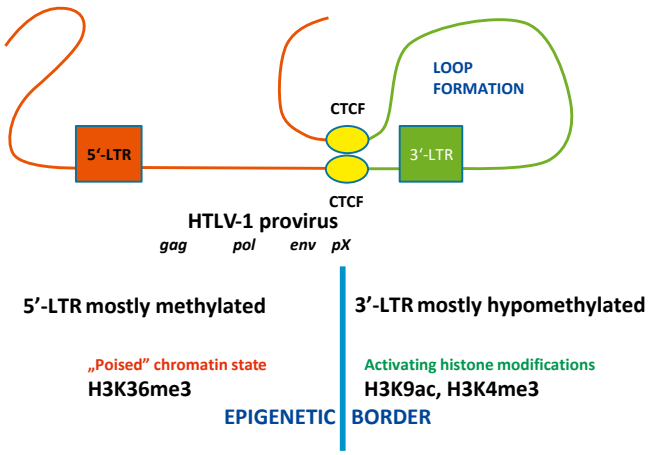
tropical spastic paraparesis patients the activation of viral gene expression using the HDAC inhibitor valproate seems to be a justified therapeutic approach, because the virus-positive cells may thereby be exposed to the host immune response, resulting in the collapse of the latent viral reservoir [261]. In ATLL patients the CGI methylation frequency of the *Src homology-2-containing protein tyrosine phosphatase* (*Shp1, PTPN6*) gene gradually increased during disease progression [262]. In parallel, the number of CGI methylated genes also increased. Because the viral oncoprotein Tax induced the dissociation of transcription factors from the *Shp1* promoter and subsequent promoter hypermethylation [263], Niller et al. speculated that Tax may act as a "hit-and-run" oncoprotein by initiating the down-regulation of Shp1 expression in an early stage of leukemogenesis [237]. This early step may be followed by silencing of additional cellular promoters and down-regulation of Tax expression itself, due to deletions of the proviral genome or its epigenetic silencing. We would like to add that recently Ichikawa et al. observed a downregulation of the putative tumor suppressor protein NDRG2 (N-myc downstream-regulated gene 2) in HTLV-1−infected cell lines and during ATLL progression, due to the binding of the *NDRG2* promoter by EZH2 (enhancer of zeste homolog 2) resulting in histone H3K27 trimethylation and subsequent promoter silencing via DNA methylation [264].

Remarkably, integrated HTLV-1 genomes carry with them a well-delineated binding site for the chromatin insulator protein CTCF within the pX gene region. Therefore, HTLV-1 has the potential to re-organize the local host cell chromatin and cause widespread abnormalities of gene expression in dependence of the specific locations of the several thousand integration sites per infected individual [265]; (reviewed by Ref. [266]). Integration of HTLV-1 genomes into the host chromatin causes insertional mutagenesis via CTCF-dependent loop formation by viral sequences and distinct regions of the cellular chromatin (Fig. 21.1). Melamed et al. observed that in the vicinity of HTLV-I integration, the altered 3D configuration of the cellular chromatin may result in dysregulated, bidirectional transcription of the host cell genome in *cis*, whereas long distance interactions, up to >300 kb or even farther from the integration site of the HTLV-I genome, may induce aberrant transcription initiated at non-contiguous loci of the host cell genome [267]. Because CTCF binding may influence the distribution of epigenetic marks as well as the transcription pattern of neighboring sequences, Miura et al.

FIG. 21.1

CTCF dependent loop formation and reorganization of chromatin in an integrated HTLV-1 provirus.

Based on the work of Satou Y., Miyazato P., Ishihara K., Yaguchi H., Melamed A., Miura M., et al. The retrovirus HTLV-1 inserts an ectopic CTCF-binding site into the human genome. Proc Natl Acad Sci USA 2016;113: 3054–3059. doi:10.1073/pnas.1423199113.



and Cheng et al. analyzed the epigenetic marks deployed to and viral transcripts initiated at integrated HTLV-I proviruses lacking the CTCF binding site [268,269]. These studies gave partly incongruent results. Further studies may reveal whether deletion of the CTCF binding site from the HTLV-I genome affects the epigenotype and transcription pattern of the host cell.

21.3.3.2 Human immunodeficiency virus: epigenetic silencing of latent viral genomes and host genes

HIV causes the acquired immunodeficiency syndrome. The virus is transmitted parenterally and, with the exception of very few long-term non-progressors, ends fatally for the infected patients due to the severe course of opportunistic infections which would normally run less severely or even subclinically. Due to the HIV-associated severe immune suppression, HIV patients have an increased risk to develop virus-associated neoplasms during all stages of HIV disease [270–272].

Shortly after primary infection, the latent viral reservoir is established both in resting and activated primary $CD4^+$ T cells [273]. Although antiretroviral therapy (ART) that combines several drugs with different viral targets significantly improved the life expectancy of HIV-infected individuals, there is still no cure for HIV infection. One reason for this failure is the existence of a dormant viral reservoir in resting memory $CD4^+$ T cells [274].

21.3.3.3 Silencing of host genes in HIV-infected cells

The chromatin structure in host cell genes is influenced by HIV infection of $CD4^+$ T cells. DNMT1 levels increased significantly in $CD4^+$ T-cell lines and primary CD4 T cells upon infection with HIV. An increase in the level of DNMT1 was observed in a T-cell line infected with an integration- and therefore replication-defective HIV mutant. Thus, the presence of viral nucleic acids was sufficient, but viral replication was not required for the increase of DNMT1 expression. The overall methylation level of the cellular DNA increased, the CGI at the $p16^{INK4A}$ promoter became hypermethylated and repressed upon HIV infection, while 5-aza-C treatment led to the induction of $p16^{INK4A}$ expression [275].

Another gene down-regulated in virus-infected immortalized T cells was UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (*GNE*). The GNE enzyme produces the sialyl-donor substrate for all cellular sialyl-transferases and thereby profoundly influences the receptor and homing functions of immune cells. In T-cell lines the CGI at the *GNE* promoter became de novo hypermethylated upon acute HIV infection, as demonstrated by bisulfite sequencing. Treatment with 5-aza-C released both the transcriptional repression and methylation, and restored sialylation function. *GNE* hypermethylation may to some degree explain the frequently persisting immune disorder of aviremic HIV patients on long-term successful HAART [276].

Infection of a T-cell line or transfection of HeLa cells with an integration-defective HIV increased DNMT1 expression. The effect was dependent on expression of HIV early proteins Tat, Rev, and Nef. The transcription factor AP1 was implicated in the HIV-dependent up-regulation of DNMT1 [277]. Furthermore, in a human neuroblastoma cell line and in human primary neuronal cells, Tat was shown to induce HDAC2. This led to the silencing of the *CREB* and *CaMKIIa* genes which contribute to synaptic plasticity and neuronal function. Thus, HIV-induced dysregulation of HDAC2 may be involved in HIV-associated neurocognitive disorder (HAND) [278] (Table 21.3).

Recently, Zeng et al. used methylated DNA immunoprecipitation sequencing (MeDIP-seq) and RNA sequencing (RNA-seq) to characterize the alterations of host cell methylome and transcriptome

Table 21.3 Epigenetic alterations induced by human retroviruses.

Microbe	Effector molecule	Epigenetic alteration	Effect
HTLV-I	Tax	*Shp1* promoter methylation	Promoter silencing
HIV-1	Tat, rev, nef	Upregulation of DNMT1	Promoter silencing
	Tat	Induction of HDAC2	Promoter silencing

in two HIV-infected T cell lines [279]. They identified a series of differentially methylated regions and differentially expressed genes and concluded that in HIV infected cells altered epigenetic regulation may upregulate or downregulate a set of distinct apoptosis-related genes [279].

Silencing of host genes in HIV-infected cells may be a potential mechanism, in addition to the destruction of target cells either by the replicating virus or by the immune response directed against HIV-infected cells, which may explain why clinical symptoms of accelerated aging were observed in case of HIV infected individuals. Based on the idea of Horvath and Raj, biological age can be estimated using an "epigenetic clock," by measuring methylation levels at distinct CpG positions in the human genome, where cytosine methylation correlates with age [280]. Horvath and Levine used 11 Illumina DNA methylation data sets generated by the analysis of whole blood samples, brain tissues, or isolated $CD4^+$ T cells and monocytes from healthy subjects, as well as whole blood samples, brain tissues and PBMCs derived from HIV infected subjects to estimate whether HIV infection affected the biological age (more exactly epigenetic age) of the cases and controls included into the study [281]. They found that the epigenetic age (EpiAge) of HIV-infected individuals, calculated on the basis of blood samples as well as brain samples, was higher than the EpiAge of healthy subjects [281]. In a recent longitudinal case/control study, Breen et al. compared the EpiAge of HIV seronegative and HIV seropositive individuals, based on the analysis of DNA samples isolated from PBMCs (peripheral blood mononuclear cells) [282]. They observed an acceleration of epigenetic age from pre- to post-HIV infection, in parallel with telomere length shortening, another indicator of biological age [282]. In contrast, there was no acceleration of epigenetic age in the corresponding HIV-seronegative controls [282].

Most studies analyzing epigenetic changes elicited by in *vivo* or in vitro HIV infection focused on various cell types carrying replicating or latent viral genomes. Several lines of evidence suggested, however, that the epigenotype of non-infected host cells interacting with viruses or other microbes may also change, i.e., due to the first encounter, the cells undergo a so called "epigenetic reprogramming" [283]. On the course of a subsequent infection by the same or even an unrelated microbe, the reprogrammed cells, typically innate immune cells including monocytes, macrophages, NK cells or dendritic cells, provide an enhanced or dampened response [283]. This phenomenon, called trained immunity is based on the acquisition of a "memory" of the first encounter [283]. It is important to emphasize, however, that depending on the strength and length of the priming stimulus, the "training" may result either in an enhanced response or in tolerance, i.e., suppression of the subsequent responses (reviewed by Refs. [284–286]). Based on a survey of the available literature, Sviridov et al. speculated that HIV may induce trained immunity in monocytes, possibly via its Nef or Gp120 protein [284].

The epigenetic alterations induced by human retroviruses are summarized in Table 21.3.

21.3.4 Local hypermethylation and global hypomethylation of the host cell DNA in hepatocellular carcinomas associated with hepatitis B virus

Hepatocellular carcinoma (HCC) is the predominant form of human liver cancer. Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are the major etiological factors for HCC. An estimated 240 million of the world population are chronically infected with HBV (reviewed by Ref. [287]). HBV-related HCC develops through distinct stages (reviewed by Refs. [288,289]). The expression of the viral genes is governed by a set of mostly hepatocyte-specific transcription factors and epigenetic mechanisms, mostly CpG-methylation of the cccDNA and acetylation of histones associated with cccDNA [290] (reviewed by Refs. [291,292]). Most interestingly, treatment with IL-6 or IFNα of infected cells leads to deacetylation and thereby transcriptional repression of the persisting viral genomes [293–295]. On the other hand, recruitment of the viral HBx protein to cccDNA reverses epigenetic repression and activates viral transcription [296,297].

Regarding the host cell genomes of the infected cells, epigenetic alterations including global hypomethylation and aberrant CGI hypermethylation at TSGs appear already in the preneoplastic lesions (cirrhotic nodules) and their frequency gradually increases during the early stages of liver carcinogenesis (low-grade dysplastic nodules, high-grade dysplastic nodules, early hepatocellular carcinoma), although the methylation level of certain genes may decrease in progressed HCCs [298]. DNMTs-1, -3A, and -3B were upregulated and overexpressed in a set of HBV-associated HCC-biopsies in comparison with cirrhotic tissue and compared with neighboring non-affected liver tissue [299,300]. Therefore, HBV replication induced methylation of viral and host DNA [299,301]. The *GSTP1* TSG was hypermethylated in HBV-associated HCC [302]. Hypermethylation of the *p16* gene started at an early stage of hepatocarcinogenesis. Sixty-two percent of cirrhotic nodules (putative preneoplastic lesions) and 70% of dysplastic nodules, surrounding HBV-positive hepatocellular carcinoma lesions, showed *p16* hypermethylation [303]. *APC, RASSF1A,* and *SOCS1* were methylated in a fraction of cirrhotic nodules but the methylation levels of *APC* and *RASSF1* increased significantly in low-grade dysplastic nodules. *SOCS1* methylation gradually increased during multistep carcinogenesis, peaked in early hepatocellular carcinoma, and decreased in progressed liver carcinomas [304]. Methylation profiling of single hepatocytes yielded seven novel hypermethylated genes (*WNK2, EMILIN2, TLX3, TM6SF1, TRIM58, HIST1H4F*, and *GRASP*) in HBV-HCC cells as compared to adjacent non-tumorous cells with the potential for use as diagnostic tumor markers [305] (reviewed by Refs. [287,306]).

A recent whole-genome profiling of methylation and hydroxymethylation comparing paired biopsies of HBV-associated tumor and neighboring tissue found significant differences in metabolic pathway genes, including gluconeogenesis, glycolysis, oxidative phosphorylation and the citrate cycle [307]. This is remarkable with respect to the work of Shaul et al. who have coined the term metabolovirus for HBV, because of the dual use of the same set of liver-specific transcription factors which are governing expression of metabolic pathway genes also by HBV enhancers and promoters [308,309].

The genome of hepatitis B virus codes for a pleiotropic regulator, called X protein (HBx or pX) which, in its wild-type (wtHBx) and truncated (trHBx) forms is thought to play a major functional role in tumorigenesis (reviewed by Ref. [291]). HBx interacts with multiple key signaling pathways and affects the regulators of cell cycle progression [310–314]. In vitro, HBx up-regulates cyclin D1 and activates DNMT1 expression via the cyclin D1-CDK4/6-pRb-E2F1 pathway, resulting in DNA-

methylation-mediated down-regulation of the tumor-suppressor protein $p16^{INK4a}$ [315,316]. In addition to DNMT1, HBx also up-regulated two variants of the de novo DNA methyltransferase DNMT3A in liver cells. DNMT3A1 and DNMT3A2 are translated from differentially spliced transcripts of the *DNMT3A* gene and induce regional hypermethylation of specific TSGs [317]. HBx down-regulated, however, another de novo DNA methyltransferase, DNMT3B, in the very same cells. Because satellite 2 repeat sequences are methylated by DNMT3B, HBx-induced down-regulation of the enzyme led to a global hypomethylation of these repeats [317]. This important observation connected the viral oncoprotein HBx both to global DNA hypomethylation, a phenomenon as widespread in neoplastic cells [318], and regional hypermethylation [319], which is another key feature of tumor cells. In hepatocellular carcinomas carrying integrated HBV genomes, CpG-rich sequences were also found to be hypomethylated in the pericentromeric regions of acrocentric chromosomes [320]. HBx was also implicated in hypermethylation-mediated-silencing of the E-cadherin promoter [321–323]. The TSG *RASSF1A* (Ras association domain family 1A) and the *GSTP1* gene, coding for the π-class glutathione S-transferase, an enzyme involved in protecting against electrophilic carcinogens, was also frequently inactivated by CGI methylation in HBV-positive hepatocellular carcinomas [302,324].

The epigenetic profile of hepatocellular carcinoma associated with HBV appears to be different from that of HCV-associated liver cancer [325,326]. Feng et al. observed that certain genes (*HOXA9, RASSF1, SFRP1*) were methylated more frequently in HBV-positive hepatocellular carcinomas than in HCV-positive liver tumors. In contrast, *CDKN2A* was significantly more frequently methylated in HCV-positive than in HBV-positive liver carcinomas [325]. Recent comparative studies of genome-scale DNA methylation profiles of sets of HBV-, HCV-, and non-viral HCC tumor biopsies revealed differentially methylated regions between all three entities, and the highest amount of methylation in HCV-associated HCC [326,327]. These data support the idea that hepatocellular carcinomas of different viral etiologies are associated with unique, virus-specific epigenetic signatures.

Several studies established that the HBV genomes, also called mini-chromosomes, occupy nonrandom positions and frequently associate with active chromatin regions in the nuclei of infected cells [328–330]. These observations suggested that such interactions may affect the viral and cellular epigenotypes and gene expression patterns in HBV-infected cells.

Hensel et al. raised the point that in host cell nuclei, HBx may stabilize the persistence of HBV minichromosomes, i.e., episomal, covalently closed circular DNA (cccDNA) molecules [331]. HBx is co-recruited to the cccDNA together with a long noncoding RNA molecule, DLEU2 (deleted in lymphocytic leukemia 2), and activates HBV transcription and replication by displacing the repressor protein EZH2 [332]. Furthermore, Salerno et al. demonstrated that a series of cellular promoters are also targeted by HBx-DLEU2 complexes upregulating their expression and facilitating hepatocyte transformation [332]. It remains to be established whether HBx-DLEU2-cccDNA-host chromatin interactions contribute to the long-range interplay of distinct interphase chromatin regions with HBV minichromosomes and the alteration of nuclear topography in HBV-infected cells.

We would like to add that certain nuclear proteins, such as FoxO4 (Forkhead O Transcription Factor 4) are capable to induce heterochromatization of HBV cccDNA, resulting in an increase of repressive chromatin marks on the viral episome [333]. Such alterations may affect the expression of the HBV genome, and one may speculate that they may influence the interaction of the HBV mini-chromosome with the host cell chromatin as well. The epigenetic alterations in HBV-associated hepatocellular carcinoma were reviewed recently by Li et al. and Yang et al. [333,334].

21.3.5 Hepatitis C virus–induced epigenetic alterations

Hepatitis C virus belongs to the genus *Hepacivirus* of the family Flaviviridae. It is transmitted through blood–blood contacts and causes inflammatory liver disease which turns chronic in about 85% of the infected patients. An estimated 130 million of the world population is infected. Chronic infection may lead to liver cirrhosis in 20%–30% of those infected and to hepatocellular carcinoma in 1%–8% of cirrhosis patients in the long term (reviewed by Ref. [287]). Several large studies described epigenetic down-regulation of numerous TSGs in HCC tissue samples [325,335–337] (reviewed by Refs. [306,338]). The overall genomic methylation levels, reflected in the methylation of LINE1 and SAT2 repeats, decreased, while methylation at CGIs of TSGs increased with the progression of hepatitis via cirrhosis to HCC [288,339,340] (Table 21.4).

DNA methylation at distinct TSG loci is significantly associated with HBV, HCV, or alcoholism, respectively [337] (reviewed by Ref. [338]). Methylation at the TSGs *CDKN2A, SOCS1, GADD45B, STAT1, APC,* and *p15* is more prevalent in HCV-positive than in HCV-negative tumors, while *HOXA9, RASSF1,* and *SFRP1* were more methylated in HBV-positive tumors [325,339,341–343] (reviewed by Ref. [338]). Methylation of the *KLK10* (kallikrein related peptidase 10) promoter correlated with HCV infection, while methylation at *KLK10* and *GSTP1* inversely correlated with HBV infection [337,344]. Another study applied methylated-DNA immunoprecipitation on chip and mass spectrometry on HCC tissue samples to discriminate between HCV- and HBV-associated HCC. While hypermethylation of only the *CYP7B1* locus was found in HBV-HCC, a multitude of 15 loci was hypermethylated in HCV-HCC: age-independent methylation markers included genes inhibitory to Ras/Raf/ERK signaling (*NPR1, DUSP4, LOX,* and *RRAD*) and inhibitory to Wnt/β-catenin signaling (*SFRP4* and *RUNX3*) [345]. The degree of hypermethylation at a set of eight TSGs (*HIC1, GSTP1, SOCS1, RASSF1, CDKN2A, APC, RUNX3*, and *PRDM2*) in HCV-infected liver tissue was a predictor for the earlier appearance of HCC [346].

In a transgenic mouse and a cell line model, epigenetic down-regulation of *SOCS1* was dependent on the expression of the HCV core protein [347]. This observation is in accordance with a clinical

Table 21.4 Epigenetic alterations induced by human hepatitis viruses.

Microbe	Effector molecule	Epigenetic alteration	Effect
HBV	pX	Upregulation of DNMT1	Promoter silencing
		Upregulation of DNMT3A1 and DNMT3A2	Promoter silencing
		Downregulation of DNMT3B	Satellite 2 repeat hypomethylation
HCV	Core protein	Induction of DNMT1 and DNMT3B	Promoter silencing
		Induction of HDAC (SIRT1)	Promoter silencing
		Induction of SMYD3, a histone methyltransferase	Silencing of RASSF1A

study showing that HCV-infection was associated with *SOCS1* methylation, whereas HBV infection was inversely correlated with *SOCS1* methylation [348]. In HepG2 cells the expression of the core protein induced DNMT1 and DNMT3B resulting in *CDH1* promoter hypermethylation and a more aggressive growth behavior [349]. DNMT dysregulation through STAT3 activation was found to be HCV-genotype dependent [350]. Furthermore, DNMT-expression was required for HCV replication in cell culture. Thus, knockdown of DNMT-1 and -3B, but not of DNMT-3A expression prevented HCV infection [351]. HCV core protein expression in the Huh cell line also led to hypermethylation and silencing of *CDH1*. In addition, the HDAC SIRT1 was induced through core protein expression. Inhibiting SIRT1 derepressed the *CDH1* promoter [352]. Again, the effect of SIRT1 inhibition through sirtinol on DNMT expression was genotype dependent [350] (reviewed by Ref. [306]). A similar mechanism may silence the promoters of interferon-stimulated genes, too [353].

Alterations of the histone modification machinery have also been associated with the progression of HCC (reviewed by Ref. [338]). HCC is unusually low in H3K4me2, due to deregulation of the trithorax protein Ash2 and histone demethylase LSD1 [354]. Inducible HCV protein expression in cell lines led to the overexpression of protein phosphatase 2A (PP2A) which influenced the histone modification machinery through binding to protein arginine methyltransferase 1 (PRMT1), and the DNA repair machinery through dephosphorylating H2AX. The deregulation of both systems is considered critical in HCC development. Accordingly, the expression of a set of indicator genes involved in HCC tumorigenesis was changed concordantly by either the expression of HCV proteins or the overexpression of PP2A in cell culture [342] (reviewed by Ref. [338]). Transfection of the HCV core protein into cholangiocarcinoma cell lines induced the SET and MYND domain containing protein, SMYD3, a novel HMT. Overexpression of SMYD3 correlated with hypermethylation of the *RASSF1A* promoter [355].

The predisposition of HCV-infected patients to develop multifocal liver tumors is highly suggestive of an epigenetic field defect caused by HCV-infection. Indeed, a comparative study of epigenetic and transcriptional profiles in HCV-infected livers at different stages of tumorigenesis showed increased methylation at liver specific enhancers containing binding sites for the FoxA1, FoxA2, HNF4A, MAFK, MAFF, CEBPB, and RXRA transcription factors, and an enrichment of H3K27me3 at polycomb target genes involved in stem cell development. Epigenetic repression seemed to be dependent on HCV infection, but not on the overall inflammatory activity. Depletion of H3K27me3 through treatment with EZH2 inhibitor GSK343 supressed the growth of cultured HepG2 cells, suggesting a potential of epigenetic drugs in the treatment of chronic HCV-hepatitis and in reversing the epigenetic field defect, before tumor formation occurs [356].

Introduction of direct-acting antiviral (DAA) therapy, i.e., a combination of inhibitors targeting key nonstructural viral proteins involved in HCV replication (NS3/4A serine protease, NS5B RNA-dependent RNA polymerase and the NS5A protein) significantly improved the life expectancy of HCV-infected individuals [357]. These developments opened new research areas as well. Perez et al. observed that in vitro treatment of HCV-infected cells by direct-acting antiviral drugs resulted in a complete elimination of HCV from the cultivated cells [358]. Perez et al. took advantage of this observation to assess whether HCV-induced epigenetic alterations could persist in host cells in vitro, even after the cure of infection [358]. They found that long term HCV infection of Huh7.5 cells or the derived hepatocyte-like cells as well as short term HCV infection of primary human hepatocytes changed the gene expression pattern of the cultivated cells, and the HCV-specific alterations of the transcriptome were maintained in “cured” cells after treatment with a combination of direct acting

antiviral drugs [358]. In parallel, genome-wide analysis of histone modifications showed a redistribution of the chromatin marks H3K4Me3, H3K9Ac and H3K9Me3 in HCV-infected versus uninfected cells. Remarkably, in "cured" cells, the HCV-induced alterations in histone marks of the differentially expressed genes were preserved, too [358]. These data suggested that the HCV-induced epigenetic changes are not fully reverted in host cells after virus eradication by DAA therapy. Because the "epigenetic signature" left after HCV elimination may affect the behavior of "cured" cells, one may speculate that such a "hit and run" scenario could contribute, in principle, to the process of hepatocarcinogenesis. A recent review by Zhao et al. gave a detailed description of HCV-associated epigenetic alterations, including the putative role of noncoding RNAs in hepatocarcinogenesis [359].

The epigenetic alterations induced by human hepatitis viruses are summarized in Table 21.4.

21.3.6 Induction of DNA methyltransferase, histone methyltransferase, and histone demethylase enzymes by the human papillomavirus oncoprotein E7

Infection with high-risk human papillomavirus (HPV) strains (especially type 16 and 18) is causally associated with the development of cervical cancer. Progression of HPV-positive premalignant lesions to invasive cancer is a rare event, however, suggesting that additional steps including chromosomal alterations and epigenetic changes are necessary for neoplastic development [360]. In an in vitro model system of cervical carcinogenesis, Henken et al. observed an accumulation of frequent methylation events involving five TSGs when primary keratinocyte cell lines transfected with HPV genomes acquired anchorage independence. In contrast, no methylation was evident in preimmortal HPV-18 transfected cells [361]. Certain cellular genes are also hypermethylated during in vivo cervical carcinogenesis in a histological type- or clinical stage-dependent manner (reviewed by Ref. [360]). The E7 oncoprotein of HPV-16 was shown to bind to DNMT1 and stimulate its methyltransferase activity [362]. This may explain the hypermethylation of selected cellular promoters in HPV-infected cells, and Laurson et al. speculated that E7−DNMT1 interaction may target DNMT1 to specific sequences, similarly to the recruitment of DNMT3A to specific genomic regions by the KSHV oncoprotein LANA [363]. Furthermore, the E6 protein of HPV-16 led to an upregulation of DNMT1 through the degradation of p53 [364].

Consistent hypermethylation patterns were described in laryngeal papillomas associated with HPV-6 as well [365]. Laryngeal papillomas are usually benign lesions, although they may progress to squamous cell carcinoma in a fraction of cases. Promoter hypermethylation affecting *TIMP3* and *CDKN2B* occurred early during tumor progression and was maintained throughout neoplastic development [365].

HPV-16 E7 activated the expression of EZH2, a Polycomb protein with histone H3K27 methyltransferase activity, too. Continuous expression of EZH2 was indispensable for the proliferation of HPV-positive tumor cells [366]. Histone H3K27me3 marks may contribute to promoter silencing either in concert with DNMTs or independently of DNA methylation. E7 is a pleiotropic regulator protein involved both in silencing and activation of certain target gene sets. HPV-16 E7 increased histone H3 acetylation at *E2F1* and *CDC25A* promoters in human foreskin keratinocytes [367], and reduced the level of the repressive histone modification H3K27me3 in primary human epithelial cells [368]. The latter change was due to induction of the histone demethylases KDM6A and KDM6B

targeting specifically histone H3K27me3 and disrupting Polycomb repressor complexes. E7-mediated reprogramming resulted in an increased expression of KDM6A- and KDM6B-responsive Homeobox genes (*HOXC5*, *HOXC8*) that are known to be upregulated in cervical carcinomas [368]. The alterations in the epigenetic regulation of the viral and host genome associated with HPV infection have recently been comprehensively reviewed [369].

The progression of cervical intraepithelial neoplasia (CIN) to more malignant invasive stages of cervical carcinoma was preceded by the development of a polycomb-mediated epigenetic field defect. This field defect was characterized by overall DNA hypomethylation at intergenic regions, and an increase of DNA hypermethylated sites involved in transcriptional regulation, with a strong enrichment of polycomb repressed target genes. Methylation changes started early in the neoplastic process, progressed with the histological grade and were also accompanied by the steady increase of polycomb protein expression. This suggests a potential for epigenetic drugs in the treatment of CIN and in reversing the epigenetic field defect, before invasive tumor formation occurs [370]. The strong PRC target gene enrichment in cervical carcinoma resembles the situation in a diverse set of hematological malignancies, especially Burkitt lymphoma [371].

Most interesting, the long control region (LCR) of HPV-18 and -16 contain consensus sequences for binding transcription factors FoxA1 and c-Myc. Indeed, FoxA1 and c-Myc were bound to the LCRs in chromatin immune precipitation and were able to regulate transcription from the LCR. FoxA1, a pioneer factor, acted strongly activating, while c-Myc acted slightly repressive on LCR-dependent transcription as examined by transfection and reporter assay experiments [372] (reviewed by Ref. [373]). A prominent binding site for the oncoprotein c-Myc was also found in the LCR of EBV, and a central pathophysiological role in the development of endemic Burkitt lymphoma ascribed to it [374–377].

We would like to add that epigenetic alterations induced by the E7 oncoprotein in cells transformed by high-risk HPV strains may contribute to immune evasion by HPV-positive malignant tumors. In a transcriptome and methylome analysis of human keratinocyte cell lines, Cicchini et al. observed that the *HLA-E* CGI, a distal CGI located upstream from the *HLA-E* gene, was hypermethylated in NIKS-16 cells expressing HPV-16 E7, compared to NIKS-16ΔE7 keratinocytes carrying the episomal HPV-16 genome but lacking E7 expression [378]. Hypermethylation of the *HLA-E* CGI correlated with the downregulation of HLA-E, a non-classical MHC class I molecule implicated in interactions with NK cells and $CD8^+$ T cells. Thus, Cicchini et al. speculated that by silencing *HLA-E* via hypermethylation of its distal regulatory region, HPV-16 E7 may prevent presentation of HPV-derived peptides to NK and cells or $CD8^+$ T cells, resulting in immune evasion [378].

In addition to the alteration of the host cell methylome, E7-induced redistribution of histone marks may also induce distinct changes in gene expression that facilitate immune evasion by HPV-positive malignant cells. As we outlined above (Section 21.3.6), HPV-16 E7 is capable to activate the expression of EZH2, a H3K27 methyltransferase [366]. A recent study by Lo Cigno et al. revealed, however, that another histone-lysine N-methyltransferase, designated SUV39H1, was also upregulated by E7 in cells carrying high-risk HPV genomes [379]. SUV39H1 trimethylates lysine 9 in histone H3, generating H3K9me3, a repressive chromatin mark that was enriched in E7 expressing cells at the promoters of so-called immune sensor genes [379]. The immune sensor genes silenced in HPV transformed cells encode cytosolic pattern recognition receptors, such as cGAS (cyclic GMP-AMP synthase), RIG-I (retinoic acid inducible gene I) and STING (stimulator of interferon genes),

interacting with viral nucleic acids. STING also functions as an adaptor protein in the type I interferon response. Thus, Lo Cigno et al. suggested that E7 may dampen the innate immune response by blocking interferon induction, via SUV39H1-mediated downregulation of cGAS, RIG-I and STING expression [379]. In addition to the research efforts focusing on HPV-associated cervix carcinoma, recently there was a significant progress as to the alterations of the methylome in HPV-positive head and neck cancer, too (reviewed by Nakagawa et al. [380]).

21.3.7 Epigenetic transcriptional silencing in Merkel cell polyomavirus–associated carcinoma of the skin

Merkel cell carcinoma (MCC), a rare neuroectodermal tumor arising from mechanoreceptor Merkel cells, frequently carries integrated genomes of a recently discovered human polyomavirus, called Merkel cell polyomavirus (MCPyV) [381]. MCCs are rare but highly aggressive neoplasms. Similarly to other polyomaviruses, the dsDNA genome of MCPyV encodes tumor antigens (large T antigen and small T antigen) implicated in oncogenesis. In MCCs, the large T antigen is regularly truncated rendering the virus replication incompetent [382]. The proneural transcription factor Atonal Homologue 1 (Atoh1) is essential for cell fate commitment of multiple neuronal lineages and acts as a developmental regulator of the mechanoreceptive Merkel cells in the skin, too [383]. ATOH1 expression was found to be reduced due to deletion or inactivation by CpG methylation in MCCs as well as colorectal carcinomas [384]. In addition, the promoter of the TSG *RASSF1A* was also hypermethylated in about half of the MCC samples [385]. These observations indicate that epigenetic events may contribute to the pathogenesis of MCPyV-associated MCC.

Compared with virus-negative neuroendocrine carcinomas of sun-damaged skin which exhibit a high number of mutations, MCPyV-positive carcinomas carry only few mutations. However, H3K27me3 was strongly reduced only in virus-positive MCCs. The loss of PRC2 which catalyzes the trimethylation of histone 3 at lysine 27 seems to play an important role in MCC formation [386]. Dysregulation of polycomb repression of the host genome is a common feature of several, but not all virus-associated malignancies (see Section 21.3.1 on EBV; Section 21.3.5 on HCV; Section 21.3.6 on HPV). PRC subunits Ezh1 and Ezh2 play an instrumental role in Merkel cell differentiation [387]. Recently, Gujar et al. analyzed the DNA methylation patterns of primary and metastatic MCCs [388]. Compared with paired normal tissues, they identified MCC-specific DNA methylation profiles distinct for primary MCCs as well as for MCC lymph node metastases and MCC skin metastases [388]. Although the authors did not expect it, comparison of eight MCPyV-positive and five MCPyV-negative tumor tissues also showed significant differential DNA methylation: as listed in supplementary table S1, hypermethylated CpGs were associated with several genes including *PAX2* (paired box gene 2) encoding a transcription factor implicated in the development of the central nervous system, and *SHANK3* (SH3 and multiple ankyrin repeat domains 3) coding for a scaffold protein implicated in synaptic plasticity, whereas a hypomethylated CpG was identified in the promoter of *PRDM9*, a gene encoding the meiotic recombination activator protein PRMD9, a lysine methyltransferase trimethylating both H3K36 and H3K4 [388,389]. The significance of these findings in the initiation and progression of MCPyV-positive MCCs remains to be established. For a recent review of MCPyV-associated epigenetic events see Rotondo et al. [390].

21.3.8 COVID-19 epigenetics

21.3.8.1 Epigenetic regulation of the SARS-CoV-2 genome

The exploration of the epigenetic regulation of SARS-CoV-2 infection is only at the beginning. Therefore, only preliminary, though most interesting data exists. Obviously, in the course of its developmental history, SARS-CoV-2 was subject to epigenetic regulation. The antiviral defense protein ZAP (zink finger antiviral protein) counteracts viruses with high CpG content by binding to and inhibiting the replication of the viral genomes. SARS-CoV-2 has the strongest CpG deficiency among all betacoronaviruses, thereby evading the antiviral defense by ZAP. This may be a hint that the SARS-CoV-2 genome has evolved in a high-ZAP tissue environment. This may also give clues to potential additional species besides bats in which the virus has dwelled before infecting humans. A canine environment might qualify [391] (reviewed by Ref. [392]). However, the virus apparently interferes with the epigenetic regulation of cellular gene expression, too.

21.3.8.2 SARS-CoV-2 interaction map on epigenetic modifiers

A comprehensive SARS-CoV-2 protein interaction map using affinity-purification mass spectrometry identified 332 high confidence interactions of 26 among all 29 viral proteins and the human proteome. These interactions comprised a couple of cellular epigenetic modifiers [393]. The main viral protease Nsp5 bound to HDAC2 that contains a cleavage site for Nsp5. Thus, it appears most likely that the virus may interfere with HDAC2 function, possibly thereby mitigating HDAC2′s effector role in inflammation and the interferon response. However, since HDAC2 deacetylates H4K16ac, there may be broad, yet unpredictable epigenetic effects of this interaction.

Furthermore, the viral transmembrane E-protein was bound to the epigenetic reader proteins bromodomain containing BRD2 and BRD4. This binding is probably due to the C-terminal part of the E-protein that mimics the N-terminal part of histone H3, a known interaction partner of bromodomains. However, since data on Nsp5 and E-protein interactions was observed in vitro, it remains to be proven that the viral proteins really interact in the same cellular compartments in vivo [393]. Furthermore, the viral gene *ORF8* was recently shown to encode a chromatin-associated protein which mimics the ARKS motifs of histone H3 to disrupt host cell epigenetic regulation. SARS-CoV-2 viruses carrying a deletion of ORF8 are less pathogenic [394].

21.3.8.3 Epigenetic effects of SARS-CoV-2 infection on cellular genes

SARS-CoV-2, the causative agent of COVID-19, interferes with the epigenetic regulation of a multitude of cellular genes, especially genes involved in immune function in peripheral blood mononuclear cells (reviewed by Refs. [395,396]). Most of those genes were found in the TGF-β, IL-1B, IL-6, and IL-17 pathways and could be influenced with dexamethasone, a proven drug against COVID-19 [397]. A transcriptomic and epigenomic analysis of peripheral monocytes revealed distinct patterns among different patient groups, corresponding to the cellular function or mal-function. The epigenetic reprogramming of cellular genomes might contribute to prolonged symptoms in some cases [398]. Another analysis investigated $CD14^{+}$ monocytes of patient groups with differing severity of COVID-19. Upon viral infection, monocytes switched their gene expression profile from a standard immune function to a pro-thrombotic phenotype. Epigenetic reprogramming was observed in Covid-19 patients, and involved increased levels of both H3K27Ac and H3K4Me3 [399]. Peripheral blood mononuclear cells from critically ill patients revealed hyper-methylation of interferon-related genes

and hypo-methylation of inflammatory genes [400]. A set of 36 differentially methylated regions (DMRs) mostly involving virus defense and interferon signaling was determined from the blood of hospitalized COVID-19 patients [401] (reviewed by Ref. [402]). While Nsp5 and the E-protein constitute potential means for the virus to make an impact on the epigenetic profile of an infected cell, the virus is also dependent on the methylation status of key cellular promoters, such as the promoter for its transmembrane receptor ACE2 (angiotensin converting enzyme type 2) which is a homolog of ACE [403].

21.3.8.4 Epigenetic regulation of the ACE2 gene

ACE2 is expressed in multiple human tissues, like kidneys, liver, the cardiovascular system, esophagus, stomach, intestines, bladder, testis, and brain, with some expression of ACE2 also in type II alveolar cells in the lower respiratory tract [404–406]. The expression levels vary widely between different tissues, the sexes, ethnic groups, individuals, and are dependent on the age (reviewed by Refs. [407,408]). This is reflected by rather diverse clinical pictures showing the involvement of organs other than the lungs in SARS-CoV-2 disease (reviewed by Refs. [392,409,410]). ACE2 expression is directed by two distinct upstream promoters, a distal and a proximal one, which are differentially used in different tissues. Some of the transcription factors binding to both promoters have been identified by chromatin immunoprecipitation (ChIP) and ChIP-Seq. The chromatin organization of the ACE2 gene locus, its topologically associated domains, and the distribution of specific histone marks within this locus has been analyzed to a limited extent (reviewed by Ref. [407]).

Coinciding with the expression status, CpG methylation of the ACE2 promoter was low in lung tissue. Furthermore, a specific CpG-dinucleotide near the transcription start site was hypomethylated during aging. There are also differences in ACE2 promoter methylation between men and women. The overall methylation level of a CpG-island of the ACE2 promoter was higher in men than women [411]. Nevertheless, in a study using single cell RNAseq, ACE2 expression was higher in male than in female lung tissues [412]. This complicated methylation and expression pattern may be connected to the fact that ACE2 is located on the X-chromosome at Xp22, and incomplete X-chromosome inactivation (XCI) plays an important role for the level of ACE2 gene expression [413] (reviewed by Refs. [395,396]). Patients with essential hypertension showed a stronger methylation and lower ACE2 expression than the healthy indicating that chronic disease may influence ACE2 methylation levels [414]. Lupus patients, on the other hand, showed hypomethylation and higher ACE2 expression on their peripheral blood T-cells, possibly leading to an easier viral spread by viremia. Furthermore, a more general tendency to hypo-methylation of interferon stimulated and other cytokine genes in lupus patients may contribute to a higher likelihood of cytokine storm in lupus patients [415,416]. A meta-analysis of published data on patients with myalgia-associated encephalomyelitis/chronic fatigue syndrome (ME/CFS) showed an imbalanced ACE expression, with higher ACE and lower ACE2 levels, also reflected in the methylation status of both, the ACE and ACE2 genes [417]. In subgroup analyses, patients with UCEC (Uterine Corpus Endometrial Carcinoma) or KIRP (Kidney Renal Papillary Cell Carcinoma) had a better prognosis when the ACE2 promoter was hypomethylated and the ACE2 protein highly expressed. This was most likely due to a better tumor infiltration by immune cells. However, upon SARS-CoV-2 infection, ACE2 levels were decreased which affects the prognosis of UCEC and KIRP patients to the worse [418].

An analysis of 700 lung transcriptome samples from patients with comorbidities, such as hypertension, diabetes, and chronic obstructive lung disease (COPD), who had died from COVID-19

showed high ACE2 mRNA levels. Correlation and network analyses revealed the potential involvement of histone modifiers, like HAT1, HDAC2, and KDM5B in the regulation of ACE2 expression. High ACE2 levels and a more general epigenetic dysregulation in those patients may lead to more severe disease courses. Therefore, a high activity of histone demethylase KDM5B may support COVID-19 prevention, and KDM5 activation may possibly constitute a pharmaceutical target [419]. The class III HDAC enzyme SIRT1 (silent information regulator T1) is also involved in ACE2 regulation, especially under cellular stress conditions [420].

In case of severe influenza, downregulation of ACE2 was associated with acute lung injury or acute respiratory distress syndrome (ARDS). ACE2 down-regulation is most likely mediated by micro-RNAs, especially miRNA-200c-3p [421]. There is a complex network of micro-RNAs involved in the regulation of ACE2 and TMPRSS2 (transmembrane serine protease 2) expression which may also be influenced by SARS-CoV-2 infection [422] (reviewed by Ref. [392]).

21.3.8.5 ACE2 expression levels and disease severity

Cells must express ACE2 on their surface in order to be infected with the virus [423,424]. ACE2 is a negative regulator of the renin-angiotensin-aldosterone-system (RAAS) (reviewed by Refs. [408,425]). Thus, high ACE2 expression increases the infectivity through SARS-CoV-2. However, once infected, ACE2 is not required anymore for virus replication. Overall, low ACE2 expression may inflict great damage upon or during SARS-CoV-2 infection. Therefore, the degree of ACE2 expression does not simply correlate with the severity of COVID-19 disease in a simple straightforward way, meaning high expression would equal severe disease. Actually, ACE2 deficiency is under circumstances connected with more severe disease states than high expression of ACE2. This is due to the counter-regulatory effects of angiotensin$_{1-7}$ against angiotensin II and the overall complex regulatory network of the RAAS. While angiotensin II increases vasoconstriction and the tendency to fibrosis, inflammation, thrombosis and pulmonary damage, angiotensin$_{1-7}$ to the contrary increases vasodilation, and decreases the tendency to fibrosis, inflammation, thrombosis and pulmonary damage and is therefore protective (reviewed by Refs. [408,425,426]). Current data suggest that the ACE2 promoter is less methylated in the lung tissues of women than of men which seems counter-intuitive, because men are more prone to severe COVID-19 disease than women [411]. However, ACE2 mRNA was higher in men's than women's lung samples [412]. Furthermore, ACE2 expression is lower in children and increases with age, corresponding with more lenient disease in children and more severe disease courses in the elderly [427]. In addition, the differences of ACE2 expression in the nasal epithelia may greatly contribute to the lower incidence of COVID-19 in children [428] (reviewed by Refs. [395,396,408,429]). ACE2 expression in smokers, and in patients with hypertension, diabetes mellitus type II, or COPD which are risk factors for severe COVID-19 was high [419,427,430]. Further comorbidities, e.g., obesity or dyslipidaemia which pose risk factors for severe Covid-19 were extensively reviewed and discussed [408].

21.3.8.6 Epigenome effects on the course of COVID-19 disease

An epigenome-wide association study (EWAS) found 44 CpG sites which were associated with COVID-19 severity. A DNA methylation EWAS signature was established which had good predictive power for COVID-19-severity and might be used for guiding the appropriate treatment [431].

21.3.8.7 The spike effect

The binding of the viral spike protein to ACE2 leads to its internalization, just like after binding its natural ligand angiotensin II. The consequence may be a deficiency of the ACE2 receptor and, thereby, the loss of its protective function [432] (reviewed by Refs. [410,433,434]). Through the loss of ACE2 receptor activity, spike-ACE2 interaction causes the above mentioned imbalance of the RAAS with an angiotensin II overactivity and an angiotensin 1-7-deficiency. Angiotensin II accumulation is detrimental and appears to be strongly implicated in all severe forms of COVID-19 [435] (reviewed by Refs. [425,433]). One prominent example for the negative consequences of ACE2 deficiency is that it may lead to the enhanced aggregation of blood platelets, thrombus formation and inflammation [436] (reviewed by Refs. [433,434]). In mice, ACE2 deficiency impaired gestational weight gain and restricted fetal growth [437]. Vaccine spike proteins have the same conformation as viral spike proteins and may, therefore, cause the same detrimental effects which are linked to angiotensin II accumulation as the viral spike proteins [436,438–442] (reviewed by Ref. [434]). The research on the epigenetics of SARS-CoV-2 infection and COVID-19 is only at its beginning. Several theoretical assumptions on the interactions of viral proteins with the epigenetic machinery of the cell need to be further experimentally confirmed. Our knowledge on the tissue-specific epigenetic regulation of the ACE2 gene and other cellular genes involved in the hyper-inflammatory responses in severe Covid-19 needs to be completed, still. Therapeutic strategies to restore the balance of the RAAS and dampen the deleterious effects of angiotensin II accumulation need to be more investigated.

21.4 Epigenetic alterations elicited in the host tissue by trematode infections

Trematode infections can lead to epigenetic alterations in the host tissue, which we will subsequently further explore.

21.4.1 Promoter hypermethylation in cholangiocarcinoma associated with *Opisthorchis viverrini* (liver fluke) infection

Cholangiocarcinoma in certain geographical areas is related to liver fluke infection. In northeast Thailand, most patients with the malignancy of the bile duct epithelium carry the macroparasite *Opisthorchis viverrini*, whereas in Cambodia, China, Japan, Korea, Laos, and Vietnam, *Clonorchis sinensis* infection is spreading via the consumption of raw freshwater fish, causing cholangiocarcinoma [443,444]. *C. sinensis* expresses an egg-specific DNMT2-like DNA methyltransferase [445]. In *O. viverrini*–associated cholangiocarcinomas downregulation of *p14ARF* expression was predominantly related to inactivation by DNA methylation, whereas $p16^{INK4A}$, another TSG of the 9p21 gene cluster, was affected by allelic loss. Both mechanisms contributed to downregulation of $p15^{INK4B}$ [446]. Sriraksa et al. analyzed the methylation pattern of 26 CGIs in *O. viverrini*–associated cholangiocarcinoma samples. There was an elevated frequency of hypermethylation at five areas including the ovarian tumor-suppressor *OPCML (*opioid-binding protein/cell adhesion molecule-like gene*)* and the immunomodulator *DcR1 (*decoy receptor 1*)* [447]. Thus, *OPCML* and *DcR1* may serve as methylation biomarkers for cholangiocarcinoma.

A recent integrated analysis of genomic, epigenomic and transcriptomic data of cholangiocarcinoma specimens derived from both liver fluke infected and liver fluke-negative patients revealed that the liver fluke-positive cholangiocarcinomas formed a distinct cluster, characterized by DNA hypermethylation, especially at CGIs, and an increased level of CpG > TpG mutations attributed to spontaneous deamination of 5-methylcytosine (5 mC) to thymine [448]. Jusakul et al. suggested that at the initial stage of carcinogenesis, liver fluke infection may induce alterations in the methylome of cholangiocytes, possibly by eliciting chronic inflammation as well as metabolic disruption and secretion of growth factors [448]. Downregulation of TET1, an enzyme involved in DNA demethylation, and upregulation of the histone-lysine methyltransferase EZH2 that facilitates heterochromatin formation may contribute to the CpG hypermethylation-mediated silencing of TSGs [448]. Genetic events, such as the increased mutation burden generated by 5 mC deamination and also due to deamination of cytosine bases by APOBEC enzymes, may contribute to tumor progression [448]. Jusakul et al. noticed that certain genetic changes, e.g., amplification of the proto-onc gene *ERBB2*, occurred in a fraction of both liver fluke-positive and liver fluke-negative cholangiocarcinomas [448]. Thus, a combination of macroparasite-induced epigenetic and genetic alterations may play a role in cholangiocarcinogenesis.

21.4.2 CpG island methylation in *Schistosoma haematobium*–associated bladder carcinoma

Squamous cell carcinoma, and to some extent also transitional cell carcinoma of the bladder in East Africa and the Middle East and other subtropical areas, is frequently associated with chronic urinary *Schistosoma haematobium* infection. Chronic mechanical irritation by calcified eggs deposited by the worms in the bladder epithelium and accumulation of carcinogenic compounds in the urine may be involved in the transformation of the uroepithelium. Contrary to earlier studies, which failed to detect cytosine methylation in *Schistosoma* flatworms, it now became clear that flatworms contain complex epigenetic machinery, and regulated DNA methylation plays an essential role in the *Schistosoma mansoni* life cycle and especially oviposition [449,450]. Epigenetic regulatory mechanisms in schistosomes are promising targets for therapeutic intervention [451]. In bladder cancer samples of most patients with probable *S. haematobium* infection from Egypt, there was a greater degree of CGI methylation than in non-*Schistosoma*–associated tumors [452]. Hypermethylation at the *RASSF1* and *TIMP3* loci in urinary sediment may serve as noninvasive biomarkers for bladder damage caused by *S. haematobium* infection [453]. Specifically, hypermethylated gene loci may serve as early detection markers both for schistosoma-associated and nonassociated bladder cancer [454].

In addition to interacting with and eliciting epigenetic alterations in uroepithelial cells, *S. haematobium* is capable to induce changes in the methylome and gene expression pattern of helper T cells as well. Di Nardo et al. studied DNA methylation changes of $CD4^+$ T cells in asymptomatic children infected with *S. haematobium* [455]. They observed that helminth infection induced hypermethylation of a battery of $CD4^+$ T cell genes associated with IFN-γ-signaling, compared to age-matched, uninfected children [455]. Using targeted real-time quantitative PCR after methyl-specific endonuclease digestion, the authors validated the methylation patterns of a subset of genes coding for transcription factors, cytokines and cytokine receptors, and demonstrated that the IFN-γ-signaling pathway was muted in $CD4^+$ T cells isolated from *S. haematobium i*nfected children [455]. These experiments and further observations suggested that even 6 months after successful *S. haematobium*

deworming the helminth-induced changes of the $CD4^+$ T cell methylome as well as the resulting immune perturbations persisted [455]. Thus, *S. haematobium*—induced epigenetic alterations may possibly induce quite a long-lasting immunological tolerance and may dampen cell-mediated immune reactions directed against other pathogens, too.

We would like to add that *S. haematobium* infection may affect the composition of the urogenital microbial community, too [456]. One may speculate that the altered microbiome may elicit local inflammation, contributing thereby, indirectly, to the schistomiasis-induced epigenetic and pathological alterations in the bladder.

21.5 Conclusions

Although most of the work related to epigenetic alterations induced by infectious agents in host cells focused on tumor-associated microbes and viruses implicated in tumorigenesis, it is clear from the overview of the literature that other important bacterial and viral pathogens directly not involved in the initiation or maintenance of neoplasia leave their epigenetic marks on their target cells as well. There is no doubt that in addition to viruses and bacteria, other microparasites, i.e., protozoa that do have their own sophisticated epigenetic regulatory systems, may also cause epigenetic dysregulation in their hosts. Furthermore, tumor-associated viruses may have a role in other diseases as well, thus the knowledge of the epigenetic control of their genomes and the epigenetic changes they elicit in neoplastic cells may help to decipher the pathoepigenetic mechanisms causing dysfunctions in non-neoplastic cells. In this respect, it is worthy to mention that the epigenetic control of EBV latency appears to be inadequate in certain autoimmune diseases and was implicated in triggering and perpetuating the pathogenic processes [457]. Other important research topics are also emerging, including the potential role of microbial and other infections in the pathoepigenetics of allergic diseases [458] and the modification of host epigenetic processes by the microbial communities inhabiting mucosal surfaces and the skin, or even by probiotic bacteria [459]. The epigenetic changes induced by the vast majority of macroparasites (helminths, arthropods) and fungi infecting humans and animals remain to be explored as well.

List of abbreviations

In the text, abbreviations, including acronyms, are used as given in the references cited. In case of synonyms, the list of **Abbreviations** also contains alternative, current versions of certain names and acronyms, wherever applicable.

3D	three-dimensional
5mC	5-Methylcytosine
ACE2	angiotensin converting enzyme type 2
AD2	acidic domain 2
AIDS	Acquired Immune Deficiency Syndrome
ankyrin A (AnkA)	an effector protein of *Anaplasma phagocytophilum*
AP-1	Activator protein 1, a transcription factor
APC	*Adenomatous polyposis coli*, a gene encoding the tumor suppressor protein APC
APOBEC	apolipoprotein B mRNA editing enzyme, catalytic polypeptide, a family of cytidine deaminases

ARDS	acute respiratory distress syndrome
Ash2	*Set1/Ash2 histone methyltransferase complex subunit*
ATLL	Adult T-cell leukemia/lymphoma
ATM	a gene encoding Ataxia Teleangiectasia Mutated, a protein kinase
Atoh1	Atonal Homologue 1, a transcription factor
BAHD1	Bromo adjacent homology domain-containing 1, a transcription repressor protein
BbSET	a SET domain protein of *Bordetella bronchiseptica*
Bcl-2	B-cell lymphoma 2, an apoptosis regulator protein
BET	Bromodomain and extraterminal domain
BET proteins	epigenetic readers carrying two tandem bromodomains and an extraterminal domain
BH3	a short peptide motif found in certain BCL-2 family proteins
BIM	Bcl-2 Interacting Mediator of cell death, a protein promoting apoptosis
Bim	also called *BIM* or *BCL2L11*, a gene encoding Bcl-2 Interacting Mediator of cell death
BLK	B lymphocyte kinase
Bmi-1	a PcG family member (Bmi-1 stands for B cell-specific Moloney murine leukemia virus integration site 1)
BRD2	Bromodomain-containing protein 2
BRD4	Bromodomain-containing protein 4
BtSET	a SET domain protein of *Burkholderia thailandensis*
c-MET	also designated *MET*, a gene encoding mesenchymal-epithelial transition factor
C. sinensis	*Clonorchis sinensis*
cag-PAI	cag-pathogenicity island
CagA	cytotoxin-associated gene A, a *Helicobacter pylori* toxin
***CaMKIIa* (also designated *CAMK2A*)**	a gene encoding calcium/calmodulin dependent protein kinase II alpha
CBP	CREB binding protein, a transcriptional coactivator with histone acetyltransferase activity
cccDNA	covalently closed circular DNA
CCL20	CC chemokine ligand 20
CCND2	a gene encoding cyclin D2
CD	cluster of differentiation
CD21	cluster of differentiation 21, also known as Epstein−Barr virus receptor and complement receptor 2
CD276	a human gene encoding the immune regulator protein Cluster of Differentiation 276
CD48	Cluster of Differentiation 48, a cellular differentiation antigen
CDC25A	a gene encoding Cell Division Cycle 25A
CDCs	cholesterol-dependent cytolysines
CDH1	a gene coding for E-cadherin
CDH13	a gene coding for H-cadherin
CDK4/6	CDK4 and CDK6
CDKN2A	a gene encoding Cyclin-Dependent Kinase Inhibitor 2A
CDKN2A	cyclin-dependent kinase inhibitor 2A
CDKN2B	a gene encoding Cyclin-Dependent Kinase Inhibitor 2B
CDKs	cyclin-dependent kinases
CEBPB	CCAAT Enhancer Binding Protein Beta
cGAS	cyclic GMP-AMP synthase
CGI	CpG island
ChIP	Chromatin immunoprecipitation

ChIP-Seq	Chromatin Immunoprecipitation Sequencing
CIMP	CGI methylator phenotype
CIN	cervical intraepithelial neoplasia
COPD	chronic obstructive lung disease
COVID-19	Coronavirus disease 2019
***COX2* (also known as *PTGS2*)**	a gene encoding cyclooxygenase-2 or COX-2 (also named Prostaglandin-endoperoxide synthase 2)
CpG dinucleotide	also called CG site, designates *5′-C-phosphate-G-3'* (i.e., cytosine and guanine separated by a phosphate group)
***CREB* (also designated *CREB1*)**	a gene encoding cAMP-response element binding *protein*
CREG	*a gene encoding* Cellular repressor of E1A stimulated genes, a glycoprotein
CtBP	C terminal binding protein
CTCF	CCCTC-binding factor (a chromatin insulator protein)
CTF1	CCAAT box binding factor 1
CXCL1	Chemokine (C-X-C motif) ligand 1
CXCL2	Chemokine (C-X-C motif) ligand 2
CYP7B1	a gene encoding Cytochrome P450, Family 7, Subfamily B, Polypeptide 1
DAA therapy	direct-acting antiviral therapy
***DAPK* (also named *DAPK1*)**	a gene encoding Death-associated protein kinase 1
DcR1	a gene coding for Decoy receptor 1
DLEU2	Deleted in lymphocytic leukemia 2, a long noncoding RNA molecule
DMR	differentially methylated region
DNMT1	DNA methyltransferase 1
DNMT3A	DNA methyltransferase 3A
DNMT3B	DNA methyltransferase 3B
DUSP4	Dual specificity protein phosphatase 4
E2F1	a member of the E2F family of transcription factors
EBER1	Epstein–Barr virus–encoded small RNA 1
EBER2	Epstein–Barr virus–encoded small RNA 2
EBNA-LP	Epstein–Barr virus nuclear antigen leader protein (also called EBNA5)
EBNA1	Epstein–Barr virus nuclear antigen 1
EBNA2	Epstein–Barr virus nuclear antigen 2
EBNA3	Epstein–Barr virus nuclear antigen 3 (also called EBNA3A)
EBNA3A	Epstein–Barr virus nuclear antigen 3A (also called EBNA3)
EBNA3B	Epstein–Barr virus nuclear antigen 3B (also called EBNA4)
EBNA3C	Epstein–Barr virus nuclear antigen 3C (also called EBNA6)
EBNA4	Epstein–Barr virus nuclear antigen 4 (also called EBNA3B)
EBNA5	Epstein–Barr virus nuclear antigen 5 (also called EBNA-LP)
EBNA6	Epstein–Barr virus nuclear antigen 6 (also called EBNA3C)
EBV	Epstein–Barr virus
EBVaGC	EBV-associated gastric carcinoma
***ECAD* (also known as *CDH1*)**	a gene coding for E-cadherin (Epithelial cadherin)

EGR1	early growth response 1, a transcription factor
EMILIN2	a gene encoding Elastin Microfibril Interfacer 2
EMT	epithelial-mesenchymal transition
EpiAge	epigenetic age
ERBB2	a gene encoding Erb-B2 Receptor Tyrosine Kinase 2
ERF	Ets-2 repressor factor (ets stands for v-ets Avian Erythroblastosis virus E26 oncogene homolog 2)
ERK	extracellular signal-regulated kinase
EZH2	enhancer of zeste homolog 2, a histone-lysine N-methyltransferase enzyme, a component of polycomb repressive complex 2
FLNc	a gene encoding Filamin C
FoxA1	Forkhead box A1, transcription factor
FoxA2	Forkhead box A2, transcription factor
FOXG1B_FKHL1	a gene encoding Forkhead box protein G1B_Forkhead-related protein 1
FoxO4	Forkhead O Transcription Factor 4
GADD45B	a gene encoding growth arrest and DNA damage-inducible 45B
GC	gastric carcinoma
GECs	gingival epithelial cells
GNE	a gene encoding UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase
Gp120 (also designated GP120 or gp120)	a HIV glycoprotein
GRASP	a gene encoding general receptor for phosphoinositides 1 associated scaffold protein
GRK5	a gene encoding G-protein-coupled receptor kinase 5
GSTP1	a gene encoding Glutathione S-Transferase Pi 1
H2AFY2	*a gene coding for* Core histone macro-H2A.2, *a variant histone,* **the current gene name is** MACROH2A2, H2AFY2 is an alias
H2AX	H2A Histone Family
H3K14	histone H3 lysine 14
H3K14ac	histone H3 lysine 14, acetylated
H3K27me3	histone H3 lysine 27, trimethylated
H3K4	histone H3 lysine 4
H3K4ac	histone H3 lysine 4, acetylated
H3K4me2	histone H3 lysine 4, dimethylated
H3K4me3	histone H3 lysine 4, trimethylated
H3K9	histone H3 lysine 9
H3K9ac	histone H3 lysine 9, acetylated
H3K9me3	histone H3 lysine 9, trimethylated
HAART	highly active antiretroviral therapy, currently it is called cART ("combination antiretroviral *therapy*")
HAND	HIV-associated neurocognitive disorder
HAT	histone acetyltransferase
HAT1	histone acetyltransferase 1
HBV	hepatitis B virus
HBx	hepatitis B virus X protein
HCC	hepatocellular carcinoma

HCMV human cytomegalovirus
HCV hepatitis C virus
HDAC histone deacetylase
HDAC1 histone deacetylase 1
HDAC2 histone deacetylase 2
HHV-8 Human herpesvirus 8 (also called Kaposi's sarcoma–associated herpesvirus, KSHV)
HIC1 a gene encoding Hypermethylated In Cancer 1 (also called HIC ZBTB Transcriptional Repressor 1)
HIST1H4F a gene encoding histone H4 (the abbreviation stands for *histone cluster 1 H4 family member f*) the current gene name is *H4C6* „*H4 Clustered Histone 6*", *HIST1HF4* is an alias
HIV human immunodeficiency virus
HIV-1 type 1 human immunodeficiency virus
HK a gene encoding hexokinase; the current gene name is *HK1*
HLA-E a gene encoding Major Histocompatibility Complex, Class I, E
HMEs histone modifying enzymes
HMT histone methyltransferase
HNF4A Hepatocyte Nuclear Factor 4 Alpha
HOXA9 a gene encoding Homeobox protein HOXA9
HOXC5 a gene encoding Homeobox protein Hox-C5
HOXC8 a gene encoding Homeobox protein Hox-C8
Hp *Helicobacter pylori*
HP1γ Heterochromatin Protein 1, isoform γ
hSET1 human SET1, a H3K4-methyltransferase
hTERT a gene coding for human telomerase reverse transcriptase
HTLV human T-cell lymphotropic virus
HTLV-1 type 1 human T-lymphotropic virus
***IE* genes** (viral) immediate-early genes
IE1 immediate-early protein 1
IE2 immediate-early protein 2
IFIT3 *a gene coding for* interferon induced protein with tetratricopeptide repeats 3*, an adaptor molecule*
IFN interferon
IFNγ interferon gamma
Igf2 a gene encoding insulin-like growth factor 2 in mice
IGSF4 a gene encoding Immunoglobulin superfamily member 4
IL-12A a gene encoding Interleukin-12 subunit alpha
IL-17 Interleukin-17
IL-1B Interleukin-1 beta
IL-6 Interleukin-6
IRF-3 IFN regulatory factor-3
JNK c-Jun N-terminal kinase
KDM5B Lysine Demethylase 5B
KDM6A Lysine Demethylase 6A
KDM6B Lysine-specific demethylase 6B, also called JMJD3 (Jumonji domain-containing protein-3)
KIRP Kidney Renal Papillary Cell Carcinoma
KLK10 a gene encoding Kallikrein related peptidase 10 (also called Kallikrein-10)
KS Kaposi's sarcoma
KSHV Kaposi's sarcoma–associated herpesvirus (also called Human herpesvirus 8 HHV-8)

L. monocytogenes	*Listeria monocytogenes*
LANA	latency-associated nuclear antigen
LCR	long control region
LDHB	a gene encoding lactate dehydrogenase B
LegAS4	a SET domain protein of *Legionella pneumophila*
LINE1 repeats	long interspersed nuclear elements, also called L1 retrotransposons
LLO	listeriolysin O
LMP1	latent membrane protein 1
LMP2A	latent membrane protein 2A
LMP2B	latent membrane protein 2B
LntA	*Listeria* nuclear targeted protein A
LOX	a gene encoding Lysyl Oxidase
LSD1	Lysine-specific demethylase 1
LY9	Lymphocyte Antigen 9
M. leprae	*Mycobacterium leprae*
M. smegmatis	*Mycobacterium smegmatis*
M. tuberculosis	*Mycobacterium tuberculosis*
M.MpeI	CpG-specific DNA methyltransferase of *Mycoplasma penetrans*
MAFF	stands for V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog F, a transcription factor
MAFK	stands for v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog K, a transcription factor
MALT	mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
MARs	matrix attachment regions
MBD2	Methyl-CpG Binding Domain Protein 2
MCC	Merkel cell carcinoma
MCD	multicentric Castleman's disease
MCPyV	Merkel cell polyomavirus
Member X	a variant histone
ME/CFS	myalgia-associated encephalomyelitis/chronic fatigue syndrome
MeCP2	methyl CpG binding protein 2
MeDIP-seq	methylated DNA immunoprecipitation sequencing
MGMT	a gene encoding O-6-methylguanine-DNA methyltransferase
Mhy1	CpG-specific DNA methyltransferase of *Mycoplasma hyorhinis*
MIEP	major immediate-early (IE) promoter
miR	microRNA gene
miRNA (also designated as miR)	microRNA
MKP2	MAPK phosphatase 2, the current name is DUSP4, dual specific phosphatase, MKP2 is an alias
MLH1	a gene encoding MutL protein homolog 1, involved in mismatch repair
MMP1	matrix metalloproteinase 1
MMP3	matrix metalloproteinase 3
MS4A1	(Membrane-Spanning 4-Domains Subfamily A Member 1), also called CD20, a B-cell marker
mSIN3A	a core component of a mammalian transcriptional corepressor complex (SIN stands for Swi-independent)
MTSS1	a gene encoding Metastasis suppressor protein 1

NALP3	an acronym standing for NACHT, leucine-rich region, and PYD domains containing protein 3, a component of the NALP3 inflammasome
NDRG2	(N-myc downstream-regulated gene 2), a putative tumor suppressor protein
Nef	Negative regulatory factor (a HIV protein; a virulence factor of other primate lentiviruses as well)
NF-κB	Nuclear Factor-κB
NK cell	natural killer cell
NPC	nasopharyngeal carcinoma
NPR1	a gene encoding Natriuretic Peptide Receptor 1
Nsp5	nonstructural protein 5; the SARS-CoV-2—encoded Nsp5 is a cysteine protease
NUE	nuclear effector
O. viverrini	*Opisthorchis viverrini*
OPCML	opioid-binding protein/cell adhesion molecule-like gene
ORF	open reading frame
OspF	outer Shigella protein F, a phosphothreonine lyase
P. gingivalis	*Porphyromonas gingivalis*
p14ARF	an alternate reading frame protein product of the CDKN2A locus (i.e., INK4a/ARF locus), encoding a tumor suppressor protein
p15	(also called CDKN2B or p15INK4B), a gene coding for Cyclin-dependent kinase 4 inhibitor B
p15INK4B	see p15
p16	(also called p16INK4a), a gene encoding the tumor suppressor protein cyclin-dependent kinase inhibitor 2A (CDKN2A)
p16INK4a	see p16
p300	a histone acetyltransferase, also called p300 HAT
PARP1	poly(ADP-ribose) polymerase 1
PAX2	paired box gene 2
PBMCs	peripheral blood mononuclear cells
PCAF	P300/CBP-associated factor, a histone acetyltransferase
PcG	Polycomb-group, a family of protein complexes
PCGF5	polycomb group ring finger protein 5
PCR	polymerase chain reaction
PDGFRA	PDGF receptor-alpha (PDGF stands for Platelet-derived growth factor)
PEL	primary effusion lymphoma
PFO	perfringolysin
PP2A	protein phosphatase 2A
pRb	Retinoblastoma protein or Retinoblastoma.associated protein, a tumor suppressor protein
PRC	polycomb repressive complex
PRC2	polycomb repressive complex 2
PRDM2	a gene encoding PR/SET domain 2, a tumor suppressor protein
PRDM9	a gene encoding PR/SET domain 9, a histone-lysine N-methyltransferase
PRMT1	protein arginine methyltransferase 1
PTEN	a gene encoding Phosphatase and tensin homolog, a tumor suppressor protein
RAAS	renin—angiotensin—aldosterone system
Raf	a word coined from Virus-induced Rapidly Accelerated Fibrosarcoma (V-RAF), a protein kinase
Ras	a word coined from Rat sarcoma virus; a GTPase
RASSF1A	a gene encoding Ras Association Domain-Containing Protein 1 isoform A
Rbl-2	a gene encoding retinoblastoma-like protein 2, the current gene name is without hyphen Rbl2

rDNA	ribosomal DNA
Rev	regulator of expression of virion proteins (a HIV protein)
RIG-I	retinoic acid inducible gene I, a pattern recognition receptor
RNA pol III	RNA polymerase III
RNA-seq	RNA sequencing
RomA	regulator of methylation A, a SET domain protein of *Legionella pneumophila*
RRAD	a gene encoding Ras Related Glycolysis Inhibitor And Calcium Channel Regulator
Rta (also designated RTA)	Replication and transcription activator
RUNX3	a gene encoding RUNX family transcription factor 3 (RUNX stands for runt-related, coined from the Drosophila segmentation gene runt)
RXRA	Retinoid X Receptor Alpha
S. haematobium	*Schistosoma haematobium*
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SAT2 repeats (also designated Sat2 repeats)	human pericentromeric repeat sequences
SET domain	a domain involved in histone lysine methylation and regulation of chromatin function, originally identified in Drosophila proteins; the SET acronym stands for Su(var)3-9, Enhancer-of-zeste and Trithorax
SFRP1	a gene encoding Secreted Frizzled-Related Protein 1
SFRP4	a gene encoding Secreted Frizzled-Related Protein 4
SHANK3	a gene coding for SH3 and multiple ankyrin repeat domains 3, a scaffold protein
SHP-2	SH2 domain-containing protein tyrosine phosphatase-2
Shp1	(also called PTPN6), a gene encoding Src homology region 2-containing phosphatase 1 (protein tyrosine phosphatase non-receptor type 6)
SIRT1	sirtuin 1, silent information regulator T1
SMYD3	SET and MYND domain containing protein 3
SOCS1	a gene encoding Suppressor Of Cytokine Signaling 1
Sox10	a transcription factor, master regulator encoded by the Sox10 gene; the acronym Sox stands for "Sry-related HMG box" (Sry sex-determining region on the Y chromosome; HMG box or high mobility group box, ensuring DNA binding)
STAT1	a gene encoding signal transducer and activator of transcription 1
STAT1	signal transducer and activator of transcription 1
STAT3	signal transducer and activator of transcription 3
STING	stimulator of interferon genes, an adaptor molecule
SUV39H1	Suppressor of variegation 3-9 homolog 1, a histone methyltransferase
Tat	trans-activator of transcription (a HIV protein)
***TbetaRII* (also designated *TGFBR2*)**	a gene coding forTGF-β type II receptor
TBP	TATA-binding protein
TET1	Ten-Eleven Translocation 1 or Tet Methylcytosine Dioxygenase 1, an enzyme involved in DNA demethylation
TGF-β	Transforming growth factor beta
THBD	a gene encoding thrombomodulin

The real gene name is *FOXG1*	*FOXG1B* or *FKHL1* are aliases (see www.genecards.org)
THP-1	acute monocytic leukemia cell line
TIMP3	a gene encoding Tissue Inhibitor of Metalloproteinase 3
TLR2	a gene encoding Toll-like receptor 2
TLRs	Toll-like receptors
TLX3	a gene encoding T Cell Leukemia Homeobox 3, a transcription factor
TM6SF1	a gene encoding Transmembrane 6 Superfamily Member 1
TMPRSS2	transmembrane serine protease 2
TNFα	tumor necrosis factor alpha
TRAF3	*tumor necrosis factor receptor–associated protein 3*
trHBx	truncated hepatitis B virus X protein
TRIM58	a gene encoding Tripartite Motif Containing 58
TRP120	tandem repeat protein 120
TRP32	tandem repeat protein 32
TRP47	tandem repeat protein 47
TRs	terminal repeats
TSG	tumor suppressor gene
UCEC	Uterine Corpus Endometrial Carcinoma
UPEC	uropathogenic *Escherichia coli*
VacA	Vacuolating cytotoxin A, a *Helicobacter pylori* toxin
vFLIP	viral FLICE.inhibitory protein (FLICE stands for Fas-associated death domain-like interleukin-1β-converting enzyme)
vIL-6	viral interleukin-6
WNK2	a gene encoding cytoplasmic serine-threonine kinase that contains cysteine in place of the lysine in subdomain II
Wnt	Wingless-Type MMTV Integration Site Family, Member 1, a signaling molecule
WT	wild-type hepatitis B virus X protein
XCI	X-chromosome inactivation
ZAP	zink finger antiviral protein

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CHAPTER

Therapy of infectious diseases using epigenetic approaches

22

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22.1 Introduction

A cell's fate is determined by both its genetic information and the patterns that affect its gene expression. The nongenetic determinants that precisely control gene expression, determine cell property, and respond to stimulation are usually referred to as epigenetics. Epigenetic changes are a bridge between genotype and phenotype, which are independent of primary DNA sequence changes. This can change the expression of a locus or chromosome without altering the underlying DNA sequence [1,2].

Many diseases, including cancers, autoimmune disorders, diabetes, and infectious diseases, are manifested because of epigenetic changes [3,4]. In recent decades, epigenetic research has become a rapidly developing and extensively studied area of research. Many studies suggest that developmental and pathological processes within the cells and organs are regulated largely by epigenetic changes. During development, epigenetic changes modify the cell phenotypes from embryonic to differentiated state without altering the DNA sequence [5]. In cancer, abnormal epigenetic states can hinder the ability of cells to exit normal self-renewal and differentiation [6]. During infection, epigenetic changes can participate in regulating the functions of the immune cells of both the innate and adaptive components of the immune systems by affecting the state of host defense [7].

Contrary to genetic abnormalities, the important characteristics of certain epigenetic changes are at least partially reversible and allow recovery of the functions of affected genes with normal DNA sequences [8]. Some epigenetic modifications seem to persist, even for decades, and some others are at least partially reversible by inhibiting the inducible agents [9]. Relying on this fact, many new epigenetic therapies and drugs are aimed to reprogram affected cells to a more normal state [10,11]. Moreover, exposure to environmental factors can induce epigenetic alterations in several ways [12]. As we know, epigenetic mechanisms are categorized as DNA methylation, histone posttranscriptional modifications, and modifications by noncoding RNAs (ncRNAs), including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) [13]. New studies suggest that transcription factors (TFs) along with the proinflammatory and antiinflammatory genes are regulated by epigenetic mechanisms after a microbial invasion [14]. These epigenetic transcriptional regulations are mediated by immune cells, including monocytes/macrophages, dendritic cells (DCs), Th1/2 cells, and so on [15,16]. Furthermore,

Epigenetics in Human Disease. https://doi.org/10.1016/B978-0-443-21863-7.00007-X
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studies have shown that miRNAs play an important role in altering the function of the immune cells by epigenetic modifications. These changes are closely related to the formation and progression of infectious diseases [17]. Thus, further understanding the epigenetic modifiers and their key role in the development of human diseases would provide an avenue for developing epigenetic-mediated therapies. This review provides a brief overview of epigenetic mechanisms, such as chromatin dynamics, the methylation patterns on DNA, and the role of RNA and proteins during gene expression. Moreover, we focus on a novel approach that uses epigenetic-modifying agents to protect from infection and inflammation.

22.2 Epigenetic modifications

Epigenetic mechanisms are increasingly recognized as having vital roles in the pathogenesis and progression of infectious diseases. These can regulate the transcriptional profile in the immune system, which contributes to the cell type−specific transcriptional response. Usually, epigenetic modifications can be grouped into three main categories: DNA methylation, histone modifications, and ncRNAs. Furthermore, it is important to realize that different types of modifications are functionally linked [18,19], and the outcome is always the result of their interactions by positive and negative feedback mechanisms. In the following subsections, we are discussing the different types of epigenetic modifications, which commonly occur within the cells.

22.2.1 DNA modifications

DNA modifications that involve methylation/demethylation of cytosine residues in DNA are major epigenetic changes in the genome and regulate crucial aspects of its function [20]. DNA methylation is a stable modification that is inherited throughout cellular divisions [21]. There are different mechanisms for the inhibition of gene expression by DNA methylation. Methylated DNA can promote the recruitment of methyl-CpG-binding domain (MBD) proteins that in turn recruit histone-modifying and chromatin-remodeling complexes to methylated sites [22]. DNA methylation can also block the recruitment of DNA-binding proteins in their target sites and thereby inhibit gene transcription [23]. Proteins that can bind to DNA-methylated sites or MBDs, acting as "readers," give functional information to the DNA modification [24]. Conversely, normal and abnormal removal of DNA methylation, a type of "erasing" mechanism, can occur passively when DNA is replicated, and the modification is not reestablished.

DNA demethylation and methylation are mediated and balanced by DNA demethyltransferases (DeMTs) and DNMTs, respectively. Overall, the functions of DNMTs and DeMTs are essential for maintaining DNA methylation patterns during cell cycle control, apoptosis, DNA damage, immune recognition, and self-renewal in cells (Fig. 22.1). DNA methylation is also mediated by the DNA methyltransferases (DNMTs) that catalyze the transfer of a methyl group from S-adenosyl methionine to DNA. Five members of the DNMT family have been found: DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L, but only DNMT1, DNMT3a, and DNMT3b have methyltransferase activity [25]. DNA methylation most often occurs at position 5 of cytosine−phosphate−guanine (CpG) dinucleotides (5-mC). These positions within the genomic sequences are called cytosine−phosphate−guanine islands or CpG sites [26]. The family of DNA methyltransferases (DNMTs) consists of four

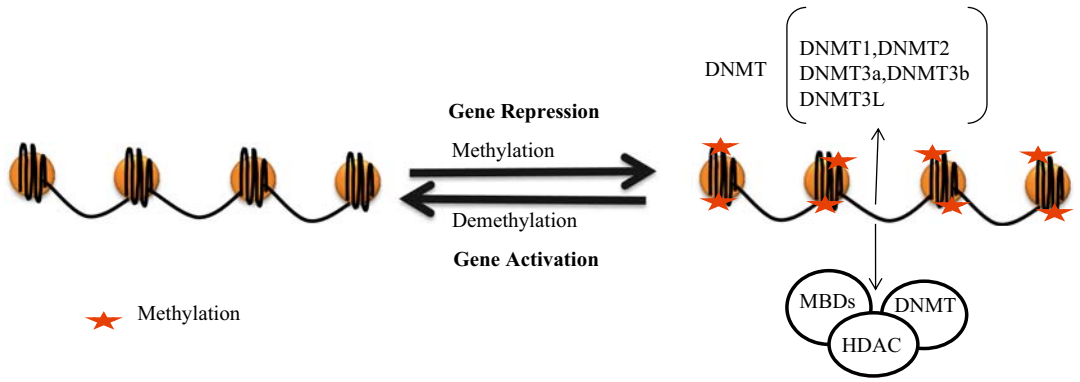

FIGURE 22.1 The Mechanism of DNA Modifications

DNA modifications involve methylation/demethylation of the cytosine residue and are major epigenetic changes in the genome. Methylated DNA promotes the recruitment of methyl-CpG-binding domain (MBD) proteins and blocks the recruitment of DNA-binding proteins from their target sites to inhibit gene transcription. DNA demethylation and methylation are mediated and balanced by DNA demethyltransferases (DeMTs) and DNA methyltransferases (DNMTs), respectively. DNMT includes DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L, but only DNMT1, DNMT3a, and DNMT3b have methyltransferase activity. The functions of DNMTs and DeMTs are essential for maintaining DNA methylation patterns during cell cycle control, apoptosis, DNA damage, immune recognition, and self-renewal in cells. *HDAC*, histone deacetylase.

catalytically active enzymes (DNMT1, DNMT3a,DNMT3b, and DNMT3c) that participate in the generation and maintenance of methylation. DNMT1 is a maintenance enzyme that methylates hemimethylated CpG dinucleotides after DNA replication and is transcribed during the S phase of the cell cycle.

Although cytosine methylation predominantly occurs at CpG sites, methylation is also found on cytosine without adjacent guanosine, particularly in differentiated tissues [27]. In general, CpG island methylation is associated with the silencing of imprinted gene alleles and transcription from repetitive elements [28]. It has been shown that methylation occurs within the gene promoters, which often contain CpG-rich regions, and it also occurs in the enhancers at the distal regulatory regions. Moreover, transcribed genes usually have low CpG methylation levels in the promoters and enhancers but can have high levels in the gene body [29,30]. In methylated cytosine, the CpG content and neighborhood content in the transcription sites for coding genes are classified as CpG islands (2%), shores (4%), shelves (6%), and open sea/others (88%) [31]. Methylation also occurs along the CpG shores; they are highly conserved and differentially methylated regions, which lie in proximity (∼2 kb) to CpG islands. Most of the tissue-specific DNA methylation seems to occur not at CpG islands but at CpG shores [32], which have the most (∼70%) methylated regions [33].

Recently, researchers discovered that cytosine hydroxy-methylation is another important DNA modification that is present in many organisms, especially human and mouse neurological tissue [34]. Similar to methylation, hydroxy-methylation replaces the hydrogen atom with a hydroxymethyl group at the C5 position in cytosine. 5-hydroxymethylcytosine (5-hmC) is speculated to be an intermediate in oxidative DNA demethylation pathways and could constitute epigenetic regulation by itself [35].

A recent exciting discovery of an active removal system of methylation marks is initiated by a set of enzymes termed ten−eleven translocation proteins (TETs) [36]. The three mammalian TET proteins generate 5-hmC from existing 5-mC, which can further process to 5-formylcytosine and 5-carboxylcytosine [37]. Until now, 5-hmC has been paid more attention as a potential epigenetic factor in many fields, including stem cell renewal, cancer development and progression, and neurological disorders [38]. Furthermore, it has been shown that the hydroxy-methylation level is found to be associated with maintaining the pluripotency of stem cells disturbed hydroxy-methylation of DNA cytosine, causing different types of cancers [39]. There are many standard methods for detecting methylated DNA, such as bisulfite conversion; however, these methods cannot distinguish between 5-mC and 5-hmC [40]. Therefore, it is necessary to accompany DNA methylation studies with experiments that can differentiate these two modifications so that the functions of DNA methylation/hydroxy-methylation can be further explored. There are two novel approaches to differentiate 5-mC from 5-hmC, oxidative bisulfite sequencing (OxBS-seq) and TET-assisted bisulfite sequencing (TAB-seq). OxBS-seq involves adding an oxidative step to bisulfite sequencing [41], whereas TAB-seq uses the TET enzyme to discriminate between 5-mC and 5-hmC [42]. These two novel approaches will facilitate the identification and help elucidate the epigenetic implications of 5-hmC.

22.2.2 Histone modifications

Chromatin conformation, positioning of nucleosomes, and access to DNA for transcription are mainly controlled by histone proteins. The nucleosome, which is the basic unit of chromatin, consists of DNA wrapped around an octamer of four histone proteins (H2A, H2B, H3, H4) and is linked together by H1 histone protein [43]. Histones are subject to a wide range of covalent modifications that occur on amino acids within the amino-terminal and carboxy-terminal tails that extend outside of the main globular histone domain. Commonly, histones can be modified by acetylation, methylation, phosphorylation, ubiquitination, or SUMOylation of amino acid tails. These modifications influence the conformation of the histone and its interactions with other proteins. Chromatin-remodeling complexes, which consist of enzymes, DNA-binding proteins, and adaptor proteins, are involved in the establishment and maintenance of nucleosome complexes and histone modifications. Furthermore, the complexes also act as readers (identification of methylated CpG islands), writers (histone acetyltransferases [HATs]), or erasers (histone deacetylases [HDACs]) of the epigenetic code [44]. Like DNA methylation, these histone modifications facilitate how cells maintain “memories” for gene expression patterns that are fundamental for proper embryonic development, cell renewal, and differentiation into adult tissues [45]. Histone modifications also play key roles in different cellular functions involved in the manipulation and expression of DNA [46]. Histone acetylation and deacetylation are mediated and balanced by the enzyme’s HATs and HDACs, respectively. Thus, histone modifications are one of the most important biological processes that are essential for epigenetic regulation (Fig. 22.2).

22.2.2.1 Histone acetylation

Histone acetylation is another important PTM, where, with the help of HAT, an acetyl group from acetyl CoA is transferred to the ε-amino group of certain lysine side chains within the histones at the N-terminal tail region [47]. Multiple lysine residues in each of the histones are subject to acetylation and are usually associated with transcriptionally active genes and deacetylation with repressed genes.

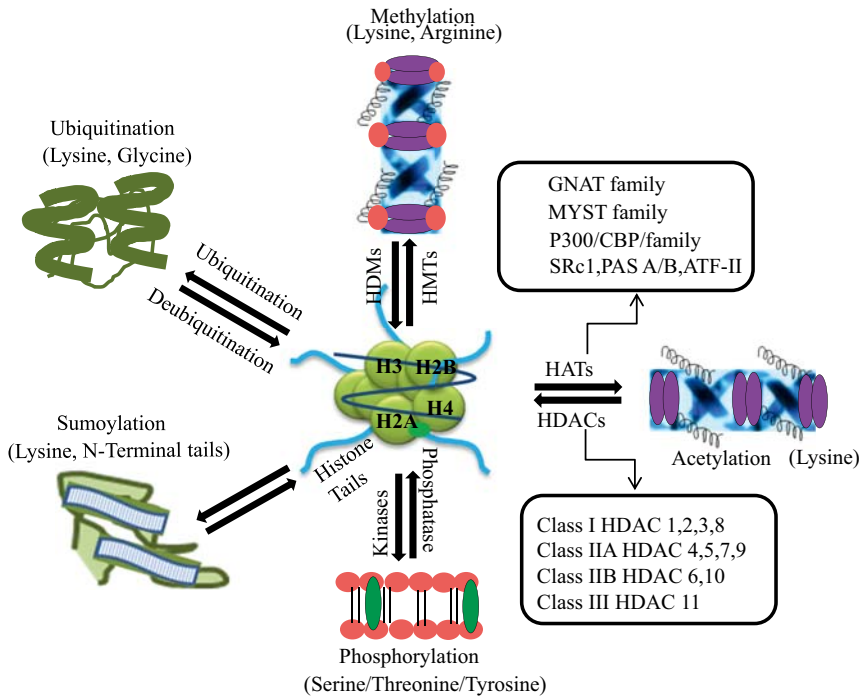

FIGURE 22.2 The Structure and Modifications of Histones

Histone modifications are posttranslational modifications (PTMs) that regulate gene expression. The nucleosome, which is the basic unit of chromatin, consists of DNA wrapped around an octamer of four histone proteins (H2A, H2B, H3, H4). The covalent PTMs of the histone tails include acetylation (Ac), methylation (Me), phosphorylation (Phos), ubiquitination (Ub), or SUMOylation (SUMO) in the certain amino acid. Acetylation is reversibly regulated by a dynamic balance between histone acetyltransferases (HATs) and histone deacetylases (HDACs). Methylation modifications can be written by histone methyltransferases (HMTs) and removed by histone demethylases (HDMs), which determine the states of gene expression.

The effects of histone acetylation on gene expression may be partly dependent on altering the net charge of the histone. This leads to the weakening of interaction with DNA to yield a chromatin structure that is more open and accessible to TFs and gene expression. These modifications control many cellular processes and have an impact on inflammatory and antiinflammatory responses [48]. The acetylation status of these proteins is usually reversibly regulated by a dynamic balance between HATs and HDACs. Thus, it is now apparent that HATs and HDACs function more widely, in addition to regulating chromatin architecture [49]. There are many available histone acetylation assays to measure global and site-specific histone acetylation; these approaches can help elucidate the role of histone acetylation in gene expression.

There are two major types of HAT enzymes that are responsible for histone acetylation. Type-A HATs are more diverse and are classified under three groups according to amino acid sequence

homology. They are Gcn5-related N-acetyltransferase; Moz, Ybf2, Sas2, and Tip60; and CBP/p300 families [50]. Type-B HATs mainly acetylate histone H4 at lysine 5 (K5) and K12 and some sites within H3. However, these acetylate-free histones, not the histones, deposited into chromatin. The type-B pattern of acetylation, which is highly conserved, is important for the deposition of histones and generally is found within the cytoplasm [51].

HDACs are considered another important class of histone-modifying enzymes that are predominantly functioning as transcriptional repressor enzymes that reverse the effects of HATs and deacetylate to restore the positive charge of the lysine. There are four different classes of HDACs: Class I contains the enzymes HDACs-1, -2, -3, and -8, and they are commonly associated with transcriptional repressive complexes known as Sin3, NuRD, corepressor proteins (HDAC-1 and -2), and SMRT/NCoR (HDAC-3) [52]. Generally, class I HDACs are considered as the repressive factors for gene expression. Class II HDACs show homology to yeast HDAC-1, contain the enzymes HDACs 4−7, 9−10, and are subdivided into classes IIa and IIb based on domain organization [53]. Class IIa HDACs (HDAC-4, -5, -7, and -9) have N-terminal domains that interact with TFs and possess C-terminal nuclear export signals in both the nucleus and cytoplasm. Class IIb HDACs are HDAC-6 and HDAC-10. HDAC-6, which is an exclusive member, mainly functions in the cytoplasm. It deacetylates cytoplasmic nonhistone substrates such as Hsp90 and α-tubulin. Additionally, HDAC-6 binds to misfolded proteins to allow these proteins to be physically transported as molecular chaperones and undergo proteasomal degradation [54]. HDAC-10 localizes and functions in the cytoplasm of the cells and is reported to be involved in cell cycle regulation and promotes cell proliferation [55]. Class III comprises of the NAD+-dependent Sirt1−7, which has been implicated in caloric restriction, aging, neuronal degeneration, and longevity [56]. HDAC-11, which is the only single member of class IV, negatively regulates interleukin (IL)-10 [57]. Moreover, the dynamic state of chromatin results from the cross-talk between HDACs and HATs. Therefore, these enzymes are playing a key controlling role in developmental processes, and their deregulation has been linked to the progression of cancers (leukemia, colorectal, and breast cancer), inflammatory diseases, and different human disorders [58].

22.2.2.2 Histone methylation

Compared with acetylation, methylation is also an important histone modification, which occurs on the side chains of lysine and arginine residues at the histone tails of H3 and H4. A myriad of methylation modifications, written by histone methyltransferases (HMTs) and removed by histone demethylases (HDMs), help to determine the states of gene expression [59]. Unlike other modifications, histone methylation/demethylation modifications are essential and associated with either transcriptional repression or activation without altering the charge on the histone protein. Moreover, lysine can be found as mono-, di-, or trimethylated, whereas arginine can be found as mono-, symmetrically, or asymmetrically dimethylated in histones [60]. Correspondingly, the effects of methylation on gene transcription are diverse, depending on various methylation patterns and the transcriptional states. For example, monomethylations of histone 3 lysine 9 (H3K9me1), H3K27me1, H4K20me1, H3K79me1, and trimethylation of histone 3 lysine 4 (H3K4me3) and H3K79me3 are responsible for gene activation, whereas H3K27me3 and H3K9me3 are responsible for gene repression [61]. Besides, it has been shown that histone methylation may influence chromatin structure by affecting histone−histone and histone−DNA interactions, as well as the binding of histones to chaperones [62].

22.2.2.3 Histone phosphorylation

Histone phosphorylation seems to be involved in various biological processes, including gene activation, mitosis, chromosome condensation, apoptosis, and DNA repair [63]. Unlike acetylation and methylation, histone phosphorylation acts as a bridge between other histone modifications and recruits effector proteins for downstream events. Primary sites for histone phosphorylation are the N-terminal tails, which can be phosphorylated by different kinases and removed by phosphatases. It is a highly dynamic process that commonly takes place on serine, threonine, and tyrosine residues of histone tails, and all core histones contain phospho-acceptor sites in their N-terminal domains. H2A is phosphorylated on Ser1, H2B on Ser14 and Ser32, H3 on Ser10 and Ser28, and H4 on Ser1 [64]. Phosphorylation has differential effects on the core histones. Most studies on histone phosphorylation have focused on the importance of Ser10 residue of histone H3. Histone H3 phosphorylation is a highly dynamic chromatin modification that impinges on the epigenetic landscape of eukaryotic cells in several ways, which contributes to the general condensation of chromatin during mitosis. In response to extracellular signals, H3 phosphorylation triggers the initiation or elongation of transcription and transiently activates the genes by overlapping repressive marks without altering the epigenetic memory [65]. Phosphorylation of H2A at Ser139 (γ-H2AX) is an important histone modification that plays a major role in the repair of DNA double-stranded breaks by helping recruit DNA damage repair proteins at the site. Moreover, phosphorylation of γ-H2AX has been identified as one of the early events after DNA damage [66]. Taken together, these studies suggest that histone phosphorylation is playing an important role in several biological processes. Overall, DNA methylation and histone deacetylation are responsible for the silencing of genes, whereas DNA demethylation and histone acetylation are responsible for activating genes [67,68].

22.2.2.4 Histone ubiquitination

Histone ubiquitination, particularly on H2A and H2B, has already been documented. H2A and H2B are mainly monoubiquitylated, which does not lead to their proteasomal degradation but changes the histone mass and affects nucleosomal dynamics [69]. Thus, H2A and H2B ubiquitination plays a critical role in regulating many processes within the nucleus, including transcription initiation and elongation, silencing, and DNA repair [70]. Commonly, ubiquitin receptors recognize the protein that is tagged with ubiquitin and removes it from the cell by the proteasomal degradation pathway. These additions and removal of ubiquitin take place in a three-step enzymatic reaction, by binding, activating, and conjugating with the help of ligating enzymes, E1, E2, and E3, respectively. There has been dramatic progress toward the identification of factors that control ubiquitin attachment to histones, which is an important chromatin modification during transcriptional regulation [71]. Histone H2A is the first protein that was identified to be ubiquitinated, and the major ubiquitination site on H2A was mapped to lysine residue 119 (K119). On the other hand, ubiquitinated histone H2B, which was discovered in 1980, and the major ubiquitination site is the C terminus in lysine 120 of mammalian H2B or lysine 123 of yeast H2B. Ubiquitination of H2A is generally associated with gene silencing, whereas ubiquitination of H2B has roles both in transcription activation and silencing. Although the function of H2A and H2B ubiquitination differs in transcriptional regulation, they both participate in DNA damage signaling by facilitating the recruitment of DNA damage response (DDR) factors to damaged chromatin [70]. Thus, in parallel with other histone modifications, histone ubiquitination plays an important role in cellular processes.

22.2.2.5 SUMOylation

The small ubiquitin-like modifier (SUMO) is a 100-amino-acid polypeptide that is conjugated to lysine residues of target proteins in a similar fashion as the ubiquitination process. SUMOylation of proteins can be reversed through the activation of SUMO-specific proteases. SUMO is generally known as a modifier of transcription and plays a key role in the DDR. Various DNA-repairing factors are SUMOylated in response to DNA damage [72]. Recently, major advances in proteomics analysis uncovered over 1000 SUMO substrates and many thousands of SUMOylated lysines during chromatin remodeling. SUMOylation regulates target proteins involved in all nuclear processes, including transcription, DNA repair, chromatin remodeling, precursor−messenger RNA (mRNA) splicing, and ribosome assembly [73]. Studies have shown that Gli2 is conjugated by SUMO at lysine residues 630 and 716 in the cell, and SUMO modification inhibited Gli2 transcriptional activity by recruiting HDAC-5 [74]. Moreover, two related HDMs, KDM5B/JARID1B and KDM5C/JARID1C, were differentially regulated by SUMOylation in response to methyl-methane sulfonates. Furthermore, SUMO conjugation mediates an epigenetic regulatory function of another HDM JMJD2A, and inhibition of JMJD2A SUMOylation may be a novel avenue for anticancer therapy [75]. In addition, SUMOylation deficiency of Sirt6, an HDAC with NAD (+)-dependent activity, also decreases Sirt6 binding with c-Myc. This decreases Sirt6 occupancy on the locus of c-Myc target genes and reduces its suppression of cell proliferation. Thus, SUMOylation has an important role in the regulation of Sirt6 deacetylation on H3K56, as well as its tumor-suppressive activity [76]. Although there is some evidence currently in this field, further studies are needed for a better understanding of the epigenetic impact of such modification.

22.2.3 Noncoding RNAs

Despite their "junk" status, studies on ncRNAs have been on a steep rise since the 1970s. Now, we know that ncRNAs are functionally important molecules in the cell and participate in a plethora of physiologic and pathologic processes [77]. These ncRNAs are classified as micro (miRNAs), long noncoding (lncRNAs), small nucleolar (snRNAs), small Cajal body−associated (SCARNAs), and circular RNAs, which can all modulate transcriptional output from the genome [78]. LncRNAs and miRNAs are the two most studied groups of the ncRNA family (Fig. 22.3).

MiRNAs are small 18−22 nucleotide strands of ncRNA that function as posttranscriptional regulators, which are transcribed into large primary miRNAs. These are further processed within the nucleus to a precursor miRNA before being exported to the cytoplasm as a mature miRNA. Thus, the mature miRNA is a part of the RNA-induced silencing complex and binds complementary regions in the 3′- or 5′ untranslated regions of mRNA encoding for specific proteins. The cross-talk between miRNA and mRNA results in transcriptional repression or degradation of the mRNA and thereby inhibits the expression of some proteins [79]. Moreover, miRNAs can target 30% of the human genes in a temporal- and tissue-specific manner. This can also regulate histone variant replacement or interact with chromatin-remodeling complexes mediating the exchange of specific subunits [80,81]. Thus, a close association between epigenetic modifications and miRNAs is just beginning to be understood and has a great importance in the field of molecular biology [82]. Besides this, studies also found that many pathogens utilize the epigenetic regulators and miRNA machinery to modify and evade the host immune responses. These studies suggest that the global epigenetic and miRNA mapping of such

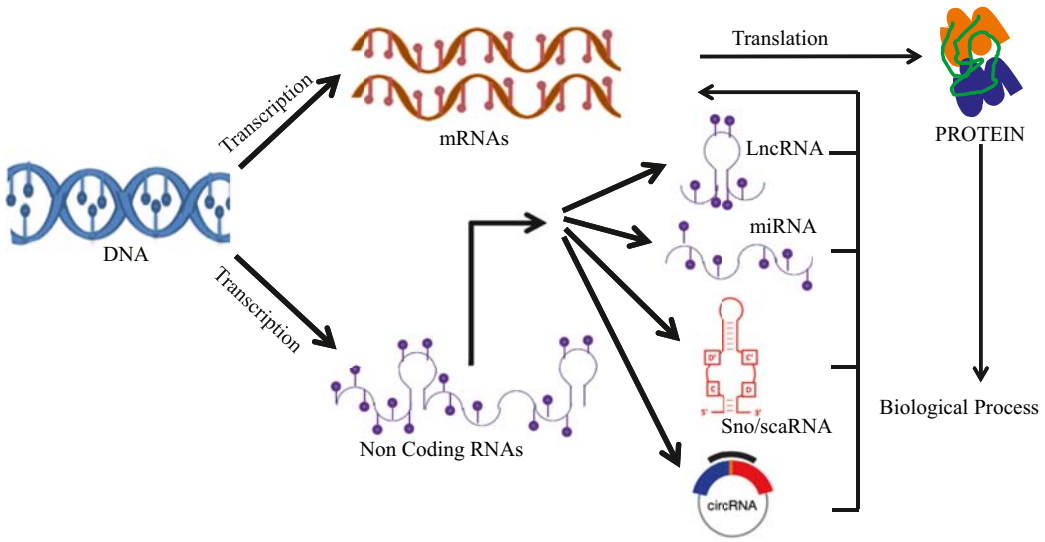

FIGURE 22.3 Regulatory Mechanisms of Noncoding RNAs (ncRNAs)

NcRNAs are functionally important molecules in the cell, which have the capacity to regulate gene expression at transcriptional or translational levels. The long noncoding RNAs (lncRNAs) and microRNAs (miRNAs), small nucleolar RNAs (snoRNAs), small Cajal body–associated RNAs (scaRNAs), and circular RNAs (circRNAs) are the important groups of the ncRNA family. LncRNAs, which show many similarities to messenger RNA (mRNA), act as key players in the epigenetic, posttranscriptional, and translational coordination of gene expression in organ development and disease processes. MiRNAs are small 18–22 nucleotide strands of ncRNA that functions as posttranscriptional regulators. The cross-talk between miRNA and mRNA results in transcriptional repression or degradation of the mRNA, thereby inhibiting the expression of some proteins. SnoRNAs are intermediate-sized ncRNAs (60–300 bp) responsible for posttranscriptional modifications and assist in folding and stability of ribosomal RNA. The scaRNAs (60–425 bp) are a subset of the snoRNA family, which is a large family of conserved ncRNAs that primarily guide biochemical modifications of particular nucleotides (e.g., methylation and pseudouridylation) of rRNAs and snoRNAs. The circRNAs, unlike the other known RNAs, the 5 and 3′ ends present in the RNA molecules, are covalently joined together and form a circular loop.

host–pathogen interactions would provide key insights into their cellular functions and help to identify various determinants for therapeutic value.

Moreover, miRNAs could act as efficient biomarkers of sepsis and distinguish the different phases of sepsis. First, it has been found that several dysregulated miRNAs, such as miRNA-23b, miRNA-143, miRNA-145, miRNA-146a, miRNA-150, miRNA-155, miRNA-182, miRNA-375, miRNA-223, and miRNA-584 are expressed in the peripheral blood mononuclear cells of sepsis patients [17,83]. More recently, a review on miRNAs as potential biomarkers of infectious disease has focused on the use of circulating miRNAs as diagnostic/prognostic biomarkers of infectious diseases such as human tuberculosis, sepsis, and viral diseases [84]. It has been shown that the level of extracellular single miRNA-21 is known to increase in many diseases, including diverse cancer types, hepatitis, multiple

sclerosis, myocardial infarction, and lupus [85]. Thus, therapeutic strategies, which employ miRNA mimics or antisense-RNA inhibitors, may represent a valid option for the treatment of patients with sepsis [86]. Therefore, targeting genes regulated by these miRNAs may represent potential new therapeutic avenues in infectious diseases.

On the other hand, lncRNAs are transcripts over 200 nucleotides long that are not translated into a protein. These lncRNAs show a lot of similarities to mRNA and act as key players in the epigenetic, posttranscriptional, and translational coordination of gene expression in organ development and disease processes. LncRNAs can function as natural antisense RNAs transcribed in gene regulatory regions to repress the canonical transcription of the gene [87]. Antisense RNAs are single-stranded RNAs complementary to a specific mRNA with which they can hybridize and block its translation into a protein. Furthermore, lncRNAs are associated with normal DNA methylation to repress gene transcription and are involved in RNA processing or differential regulation in cancer and embryogenesis [88]. Mammalian cells express thousands of such lncRNAs mostly in a tissue- and cell type−specific manner. According to their biological function, some lncRNAs are confined to distinct cellular compartments, demonstrating their functional relevance [89]. One significant role of lncRNAs is the regulation of gene expression via the interaction with the histone-repressive proteins of the polycomb repressive complex 2 (PCR2), an epigenetic silencing complex estimated to bind to ∼20% of all lncRNA transcripts [90]. The PRC2 complex is made of three core protein subunits EZH2, EED, and SUZ12. EZH2 contains a SET domain that catalyzes the trimethylation of histone H3 at lysine 27 (H3K27 → H3K27Me3), a mark associated with transcriptional repression; SUZ12 and EED are also required for this EZH2 methyltransferase activity to occur [90]. Since the PCR2 complex is unable to target and silence genes by itself, the lncRNAs aid in the recruitment of the PCR2 complex to the gene loci.

22.3 Posttranscriptional RNA methylation

The most abundant RNA methylation modification is the 6-methyl adenosine (m^6A), which occurs in all types of RNA (mRNA, rRNA, tRNA, snRNA, and lncRNA) [91]. The m^6A modification alters the local structure of mRNA or lncRNA, to facilitate the binding of heterogeneous nuclear ribonucleoproteins (HNRNPs) for biological regulation [92]. The group of enzymes responsible for this modification is the METTL3/METTL14 m^6A methyltransferase complex. METTL3 is an S-adenosylmethionine methyltransferase with catalytic properties, while METTL14 serves as an RNA-binding platform [91]. Recently, METTL16 was identified as another methylating enzyme, which interacts with several types of RNAs: mRNA, U6, and lncRNA [93]. METTL16 is known to play an important role in MALAT1 epigenetics [91]. The m^6A modification has two specific erasers, FTO and ALKBH5 [94]. FTO removes the methylation by oxidizing m^6A to N^6-hydroxymethyladosine or N^6-formyladenosine, which are chemically unstable and can hydrolyze to adenine, whereas ALKBH5 catalyzes the direct removal of the methyl group from adenine [95]. In general, the methylation of RNA is a reversible modification that plays an important role in the regulation of gene expression and impacts numerous biological processes.

22.4 Posttranscriptional RNA pseudouridylation

Pseudouridylation (Ψ) is the most common type of epigenetic modification in RNA; however, its biological significance remains poorly understood mainly due to the lack of appropriate pseudouridine

identification techniques. Pseudouridine is catalyzed by human pseudouridine synthases (PUS) and catalytic aspartate, which acts as a nucleophile to achieve catalysis [96]. The isomerization of uridine to pseudouridine requires a glycosidic bond cleavage between N1 and C1′, a 180-degree rotation of the base and reattachment of the base to the ribose at C5 [96]. Some of the known molecular functions of pseudouridylation include stabilization of RNA conformations and destabilization of RNA−protein interactions, which could play a significant role in RNA metabolism and gene expression.

Ψ can occur in tRNA, rRNA, snRNA, lncRNA, and mRNA. Seventeen positions of cytoplasmic and mitochondrial tRNA Ψ have been identified in human HEK23 cells; however, their role remains unknown. A recent study suggests that dysregulation of tRNA Ψ during embryogenesis has been linked to poor development and disease [97]. For rRNA, 104 Ψ sites have been experimentally validated, and these sites are predicted to base pair with H/ACA snoRNA guide sequences [98]. Twenty-seven Ψ sites have been identified in the mammalian U1, U2, U4, U5, and U5 snRNAs. The most modified snRNA, with a total of 14 Ψ sites, is U2. Additionally, Ψ sites have been identified in mRNA at the 5′-UTR, 3′UTR, and coding regions. There have also been identified cell type−specific mRNA Ψ in different human cell lines.

22.5 Epigenetic regulations and infection

Host defense against infectious pathogens requires a coordinated immune response, which contains two systems—innate and adaptive immune systems. The innate immunity is generic and rapid but lacks immunological memory and has similar changes on reactivation. The adaptive immunity is responsible for specific response and memory but requires more time to develop. Most innate immune systems do not display such specificity. The adaptive immune system consists of functionally distinct modules such as antibodies that provide different forms of protection against pathogens. Specifically, pathogens trigger the activation of antimicrobial defenses and stimulate the innate immune response through pathogen recognition receptors, including Toll-like receptors (TLRs), Nod-like receptors, and nucleic acid sensors. These receptors allow the innate immune responses to be activated by pattern-associated molecular patterns and endogenous danger signals from danger-associated molecular patterns [99]. The innate immune system, in turn, activates adaptive effector mechanisms in an antigen-specific manner. Currently, with the technological advancement in the sequencing of the human genome, over 240 specific DNA mutations that induce primary immunodeficiency have been identified [100]. Epigenetic modifications affect a spectrum of immune functions including triggering or preventing the development of immune cell functions that cause increased susceptibility or resistance to specific organisms. There is an interindividual variation at least partly dependent on both the individual's genome and epigenetic regulation of the genome. The overall epigenetic modifications and their effects in a spectrum of innate and adaptive immune system are summarized in the following sections (Fig. 22.4).

22.5.1 Innate immune response

Innate immunity is the first host defense system, it is not specific to a particular pathogen, and its recognition is based on the detection of molecular structures that are unique to microorganisms. The recognition of pathogen-associated immunostimulants stimulates two types of innate immune responses, the inflammatory response, and phagocytosis of cells such as macrophages and neutrophils

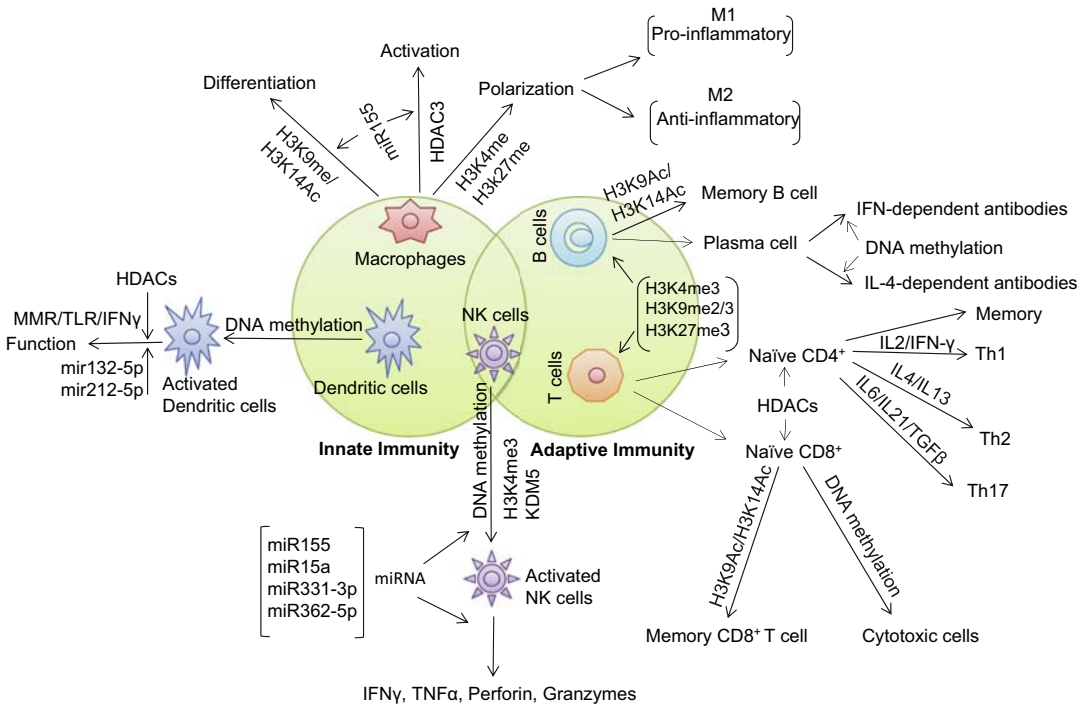

FIGURE 22.4 Epigenetic Modifications Affect a Spectrum of Innate and Adaptive Immune Functions

Innate immune response: Macrophages and dendritic cells (DCs) are the two most important types of cells in the innate immune system. Differentiation, activation, and polarization of macrophages are characterized by DNA hypomethylation and involve in histone posttranslational modifications (PTMs); DNA methylation plays an important role in activating DCs' ability to promote a Th2 response. Histone deacetylases (HDACs) and micro-RNAs (miRNAs) also regulate DCs function by controlling inflammatory mediator production. Adaptive immune response: Adaptive immune recognition is mediated by two types of antigen receptors, T cell receptors and B cell receptors, and includes conventional T cells (B cells and T cells) and innate-like lymphocytes (natural killer cells, NK cells). Methylation of DNA and histones modifications regulates the gene expression of CD4+ T cells and CD8+ T cells. Epigenetic modifications (methylation, acetylation) and factors influence gene expression and modulate critical B cell processes, and epigenetic dysregulation in B cells can result in aberrant antibody responses to foreign antigens. The epigenetic diversification in NK cell subsets may represent a broader adaptive cellular immune response.

[101]. The two main types of pathogen-associated immunostimulants of bacterial origin are the presence of formylated methionine, instead of regular methionine in prokaryotic translation initiation and cell wall components of gram-negative and gram-positive bacteria. Additionally, immunostimulants are often present in repeating patterns, which facilitates their recognition by host receptors called pattern recognition receptors [101]. These receptors are key for the initiation of the innate immune response. The interactions between the innate immune system and the pathogen are key factors in determining susceptibility to infection and the progression of infectious diseases.

Macrophages and DCs are the two most important types of cells in the innate immune system. The innate immune system is clearly dependent on how effective the posttranscriptional and translational regulations of these cells are and the early responses to intracellular pathogens such as viruses and bacteria [102]. Postseptic lymphocytes exhibit their own modulations in epigenetic modifications, and these changes are correlated with cell-intrinsic defects in lineage commitment and effector function.

22.5.1.1 Macrophages

Macrophages are one of the most important cells during the development of innate immune respose in tissues. These cells are responsible for identifying, engulfing, and destroying pathogens and apoptotic cells. Resident macrophages from fetal origin are supplemented by monocyte-derived macrophages and are recruited to the sites of inflammation [103]. The functional fate of monocyte-derived macrophages is determined by the immunological imprinting of either immunosuppression or innate immune response [104]. Macrophages are categorized into two types: "classically" activated macrophages (M1 phenotype) and "alternatively" activated macrophages (M2 phenotype). The M1 phenotype is particularly important for the immune response to intracellular bacteria, generating reactive oxygen species (ROS) and nitric oxide (NO) and inducing the proinflammatory response. The M2 phenotype not only plays a key role in wound healing but also provides immunity to helminths and other parasites by trapping and killing them and activates the antiinflammatory response [105]. Recent studies suggest that rather than going to either M1 or M2 phenotype, macrophage polarization is more complex with a spectrum of activation states depending on the mediator or stimuli [106].

Macrophage differentiation is characterized by DNA hypomethylation and involves changes in histone PTMs, which repress macrophage-specific genes through the lineage-specific TFs CAAT/enhancer-binding protein-α acting in concert with the PU-1 TFs [107]. The macrophage differentiation process is associated with the combinatorial modification of lysine 9 methylation, serine 10 phosphorylation, and lysine 14 acetylation on histone H3 (H3K9me−S10phos−K14ac) [108]. The importance of epigenetic regulation in macrophage activation is exemplified by the effects from the loss of HDAC-3 in mouse macrophages. These render the hyperresponsive to IL-4 and transfer toward an M2 phenotype, highlighting the relevance of histone acetylation status for controlling the functional responses of macrophages [109]. Thus, reciprocal changes in methylation of histone H3 lysine 4 (H3K4) and histone H3 lysine 27 (H3K27) can regulate M2-macrophage genes [110]. In addition, miRNA-155, which is induced by TLR-2, TLR-3, TLR-4, and TLR-9 ligands, and tumor necrosis factor alpha regulate macrophage differentiation and innate inflammatory response [111]. Taken together, these studies suggest that the epigenetic changes govern differentiation, polarization of macrophage phenotype, and function at the time of infection.

22.5.1.2 Dendritic cells

DCs are critical for the immune defense against infection and build a bridge between the innate and the adaptive immune system. These antigen-presenting cells are involved in pathogen recognition and are solely able to prime naive T cells in the lymph nodes and then affect the progress of the host immune response. Human and mouse studies have identified myeloid, lymphoid, and plasmacytoid dendritic cells; these cells stimulate T cells differently and produce different cytokines, altering the T cell response [112]. DNA methylation plays an important role in activating DCs' ability to promote a Th2 response [113]. In human DCs, infection by pathogenic bacteria is associated with rapid and active demethylation at thousands of loci, independent of cell division. Moreover, demethylation induces

extensive epigenetic remodeling that includes the gain of histone activation marks and increased chromatin accessibility. Furthermore, CXXC5 is a zinc finger CXXC family epigenetic regulator and is highly expressed in DCs, where it plays a crucial role in TLR7/9- and virus-induced interferon (IFN) response. The genetic ablation of CXXC5 resulted in aberrant methylation of the CpG-containing island within the IRF7 gene and impaired IRF7 expression in steady-state DCs [114]. HDACs also regulate DCs function by controlling the production of inflammatory mediators, including cytokines, chemokines, matrix metalloproteinases, TLR, and IFN-γ signaling pathways [115]. In addition, miRNAs in DCs play a regulatory role in response to fungal infections. The study of miRNAs and their predicted target genes reveals a fine-tuning regulation by miR-132-5p and miR-212-5p in the anti-fungal immune response [116]. Thus, epigenetic changes can influence host immunity by regulating the function of DCs in infectious diseases.

22.5.2 Adaptive immune response

Adaptive immune recognition is mediated by two types of T cell and B cell antigen receptors. The DNA cleavage events that encode antigen receptors are assembled in variable and constant fragments through gene recombination−activation [117]. Lymphocytes are poised to respond to antigen recognition with a transcriptional program that involves metabolism, gene regulation, proliferation, and clonal expansion. The epigenetic landscape that is essential for lymphocyte subset specialization is also critical during the development of immunological memory. In the following sections, we will discuss the role of epigenetics on different types of immune cells such as B-, T-, and natural killer cells (NK cells).

22.5.2.1 T cells

T cells, unlike macrophages that can attack any foreign cell or virus, possess highly specific cell surface receptors for a unique foreign antigen. The function of T cells changes over time, during infancy, for example, they will function to develop immunity toward common pathogens, whereas in adulthood, when fewer novel antigens are found, the T cells are in charge of maintaining homeostasis and the immunoregulation of chronically encountered antigens [112]. The functionality of T cells decreases with aging, which leads to an impaired immune response and immune-associated pathologies.

There are two types of conventional T cells: T-helper (Th) cells, which are marked by the coreceptor CD4 on the cell surface, and cytotoxic T cells, which express coreceptor CD8. In general, methylation of DNA and modifications to histones through acetylation and methylation such as H3K4me3, H3K9me2, H3K9me3, and H3K27me3 play an important role in the regulation of gene expression in many different cell types; for T cells, these modifications particularly influence the gene expression of CD4+ T cells and CD8+ T cells [118]. CD4+ T cells are regarded as the most prolific cytokine producers; they subdivide in lineages Th1, Th2, and Th17. These different lineages are associated with different acetylation statuses of the promoter regions of IL-4 and IFN-γ genes, which are regulated by H3K4me3 and H3K27me3 in a lineage-specific manner [119]. TFs controlled by epigenetics are clearly involved in both the establishment and maintenance of CD4+ T cell differentiation states. Th1-type cytokines tend to produce proinflammatory responses responsible for killing intracellular parasites, and for perpetuating autoimmune responses, interferon gamma is the main Th1 cytokine [120]. Excess in proinflammatory responses could lead to tissue damage; Th2-type cytokines

promote antiinflammatory responses to counteract the Th1-mediated inflammation; interleukin 10 (IL-10) in the main Th2 antiinflammatory cytokine [120]. Optimally, there should be a good balance of Th1 and Th2 response during an immune challenge; however, an imbalance of Th2 response could lead to hypersensitivity and allergic reactions.

On the contrary, during the development and differentiation of memory CD8+ T cells, there is an increase in the levels of acetylation on H3K9 and H3K14, which cause a faster response on reexposure to the pathogen. Besides this, DNA methylation also changes dynamically during infection and correlates inversely with gene expression. For example, granzyme B, IFN-γ, and genes silenced by DNA methylation are all associated with the acquisition of differential gene expression profiles unique to effector CD8+ T cells [121].

22.5.2.2 B cells

Unlike T cells, which can identify specific foreign pathogens and act on them directly, B cells function by releasing antibodies that act against specific antigens. Epigenetic modifications and factors influence gene expression and modulate critical B cell processes, such as class switch DNA recombination, somatic hypermutation, and differentiation to memory B cells or plasma cells (PCs), thereby generating the antibody response. Epigenetic dysregulation in B cells, including the aberrant expression of ncRNAs and alterations of histone modifications and DNA methylation, can result in an abnormal antibody response against foreign antigens [122]. Upon immune activation, DNA methylation of B cells is reprogrammed in a dramatic fashion at the germinal center (GC) stage. This transition permits GC B cells to acquire the potential to differentiate toward memory or PCs fates in response to antigenic challenges [123]. In addition, histone modifiers are also involved throughout the development and activation of B cells. Studies have shown that early B cell development is almost completely blocked by cell-specific deletion of both HDAC-1 and HDAC-2, whereas deletion of either gene alone has no effect. In contrast, the B cell compartment is normal when HDAC-1 and HDAC-2 are selectively deleted from the mature nondividing B cells. As a result, these cells fail to proliferate in the presence of lipopolysaccharide (LPS) and IL-4, rather undergoing apoptosis [124]. It is known that HDAC-5 and HDAC-7 are phosphorylated by the protein kinases D1 and D3 and are exported from the nucleus in response to B-cell receptor signaling [125]. However, it is unknown what type of functional consequences other HDACs could have on B cell development and/or function.

22.5.2.3 Natural killer cells

NK cells are cytotoxic; their cytoplasm contains perforin and proteases known as granzymes. When they are near to a cell targeted for killing, perforin is released and forms pores on the targeted cell membrane allowing the granzymes and associated molecules to enter the cell to induce either apoptosis or cell lysis [126]. Historically, NK cells are short-lived cytolytic cells that can rapidly respond against pathogens in an antigen-independent manner. However, it has recently been shown that NK cells possess traits of adaptive immunity and can acquire immunological memory such as T- and B cells. Moreover, NK cells express germline-encoded activating receptors capable of directly binding to pathogen-derived or stress-induced self-antigens and have an immunological response to a second antigen exposure for trained and memory responses, respectively [127]. The epigenetic diversification in NK cell subsets may represent a broader adaptive cellular immune response hitherto unappreciated. Some studies showed that genome-wide DNA methylation patterns play a key role for the activation and function of NK cells. In cytomegalovirus infection, certain stochastic promoter

methylation results in the generation of a diverse NK cell repertoire and reduced expression of FcεRγ, spleen tyrosine kinase, and Ewing's sarcoma−associated transcript 2. These alter target cell specificity by attenuating NK cell signaling lymphocytic activation molecule family receptor, NKp30, and NKp46 receptor signaling [128]. It has been shown recently that H3K4me3 demethylase Kdm5a that associates with p50 binds to the Socs1 promoter region and is required for priming activation of NK cells [129]. In addition, NKG2D, which is a major activating receptor of NK cells, plays a critical role in immune surveillance. This can be regulated by cooperation of histone acetylation and STAT3 tyrosine705 phosphorylation in NK cells [130]. Other investigations include that of miRNAs, such as miR-155, miR-15a, miR-1246, miR-331-3p, and miR-362-5p, which have been found to have a differential expression in activated human NK cells by IL-2, IL-15, or IL-21 [131].

22.6 Epigenetic drugs and their applications during infection

Development of "epigenetic therapies" now has the immense potential in the treatment of many diseases, including cancer, cardiovascular disorders, and inflammation [13]. Different types of epigenetic drugs will be discussed in the following subsections.

22.6.1 DNA methylating agents

DNA methylating agents or DNMT inhibitors (DNMTis) are drugs that inhibit DNA methylation. These are essentially hypomethylating agents that block the activity of DNMT. Two types of methylating agents are currently approved by the Food and Drug Administration (FDA) for treatment of myelodysplastic syndrome, the pan-DNMTi 5-aza-2′-deoxycytidine (decitabine), and 5-azacytidine (Vidaza). They are pan inhibitors that are broad acting and target nonspecific DNMTs. Azacitidine also shows direct cytotoxicity to abnormal hematopoietic cells in the bone marrow [132]. In 2020, a novel oral formulation of azacytidine as film-coated tablets (ONUREG) was approved by the FDA for continued treatment of adult patients in remission with acute myeloid leukemia [132]. Additionally, pan inhibitors are now used to increase fetal hemoglobin production by causing hypomethylation of γ-globin genes in patients with sickle cell disease [133]. Apart from the pan inhibitors, specific inhibitors for DNMT1 (RG108, MG98, or procainamide), DNMT3a (theaflavin3,3′-digallate), and DNMT3b (nanaomycin A) are more potent epigenetic molecules that modify the genes [134]. Some studies have shown that DNMTi has an important effect on embryo development and neoplasm invasion, whereas microbial infection−induced DNA hypermethylation is associated with the augmentation of inflammation and oxidative stress [135]. DNMTis are widely used in preclinical and clinical studies, but their therapeutic use is limited because of the side effects and low efficacy in solid tumors. Recently, Papparlardi and colleagues described the development of a novel, reversible, and selective DNMT1 inhibitor called GSK3685032. This inhibitor is less toxic and has a higher and long-lasting hypomethylating activity compared with current DNMTi in the market [136].

Many viral infections, such as Epstein−Barr virus (EBV), human papillomavirus (HPV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV), often trigger the host immune response, particularly through methylation of foreign movable viral genome, and DNA methylation of these viral DNAs is an effective method to silence viral gene expression [137]. The viral genome is silenced by host-driven DNA methylation, which allows viruses to escape from the host immune system [138], and

the virus infection can also result in epigenetic changes to the host [139]. For example, downregulation of DNMT1 or DNMT3B expression in Huh7.5.1 cells severely impaired cell culture–produced HCV (HCVcc) infection, and DNMTi such as 5-aza-C significantly suppressed HCVcc infection, viral RNA replication, and protein expression [140]. A subset of differentially methylated genes was identified, which correlated the acquired DNA methylation to altered transcript levels following EBV infection. Loss of DNA methylation using the DNMTi decitabine-restored RNA levels induced G2/M arrest and apoptosis in EBV-associated gastric cancer cell lines [141]. CpG island methylator phenotype is where CpG islands upstream of tumor suppressors are hypermethylated, and this phenotype has a strong correlation with EBV [142]. The study reported that DNA methylation was involved in controlling viral genome of HPV, and repression of tumor suppressor genes by inducing promoter hypermethylation was an important reason for carcinogenesis [143]. In addition, decreased DNA methylation of the PD1 locus in CD8+ T cells happened in both chronic lymphocytic choriomeningitis virus and HIV infections [144].

The upregulation of IL-6 was modulated by promoter demethylation in response to influenza virus infection or dsRNA treatment. DNMT1, but not DNMT3a or DNMT3b, was responsible for IL-6 expression in this process [145]. Then it was also demonstrated that downregulation of DNMT1 or DNMT3B expression in Huh7.5.1 cells severely impaired cell culture–produced HCV infection [140]. In addition, it has been found that the beneficial effects of DNMT1i procainamide could be attributed to the suppression of DNA methylation, neutrophil infiltration, superoxide production, and NO formation to alleviate multiple organ dysfunctions in rats with septic shock [146]. Therefore, further research should be conducted regarding DNMTi, especially DNMT1i, in infectious diseases.

22.6.2 Histone deacetylation inhibitors

HDAC inhibitors (HDACis) induce the hyperacetylation and relaxation of chromatin, which leads to cellular differentiation and apoptosis of cancer cells. They also promote cell cycle arrest, inhibit angiogenesis of cancer vasculature, and regulate host immune responses [132]. HDACis are able to decrease the expression of PU-l TFs, which regulate constitutive expression of HDACi target genes encoding for integrins, scavenger receptors, TLR-4, CD 14, and p40/47/67 NADPH oxidase subunits [147]. Moreover, HDACi regulates the expression of innate immune genes and host defenses against microbial pathogens and impairs gene expression through acetylation-dependent recruitment or chromatin modifications of transcriptional repressors. Trichostatin (TSA), a nonspecific HDACi, inhibits the expression of proinflammatory cytokines through a local deacetylation of migration inhibitory factor promoter–associated histones impairing the recruitment of the basal transcriptional machinery [148]. This inhibits macrophage activation to LPS through inducing Mi-2β and Mi-2/NuRD transcriptional repressor complex [149]. Many studies have shown that some HDACis have a therapeutic effect on viral infectious diseases. In HIV infection, for example, a selective HDACi could activate HIV infection from latency, which suggests a clinical strategy to target persistent HIV infection. This strategy is based on the fact that HIV can evade the immune system by hiding out in its dormant form; therefore, reactivating the latent virus can make it vulnerable to immune-mediated death, a concept termed "shock and kill." The theory behind the "shock and kill" strategy involves the use of drugs that reverse the latency of HIV and could increase viral gene expression (shock), rendering the viral reservoir cells vulnerable to elimination (kill) by other cells of the immune system [150]. So far, the administration of vorinostat or suberoylanilide hydroxamic acid (SAHA) disrupted

HIV-1 latency in patients on antiretroviral therapy [151,152]. In addition, studies have shown that these HDACis significantly induce EBV lytic cycle in EBV-positive gastric carcinoma cells [153]. Orchestrated epigenetic alterations are important in gene regulation during EBV infection, and histone modification status in promoter regions is significantly associated with the acquisition of de novo DNA methylation or protection of the unmethylated status of the transcription start site [154]. Studies have shown that HPV oncoproteins interact with both HATs and HDACs, and HDACi induces apoptosis in HPV-positive cell lines and affects their growth independently of E6/E7 oncogene expression [155,156].

In addition, abundant preclinical and clinical studies indicate that these epigenetic modifiers interfere with critical mediators of innate or adaptive immunity to minimize the risk of infections. Researchers have shown an increasing interest in using of HDACi in sepsis. It has been shown that the macrophages exposed to HDACi had decreased levels of ROS and NO, which impeded their microbicidal responses to *Escherichia coli* and *Salmonella Typhimurium* [157]. Furthermore, in septic mouse model, SAHA is associated with clear survival benefits, following cecal ligation and puncture [158]. Moreover, mice treated with the SIRT1/2 inhibitor—cambinol were protected from endotoxic and septic shock [159]. Additionally, in phase I and II clinical trials, the most severe adverse effects that cancer patients treated with HDACi (SAHA, MS-275, valproate, and ITF2357) along with chemotherapy developed were myelosuppression, gastrointestinal, hepatic, and neurological toxicity, which were resolved adjusting the HDACi dosages. Only a few patients developed severe pneumonia even in the absence of treatment-induced neutropenia, suggesting that it was necessary to monitor the immune status and susceptibility to infection during treatment with HDACi, especially in immunosuppressed patients [160,161].

Tubastatin A, a specific HDAC-6 inhibitor, promotes natural resolution of pathological inflammation by stimulating IL-10 production while permitting the initiation of the proinflammatory response. This significantly protects mice from inflammatory shock induced by LPS and enhances mitochondrial ROS, a TLR-generated microbicidal response in human macrophages and increased intracellular killing of pathogens [162]. Thus, there is a need for more carefully designed and analyzed HDACi studies in infection to provide further insight into the mechanisms of infectious disease.

Recent studies have shown that chromatin-modifying agents can induce stemness by reprogramming the silenced genes in the somatic cells and increasing the developmental plasticity. For instance, valproic acid (VPA), an HDACi, has been shown to promote self-renewal/expansion of hematopoietic stem cells and facilitate the generation of induced pluripotent stem cells (iPSCs) [163]. Furthermore, studies have shown that reprogramming of adult somatic cells into iPSCs by epigenetic drugs could be a safe method and may provide an attractive source of stem cells for regenerative medicine [164]. Moreover, we have shown that chromatin-modifying agents Aza and TSA together induced pluripotent genes that are silenced in bone marrow progenitor cells, which could be an attractive source of cells for therapy [165].

Our recent two studies found that combinations of epigenetic modifiers played an important role in the reduction of inflammation and protected animals from death during septic shock. A single dose of combinatorial administration of Aza plus TSA after the onset of acute lung injury (ALI) can significantly attenuate lung vascular hyperpermeability and inflammatory lung injury. Moreover, the combined treatment also reduced inflammation and promoted antiinflammatory M2 macrophages during ALI [166,167]. These two studies clearly demonstrated that epigenetic modifiers have a therapeutic potential for patients with sepsis-induced vascular injury and inflammation. Two general

types of epigenetic drugs such as DNMTi and HDACi are commonly used in the disease treatment. DNMTi, 5-azacytidine (Vidaza), and 5-aza-2′deoxy-5-azacytidine (decitabine) are pan inhibitors being used to increase fetal hemoglobin production by causing hypomethylation of γ-globin genes in patients with sickle cell disease [168]. HDACis have been found to be a novel category of anticancer drugs. To date, the FDA has approved four HDACis, vorinostat, romidepsin, panobinostat, and belinostat. More specifically, these HDACis have the least severe side effects and are mostly used for hematologic cancers. More clinical trials are continuously expanding to address other types of cancer and nonmalignant diseases. HDACis are also used for various types of neurodegenerative diseases, inflammation disorders, and cardiovascular diseases [13,169].

22.6.3 Toxicity of epigenetic drugs

Despite DNMTi and HDACi are FDA-approved drugs, their use in the clinics is limited because of toxicity such as cardiotoxicity, thrombocytopenia, and teratogenic effects [170–173]. According to associated clinical trials, these epigenetic drugs, including DNMTi (azacytidine, decitabine, panobinostat) and HDACi (VPA, sodium phenylbutyrate, vorinostat, romidepsin), have certain adverse effects. The adverse effects from these drugs include myelosuppression, gastrointestinal toxicity, neurological toxicity, liver toxicity, and even severe infection, and many of them have dose-limiting toxicities [174,175]. Even though these drugs are shown to be beneficial in some cancer treatments and hematological malignancies, limitations about their administration and efficacy remain. In vivo, they have a short half-life of around 30–50 min [136]. In addition, there are limited studies regarding epigenetic drugs in infectious diseases. The main focus of epigenetic-targeted therapy for viral infection is to repress the virus lytic and reactivation phases in a “block” and “lock” manner. This approach could be applied to any virus and seems like an encouraging option to overcome latency and viral shedding, perhaps block viral replication and infectivity [176]. However, most of the currently available studies are on animal-based experiments and not on clinical trials. Thus, more studies are needed to figure out the appropriate dosage of drugs without eliciting toxicity and to elucidate the optimal therapy with different drugs in the field of infectious disease.

22.7 Future perspective of epigenetic modifiers in clinical treatment

Epigenetic studies have become an important field in biomedical research and drug discovery. Although epigenetic mechanisms have been studied for quite a long time, there is still a great deal to know about the specificity and molecular target of each of the epigenetic-modifying drugs. Because of the reversible nature of epigenetic modifications, epigenetic drugs have great potential for therapeutic use. As described earlier, epigenetic modifications have been implicated in infectious diseases by regulating the host innate and adaptive immune responses. Studies regarding these epigenetic changes in immune functions not only provide a better understanding of postinfection-mediated immunosuppression but also may supply important biomarkers that can be used to assay the functionality of a postseptic patient’s immune condition. Moreover, a better understanding of the relationship between epigenetics and postseptic immunosuppression can improve diagnostic tools and may serve as a novel important target for new therapies in infection. Studies have shown that DNA and histone epigenetic modifications act synergistically and promote the gene on/off switch for various cellular events. It has been shown that epigenetic drugs are good for treating a certain type of cancers. Most importantly, studies are focusing on

the importance of these drugs in infectious diseases, including infection and inflammation. The currently available epigenetic drugs in clinical use are having a broad spectrum of inhibition rather than specific inhibitory activity. This could result in widespread changes in the epigenetic pattern in several other genes, including diseased genes or protooncogenes. Thus, in the future, studies should focus on specific inhibitors that will target a specific gene or a set of genes. Recent efforts in bioinformatics dataset collection in the form of epigenetic enzymes and epigenetic drug databases will also provide the foundation for the future of epigenetic drug discovery and development [177]. Thus, the study of the epigenetic mechanisms on infection has profound clinical significance.

22.8 Conclusion

Alterations in epigenetic mechanisms lead to expression level changes within the genes and cause various diseases. The level of understanding of the role of epigenetic aberrations in infection and inflammation is still primitive. Currently, there are few strong studies demonstrating how genetic variants and epigenetic processes impact infectious diseases. However, the number of studies in this field is continuously increasing. For diseases, timely diagnosis and early treatment are important factors and improve the prognosis. Carefully designed and analyzed epigenetic studies are needed to provide further insight into the mechanisms and treatment of infection. Thus, from the available literature, studies relating to epigenetics, especially combination therapy, have profound clinical significance.

List of abbreviations

5-hmC	5-Hydroxymethylcytosine
C/EBPα	CAAT/enhancer-binding protein-α
CGI	CpG-containing island
CIMP	CpG island methylator phenotype
CMV	Cytomegalovirus
CoRest	Corepressor proteins
CpG	Cytosine−phosphate−guanine
CSR	Class switch DNA recombination
CXXC5	CXXC finger protein 5
DAMPs	Danger-associated molecular patterns
DCs	Dendritic cells
DDR	DNA damage response
DN	Deoxyribonucleic acid
DNMTi	DNA methyltransferases inhibitor
DNMTs	DNA methyltransferases
EAT-2	Ewing's sarcoma−associated transcript 2
EBV	Epstein−Barr virus
GC	Germinal center
GNAT	Gcn5-related N-acetyltransferase
HATs	Histone acetyltransferase
HCV	Hepatitis C virus
HCVcc	Cell culture−produced HCV
HDA1	Histone deacetylase-A1

HDACi Histone deacetylases inhibitor
HDACs Histone deacetylases
HDMs Histone demethylases
HIV Human immunodeficiency virus
HMTs Histone methyltransferases
HPV Human papillomavirus
JARID Jumonji and AT-rich interaction domain
JMJD Jumonji domain-containing proteins
LCMV Lymphocytic choriomeningitis virus
lncRNA Long noncoding RNA
LPS Lipopolysaccharide
MBD Methyl-CpG-binding domain
miRNAs MicroRNAs
MMPs Matrix metalloproteinases
MMS Methyl methane sulfonates
mRNA Messenger RNA
mROS Mitochondrial ROS
NCoR Nuclear receptor corepressor
ncRNA Noncoding RNA
NLR Nod-like receptors
NO Nitric oxide
NuRD Nucleosome remodeling deacetylase
PAMPs Pattern-associated molecular patterns
PBMCs Peripheral blood mononuclear cells
PCs Plasma cells
pri-miRNA Primary miRNA
PRRs Pathogen recognition receptors
PTMs Posttranslational modifications
RAG Recombination-activating gene
RNA Ribonucleic acid
ROS Reactive oxygen species
SAHA Suberoylanilide hydroxamic acid
SHM Somatic hypermutation
SLAMFR SLAM family receptor
SMRT Silencing mediator of retinoid and thyroid hormone receptors
snRNAs Small nuclear RNAs
SUMO Small ubiquitin-like modifier
SYK Spleen tyrosine kinase
TBSA Tubastatin A
TETs Ten−eleven translocation proteins
TFs Transcription factors
TLRs Toll-like receptors

Acknowledgements

This work was supported, in part, by the American Heart Association Transformational Project Award 20TPA35490215 and the National Institute of Health R01 grant HL141345 (to JR).

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CHAPTER

Translational aspects of the endometriosis epigenome

23

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23.1 Introduction

23.1.1 Endometriosis

Endometriosis is a painful, inflammatory disease defined as the presence of lesions consisting of glands and stroma outside of the uterine cavity [1]. Ectopic growth of this endometrial-like tissue causes pelvic inflammation, fibrosis, and ovarian cysts, resulting in the hallmark symptoms of endometriosis: pain (dysmenorrhea, dyspareunia, dyschezia) and infertility [2]. Endometriosis is a common gynecologic diagnosis, with an estimated prevalence of up to 10% among all pre-menopausal women and persons with uterus [3]. It has been estimated that 176 million women and teens are affected by endometriosis around the world [4], with an impact on health care that reaches 22 billion in the United States only [5]. Many qualitative and quantitative studies conducted around the world have documented its negative impact on the quality of life (QoL) of affected women in aspects related to physical and mental health, wellbeing, relationships, reproduction, and work or study [reviewed in Ref. [6]]. However, despite decades of research efforts, the etiology of endometriosis has not been fully elucidated. Inadequate understanding of its pathogenesis results in a lack of non-invasive diagnostic markers and limited therapeutic options, none of which is curative—recurrence rates after surgical and drug therapy are typically 20% at 6 months and 50% in 1 year [7,8]. The few hormonal drugs available (e.g., oral contraceptives, androgens, progestins, and gonadotropin-releasing hormone agonists/antagonists) are not indicated to treat infertility but rather to alleviate pain symptoms [9]. Moreover, some of these treatments are expensive, are not effective in all patients, and have considerable side effects—mainly related to hypoestrogenic effects—including hot flashes, bone loss, weight gain, and mood swings. Therefore, finding effective new therapeutics for endometriosis involving novel targets and a multidisciplinary approach is considered a priority in the field [10,11].

Endometriosis is diagnosed surgically, most often via laparoscopy during which the peritoneal structures and organs are scanned for visible lesions, and by histopathological analysis of endometriotic biopsies. Currently, the only non-invasive diagnostic marker that is clinically used is serum CA-125 levels, which have low specificity and sensitivity [12,13]. Because of the lack of specific

Epigenetics in Human Disease. https://doi.org/10.1016/B978-0-443-21863-7.00008-1
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diagnostic markers for endometriosis and the requirement for surgical identification of lesions, the diagnosis of endometriosis is typically delayed an average of 7 years [14]. The diagnostic delays seen worldwide contribute to the uncertainties of not knowing the cause of symptoms, excessive medical expenses, and frustrations associated with hit-and-miss treatments [15,16]. Moreover, delays in receiving the appropriate medical care makes affected women more prone to serious complications such as infertility, frozen pelvis, need for aggressive and complex surgeries, hysterectomy, chronic and incapacitating pain, and even cancer [17]. Thus, it is imperative to find better ways to diagnose, preferably via non-invasive methods, and intervene clinically, preferably with non-hormonal treatments, in a timely fashion.

The most common clinical presentations of endometriosis include (1) lesions in the peritoneum and the surface of the uterus and ovaries, (2) blood-filled ovarian cysts (*endometriomas*), and (3) rectovaginal endometriotic nodules (deeply infiltrating lesions or DIE). DIE are solid masses of endometriotic tissue composed of adipose and fibromuscular tissue commonly found invading the retroperitoneum and cul-de-sacs [18]. Endometriosis is considered a "benign neoplastic disorder": endometrial cells are highly proliferative and invasive, resistant to apoptosis, and able to induce angiogenesis without losing cell cycle control. Yet, a twofold increase in the risk of ovarian cancer (endometrioid and clear cell types) has been reported in women with ovarian endometriosis [19–21]. Several studies have reported a high burden of cancer driver mutations in endometriosis lesions, despite not being malignant [22–24]. A study by Anglesio et al. (2017) showed that somatic mutations were present in 79% of deep infiltrating endometriosis lesions, which included non-silent mutations in the cancer-associated genes *ARID1A*, *PIK3CA*, *KRAS*, *FBXW7*, *PPP2R1A*, and *PIK3R* [22]. Follow-up studies by Noe et al. (2018) and Suda et al. (2018) also identified recurrent non-silent somatic mutations in cancer-driver genes [23,24]. Whether the presence of these mutations represents a more aggressive, potentially transforming phenotype is still unknown [25].

Although the origin of endometriosis remains elusive, several theories have been proposed to explain the etiology of this debilitating gynecological condition [18]. The retrograde menstruation theory, also known as Sampson's theory [26], states that endometrial cells migrate outside the uterine cavity toward the peritoneum via the fallopian tubes, where they survive and grow as superficial or infiltrative lesions. Since it is known that most women experience retrograde menstruation without all having endometriosis, this theory has been challenged. The coelomic metaplasia theory suggests that endometriosis results from metaplastic changes at the level of the mesothelium or the ovarian surface epithelium causing their differentiation into endometrial glands [27]. Different factors may trigger this process including environmental toxins, ovarian steroid hormones, and immune factors. A recent theory states that endometrial mesenchymal and progenitor stem cells could attached and invade the mesothelium forming a niche that eventually leads to the development of an endometriotic lesion. Thus, these cells could constitute a primary event in the etiology of endometriosis [28–30]. It has also been proposed that under the influence of E2 or E2 mimetics, residual cells from Mullerian ducts, from which the mammalian female reproductive tract develops, maintain the capacity to further develop into endometrial tissue ectopically via activation of key developmental genes [31,32]. The lymphatic/circulatory spread theory proposes that endometriotic cells may be transported by blood or the lymphatic system and eventually transplanted into extra-pelvic organs or anatomical structures (i.e., lungs, brain, and nostrils), via processes akin to those activated during metastatic spread of cancer cells [33]. In addition, it has been proposed that a deficient immune response fails to clear endometrial cells reaching the peritoneal cavity during retrograde menstruation, leading to their implantation and

growth. In fact, abnormalities in T cells, NK cells, macrophages, and humoral responses have been documented in peripheral blood and peritoneal fluid of women with endometriosis [34]. Environmental factors such as estrogenic toxicants have been implicated as a potential cause of endometriosis, based on the observation of high levels of dioxin, phthalates, and bisphenol A in biofluids from women with endometriosis compared to controls [35]. In addition, exposure to dioxins through the diet has been shown to cause endometriosis in rhesus macaques in a dose-dependent manner [36,37].

Like other complex traits, endometriosis is a polygenic disorder, resulting from interactions of multiple susceptibility genes and environmental factors [38]. The prevalence of endometriosis is up to 10 times higher in women with an affected first degree relative, and severe disease is more prevalent in women with family history of endometriosis. It has been estimated that the genetic contribution to endometriosis risk is 50%, with the remaining risk being contributed by the environment. Recent Genome Wide Association Studies (GWAS) of thousands of patients and controls have shown the association of endometriosis and 34 genetic loci, many of which have been replicated in independent studies [39]. However, these loci explain only a small percentage of the expected heritability of endometriosis, are unlikely to be the actual causal variants, and do not have the power to be diagnostic. Estimates range from 26% genetic risks overall, to 34% for severe disease, and 15% for minimal disease. Thus, more studies on the genetics of endometriosis are warranted to search for genetic variants that predict risk across racial groups. In addition, GWAS findings need to be followed up with studies to determine the biological function of the mutations identified [40]. Functional studies have already confirmed that some of these SNPs lead to dysregulation in the expression of key genes including *ESR1*, *FSHB*, *CDKN2BAS*, *GREB1*, *CDC42*, *VEZT*, and *p16INK2A* [39]. Importantly, understanding the functional effects of these genetic variants will require an integrated view encompassing genomic, transcriptomic, and epigenomic determinants of the observed molecular perturbations [41].

Global gene and protein profiling technologies have identified differences in the molecular profile of endometriotic tissues and eutopic endometrium, involving activation of cellular and molecular pathways (e.g., adhesion, invasion, extracellular matrix remodeling, angiogenic, neurogenic, anti-apoptotic, and inflammatory) [20,30,42]. At the molecular level, specific alterations have been reported including loss of E-cadherin, decreased or null expression of the progesterone receptor isoforms (PGR-A and PGR-B), increased expression of aromatase, and of some matrix metalloproteinases (MMP3, MMP9), and dysregulation of cell−cell adhesion processes. Unique mechanisms must also play a role since endometriotic cells develop invasive/metastatic behavior independently of malignant transformation, which would require alterations in cell growth, cell migration, and cell death pathways. The factors that trigger the activation of these mechanisms remain elusive.

Recent advances using single-cell genomics platforms have characterized the endometriotic lesions and eutopic endometrium from women with endometriosis at the single-cell resolution level. These studies have profiled the different cell types (fibroblasts, epithelial, and immune cells) that reside in the endometriotic niche. Ma et al. (2021) identified different subsets of endometrial fibroblasts in ovarian endometriotic lesions [43]. Gene ontology and KEGG enrichment analyses showed that endometriotic fibroblasts were enriched for genes associated with endometriosis progression (e.g., cell motility, extracellular matrix structure reorganization, angiogenesis, and inflammation). Also, the composition of immune cell populations was different in the endometriotic lesions compared to endometria from patient and controls. In the second study, Tan and colleagues profiled single cell populations in peritoneal and ovarian endometriotic lesions [44]. Interestingly, they found (1) a perivascular mural

cell population unique in peritoneal lesions involved in angiogenesis and immune cell responses and (2) a novel progenitor-like epithelial cell type in endometriosis. Collectively, these studies identified cell subtypes implicated in the development of endometriosis and provide a more in-depth understanding of the disease by highlighting differences in the transcriptomic profile of different endometriotic cellular entities.

Epigenetics has emerged as an important etiological mechanism in chronic, inflammatory pathological states and in tumorigenesis [45], and epigenetic drugs ("epidrugs") are already approved or in clinical trials for cancer (www.clinicaltrials.gov). Epigenetics has been proposed to be a significant yet understudied factor that may explain the complexities of the pathophysiology of endometriosis, which involves hormonal, inflammatory, and environmental triggers. Many of the proposed theories for the origin of endometriosis could be explained by the action of epigenetic factors. Activation of adhesive, proliferative, and invasive mechanisms in refluxed endometrium as proposed by the Sampson's theory, may be explained by epigenetic reprogramming. During coelemic metaplasia it is plausible that epigenetic modifications are activated by environmental and hormonal cues. Stem cells are known to depend on repressive epigenetic marks to maintain stemness. Steroid hormones produced once puberty ensues can activate the expression of genes involved in Mullerian duct differentiation, including *WNT* and *HOX*, via epigenetic mechanisms [32]. Genetic and epigenetic mechanisms have been implicated as triggers of key genes underlying metastatic potential and immunodeficiencies [46]. And estrogenic toxicants are well known to induce epigenetic effects in different pathologies providing a direct link between gene expression and the environment [47].

Identification of the underlying mechanisms of endometriosis is crucial for the development of new treatments, risk assessment tools, and specific diagnostic assays for this enigmatic, incapacitating disease. As a complex, chronic condition, multiple factors are expected to be involved in its pathophysiology. As already accomplished in other fields, understanding the molecular phenome of lesions and recognition of the interplay between genomics, transcriptomics, and epigenomics will promote a better understanding of relevant biological pathways, and ultimately allow the discovery of novel, targeted ways to diagnose and cure this condition [41,42].

23.1.2 Endometrium

Understanding of endometriosis must rely on knowledge of the normal structure and function of the endometrium, the tissue from which, according to some theories, lesions arise. The endometrium is a unique tissue that has the capacity of monthly regeneration during a woman's reproductive life span [48]. Recent transcriptomic studies at the single cell resolution level have identified different endometrial cell types and states along the different phases of the menstrual cycle [49,50]. The main cellular components of the endometrium are stromal cells that provide support and structure, and epithelial cells that invaginate to form tubular shaped glands, composed of simple columnar epithelium with basally located nuclei. The layer facing toward the uterine lumen consists of ciliated epithelium conforming simple tubular glands supported by the endometrial stroma. The endometrium also contains endothelial cells, immune cells, and nerve fibers (Fig. 23.1). The primordial function of the endometrium is to provide the optimal environment for the implantation of the blastocyst approximately 1 week after fertilization. If fertilization does not occur, menses ensues.

The endometrium undergoes structural, molecular, and functional changes during the menstrual cycle, which are tightly regulated by gonadotropin and ovarian steroid hormones upon specific

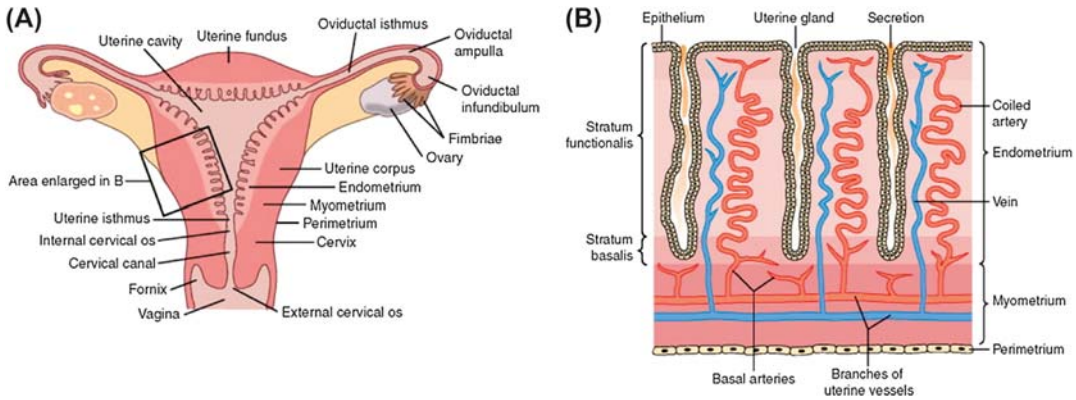

FIGURE 23.1

The uterus and uterine wall. Picture from: Jones, R., Lopez K (2006) [51]. Human reproductive biology (third edition). Chapter 2, Fig. 2.2. (A) Anatomy of the uterus. Drawing that shows the uterus and its parts. (B) Uterine wall. Enlarged area from Fig. 23.1 A showing the uterus wall and its three layers: Endometrium, myometrium, and perimetrium.

receptor binding and activation [51]. The menstrual cycle comprises a highly coordinated series of events leading to ovulation (release of an ovum from an ovarian follicle) and the subsequent preparation of the uterus to allow for embryo implantation if fertilization has occurred. In healthy women, the menstrual cycle occurs every 28 days (on average) from menarche until menopause, which may vary intra- and inter-person during the lifetime. The menstrual cycle consists of four different phases: menses, follicular (proliferative or estrogenic), ovulatory, and luteal (secretory or progestational). The endometrium normal functionality is controlled by activation of specific molecular processes in response to hormonal cues. A Gonadotropin Release Hormone (GnRH) pulse is generated in the hypothalamus followed by the release of the gonadotropin hormones, Luteinizing Hormone (LH) and Follicular Stimulating Hormone (FSH), by the anterior pituitary. As a result, the ovaries secrete estrogen (E2) and progesterone (P4), leading to hormonal fluctuations and structural changes in the ovaries and uterine endometrium. During each menstrual cycle phase the endometrium expresses a characteristic transcriptome reflecting the continuously cycling phenome of this tissue in response to ovarian steroid hormones [52,53]. Therefore, factors (e.g., hormonal, environmental, oxidative stress, inflammatory) that alter the transcriptomic program of the endometrium at each phase may lead to gynecological pathologies including uterine fibroids, infertility, pregnancy complications, endometrial cancer, and endometriosis.

Mechanism of action of estrogen and progesterone receptors: In the endometrium, estrogen and progesterone signaling events are critical to support fertility and pregnancy success. The biological actions of estrogen and progesterone are mediated through canonical and non-canonical signaling pathways. The most well understood mechanism involves hormone activity through their cognate's nuclear receptors, the estrogen receptor (ESR1/ERα and ESR2/ERβ) and the progesterone receptor (PGR-A and PGR-B), respectively (for a comprehensive review for ER and PGR roles in the endometrium, please refer to Refs. [54,55]). Estrogen receptor: Both ER isoforms bind to E2 ligands in the

cytosol causing receptor activation and dimerization and further nuclear translocation. Once in the nucleus, the ER complex binds to estrogen response elements (ERE) and effector proteins (e.g., transcription factors and chromatin modifiers) to induce transcription of target genes. Cell type differences in ERα and ERβ expression have been documented in the human endometrium throughout the menstrual cycle phases [56–58]. ERα is primarily involved in cellular proliferation processes, glandular–stromal interactions, and is required for embryo implantation [59–61]. ERβ knock-out mice models have shown a sub-fertile phenotype suggesting a key role of ERβ during ovulation [61]. A recent study comparing the effect of estrogen signaling in human organoids and endometrial biopsies via chromatin immunoprecipitation and RNA-sequencing showed that ESR1 cistrome regulates the expression of a subset of genes involved in chromatin modification and cell proliferation [62].

Progesterone receptor: The PGR isoforms (PGR-A and PGR-B) are encoded by two different promoters found in the same gene [63]. The combined actions of progesterone are exerted via ligand mediated receptor homodimerization (PR-A or PR-B) or heterodimerization (PR-A/B), ligand-independent receptor signaling, and non-genomic signaling (via the cell membrane associated PGRs) (reviewed in Ref. [64]). Differences in PGR expression levels during the menstrual cycle have been reported in the human endometrium [65,66]. In the mouse, deletion of progesterone receptor demonstrated the importance of PGR for female reproduction [67] and identified PGR-A as the main driver of progesterone action in the uterus [68].

Decidualization and role of steroid hormones: To promote optimal implantation, cyclic changes in estrogen and progesterone levels are required to induce transcriptional, biochemical, and immunological cues in the endometrium (as comprehensively reviewed in Ref. [69]). During the follicular phase, estrogen is actively secreted by the ovarian granulosa cells causing an increase in cell proliferation and endometrial thickness. In addition, the surge of E2 promotes the expression of the leukemia inhibitory factor (LIF) and the progesterone receptor (PGR) molecules, both required to induce the endometrial receptive state needed prior implantation [64,70]. After ovulation, the endometrium begins a process known as decidualization characterized by a dramatic morphological and functional differentiation of endometrial fibroblasts into secretory decidual cells. In vitro experimentation using human endometrial stromal cells demonstrated that this event is driven by the action of PGR-B via the canonical nuclear progesterone receptor (nPGR) signaling pathway [71]. Recently, Chi et al. (2020) showed that genes involved in uterine receptivity and function are regulated by nPGR at the chromatin level in the human endometrium [72]. If implantation occurs, progesterone production is maintained until placentation begins. In the absence of a conceptus, progesterone withdrawal ends the decidualization process and shedding of the endometrium occurs resulting in menses.

23.1.3 Endometrial extracellular matrix

The structure and composition of the extracellular matrix (ECM) provides physical support, biochemical cues, and mechanical stimulation to sustain tissue homeostasis [73]. Disruption of ECM homeostasis can lead to aberrant deposition of fibrillar collagen resulting in tissue fibrosis, or increase degradation of ECM proteins which facilitates invasion and metastasis progression [74]. The endometrial ECM is complex and composed of structural (collagen fibrils, elastic fibers-EFs) and non-structural proteins (small leucine rich proteoglycans-SLRPs such as decorin, biglycan and lumican; matricellular proteins) that safeguard the integrity and physiological functions of the endometrium.

Remodeling of the ECM is critical to maintain the physiological changes that occur in the endometrium throughout the menstrual cycle [75].

Collagen and EFs: These structural proteins are primarily involved in providing mechanical support and stability to the ECM [74,76]. Dynamic changes in the distribution of collagen and EFs have been reported in the endometrium highlighting the importance of ECM remodeling to sustain uterine reproductive functions [77–79].

SLRPS: These non-structural proteins regulate a wide variety of matrix assembly processes such collagen fibrillogenesis, inflammation, and cell migration, adding complexity to the mechanisms that govern ECM remodeling (reviewed in Refs. [80,81]). Spatial and temporal alterations in deposition is observed during the pre-and post-implantation period in the uterus [82]. Differential expression of SLRPs have been reported in human endometrial samples during the different phases of the menstrual cycle [83]. Collectively, deposition of ECM components mediates cell–ECM interactions and allows the coordinated cyclic remodeling of the endometrium.

Collagen turnover in endometriosis: Fibrosis is one of the major pathological features that characterizes endometriosis. As reviewed by Vigano et al. (2018) [84], activation of fibroblasts into myofibroblasts in the endometriotic niche mediates the aberrant deposition of type I collagen, leading to endometriosis-associated fibrosis. It has been suggested that there is an imbalance in the activation of processes required for the clearance and degradation of collagen. The molecular mechanisms that lead to dysregulation in collagen turnover are unknown. Additional studies are required to better understand the molecular cues that trigger the endometrial myofibroblast activation and remodeling of the ECM. In this context, epigenetic regulation of ECM components may be an area of further investigation. Below, we discuss the few studies that explore the potential implication of epigenetic regulation of the ECM in endometriosis.

Integrins and MMPs/TIMPs in endometriosis: An imbalance in cell–ECM interactions and in the expression and activity of enzymes that mediate endometrial turnover results in higher adhesive, migratory, and invasive capacity of endometriotic tissues. Klemmt et al. (2007) reported that endometrial stromal cells from women with endometriosis (1) are more adhesive to the ECM and (2) expressed an aberrant integrin profile, compared to endometrium from healthy controls [85]. Recently, Joshi and colleagues (2021) showed that mid-secretory endometria from women with endometriosis expressed low levels of $\alpha V\beta 3$ integrins compared to disease free controls [86]. Work by Luddi et al. (2020) found an altered pattern in MMP and TIMP expression favoring the increased migration, attachment, and tissue invasion/remodeling observed in endometriosis [87].

SLRPs in endometriosis: Few studies have implicated decorin, biglycan, and lumican in the development of endometriosis, showing increased levels in peritoneal fluid of patients, eutopic endometrium of patients, endometriotic lesions, and endometriotic cells [88,89]. It has been reported that ovarian steroid hormones (E2 and P4) differentially influence the expression of decorin and biglycan [88,90]. Additional studies are warranted to fully elucidate the role of SLRPs in the development of endometriosis.

23.2 Epigenetics mechanisms underlying endometriosis

It is generally accepted that, regardless of the etiology, activation of cellular and molecular pathways must occur to allow endometriotic tissue to survive, evade immune clearance and apoptosis, attach, grow, and invade peritoneal tissues and organs [91]. Epigenetics has been brought to the spotlight as a

mechanism that could explain the dysregulation of normal cellular pathways occurring in ectopically growing endometrial-like tissue [92,93], resulting in endometriosis [94,95]. This includes dysregulation of hormonal, proliferative, immunological, histological, and signaling processes leading to survival and ectopic growth. If proven so, this would open the possibility for therapeutic intervention with specific inhibitors of enzymes that modulate disease-specific epigenetic signatures and their resulting transcriptomic program.

Epigenetics refers to heritable alterations in gene expression that are not caused by changes in the DNA sequence. Epigenetic mechanisms are known to be involved in normal cellular processes including development, cell-differentiation, and aging by regulating gene expression and consequently the cellular and tissue phenome. They include methylation at CpG islands within gene promoter regions, covalent modifications of histones (e.g., acetylation, methylation, phosphorylation), and short non-coding RNA molecules (microRNAs or miRNAs, long-non-coding RNAs, circular RNAs) [96]. In eukaryotic cells, DNA is packed in the nucleus in a highly conserved and malleable structure with associated proteins, collectively known as *chromatin*. The main building block of the chromatin is the nucleosome which consists of DNA and histones (H2A, H2B, H3, and H4), held together by electrostatic interactions. At the level of the nucleosome, two epigenetic mechanisms collide in space and time, collaborating to regulate gene expression and thus cellular phenotype: DNA methylation and histone modifications. Together, these two mechanisms determine if the chromatin is open (*euchromatin*) or closed (*heterochromatin*), and whether gene promoter regions are accessible to the transcription machinery [97]. miRNAs, on the other hand, regulate gene expression post-transcriptionally, by mediating specific degradation of transcripts thus preventing their translation into protein [98].

As reviewed previously [93,99], menstrual cycle—related changes in the endometrium are tightly regulated by epigenetic mechanisms. Processes related to normal endometrial function (proliferation, differentiation, angiogenesis), including cyclic tissue degradation and regeneration akin to stem cell differentiation, are epigenetically regulated. There is evidence that the endometrial methylome and histone mark profile is influenced by levels of ovarian steroid hormones [99]. These processes are also involved in endometriosis, suggesting that endometriotic tissues (both ectopic lesions and eutopic endometrium) could potentially be treated with epigenetic-based therapeutics.

Most of the available literature on epigenetics of endometriosis pertains to identification of the most relevant candidate genes regulated by DNA methylation. Recently, histone modifications have also been shown to be involved in expression regulation of the same genes. These two epigenetic mechanisms often work together to determine whether a gene is expressed or silent. miRNAs have also gained a lot of attention in the endometriosis field, though most of the research is focused on their potential role as diagnostic biomarkers. Despite being a relatively recent topic of study in the endometriosis field, the available literature strongly supports that epigenetics are involved in the pathophysiology of this condition. Furthermore, mechanistically, epigenetics provides a unified model to explain the complex basis of this disease, influenced by hormonal, immune, inflammatory, and environmental factors [95,100].

23.2.1 DNA methylation in endometriosis and endometrium

The first published studies suggesting that endometriosis has an epigenetic basis were published over a decade ago. In 2005, Wu et al. showed that the Homeobox A10 (*HOXA10*) gene, involved in endometrial development and implantation, and known to be under expressed in the endometrium of

women with endometriosis-associated infertility, was regulated by DNA methylation [101]. In 2006, MacLachlan linked this mechanism with the observed effects of environmental endocrine disruptors in reproductive conditions including endometriosis [102]. Soon followed additional reports of differences in DNA methylation in candidate genes including: progesterone receptor B (*PGR*-B) [103], E-cadherin (*CHD1*) [104], estrogen receptor alpha (*ESR1*), estrogen receptor beta (*ESR2*) [105], steroidogenic factor-1 (*SF-1*, *NR5A1*) [106] and aromatase (*CYP19A1*) [107]. Higher expression of DNA methyltransferases (DNMTs; e.g., DNMT1, DNMT3A, and DNMT3B)—the enzymes that catalyze the methylation of CpG islands—were also shown to be elevated in endometriotic lesions (peritoneal and ovarian) compared to controls (endometrium from women with and without endometriosis) [108]. Also, using chromatin immunoprecipitation (ChIP), differential DNMT3B enrichment was observed at the promoter and non-promoter regions (e.g., intronic regions) for *SF*-1 and *ESR1* [109]. In addition, treatment of endometrial cells with a DNA methylation inhibitor (5-Aza-deoxycytine) resulted in activation in the expression of the aromatase gene [107]. Since then, there has been cumulative evidence indicating that key genes known to be dysregulated in endometriotic lesions and endometrium of women with endometriosis and infertility are epigenetically regulated by DNA methylation. These genes are described next (Table 23.1).

- Homeobox (*HOX*)*: HOX* genes encode transcription factors that regulate genes involved primarily in embryonic development. Genes found at *HOXA* cluster including *HOXA9*, *HOXA10*, *HOXA11*, and *HOXA13* are differentially expressed along the female reproductive tract beyond the embryonic development into adulthood. Dysregulation in *HOXA* genes expression has been linked to endometrial abnormalities including polycystic ovarian syndrome, leiomyomas, infertility, and endometriosis [110]. In the context of endometriosis, *HOXA10* is of high relevance since it is involved in uterine developmental processes (growth, proliferation, differentiation) as well as in implantation [111]. It has been demonstrated that *HOXA10* expression is regulated by E2 and P4, and *HOXA10* levels are highest during the mid- and late secretory phases of the menstrual cycle [112]. In contrast, the endometrium of women with endometriosis associated infertility showed a reduced expression of *HOXA10* during the mid-secretory phase, which may lead to defects in endometrial receptivity and implantation [113]. Similarly, it has been demonstrated that silencing of *HOXA10* may impair the responsiveness to P4 by down-regulation of *PGR* in uterine stromal cells during decidualization and implantation [114]. The *HOXA10* promoter is hypermethylated in endometrium from patients with endometriosis compared to controls [101,115,116]. At the protein level, HOXA10 has been shown to be downregulated in endometriotic lesions [112,117]. More recently, using a ChIP array Esfandiari et al. (2021) reported on a pattern of methylation of HOX clusters (A-D) and HOX cofactor genes in endometriotic tissues that was conserved in organoids [118].
- Progesterone Receptor (*PGR*): This member of the steroid-nuclear receptor superfamily has two isoforms, PR-A and PR-B, arising through alternative promoters and translational start sites of the same gene [119]. PGR and its ligand, P4, are involved in endometrial stromal cell proliferation and decidualization, which is required for normal pregnancy. Endometriosis is generally regarded as a P4-resistant disease, resulting from decreased *PGR* mRNA and protein levels [120]. Dysregulation in P4 response may in turn decrease the expression of P4-responsive genes such as *HOXA10* in eutopic endometrium of patients with endometriosis, potentially affecting fertility [121]. The promoter region of PGR isoform B, but not PGR-A, is

Table 23.1 Endometriosis candidate genes and their functions.

Gene	Function	Status in endometriosis
Homeobox (*HOXA10*)	Involved in uterine developmental processes such as growth, proliferation, and differentiation as well as in normal uterine function including implantation.	DNA hypermethylation in ectopic and eutopic endometrium from patients
Progesterone receptor (*PGR*)	Essential for uterine growth and endometrial stromal cell decidualization. Also, it may be involved in anti-proliferative and apoptotic pathways in endometriotic cells and ovarian epithelial cells. Endometriosis is known to be a progesterone-resistant disease.	DNA hypermethylation in endometriotic cells
E-cadherin (*CDH1*)	Involved in normal tissue development, cell adhesion, proliferation and differentiation, vascularization, and carcinogenesis (acting as a tumor suppressor gene).	Inactivated by DNA methylation H3 hypoacetylation
Estrogen receptor 2 (*ESR2*)	Acts as a transcription factor that becomes associated with the promoter of E2 responsive genes. Also, it may be involved in proinflammatory, proliferative, and anti-apoptotic outcomes in endometriotic cells.	DNA hypomethylation in endometriotic cells
Transcription factors GATA-binding protein 2/6 (*GATA2* and *GATA6)*	Overexpression of *GATA6* results in reduced PR and increased in ERβ gene expression. These data provide a molecular mechanism underlying the observed upregulation of *ESR2*,	Hypermethylation of *GATA2* and hypomethylation of *GATA6* in endometriotic tissues

Table 23.1 Endometriosis candidate genes and their functions.—cont'd

Gene	Function	Status in endometriosis
	NR5A1, *HOXC6*, and *CYPR19A1* and downregulation of *ESR1*, *PGR*, *MMP11*, and *ALD1A2* in lesions.	
Steroidogenic factor 1 (*SF-1*, *NR5A1*)	Acts as a transcription factor that regulates the expression of various steroidogenic genes involved in estrogen biosynthesis including aromatase.	DNA hypomethylation in endometriotic cells H3 and H4 hyperacetylation

hypermethylated in ectopic endometrium and endometriotic epithelial cells [103], and in deep infiltrative intestinal endometriosis [122]. Li and colleagues (2016) [123] showed that *PGR* is hypermethylated in ectopic lesions of a murine model of endometriosis, and that treatment with a DNMT inhibitor (DNMTi) led to reductions in the methylation levels and increments in *PGR* expression levels in lesions. A recent review discusses the evidence in support of epigenetic regulation of PGR and also ESR, including not only promoter methylation but also histone modifications and miRNAs [124]. However, PGR expression has been shown to vary substantially across lesion types [125], which may explain the variable effectiveness in pain alleviation across patients. Recent studies have shown that in patients with endometriosis failing progestin treatment, lesions lacked expression of PGR [126]. This finding uncovered the molecular reasons underlying responsive (non-resistant) versus unresponsive (resistant) lesions, with direct implications for precision medicine. As is the standard of care in other hormonal conditions such as breast cancer, endometriotic tissues should be evaluated for the expression of hormone receptors to decide a personalized treatment plan.

- E-cadherin (*CDH1*): This calcium-dependent cell surface glycoprotein plays diverse roles during normal tissue development, cell adhesion, proliferation and differentiation, vascularization, and carcinogenesis [127]. *CDH1* is a key mediator of EMT, contributing to initiation, progression, and metastasis of cancer cells. *CDH1* expression is regulated by transcriptional repression, gene mutations, and epigenetics, including promoter hypermethylation and histone deacetylation and methylation [128]. In the context of the endometrium, *CDH1* plays an important role in endometrial receptivity and implantation [129]. High expression of *CDH1* has been reported in normal endometrium, while loss of expression is associated in endometrial abnormalities [130]. Treatment of endometriotic epithelial cells with HDACi (Trichostatin A—TSA) reactivated *CDH1* expression [104]. In endometriosis, the majority of studies show that *CDH1* expression is reduced in endometriotic lesions and to some extent in eutopic endometrium from patients compared to endometrium from controls [131]. Li et al. (2017) demonstrated that DNA hypermethylation of the *CDH1* promoter region was more frequently seen in endometriotic lesions (32%) and endometrium from patients with endometriosis (26%) compared to endometrium of women without endometriosis (8%) [132].

- Estrogen Receptors (*ESR1* and *ESR2*)*:* Two estrogen receptor isoforms, ER-α and ER-β, encoded by the *ESR1* and *ESR2* genes, respectively, play critical roles in proliferative responses of eutopic and ectopic endometrium [133]. Evidence from knockout and transgenic animal models supports the role of ER-α as the principal mediator of estrogen actions in the endometrium [134], whereas ER-β is the main E2 receptor in endometriotic tissues and in primary endometriotic stromal cells [135]. In vitro studies showed that *ESR1* mRNA levels were drastically reduced (7-fold), whereas *ESR2* mRNA levels were significantly increased (34-fold) in endometriotic stromal cells compared to endometrial stromal cells [120]. A tissue microarray study showed a very high level of expression of ER-β across lesion types [125]. DNA methylation is also well known to regulate expression of *ESR2* in endometriosis. As first evidenced by Xue et al. (2007), the *ESR2* promoter is hypomethylated in endometriotic lesions, leading to the overexpression of ER-β and suppression of *ESR1* promoter activity in endometriosis, disturbing the ER-β:ER-α ratio [105]. In addition, Bulun et al. (2010) showed that methylation of the *ESR2* promoter is the main driver of ER-β upregulation in endometriotic cells [120]. Regarding *ESR1* regulation in endometriosis, recent global DNA methylation studies have shown hypermethylation at tissue-dependent and tissue differentiated methylated regions (T-DMR) in ovarian lesions compared to eutopic endometrium, which was associated with downregulated gene expression [136,137]. There is strong evidence for a role of aberrant epigenetic regulation of ERs in the pathogenesis of endometriosis [138].
- Steroidogenic Factor 1 (*SF-1*): Also known as *NR5A1*, this gene is a key regulator involved in adrenal, hypothalamic, splenic, and reproductive development and function [139]. SF-1 acts as a transcription factor that regulates the expression of steroidogenic genes including aromatase, involved in E2 and P4 biosynthesis. The expression of *SF-1* is higher in ectopic endometrium (i.e., peritoneal and ovarian lesions), ovarian endometriosis stroma, and stromal endometriotic cells compared to their counterparts (eutopic endometrium and endometrial stromal cells, respectively), resulting in a high estrogen microenvironment and enhanced survival [140,141]. Aberrant DNA methylation at CpG islands was found in the promoter region as well as in *SF-1* coding and intronic regions, causing either its complete repression in endometrial cells or aberrant expression in endometriotic cells [142].
- *Transcription factors GATA 2 and GATA 6:* GATA 2 and GATA6 are zinc finger DNA binding proteins that regulates embryonic development, cell differentiation, and tissue morphogenesis. GATA2 regulates the action and expression of PGR in the uterus and plays an important role during endometrial stomal cell decidualization [143,144]. GATA6 protein synergistically mediates transformation of endometrial cells into endometriotic-like cells by activation of the estrogen receptor (ER) signaling pathway [145]. A recent study showed that in ovarian endometriosis GATA6 mRNA expression is sustained in an N6-methyladenosine RNA methylation-dependent manner [146]. DNA methylation fingerprint of endometriotic stromal cells versus Healthy eutopic endometrium uncovered hypermethylation of *GATA2* and hypomethylation of *GATA6.* In vitro studies demonstrated a functional effect of overexpression of *GATA6* resulting in reduction of PR and increases in Erβ gene expression. These data provide a molecular mechanism underlying the observed upregulation of *ESR2*, *NR5A1*, *HOXC6*, and *CYPR19A1* and downregulation of *ESR1*, *PGR*, *MMP11*, and *ALD1A2* observed in lesions [147,148].

A recent review by Wang et al. (2020) on the etiology and pathogenesis of endometriosis summarizes key genes that are known to be differentially methylated in endometriotic tissues including *ESR1*, *ESR2*, *PGR*, *NR5A1*, *CYP19A1*, *HOX* gene family, and *GATA* gene family, with concomitant protein expression changes [30]. The epigenetic mechanisms underlying ovarian steroid hormone receptor expression regulation in endometriosis lesions—DNA methylation, histone modification and miRNAs—were recently reviewed by Chen et al. (2020) [124]. Other genes that have been recently shown to be regulated by DNA methylation are summarized in Table 23.2.

Technological advances allow unbiased genome-wide studies to identify differentially methylated regions in lesions and endometrium from patients with endometriosis compared to controls. Studies conducted from 2010 to 2017 discovered novel differentially expressed genes under the regulation of promoter methylation status and also confirmed the role of previously identified candidate genes [147,159–163].

More recent global promoter methylation studies have validated the role of DNA methylation as regulator of processes that promote the endometriotic phenotype. This includes inflammatory responses, apoptosis, cell adhesion, cell proliferation and hormonal pathways. A study by Wang et al. (2019) using methylation chip arrays identified 375 differentially methylated regions in endometriotic lesions; genes involved in immune and inflammatory response, steroid hormone responses, cell adhesion, apoptosis, and MAPK signaling [100]. A study by Barjaste et al. (2019) identified a group of methylated genes involved in signaling, development, cell adhesion, PI3K-Akt, oxytocin, and relaxin signaling in ectopic compared to endometrium from both patients and controls [121]. In contrast, Li et al. (2021) reported patient-specific methylation patterns observed in matched ectopic and eutopic endometrium, pointing to a clonal origin of disease [164]. Another study by Zhang et al. (2022) identified 37 hypermethylated low-expression genes and 66 hypomethylated high-expression genes in ovarian endometriosis, including *KRT19*, *KRT8*, *ESR1*, *PRL*, *SFN*, *IL20RA*, *IL2RB*, and *PAX8* (hypermethylated, low expression) and *CYP11A1*, *NR5A1*, *ME1*, and *GSTM* (hypomethylated, highly expressed) [137]. The most relevant pathways regulated in this study were JAK-STAT (*IL20RA* and *IL2RB*), prolactin (*PRL* and *ESR1*), and drug metabolism-cytochrome P450 (*GSTM1*). Despite methodological issues that have resulted in conflicting or inconclusive results, and the influence of tissue heterogeneity, hormonal treatments and menstrual phase variability in the DNA methylome, evidence continues to accumulate for common genes and pathways across studies [20,30,42], and novel potentially important pathways are discovered. Table 23.3 summarizes the findings of global DNA methylation studies on endometriosis conducted to date.

In sum, similarities among global methylation studies support the relevance of both novel or previously linked pathways to the endometriosis phenotype and have advanced knowledge of mechanisms involved [100]. The available evidence to date supports differential methylation as a key mechanism in endometriosis, via regulation of genes involved in cell-cell adhesion (*CDH1*), steroidogenic pathways (*ESR1*, *ESR2, NR5A1, StAR*, *CYP19A1*), transcription factors involved in estrogenic responses (*GATA2, GAT6)*, immunity (*TGFBR1, IL20RA, IL2RB*), neurogenesis (*BDNF*), cellular proliferation (*JAZF1*), hormonal responses (*PR*, *ORS2*, *HAND2*, *SGK1*), cell signaling (*DAPK1*, *WNT)*, angiogenesis, and apoptosis.

Regarding the endometrium, several studies have used genome-wide methylation screening analysis to identify the regions that are differentially methylated across the menstrual cycle and between women with and without endometriosis (reviewed by Adamczyk et al. [166]). Earlier

Table 23.2 Other genes regulated by DNA methylation in endometriosis.

Gene	Finding	References
Ras Association Domain Family Member 1 *(RASSF1A)*	Promoter hypermethylation results in silencing of this tumor suppressor gene.	[149]
Catechol-O-methyltransferase (*COMT*)	Hypermethylation of its promoter involved in the degradation of cathecolamines or cathecolestrogens, and results in impairment in the degradation of 2-hydroxyestradiol (2-OHE2) sustaining increased E2 levels.	[150]
Cytochrome C oxidase 2 (*COX*-2)	Hypomethylation of its promoter correlates with higher level of protein expression in eutopic and ectopic endometrium from patients compared to endometrium from controls.	[151,152]
Prostacycle (*PGI2*)	Hypoxia-promoted hypermethylation is associated with decreased protein expression; impacts its roles to modulate the immune microenvironment, induce adhesion and inhibit NK cell activity.	[153]
Ten-eleven translocation methylcytosine dioxygenase 1 (*TET1*)	Diminished expression due to DNA hypermethylation may lead to implantation abnormalities in infertile endometriosis patients.	[154]
Grainyhead-like 2 gene (*GRHL2*)	Is involved in suppression of EMT, its promoter is hypermethylated in lesions leading to increased invasion and migration.	[155]

Table 23.2 Other genes regulated by DNA methylation in endometriosis.—cont'd

Gene	Finding	References
Thymic stromal lymphopoietin (*TSLP*)	The promoter of this interleukin 7-like cytokine gene is hypomethylated which may trigger dendritic cell-mediated T helper2 inflammatory responses.	[156]
Interleukin 12 B (*IL12B*)	Its promoter region is hypomethylated in lesions and eutopic endometrium of patients, pointing to a defect in T cell and NK cell responses.	[157]
Glutathione S-Transferase Mu 1 (*GSTM1*)	Its promoter region is hypomethylated and its expression is increased in ectopic and eutopic endometrium of patients with ovarian endometriosis; GSTM1 is primarily a detoxification enzyme, but it is also known to negatively regulate apoptosis.	[158]

Other genes known to be epigenetically regulated in endometriosis.
GdA, PAEP, *glycodelin A;* HAND2, *heart and neural crest derivatives expressed 2;* LIF, *Leukemia inhibitory factor;* MMP3, MMP-9, *metalloproteinases;* OPN, SPP1, *osteopontin;* PAX2, *paired box 2;* S100A4, *S100 calcium-binding protein A4;* TIMP, *tissue inhibitors of MMP.*

studies conducted before 2018 provided evidence for differentially methylated genes related to angiogenesis, apoptosis, DNA repair, chromatin remodeling pathways, ovarian steroid hormone responses, proinflammatory and transcription factor regulatory pathways in lesions [165,167]. There were striking differences depending on the menstrual cycle phase, with higher DNA methylation levels were found in endometrium from women with endometriosis during the mid-secretory phase [99,165]. Unique DNA methylation patterns were identified in healthy pre-receptive versus receptive stage of endometrial samples in genes involved in chemotaxis, wound healing, apoptosis, and cell adhesion [168]. These results suggest a potential involvement of the hormone milieu (and of P4 in particular) as a key regulator of gene expression through DNA methylation across the menstrual cycle in the endometrium that is different in women with endometriosis compared to controls. These studies provided a baseline knowledge to better understand the mechanisms whereby the epigenetic landscape could result in dysfunctions leading to endometrial pathologies such as endometriosis.

Table 23.3 Genome-wide studies of differentially methylated genes in endometriotic tissues (2010−22).

References	Methodology	Key findings
Borghese et al. [159]	DNA immunoprecipitation (MeDiP) profiled the global DNA methylation; superficial endometriosis, endometriomas, and deep infiltrating endometriosis	Hypermethylated regions were more commonly found at sub-telomeric regions which is associated with chromosomal stability; differential methylation of ***CDH1***, ***ESR1***, ***ESR2***, ***NR5A1***, ***StAR***, ***CYP19A1***, ***GATA2***, ***TGFBR1, IL20RA***, ***IL2RB, PR***, ***ORS2***, ***HAND2***, ***SGK1***
Houshdaran et al. [165]	DNA methylome and gene expression in three phases of the menstrual cycle in endometrium of women with severe endometriosis compared to controls using Illumina Infinium Human Methylation 27K platform	DNA methylation changes were associated with altered gene expression relevant to endometrial function/ dysfunction, including cell proliferation, inflammation/immune response, angiogenesis, and steroid hormone response
Rahmioglu N et al. [163]	DNA methylation and RNA expression profile variability in endometrium, endometriotic tissues, and subcutaneous abdominal fat samples using Illumina Human Methylation 450 BeadChip and gene expression arrays	Endometriotic tissues have a different methylation signature compared to endometrium; WNT signaling, angiogenesis, **cadherin** signaling, and gonadotropin-releasing-hormone-receptor pathways were enriched in endometriosis. Menstrual phase accounted for most variability in methylation/expression patterns in endometrium only
Yamagata et al. [160]	Global methylation status of 27,578 CpG islands at the promoter regions in endometrial stromal cells derived	Differentially methylated genes are involved in signal transduction and cell-cell adhesion pathways;

Table 23.3 Genome-wide studies of differentially methylated genes in endometriotic tissues (2010–22).—cont'd

References	Methodology	Key findings
	from eutopic and ectopic endometrium using Illumina Infinium human methylation array	steroidogenic enzyme genes ***NR5A1*** and ***StAR*** were hypomethylated in endometriotic lesions. Differentially methylated and/or expressed genes (***NR5A1***, ***StAR***, ***STRA6***, and ***HSD17B2***), which are related with steroidogenesis, were independently validated
Yotova et al. [162]	Global analysis of differential DNA methylation in ovarian endometriotic stromal cells and linked gene expression levels using Illumina Human Methylation 450 K BeadChip Array	Differential methylation in promoter sequences of genes involved in immunity (***TGFBR1***), neurogenesis (***BDNF***), cellular proliferation (***JAZF1***), hormonal responses (***ORS2***, ***HAND2***, ***SGK1***) and cell signaling (***DAPK1***)
Wang et al. [100]	Genome-wide analysis of endometriotic lesions and eutopic endometria of patients (with ovarian endometriosis) and endometria from controls using Illumina Human Methylation 450 K BeadChip Array	Methylated genes were primarily associated with immune response, inflammatory response, response to steroid hormone stimulus, cell adhesion, negative regulation of apoptosis, and activation of the MAPK activity. DNMT1, DNMT3A, DNMT3B, and MBD2 expression levels were lower in endometriotic lesions and eutopic endometria compared with control endometria
Barjaste et al. [161]	Genome-wide DNA methylation profile of ectopic and ectopic endometria using Infinium Human Methylation 450K BeadChip arrays	Methylated genes were related to cell signaling abnormalities, development, adhesion, PI3K-Akt, relaxin signaling and autoimmune disease

Continued

Table 23.3 Genome-wide studies of differentially methylated genes in endometriotic tissues (2010−22).—cont'd

References	Methodology	Key findings
Zhang et al. [137]	Differentially methylated and gene expression profiles of ovarian endometriosis with Illumina 450K methylation assay and analysis of GSE141549 microarray dataset	They found 37 hypermethylated low-expression genes (***KRT19***, ***ESR1***, ***PRL***, ***IL2RB***, ***IL20RA***, ***PAX8***) and 66 hypomethylated high-expression genes (***CYP11A1***, ***NR5A1***, ***GSTM1***) were identified in ovarian endometriosis. Signaling pathways included Jak-STAT, prolactin, and cytokine-receptor interaction
Li et al. [164]	Global methylation and mutation profiles of microdissected epithelium and stroma from peritoneal lesions compared to endometria using methylation EPI Bead Chip platform	Patient-specific methylation profiles were identified; somatic mutation profiles indicate monoclonal development of lesions

Genes that are underlined and bold are repeated in one or more studies.

More recently, Adamczyk et al. (2022) reported diminished expression of ten eleven translocation 1 (TET1) gene during the secretory phase due to DNA hypermethylation, which may be linked infertility due to aberrant embryonic implantation in infertile endometriosis patients [154]. TET1 protein catalyzes DNA demethylation at the CpG islands via the hydroxylation of DNA methylcytosine (5 mC) into 5-hydroxymethylcytosine (5hmC) [169]. TET1 has a dual function during transcription by 1) maintaining a DNA hypomethylated state of active genes and 2) mediating direct binding of PCR2 complex to chromatin leading to gene repression [170]. A study by Joshi et al. (2021) showed that the aryl hydrocarbon receptor (AHR) gene was hypomethylated and its expression increased in endometrial samples from women with endometriosis and low expression of integrin αvβ3, an integrin that has been previously associated with implantation failure [86]. AHR is a basic helix-loop-helix (bHLH) transcription factor protein that dimerizes with the AhR nuclear translocator (ARNT) protein. Nuclear translocation of AHR complex binds to the xenobiotic response elements (XREs) at the promoter regions to integrate environmental and metabolic signals that control transcriptional programs that may evoke in abnormal immune responses, toxicity, and carcinogenesis [171−173]. The putative role of AHR in endometriosis involves activation of migration and invasion of endometrial stromal cells by exposure to endocrine disrupting chemicals [174].

An in vitro study of endometrial stromal cells showed that E2 can extensively change the DNA methylome resulting in an "open" state, and modify the transcriptome of these cells, with implications for implantation success. In endometrial stromal cells from patients with endometriosis the changes induced by E2 were different but involved genes and pathways known to be associated with the endometriotic phenotype. These data implicate inherent aberrant responses to hormones that impact the epigenetic landscape and resulting transcriptome of endometrial stromal cells leading to infertility in patients with endometriosis [175].

In conclusion, high-throughput technologies have allowed comprehensive and unbiased genome-wide profiling of endometriosis lesions, primary stromal endometriotic cells, and in endometrium from patients and controls, uncovering differentially methylated genomic regions and novel DNA methylation signatures that could be linked to disease. The most predominant changes in DNA methylation seen among the different studies were in genes encoding transcription factors, hormonal regulators, and immune factors. However, generalized conclusions are difficult to generate due to the broad differences in the implicated genes seen when comparing studies. Reasons for the observed differences include heterogeneity of lesions and alterations due to the influence of menstrual cycle hormones or hormonal treatments [99]. Guidelines to ensure that tissues are obtained in a standardized fashion across research centers to allow for meaningful comparisons have been discussed elsewhere [176,177]. Despite these limitations, the available evidence gathered by the genome-wide assessments of DNA methylation validate previous observations while also uncovering new players in endometriosis that could serve as therapeutic targets of a new generation of epidrugs that target specifically DNMTs [178]. Importantly, these studies provide evidence for a crucial role of epigenetics in the regulation on the normal cycling endometrium and suggest that perturbations in these processes could result in endometrial pathologies such as implantation failure, pregnancy loss, and endometriosis.

23.2.2 Histone modifications in endometriosis and endometrium

Histones are basic proteins with a globular shape except their protruding tails, which are rich in the positively charged amino acids arginine and lysine. Post-translational modifications of histones (PTMs) determine chromatin states and regulate gene expression. The most common PTMs include methylation, acetylation, phosphorylation, sumoylation and ubiquitylation, but other PTM of relevance have been reported (as reviewed in Ref. [179]). The covalent modifications of histones, referred to as *histone marks*, are mediated by the balanced interplay of enzymes including histone deacetylases (HDACs), histone acetylates (HATs), histone demethylases (HDMTs), and histone methylases (HMTs) [180].

Histone acetylation is a versatile and widespread PTM that involves the deposition of acetyl groups into the ε-amino groups of distinct lysine residues at the N-terminal [181]. Acetylation occurs in selected lysine (K) residues such as K5, K8, and K12 at histone 4 (H4), and K9, K14, K18, and K27 at histone 3 (H3). The addition of an acetyl group alters the charge of the histone protein by neutralizing the lysine positive charge, weakening the histone: DNA interaction. The acetylation of histones is a highly dynamic process that is regulated by the opposing action of HATs and HDACs, which add or remove acetyl groups from amino acid residues on the histones, respectively. Hyperacetylation of lysine residues changes the chromatin structure to an open state (*euchromatin)*, which facilitates the access of transcriptional machinery promoting gene expression. In contrast, deacetylation accompanied by methylation of amino acids in histone tails causes a closed and inaccessible state of the

chromatin (*heterochromatin)*, causing gene repression. Deacetylation of histones is an important mechanism for silencing the expression of tumor suppressor, cell-cycle progression, proliferation, differentiation, and apoptosis-related genes. Aberrant HDAC levels or sustained HDAC activity results in histone deacetylation, closed chromatin states, and silencing of gene transcription, leading to the activation of pro-proliferative and pro-survival mechanisms [181]. Numerous HDAC inhibitors (HDACi) have been approved to be used in the clinic by their potential to evoke cell growth arrest, apoptosis, and differentiation in different cancer types [182].

Histone methylation is another key epigenetic modification that plays a role in several cellular processes via modulation (activation or silencing) of transcriptional activity [183,184]. Individual lysine, arginine, and histidine residues may be methylated at different levels: mono, di, or tri-methylation. Each methylation level, in combination with other neighboring methylated residues, has been shown to induce particular effects on the level of expression of nearby genes [185]. The methylation of lysine residues occurs at the ε-amino groups at the N-terminal in histones, causing changes in the chromatin structure to allow (or block) access to the transcriptional machinery. In general, methylation in histone 3 lysine 4, 36 and 79 (H3K4me, H3K36me, H3K79me) is related to gene expression, whereas methylation in histone 3 lysines 9, 20 and 27 (H3K9me, H3K27me, and H4K20me) are associated with gene repression [186]. In combination, histone marks exert direct consequences in the cellular transcriptome and therefore modulate the phenome [187] (Fig. 23.2).

Histone methyltransferases (HMTs) are the regulatory enzymes responsible for catalyzing the addition of one, two, or three methyl groups from s-adenosylmethionine to ε-amino group of specific amino acid (lysine, arginine) histone residues. Lysine methyltransferases (KMTs) are structurally characterized by a conserved SET (Su(var)3-9, Enhancer of Zeste, Trithorax) domain that confers the specific enzymatic activity. KMTs are classified into eight families (KMT1−KMT8) according to substrates specificities and enzymatic activities, and include EZH2, SET-9, SETDB1, and SUV39H2 [188]. Substantial efforts in the cancer research field are geared toward elucidating whether HMTs

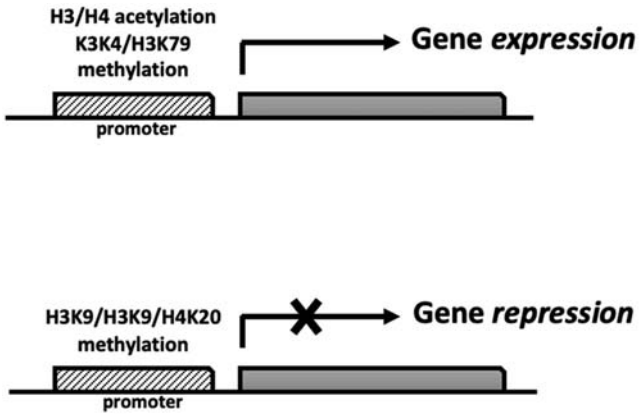

FIGURE 23.2

Distinct active and repressive histone marks at the promoter regions of genes. (A) H3/H4 acetylation and H3K4 or H3K79 methylation are histone marks associated with gene expression. (B) H3K9, H3K27, and H4K20 methylation are histone marks associated with gene repression.

represent effective treatment targets with a relatively safe toxicity profile [189]. In fact, blocking HMTs have been shown to change the expression of less than 2% of the transcriptome [190].

Global levels of histone methylation and enrichment in specific histone marks have been shown to be associated with clinical outcomes in various cancers [191]. In combination, post-translational modifications of histones regulate the cellular transcriptome and phenome. The histone modifications that have been predominantly implicated in endometriosis, namely acetylation and methylation, are discussed next.

23.2.2.1 Histone acetylation in endometriosis

Although there is robust evidence suggesting a role of histone modifications in cancer, their role in benign disorders such as endometriosis has not been thoroughly investigated. Munro and colleagues were the first to report in 2010 on the global histone acetylation levels in normal endometrium during the menstrual cycle [93,192]. They reported that acetylation levels of H2AK5, H3K9, H3K14/18 and H4K8 were increased in the early proliferative phase and post-ovulation when relatively higher gene transcription levels occur compared to the late secretory phase. We have also studied selected histone marks in endometrium from patients and controls and observed statistically significant differences in the levels of H3K9ac and H3K16ac in lesions compared to endometrial samples, adding to the growing body of evidence that histone modifications are also involved in the regulation of the endometriosis transcriptome. A better understanding of the role of these histone marks on normal endometrial physiology will allow the identification of potential new therapeutic targets for diseases involving the endometrium, including endometriosis.

Studies that support the role of histone acetylation as a mechanism that may contribute to the molecular dysregulations in endometriosis have been reviewed elsewhere [154,193]. We have previously shown by immunohistochemistry (IHC) of a tissue microarray (TMA) that HDAC1/2 proteins were expressed in a substantial proportion of lesions and also eutopic endometrium from patients, and their expression levels varied according to lesion localization [194]. The highest proportion of strong HDAC1 immunostaining was seen in ovarian, skin, and gastrointestinal lesions, and of HDAC2 in skin lesions and endometrium from patients with endometriosis. Other studies also utilizing a TMA [195] have confirmed the overexpression of HDAC1 (but not HDAC2) in endometriotic lesions compared to control endometrium. The expression levels of HDAC1 and HDAC2 were significantly correlated in endometriotic lesions but not in endometrium of women with endometriosis or controls. This suggests that HDAC1 and HDAC2 are co-regulated within the lesions by the same inflammatory and hormonal cues. HDAC1 and HDAC2 are conserved enzymes that are part of the Sin3a repressor complex, thus their combined expression and regulation is therefore key to their function as co-regulators of gene expression [196]. The Sin3a-HDAC complex regulates expression of steroidogenic acute regulatory protein (*StAR*) gene and Snail-mediated repression of *CDH1* [197,198]. Loss of Krupper like factor 11 (KLF11)-Sin3a/HDAC epigenetic regulation triggers a profibrotic state via increase expression of Collagen 1 alpha 1 (Col1a1) in an animal model of endometriosis [199]. The profibrotic phenotype and endometriotic lesion development in a *KLF11* knockout mice model was reverted by using a histone acetylase inhibitor (HATi). Adding complexity to the previous defined Sin3a-HDAC deacetylation mechanism, the Sin3a/HDAC complex was shown to interact with the lysine specific-demethylase 1 (LSD1) leading to dysregulation in the expression of the cyclin-dependent kinase inhibitor p21 [200]. This study uncovered the complex interplay between histone epigenetic erasers (HDACs and LSDs) to control gene transcriptional activity. Other lines of evidence demonstrated that HDAC1 and HDAC2

have overlapping functions in cell cycle regulation [201,202]. Silencing of *HDAC1* and *HDAC2* leads to cell cycle arrest and up-regulation of the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$ [203–205]. This is in accord with reports of HDACi inducing decreased proliferation of an endometriotic cell line by upregulating $p21^{waf1/cip1}$ [206,207]. We have reported that the basal HDAC1/2 gene expression was higher in endometriotic versus normal endometrial or epithelial cells [194]. Also, treatment with ovarian steroid hormones—estradiol (E2) and progesterone (P4)—significantly downregulated the expression of HDAC1 in endometrial epithelial cells. In contrast, HDAC2 levels were upregulated by E2 and downregulated by E2+P4 in endometrial stromal cells. This indicates that HDACs are responsive to hormones and thus aberrant expression of hormone receptors, as seen in lesions, would lead to their inappropriate regulation. Recently, Mai et al. (2021) showed that silencing of HDAC2 diminished endometriotic lesions size and suppressed cell proliferation and induced apoptosis in endometriotic cells via upregulation of the hepatocyte nuclear factor 4α/AT-rich interactive domain 1A (HNF4A/ARID1A) axis [208]. Work by Kim et al. (2019) has reported a reduced expression of HDAC3 in eutopic endometrium of infertile women with endometriosis [209]. In addition, they found an increased expression and deposition of fibrillar collagen in the uterus of HDAC3 conditional KO mice, suggesting that loss of HDAC3 may trigger fibrogenesis and impairs ECM turnover in endometriosis.

Evidence continues accumulating in support for a role of HDACs in the pathophysiology of endometriosis. Our previous work showed that endometriotic lesions are characterized by H3K9 and H3K16 hypoacetylation and that the promoter regions of *HOXA10*, *ESR1*, $p21^{waf1/cip1}$, and *CDH1* were associated with histone hypoacetylation, while *SF-1* was hyperacetylated, which correlated with their status in lesions (silenced vs. expressed, respectively) [210]. These studies suggest that endometriosis etiology may be partially explained by epigenetic regulation of gene expression due to dysregulations in the expression of HDACs and point to the possibility of using HDACi for treatment of endometriosis, as discussed in Section 3.1.

23.2.2.2 Histone methylation in endometriosis

Research on the expression profile of HMTs in endometriosis is still limited [211–217]. In order to assess whether histone methylation dysregulations could play a role in endometriosis, we first measured the levels of histone methylation marks in tissues from patients (lesions, endometrial biopsies), and endometrial biopsies from control women (surgically shown not to have endometriosis) via commercially available immunoassays [210]. We observed that endometriosis tissues are characterized by hypoacetylation of H3K9 and H3K16, and hypermethylation of H3K9, H3K4, H3K27 compared to control tissues (obtained from women without endometriosis) [218]. We also determined EZH2 and H3K27me3 nuclear positivity in endometriotic and endometrial tissues from cases and controls by immunohistochemistry (IHC) of an endometriosis-focused TMA. The proportion of EZH2 and H3K27me3 positive nuclei was measured by histological type (glands vs. stroma) and by lesion type (ovarian, peritoneal, fallopian, and extra-pelvic), and compared to endometrium from cases and controls. EZH2 positive immunostaining was higher in the glands and stroma of the endometriotic lesions relative to endometria from patients and controls [214]. Similarly, we observed a high percentage of H3K27me3-positive nuclei in stroma of lesions compared to proliferative endometrium from cases [212]. Next by ChIP-qPCR approach, we observed differential enrichment for H3K27me3-repressive histone mark in promoter regions of candidate genes in endometriotic lesions relative to endometria from healthy control. In particular, *ESR1* had the

highest level of H3K27me3 enrichment (69%, 9/13), followed by *CDH1* (46%, 6/13) and *PGR* (31%, 4/13) in the endometriotic lesion cohort [214]. In support of our work, Samadieh et al. (2019) observed high enrichment of H3K27me3 repressive mark on the *HOXA10* promoter region in ovarian endometriotic lesions relative to both eutopic endometria from patients and healthy controls in the secretory phase [219]. A recent study reported high expression of EZH2-H3K27me3 axis and known regulators of the PRC2 complex (e.g., JARID2 and miRNA-155) in endometriotic lesions and endometrial epithelial cells treated with peritoneal fluid from women with endometriosis [217]. Lin et al. (2019) found that hypoxic conditions modify the epigenetic profile of primary endometriotic stromal cells leading to abnormal expression of the anthrax toxin receptor 2 (ANTRX2) by decreasing EZH2 expression and further loss of H3K27me3 repressive mark at the gene promoter level [220]. Work by Wen et al. (2020) found a potential involvement of the mixed lineage leukemia 1 (MLL1), a histone methyltransferase, in endometriosis-associated infertility. Expression of MLL1 and enrichment of its catalytic product-H3K4me3 mark were reduced in eutopic endometria from patients relative to the eutopic endometria from controls [215]. Using ChIP-Seq and ChIP-qPCR approaches, the cytochrome *c* oxidase subunit 4I 2 (COX4I2) and hypoxia inducible factor alpha 2 (HIF-2α) were identified as epigenetic targets of MLL1 in human endometrial cells. Loss of the MLL1/COX4I2/HIF-2α axis impaired endometrial decidualization and impacts pregnancy success in mice.

Recently, Rytokonen et al. (2022) found that H3K4 demethylases (KDM5A-C) were upregulated in normoxia, whereas KDM5A showed the most significant hypoxic downregulation in endometrial stromal cells. ChIP-Seq approach showed H3K4me enrichment in promoter regions of hypoxic upregulated genes. Further bioinformatic analyses using available endometriosis transcriptomic datasets revealed that a subset of genes involved in cell survival (e.g., *KLF2*, *NUAK1*, *NDRG1*, and *SGK1*) and angiogenesis and fibrosis (*SERPINE2*, *S100A10*, *TNFSF4*, and *ACTA2*) pathways were enriched by H3K4me3 and upregulated in the endometriosis [221]. Thus far, the available data suggest that HMTs and its post-translational modifications in histone tails contribute to the etiology of endometriosis, and HMTs such as EZH2 represents a potential epigenetic therapeutic target, as discussed further in Section 3.

23.2.3 Non-coding RNAs in endometriosis and endometrium

MicroRNAs (miRNAs) are short non-coding RNA sequences, usually 21-22 nucleotides in length, which play important roles in cellular processes by regulating gene expression. miRNAs bind to the 3′ untranslated region (3′-UTR) of the targeted transcript, which leads to its specific degradation thus preventing their translation. In the normal endometrium, there are menstrual cycle-specific miRNAs expression profiles. Aberrant expression of miRNAs has been proposed as a key mechanism whereby cellular pathways are dysregulated, leading to pathologies including endometrial cancers, infertility, and endometriosis [222,223]. Other non-coding RNA moieties involved in gene regulation include long-non-coding RNAs (lncRNA) and circular RNAs (circRNA).

In recent years, there has been increasing focus on understanding the role of miRNAs, lncRNAs and cirRNAs in the pathogenesis of endometriosis via global profiling technologies in endometriotic lesions and in the endometrium of women with or without endometriosis (reviewed elsewhere [224–227]. Early studies showed that pathways with relevance for endometriosis, including inflammation, pain, proliferation, apoptosis, and signaling pathways (e.g., PI3K/AKT, P38-MAPK, ERK1/2-

MAPK and Wnt-β catenin, c-Jun, CREB-binding protein, AKT, and cyclin D1) are regulated by miRNAs [225,228,229]. A recent review by Kolanska et al. (2021) provides a comprehensive summary of the early findings as they relate to normal endometrial function and endometrial disorders [222]. More recently, lncRNAs LINC01133 and LncRNAH19 were shown to be overexpressed in lesions; the former was shown to promote proliferation inhibit migration, and to affect the protein expression of p21 and Cyclin A [230,231]. Hu et al. (2022) reported that expression of miR-106b-5p was upregulated in endometriotic lesions and its overexpression increased the proliferation, migration, and EMT of human endometrial stromal cells, while downregulating the levels of PTEN [232]. A study by Hajimaqsoudi et al. (2020) founds that miR-125b was significantly overexpressed in ectopic endometrium and negatively correlated with TP53 expression in both the ectopic endometrium and eutopic samples compared with endometrium from controls [233]. A study by Moustafa et al. (2020) evaluated circulating miRNAs in serum of patients prior to surgery. They found a significantly higher expression levels of miR-125b-5p, miR-150-5p, miR-342-3p, and miR-451a and lower expression of miR-3613-5p and let-7b in serum from women with endometriosis compared to controls, with no evidence for influence of menstrual cycle phase or hormonal medications on their expression levels [234]. Finally, Yin et al. (2022) recently identified a network of lncRNAs/circRNAs-mRNA modulating EMT and immune pathways [235].

Together, these studies have provided strong evidence to show that non-coding RNAs regulate endometriotic-specific mechanisms, including anti-apoptotic, angiogenic, inflammatory, and invasive cellular pathways. However, although several of these RNA moieties have been shown to be aberrantly expressed in biospecimens (cells/tissues and serum/plasma) of endometriosis patients, further investigations are needed to obtain a strong consensus about the specific miRNAs molecules that are ultimately involved in endometriosis. The review by Kolanska et al. (2021) provides a comprehensive summary of the miRNAs that have been shown to be deregulated in endometriosis, their function, and hormonal regulation [222].

Compared to endometriotic lesion profiling, fewer studies have shown significant differences between eutopic endometrium from women with endometriosis compared to endometrium from controls. Early studies have identified miRNAs that are downregulated in early secretory endometrium from women with endometriosis compared with endometrium from controls (miR-9, miR-34b and miR-34c-5p, let-b7, miR-15, miR16, miR-125, miR-483-5p, miR-629-3p). The predicted targets include genes involved in cell-cycle regulation (cyclin E1, cyclin E2, *CDK4*, *CDK6*, and *CDC25A*), apoptosis (*BCL*-2), *IGF2,* inflammation, and NK cell cytotoxicity [222]. Recently, Rezk et al. (2021) reported that miRNA 34a regulates expression of *SIRT-1*, *BCL2*, *BAX*, *BCL-xL*, and *FOXO-1* in endometrial tissues from patients compared to controls [236]. A study by De Silva et al. (2020) showed that miR-532-3p was down-regulated in cumulus cells from severe endometriosis patients compared with both mild endometriosis and infertile controls, suggesting its role in endometriosis-related infertility [237].

Although collectively these studies have uncovered non-coding RNAs that are differentially expressed in tissues from women with endometriosis it is noteworthy that only a few of them were identified by more than one study. These differences may be ascribed to multiple factors, including selection of patients and controls and technologies used for the molecular profiling, tissue heterogeneity, and hormonal influences [238,239]. Despite the observed differences, follow up studies have shown that non-coding RNA-driven regulation appears to be involved in processes relevant to the endometriotic phenome, including cell adhesion and invasion, inflammation, cellular

proliferation, apoptosis, angiogenesis, progesterone resistance and extracellular matrix remodeling [240].

23.3 Translational aspects of the endometriosis epigenome

Despite decades of research, there is a gap in the knowledge of the molecular mechanisms at play in endometriosis, and as a result this debilitating condition has no cure and non-invasive specific diagnostic biomarkers have not been yet identified. The evidence summarized above support the notion that endometriosis is an epigenetic disease. Beyond contributing to our current understanding of how endometriosis develops, this rapidly increasing body of knowledge can be translated to solutions for women with the disease and for the health practitioners in charge of their clinical care, who currently have few tools available to diagnose, treat and determine risk of development of endometriosis. Following, a review of the literature on the potential use of the epigenome of endometriosis for development of new treatments and improved, highly specific diagnostic methods.

23.3.1 The endometriosis epigenome as therapeutic target

It is well established that epigenetic signatures (differential methylation in promoter regions, specific histone mark, miRNAs profiles) vary between diseased and normal tissues in conditions such as cancer, and that these patterns are reversible, contrary to DNA mutations. Whether epigenetic factors are a cause or an effect of endometriosis development has not been fully determined, and warrants future investigations. Additional evidence that there are distinct epigenetic signatures in endometriosis will result in more support for investigating pharmacologic modulation with epidrugs and mechanistic studies to fully understand the molecular pathways involved and discover novel therapeutic targets. For many of the epigenetic enzymes known highly specific small molecule inhibitors have been developed, and several are already approved by the Federal Drug Administration (FDA) (e.g., DNA methyltransferase inhibitor 5-azacytidine, Vidaza) or in clinical trials mainly for cancer (clinicaltrials.gov). Despite limitations and challenges related to epidrugs, mainly due to their non-canonical effects, research efforts still continue to determine the optimal doses required for clearly defined molecular outcomes (e.g., global vs., targeted effects), understanding interactions with other epigenetic or molecular pathways, and maximizing tissue specificity [241].

23.3.1.1 DNA methyltransferases

As reviewed by Ding et al. (2020), DNMT's are aberrantly expressed in endometriotic cells and tissues [242], and animal model studies have shown that treatment with DNMTi leads to re-expression of genes that were silenced in lesions [123]. Therefore, these studies suggest that DNMTi could potentially be used to reset DNA methylation signatures in the lesions, thereby changing expression levels of genes involved in its etiopathology. A search in clinicaltrials.gov for "endometriosis" and "epigenetic drug" uncovered only one trial of the drug EX-527 (Selisistat), a specific inhibitor of the histone deacetylase Sirtuin-1 (SirT1), entitled *SIRT-1 Antagonism for Endometrial Receptivity (SAFER)* (NCT NCT04184323), which was withdrawn due to lack of funding. There are, however, 12 ongoing studies on azacytidine for cancer. From a biological standpoint, endometriosis and cancer share key hallmarks, including increased migration and invasion, angiogenesis, and EMT, in addition to decreased apoptosis and altered immunological and oxidative stress responses [243], indicating the

potential for cancer epigenetic drugs to be effective also for endometriosis. Despite the fact that there are clinically available DNMTi for cancer, there are still unresolved issues (e.g., side effects profile) that preclude their general use for benign, non-lethal conditions such as endometriosis [244]. As the epigenetics field continues to evolve more specific targets may be discovered that are at play in both cancer and endometriosis, making it possible in the future to implement epigenetic-based treatments with less side effects affecting compliance.

23.3.1.2 Histone deacetylases

Several HDAC inhibitors have been approved for treatment of multiple cancers, as reviewed in Ref. [182]. Silencing of HDAC activity induce apoptosis, activation of tumor suppressor genes, and cell-cycle arrest. However, due to lack of specificity and substantial side effects, the clinical use of HDACi is still controversial. Recent efforts to overcome this limitation intend to use HDACi in combination therapy. Thus far, the pharmacological benefits of HDACi in endometriosis have been tested as a single agent using in vitro and in vivo models. HDACs are aberrantly expressed in endometriosis tissues, leading to a diseased phenotype characterized by increased cell proliferation and invasion [201]. In vitro studies have shown that treatment with HDACi (Trichostatin A—TSA and Valproic acid—VPA) leads to histone acetylation increments resulting in reactivation of silenced genes (*CDH1*, *p16*, *p21*, and cycle checkpoint kinase 2, *CHK2*), suppression of *BCL2* and *BCL*-XL, and attenuation of invasion and proliferation of endometriotic cells [206,245]. In addition, treatment of endometriotic cyst stromal cells with VPA reduced proliferation and induced apoptosis [245], and upregulated the expression of death receptor 6 (DR6), involved in the anti-apoptotic phenotype of endometriotic lesions [246]. Similarly, VPA treatment in endometrial stromal cells inhibit histone acetylation in the CYP19 (aromatase) promoter region, reducing the cell proliferation capacity, while inducing apoptosis [247]. Work by Seo et al. (2018) showed that TSA induces apoptosis by upregulation of the nonsteroidal anti-inflammatory drug-activated gene 1 (NAG-1) in human endometriotic stromal cells (HESC) [248]. Treatment with Suberoylanilide hydroxamic acid (SAHA) and romidepsin led to decreases in the expression of *GPER*, a G-protein estrogen receptor, in endometriotic epithelial cells [207]. Moreover, studies using animal models have shown significant reductions in lesion development, decrease in fibrosis and deposition of collagenous ECM, and improvement of hyperalgesia by TSA and SAHA treatment [199,245,249]. Collectively, these data provide additional evidence in support of the use of HDACi as therapeutic agents for endometriosis.

23.3.1.3 Histone methyltransferases

Inhibitors to histone methyltransferase (HMTi) are emerging as the most recent innovation in cancer therapeutics. In 2020, Tazemostat (EPZ-6438), an EZH2 inhibitor was approved by the FDA as the first marketed "epidrug" for the treatment of follicular lymphoma and soft tissue sarcoma [250]. Other pharmacological agents are still in developmental stages or undergoing clinical trials [251]. Current investigations in the epigenetics field are moving forward with the development of allosteric modulators and proteolysis targeting chimeras (PROTACs) as promising therapeutic strategies for targeting HMTs (as reviewed in Refs. [252,253]). Among all the HMTs enzymes, EZH2 is the most promising target considering its oncogenic role during carcinogenesis. EZH2 is the HMT responsible for catalyzing the addition of three methyl groups to the lysine residue 27 at histone 3. Its canonical function is to mediate a transcriptional repressed state [254]. EZH2 is commonly used as a pathophysiological marker for detection of precancerous and cancerous lesions [255,256]. As reviewed by Duan et al.

EZH2 regulates endometriosis-relevant biological functions such as proliferation, migration, epithelial to-mesenchymal transition (EMT), and angiogenesis during the onset and progression of cancer [257]. Silencing of EZH2 was shown to significantly decrease proliferation and invasion rates, and to increase the expression of pro-apoptotic related genes in endometrial and ovarian cancer [258,259]. Work by Zhang et al. (2017) found that EZH2 induces EMT in endometriotic epithelial cells [260]. Pharmacological inhibition of EZH2 with GSK126 and 3-Deazaneplanocin A (DZNep) reduced the expression of EMT related genes (e.g., snail, slug, vimentin, and N-cadherin) in human endometriotic cells. In vivo, administration of DZNep, significantly attenuated endometriotic lesion development, improved hyperalgesia, and diminished fibrosis. We have shown that EZH2 was highly expressed in endometriotic cells and that there was significantly higher proportion of H3K27me3 nuclear positivity in the stroma of lesions compared to control tissues (endometrium from cases and controls) [212]. In addition, we showed that pharmacological inhibition of EZH2 via GSK343, a selective EZH2 inhibitor, decreased the proliferative and migratory capacity but not the invasive behavior of human endometriotic stromal cells [214]. Using a pre-clinical model of endometriosis, we observed that GSK343 reduced the size and progression of endometriotic lesions [216]. These results suggest that H3K27me3 plays an important role in regulating the expression of candidate genes in endometriosis and thus a potential therapeutic target. However, additional studies are warranted to uncover new targets that are epigenetically regulated by EZH2 relevant to endometriosis pathophysiology. Moreover, the new EZH2 selective inhibitor SHR2554 already undergoing clinical trials for lymphoma, prostate, and breast cancer (www.clinicaltrials.gov; NCT 04407741) could be tested in preclinical in vitro and in vivo models of endometriosis. Other HMTi currently under investigation include Chaetocin, which specifically targets SUV39H1, the HMT responsible for setting H3K9me3 [261]; and 5′-deoxy-5′-methylthioadenosine (MTA) and BIX-01294, both targeting SET-1, the HMT that sets H3K4me3 [262,263]. To date, there are no published pre-clinical studies of HMTi in endometriosis.

23.3.1.4 Histone demethylase 1 (LSD1)

The lysine specific demethylase for H3K4me3, Histone demethylase 1 (LSD1), has been shown to be elevated in endometriosis, and therefore suggested as therapeutic target. Pharmacological inhibition of LSD1 by tranylcypromine (TCA) modulated invasion and proliferation of endometriotic cells in vitro, reduced lesions' size, and alleviated hyperalgesia in a murine model [211,213]. Thus far, this is the only study investigating the potential use of HDMi as a therapeutic target in endometriosis. A search in clinicaltrials.gov for "LSD1" reveals nine trials in the recruitment phase for the drug seclidemstat (SP-2577), a reversible, noncompetitive inhibitor of lysine-specific demethylase. These studies aim to determine the pharmacological benefits of LSD1 in thrombocytopenia, Erwing sarcomas, chronic myelomonocytic leukemia, and small-cell lung and prostate cancer. Additional studies are warranted to elucidate the potential use of seclidemstat as an "epidrug" in endometriosis.

23.3.1.5 miRNA-based therapies

The potential for developing molecular therapies based on differential miRNA levels has been discussed elsewhere [224,225]. The general notion of this approach is that once it is convincingly shown by many well-designed studies that one or more miRNA molecules are either over or under-expressed in the disease tissue, it would be feasible to target them by either blocking or mimicking their action. Progress in other fields (cancer, inflammatory bowel disease) has been driven by studies that convincingly show differential expression compared to healthy tissue counterparts and gather data

confirming miRNA molecules' functionality in regulating relevant pathogenic pathways. In vitro and in vivo studies will be necessary to test the efficacy of libraries of synthetically designed oligonucleotides, before advancing to the clinical trials phase. Other challenges of targeting miRNAs therapeutically include ensuring their stability in serum, finding the appropriate vector and administration route to prevent degradation and rapid renal clearance, limiting off-target effects in other organs or tissues (endometrium, GI), and increasing their cellular uptake [264]. Due to the increasing interest in studying the levels of and functional effects of miRNAs in endometriosis [265–268], the base knowledge that is required to move forward the possibility of using mi-RNA-based therapies is expected to increase substantially in the future [269].

Recently, two studies have assessed the potential use of miRNA molecules as therapy for endometriosis. A pre-clinical study by Sahin et al. (2018) found that treatment of mice with endometriosis miRNA Let-7b resulted in smaller lesions and downregulated expression was noted in several on genes known to promote the endometriosis phenotype (ER-α, ER-β, Cyp19a, KRAS 4A, KRAS 4B, and IL-6) [270]. The authors concluded that treatment with let-7b is a promising novel approach based on its pleiotropic role regulating key pathways such as estrogen signaling, inflammation and growth factor receptors. Another pre-clinical study found that treatment with a miR-451 inhibitor reduced endometriosis lesion size despite not causing a difference in the number of lesions that developed compared to controls in mice [271]. Treatment significantly changed the expression levels of genes related to endometriosis including *YWHAZ*, *CAB39*, *MAPK1*, β-catenin, and *IL*-6. Authors concluded that systemic treatment with a miR-451a inhibitor is a promising therapy for endometriosis that simultaneously affects multiple pathways driving the disease. Regarding clinical trials in humans, a search in clinicaltrials.gov showed that there are no ongoing studies of miRNAs as therapeutics for endometriosis registered.

23.3.2 The epigenome as diagnostic target for endometriosis

The standard method currently employed for detecting endometriosis is laparoscopic surgery, during which the pelvis is scanned for visible lesions, and histopathological confirmation of the ectopically growing lesions (e.g., presence of glands and stroma). Because a definitive diagnosis of endometriosis requires a surgical intervention that may not be accessible to all, combined with other social and cultural barriers to diagnosis, it is not surprising that there are substantial delays in the diagnosis of endometriosis (7 years in average worldwide) [14]. Thus, there is a critical need to discover specific non-invasive markers to help reach an early and precise diagnosis in affected women and teens [272–274]. Epigenetic signatures represent a novel opportunity to develop specific tests for endometriosis, as discussed next.

23.3.2.1 miRNAs as non-invasive diagnostic biomarkers

The study of circulating miRNAs as biomarkers of endometriosis is an emerging field of research, with great potential to not only inform the pathophysiology but also to become the basis for non-invasive diagnostic assays. Efforts to profile miRNA in tissues and biofluids obtained from patients with endometriosis have been described in great detail in Refs. [226,275,276]. Detection of miRNAs can be done using either endometriotic tissues or, preferably samples that would allow for a non-invasive assay (plasma, peritoneal fluid, urine, saliva, or menstrual blood). The mechanisms whereby miRNAs are released into different biofluids have been reviewed elsewhere in this book. Regardless of

their source—tissue injury, chronic inflammation, cell death, or microvesicles—their stability and accessibility make miRNAs ideal diagnostic biomarkers. From 2013 to 2018 there were few published studies that identified differential circulating levels of miRNAs in serum or plasma of women with endometriosis that have been reviewed in detail elsewhere [226,229,275,276]. Since 2018, there have been several new reports of diagnostic signatures based on miRNA expression profiles. Zafari et al. (2021) showed that the levels of miRNAs 199b-3p, 224-5p, and Let-7d-3p are higher in plasma of endometriosis patients compared to controls [277]. A study by Pateisky et al. (2018) measured changes in plasma miRNA and assessed their potential as non-invasive biomarkers for endometriosis. They showed that has-miR-154-5p, which alone or in combination with has-miR-196b-5p, has-miR-378a-3p, and has-miR-33a-5p and the clinical parameters of body mass index and age, are potentially applicable for non-invasive diagnosis of the disease [278]. A study by Qi et al. (2019) measured miRNA levels and level of expression of target genes in tubal endometriosis samples. They found 17 significantly differentially expressed miRNAs and 4343 potential miRNA-target genes. miRNA-21 was overexpressed and was found to directly participate in regulating and activating a number of EM-related signaling pathways such as Wnt and TGF-β [279]. A study by Farsimadan et al. (2021) reported a diagnostic signature composed of SNP variants of three miR-146a, miR-149, and miR-499 detected in genomic DNA from endometriosis patients [280]. A study by Wu et al. (2022) investigated the miRNA expression profile of serum exosomes from women with endometriosis compared with normal controls and uncovered 26 genes as significantly up-regulated and 19 genes as significantly down-regulated in endometriosis patients. Further analysis indicated that these differentially expressed miRNAs in serum exosomes may be involved in the pathogenesis of endometriosis and are related to the severity and certain symptoms of endometriosis [281].

A close examination of the reported miRNAs shows that none were identified by more than one study, and for some (miR 542-3p and miR 141*) there were discrepancies in the direction of the regulation. As discussed in Section 2.3, there are also conflicting reports about miRNA molecules that are differentially expressed in endometriotic tissues, and only a few (e.g., miR-125b, let-7b) have been identified by multiple studies. As recently stated by Saare et al. (2017) and Muga et al. (2019), the challenge in this field remains the heterogeneity of endometriotic tissues, the influence of ovarian steroid hormones, and the known differences in the expression of miRNAs depending on the biospecimen studied (tissues, blood), with important implications for the development of a specific tests based on these molecules [226,239]. As reviewed by Nisenblat et al. (2019), miRNAs demonstrated modest sensitivity and specificity as diagnostic tests or triage tools for endometriosis [282]. Circulating miRNAs demonstrate diagnostic potential, but stringent, standardized methodological approaches are required for the development of a clinically applicable tool.

A search in clinicaltrials.gov uncovered three clinical studies of the potential of blood miRNAs as diagnostic biomarkers for endometriosis: *Evaluation of miRNAs in Endometriosis* (NCT04728152), *Multicenter Validation of the Salivary miRNA Signature of Endometriosis* (NCT05244668), and *The EMPOWER Study: Endometriosis Diagnosis Using microRNA (EMPOWER)* (NCT04598698) are all active and recruiting. The former is based on results of a saliva miRNA diagnostic profile recently published [283–286]. The authors showed that a panel of 109 miRNA had 96.7% sensitivity, 100% specificity, and 98.3% AUC as a diagnostic test for endometriosis. The latter is based on findings of a diagnostic panel consisting of six miRNAs in serum (high expression: miR-125b-5p, miR-150-5p, miR-342-3p, and miR-451a; low expression: miR-3613-5p and let-7b. The authors report an AUC of 94% for a classifier combining these differentially expressed miRNAs. However, these results are

limited in that they have not been validated in an independent patient cohort. In sum, it is encouraging to see three recent trials attempting to validate miRNA signatures in non-invasive biofluids in a larger sample size and a more representative patient population. Understanding the role of miRNAs in the pathogenesis of endometriosis and determining its potential applicability as biomarkers are considered research priorities in this field [287].

23.3.2.2 Histone marks as diagnostic biomarkers

Because of their stability and heritability, covalent histone modifications, alone or in combination, have been proposed to have diagnostic and prognostic potential [288]. In combination, histone marks function as regulators of the cellular transcriptome and phenome not only of lesions but also of endometrial tissues, which are accessible in a minimally invasive way via an endometrial biopsy. Recent proteomics work using urine samples found elevated levels of H4 with a sensitivity of 70% and specificity of 80% in patients with ovarian endometriosis. Therefore, measurement of H4 levels in body fluids can be further explored to be considered as a potential diagnostic biomarker in endometriosis [289]. In addition, modified histones have been detected in circulating nucleosomes in blood, thus opening the possibility of using them as non-invasive biomarkers [290]. For instance, immunoassays have been developed to measure levels of selected histone marks (H3K4Me2, H3K9Ac, H4PanAc, H3K27Me3, and H3K9Me3) in nucleosomes isolated from serum (NuQ, VolitionRx, Belgium). There are published reports on the value of this test for the diagnosis of colon and pancreatic cancers [291,292], and recently as biomarker to monitor transplant rejection and COVID-19 progression [293–296]. The company, VolitionRx, claims that these assays can identify histone mark signatures that can be specific to cancer cells and that could be used for population screening, diagnosis, risk stratification, and treatment monitoring (www.volition.com). In 2018, VolitionRx completed a prospective study in collaboration with the University of Oxford, England, to was to assess the potential use of the NuQ technology in the diagnosis of endometriosis using a large patient cohort. Results from this study are still unpublished.

Moving forward toward the development of a specific and universal diagnostic assay for endometriosis, it is important to conduct well-designed studies with large sample sizes that would allow the statistical power needed to reach valid conclusions. A call for the standardization of methods used to collect, process and store biospecimens used for endometriosis research has been published by the World Endometriosis Research Foundation (WERF) and the World Endometriosis Society (WES), to ensure that future studies conducted in different research centers around the world are comparable and thus data can be combined in efforts to validate the most significant findings across populations [176,177].

23.4 Summary

Although compared to other fields, the number of studies linking endometriosis to epigenetic mechanisms is not extensive, there is nevertheless convincing evidence in support of a role of epigenetics in this gynecologic condition. The initial observations of higher levels of epigenetic enzymes and its products in cell lines and lesions has been followed up by pre-clinical studies confirming the effects of epidrugs on the re-setting of epigenetic signatures and modulation of key cellular pathways. Lesions appear to have a characteristic histone modification (e.g., methylation, acetylation) pattern that contributes to gene expression programs and phenotype, and there is strong evidence for DNA methylation

and miRNA signatures in this disease. When deriving conclusions of research conducted using whole lesion tissue it is critical to take in consideration the heterogeneous cellular composition of the endometriotic lesions. These usually consist of a mix or epithelial and stromal endometrial cells, as well as infiltrating inflammatory cells and endothelial cells, thus the observed gene expression or epigenomic signatures represent the result of those complex interactions inherent to this condition. This matter of tissue heterogeneity is discussed by Saare and colleagues (2017) who highlight the importance of standardized guidelines for tissue dissection [e.g., laser capture microdissection (LCM)] and the use of antibodies for proper identification and further isolation of individual cell types that comprise the endometriotic lesions in order to assess their specific epigenetic profiles [239]. In addition, Munro and colleagues (2010) noted that the data obtained when studying whole endometriosis or endometrial tissues are derived from a mixed cell population, which is likely to differ from patient to patient, lesion to lesion, and according to menstrual phase [93]. While it can be argued that it would be necessary to macrodissect tissues to obtain proper conclusions, many downstream molecular studies will be limited by the actual size of samples and studying the cell mix that constitutes a lesion can also be quite informative. New technologies (scRNA-Seq, ATAC-Seq, 10X Transcriptomics) are promising tools to better understand the heterogeneity of endometriotic lesions and uncover novel targets at the cellular level that will offer greater specificity and improved outcomes of targeted therapies. These high throughput technologies together with the standardization of methods for tissue collection, storage and processing published in 2014 by the World Endometriosis Research Foundation will allow a more thorough understanding of the role of epigenetics in endometriosis and identification of the key molecules and pathways at play [176].

Further research is warranted to better define the expression patterns of epigenetic enzymes in different types of lesions and their association to clinical symptoms, including associations between specific epigenetic states and disease severity. Additional work is also needed to confirm and validate endometriosis-specific epigenetic signatures, and to determine whether there are variations among lesion types and clinical presentations (e.g., pain, infertility, pain and infertility). This is relevant for miRNA profiling studies which until now have not yielded a consensus profile with the specificity and sensitivity required for its clinical application. Other potential areas for further research include the role of epigenetic in modulating pain mechanisms, including epigenetic control of nociceptive pathways with obvious translational application [297]. Finally, whether epidrugs could represent a novel, non-hormonal approach for the treatment of endometriosis requires application of knowledge gathered from the cancer epigenomics research field and further experimentation in pre-clinical models to identify unfavorable effects, before they can eventually be tested in clinical trials.

List of abbreviations

3′ untranslated region	3′-UTR
Assay for Transposase-Accessible Chromatin with high-throughput sequencing	ATAC-Seq
Candidate Genes Association Studies	CGAS
Circular RNAs	circRNAs
Deazaneplanocin A	DZNep
DNA methyltransferases	DNMTs

E-cadherin	CDH1
Embryonic ectoderm development domain	EED
Enhancer of zeste homolog 2	EZH2
Epithelial-to-mesenchymal transition	EMT
Estrogen receptor alpha	ESR1
Estrogen receptor beta	ESR2
Estrogen	E2
Extracellular matrix	ECM
Follicular stimulating hormone	FSH
Genome Wide Association Studies	GWAS
Gonadotropin releasing hormone	GnRH
Histone deacetylase inhibitor	HDACi
Histone deacetylases	HDACs
Histone methyltransferase inhibitors	HMTi
Histone methyltransferases	HMTs
Homeobox	HOX
Immunohistochemistry	IHC
Long non-coding RNA	lncRNA
Luteinizing hormone	LH
Matrix metalloproteinases	MMPs
MicroRNAs	miRNAs
Polycomb repressive complex 2	PCR2
Progesterone receptor	PGR
Progesterone	P4
Single cell RNA sequencing	scRNA-Seq
Small leucine rich proteoglycans	SLRPs
Steroidogenic factor 1	SF1
Suberoylanilide hydroxamic acid	SAHA
Tissue inhibitors of matrix metalloproteinases	TIMPs
Tissue microarray	TMA
Valproic acid	VPA
Vascular endothelial growth factor	VEGF
Zinc finger protein suppressor zeste 12	SUZ12

Acknowledgments

I.F.C is supported by grant #R21HD098481 from the Eunice Kennedy Shriver National Institute of Child Health and Human Development and the Puerto Rico Science, Research and Technology Trust. M.C.C is supported by the Ruth L. Kirschstein National Research Service Postdoctoral Fellowship (1F32 HD103314-01A1). We acknowledge the contribution of Dr. Mónica Rodríguez, San Juan City Hospital Ob-Gyn Residency Program, to the literature review.

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CHAPTER

24

Aberrant DNA methylation signatures in gynecological malignancies: Implications for early diagnosis, prognosis, and therapy

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24.1 Introduction

The dawn of molecular biology in the field of epigenetics paved the way for DNA methylation to answer biological phenomena such as genomic imprinting, transposon silencing, X-chromosome inactivation, genomic stability, and diseases. There is cumulative proof that cancerous cells exhibit significantly altered 5mC (5-methylcytosine) patterns as opposed to normal cells [1]. The significance of DNA methylation in cancer epigenome may one day be developed as a potent methylation-based biomarker due to recent breakthroughs, backed up by several traits, including their early and frequent onset in cancer, their simplicity in identification by existing techniques, their stability in fixed specimens over time, their presence in a variety of human fluids, and their cell-type precision nature. However, the latest research has revealed this change to be more dynamic and complex than previously believed as exemplified with the discovery of sixth base of the genome 5-hydroxymethycytosine (5-hmC). It is an intermediate formed during recently discovered DNA demethylation process catalyzed via ten−eleven translocation (TET) family of enzymes [2]. It also plays a significant role in regulation of gene expression in several gynecological malignancy. It is still the most extensively researched epigenetic mechanism and is frequently cited as the classic example of epigenetic imprints.

The female uterus or womb comprises the cervix and is the focal point of a woman's reproductive system. The uterus is linked to the exterior of the body by the vagina through the cervix and the ovaries at its top. The term "vulva" refers to the external genitalia. Gynecological malignancies are caused by the rapid development and division of tumor cells in one of these organs within the pelvis. The five main types are cervical, uterine, ovarian, vaginal, and vulvar cancers. Among all gynecological malignancies, vaginal and vulvar cancer is highly uncommon. All gynecological cancers, except for vulvar, exhibit abnormal vaginal bleeding or discharge as a common symptom. Only ovarian cancer

Epigenetics in Human Disease. https://doi.org/10.1016/B978-0-443-21863-7.00009-3
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(OC) patients experience the usual symptoms of bloating, trouble eating, feeling heavier quickly, and back- or stomachache. Patients with ovarian or uterine cancer typically experience pain within the pelvis. Constipation or more frequent urgent urine pressures are common symptoms of ovarian and vaginal cancer. Alteration in the vulva's color or skin, such as rashes, ulceration, and warts, and vulvar inflammation, itching, discomfort, or soreness are only signs of vulvar cancer [3].

Gynecological oncology is high on the global health agenda as exemplified by the SEER (Surveillance, Epidemiology, and End Results) program:13,000 cases of cervical, 19,571 cases of ovarian, 59,540 cases of uterine, and 1368 cases of vaginal and vulvar cancer were reported in the United States in 2019. The mortality statistics for the same are roughly 4000, 13,445, 11,556, and 431, respectively [3]. The global cancer observatory (WHO) or Globocan 2020 reported 604,127, 417,367, 313,959, 45,240, and 17,908 cases of cervical, uterine, ovarian, vulvar, and vaginal cancer, respectively [4]. It is an alarming call with current statistics for better screening, early diagnosis, prognosis, and treatment for women in low- and middle-income developing countries. This chapter highlights the background of altered DNA methylation and demethylation profile in gynecological cancer, their clinical applications, and the emerging domain of liquid biopsy utilization for early detection and assessment of malignancy.

24.2 Epigenetics behind gynecological malignancy

"Epigenetics" refers to core genomic changes that impact gene expression devoid of altering the fundamental DNA sequence. Epigenetic alterations seem to be crucial in the development of gynecological cancers. Approaches to epigenetic regulation of gene expression comprise noncoding RNAs, histone alterations, and DNA methylation (Fig. 24.1) [5,6].

In a CpG (dinucleotide), DNA methylation typically takes place due to genetic fragility brought on by the dinucleotide environment. Methylation of promoter and passive heterochromatin domains is typically linked to reduced gene transcription, and abnormal methylation of the CpG island has been identified as a critical genetic change in some cancers in humans. Both availability of DNA and the engagement of DNA-binding proteins (DBPs) are indeed impacted by covalent changes, such as acetylation, methylation, SUMOylation, and phosphorylation, which control transcriptional activity. DNA methyltransferases (DNMTs), histone deacetylases (HDACs), and histone acetyltransferases (HATs) are the enzymes involved in chromatin alteration that has been most comprehensively researched [7]. Scientific investigations have demonstrated that the non−protein-coding region of the genome may have a significant biological influence on health and disease, notably gynecological cancer. Now, this genomic region is not just false-transcribed junk. Among these noncoding RNAs (ncRNAs), there are two main groups, namely microRNAs (miRNAs) together with long noncoding RNAs (lncRNAs) [8,9].

24.2.1 Carcinogenesis: Interlink between DNA methylation and demethylation mechanism

The complex and dynamic procedure of DNA methylation primarily takes place on the CpG islands of genes or individual CpGs in the genome. The 5-methylcytosine (5-mC) is produced when DNA methyltransferases (DNMTs) add a methyl group onto alternating cytosines. The maintenance methyltransferase (DNMT1) along with de novo methyltransferases (DNMT3a and DNMT3b)

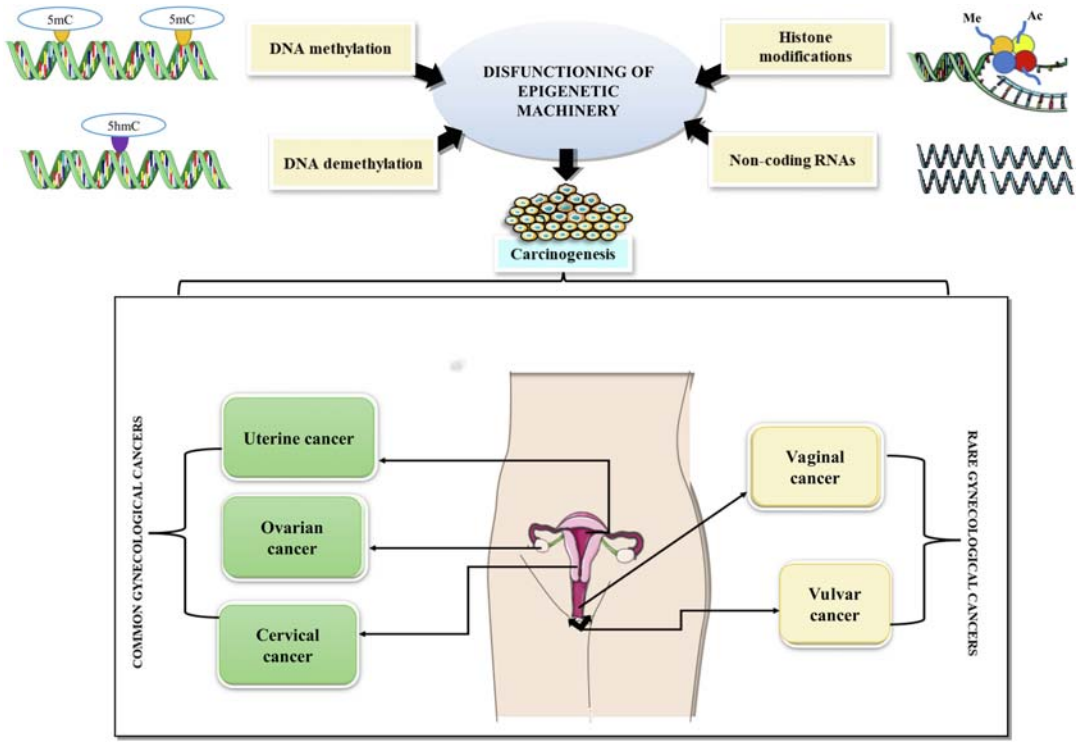

FIGURE 24.1

Dysfunctioning of normal epigenetic machinery during the development of common and rare gynecological cancer [6].

families of DNMTs were recognized. DNMT1 contributes to the maintenance of the methylation patterns of genes, whereas "de novo DNA methylation" is brought out by DNMT3a and DNMT3b.

Despite the permanence, DNA methylation can still be reversed to its original form. TET or DNA demethylase enzymes catalyze the process of conversion of five methyl-cytosine to 5-formylcytosine (5-fC), 5-hydroxymethylcytosine(5-hmC), and 5-carboxylcytosine (5-caC). For carcinogenesis, the equilibrium involving DNA methylation and demethylation is often disrupted, resulting in abnormal alteration patterns [10].

The changed amounts of 5-hmC in cancer may entail a separate mechanism since the genome-inclusive reduction of 5-hmC does not coincide with an overall loss of 5-mC. On the contrary, some research has shown a connection between DNA methylation and hydroxymethylation. In cervical cancer patients, the same 5-mC pool is diluted when the DNA profile comprises 5-hmC because DNMT1 activity drastically gets reduced. Lower 5-hmC concentrations encourage DNMT1 activation. 5-hmC adversely correlates with the advancement of cervical cancer and is downregulated under the control of 5-mC. As a result, the process of demethylation was blocked, and abnormal DNA hypermethylation accumulates globally in cervical cancer [11]. Such interlinking studies are yet to be explored in other gynecological malignancies.

24.3 Analysis of altered DNA methylation profile in different types of gynecological cancer

Cellular multifaceted machinery, including DNMTs, the DNA repair system, epigenetic regulatory elements, external environmental exposures, and others are responsible for DNA methylation [12].

24.3.1 Cervical cancer

Both methylation and hypermethylation depend on DNMTs specifically on DNMT3b activity level, which is downregulated in several malignancies [13]. Research has evolved to pinpoint specific genes getting methylated due to the identification of the enzyme that triggers DNA hypermethylation. As per Clarke et al. (2017), elevated methylation was present in *ADCYAP1*, *CADM1*, *ASCL1*, *ATP10*, *HS3ST2*, *DBC1*, *MYOD1*, *SOX1*, *MOS*, *SOX17*, and *TMEFF2* genes versus controls [14]. Higher DNA methylation was observed in *LHX8*, *ASCL1*, and *ST6GALNAC5* and led to intensifying cervical cancer aggressiveness [15]. Additionally, Clarke et al. (2018) discovered that all 12 kinds of carcinogenic human papillomavirus (HPV) forms undergo DNA methylation as they advance from HPV to the precancerous phase [16].

Furthermore, epigenetic alterations are connected to ribosomal DNA (rDNA) promoter decondensation plus hypomethylation. DNA methylation hinders transcription by preventing proteins from binding to the DNA. Numerous cancers, such as ovarian or lung, have similar problems. Because rRNA levels are negatively correlated with rDNA methylation levels, rDNA hypomethylation impacts the development of cervical cancer [17].

To stimulate tumorigenesis, *STK31* gene prevents apoptosis via enhancing cellular proliferation and metastatic spread. Hypermethylation and gene inactivation are strongly driven by *STK31*. Cancers characteristically exhibit large focal DNA hypermethylation. STK31 drives the transcriptional inactivation via promoter and exon hypermethylation affecting genes involved in the main cellular pathways [18].

It is undeniably possible to perform DNA methylation research in cervicovaginal lavages using self-sampling equipment, and its diagnostic performance appeared to be at a minimum in line with the diagnosis of the cervical tumor via hr-HPV and cytomorphology. Eijsink et al. (2011) showed that the use of DNA methylation analysis in cervicovaginal lavages for the diagnosis of cervical neoplasia demands investigation in a large prospective cohort [19].

Poomipark et al. (2016) proposed a new theory in which the methyl donor status inside cellular machinery regulates DNA methylation and impacts the development of cervical cancer. Human cervical cancer cells (C4-II) were nurtured in the medium following a substantial decrease in the methyl donor, and it was noticed that DNMT3a and DNMT3b were stunningly dysregulated via DNA hypomethylation [20].

One prominent yet initial occurrence in cervical cancer development is the hypermethylation of the CpG island connected with the promoter domain, which is a crucial element of gene suppress. Tumor suppressor gene ablation is connected to all explicit clinicopathological features of cervical cancer. This has been demonstrated throughout the course of the past few years that DNA methylation modifications certainly play a role in the pathogenesis of cervical cancer and can be used for early identification, prognostic assessment, and the creation of innovative treatment interventions [21]. Additionally, it has

been postulated that during the multilayered carcinogenic advancements, methylation commences at 1% of CpG islands and extends beyond 10% of the overall CpG islands [8,22].

Profoundly all signaling pathways affecting cell cycle management, apoptotic and cell growth, DNA repair mechanism, cell-to-cell communication, revascularization, and metastatic spread are known to be marked by hypermethylation and gene suppression, independent of HPV infection. As exemplified by hypermethylated genes involved in inhibition of apoptosis, viz., *DAPK1*, *TP73*, *RARB*, and *POU2F3*, a few hypermethylated genes implicated in cellular growth and cycle are *SLIT2*, *HIC1*, *CTNNB1*, *CDKN2B*, *CADM1*, *CDH13*, etc. *BRCA1*, *FANCF*, *BRCA2*, and *RASSF1* are the hypermethylated genes implicated in DNA repair machinery of the cell. In the same line, the hypermethylated genes such as MGMT, RUNX3, and HLTF affect tumor suppressor genes via hypermethylation [21,23].

Numerous investigations have uncovered that hypomethylation occurs in both the genetic material and the HPV genomes during cervical cancer progression. The methylation of the HPV 16 genome now has been revealed to be transcriptionally hindered in the past [24]. Research has shown that cervical cancer also has hypomethylation of the *LINE1*, *RAPGEF1*, and *CAGE* promoters in addition to the HPV genome [25].

24.3.2 Ovarian cancer

Epithelial ovarian cancer (EOC) constitutes nearly 90% of all kinds of OC incidents. Its late-stage representation at the time of preliminary diagnosis is the major cause of its high mortality rate. DNA methylation research has either focused on the methylation status of certain gene groups or with the help of genome-wide DNA methylation by contrasting (EOC) histotypes in cancerous versus normal tissues. The size of samples tested, how histotypes are studied, and laboratory analysis procedures used in this research vary greatly. DNA methylation of EOC principally focused on genes implicated in cell cycle, DNA repair, and development modulation, including *BRCA1*, *CDH1*, *PTEN*, *RASSF1A, CDKN2A*, *MLH1*, and others.

Most studies on DNA methylation in the ground of candidate genes are done and reported in (HGSOC) high-grade serous OC. The fact that so few studies have considered uncommon histotypes shows how challenging it is to collect sufficient sample sizes. Methylation-specific PCR (MSP), which assesses the product formation using primers that address the protected methylated cytosines within bisulfite-converted DNA, was employed in most investigations [26,27].

Numerous tumor suppressor genes deregulated by promoter hypermethylation, including *DAPK*, *TMS1*, *LOT1*, and *PAR4* involved in cellular proliferation in addition to proapoptotic features. Similarly, *ICAM-1* and *CDH1* (which controls cell adhesion), and *EG31* (starring role in imprinting), are also silenced by promoter hypermethylation. In OCs, hypomethylation-induced anomalous expression of many oncogenes, comprising *CLDN4*, *BORIS*, *MAL*, and *IGF2* was reported [28].

Over the past years, researchers with more samples have used superresolution methylation-built arrays, such as Human Methylation 450 K, Illumina's Human Methylation 27 K, and tailored microarrays. Inclusive assessments of genetic, epigenetic, and gene expression statistics discovered consistent associations between 12 CpG allied to five genes and their EOC risk; it suggests that methylation at 12 CpG might mark EOC risk by governing expression amid these five genes, which are *HOXB3*, *ABHD8*, *ARHGAP27*, *MAPT*, and *SKAP1*. It is still grounded with ongoing consolidative research for targeting CpGs associated with EOC risk for the discovery of potent diagnostic biomarkers [29].

24.3.3 Uterine cancer

Cancers of the uterus or womb are generally referred to as uterine cancer. The endometrium, or inner layer of the womb, is where common endometrial cancer (EC) begins in contrast to the myometrium where uterine sarcoma grows [30].

To accurately control endometrial cell growth and death during the routine menstrual cycle, elements of the DNA methylation mechanism must be kept under strict control. Deregulation of DNA methylation proteins can thereby impair the endometrium's cell homeostasis and in this manner result in the establishment of EC. There is expanding proof that many diseased endometrial types and the progression of EC emerge from an abnormal DNA methylation profile [31].

EC cells primarily adopt two atypical methylation patterns with tumor progression: global DNA hypomethylation and localized hypermethylation. DNA hypermethylation of CpG islands is affiliated with the gene promoters of primarily tumor suppressor genes. It, therefore, can happen early in endometrial oncogenesis, while DNA hypomethylation of CpG islands is attributed to repetitive, inter-, and intragenic areas and led directly to microsatellite destabilization and increased expression of many genes particularly oncogenes [32,33].

A collection of often hypomethylated oncogenes and hypermethylated tumor suppressor genes in EC was developed in support of numerous methylation studies. And over 50 hypermethylated tumor suppressor genes have already been found, together with some of the most well-established ones, such as *RASSF1, MLH1, p16, APC, PR, PTEN, MGMT,* and *CDH1*. Contrarily, just a small number of studies have discussed hypomethylated oncogenes with EC including *CTCFL*, *PARP1*, *BMP*, and *CASP8*. Genes exhibiting abnormal DNA methylation were involved in various biological processes, including apoptosis, cell growth and adhesion, signal transduction, cell cycle control, and microtubule maintenance [31,34,35].

In addition, 50% of the hyperplasia specimens had RASSF1A methylation, which, Arafa et al. (2008) argued, would be an early indicator of endometrial tumorigenesis [36].

ECs have been found to commonly have the APC gene promoter methylated, as they have malignancies with microsatellite disruption. 18%−19% of the total sporadic endometrium malignancies were found to have promoter hypermethylation of the tumor suppressor PTEN (phosphatase and tensin homolog), which was linked to the advanced stages of the disease. The protein suppresses the PI3 kinase/AKT pathway, which controls cellular proliferation and prevents the development of focal adhesions and motility of cells [37,38].

Contrary to these findings of DNA hypermethylation, Sánchez-Vega et al. (2013) found that endometrioid cancer had hypomethylation in a genomic section close to the CASP8 tumor suppressor promoter in comparison with healthy cells [39].

24.3.4 Vaginal and vulvar cancer

Primarily, cancer beginning in the vagina is called vaginal cancer. It is the rarest among all gynecological malignancies, and there are no reports of abnormal methylation profile in vaginal cancer.

Only around 5% of gynecological malignancies were vulvar cancers, which are an unusual kind of malignancy. Such tumors primarily consist of vulvar squamous cell carcinoma (VSCC). Among affluent nations, the overall prevalence rate of VSCC is two to three in every 100,000 women and rises with age, peaking between 60 and 70 years. HPV-associated and HPV-independent VSCC are the two

primary etiological subgroups of the disease [40,41]. It has also been recognized that in these subtypes' demographic, therapeutic, pathological, and genetic aspects vary. For instance, the less common subtype of HPV-associated VSCC, which affects women in their 50 and 60s, has a good prognosis [42]. Contrarily, the more common HPV-independent VSCC is linked to high relapse rates and classically occurs in women over 70 years old under the backdrop of chronic skin disorders. The "tumorigenic driver" for this subgroup has been identified as somatic TP53 mutations found in many HPV-independent VSCCs [43]. Nevertheless, since some of these tumors can be TP53-positive, current research has found molecular diversity among HPV-independent VSCCs. The most well-studied epigenetic alteration is the hypermethylation of CpG islands in the tumor suppressor gene—promoter sequences, which results in the gene's inactivity. *MGMT*, *RASSF2A*, and *TSP1* promoter hypermethylation have just been reported in vulvar cancer [44,45].

24.4 Biological implications of DNA demethylation in the landscape of gynecological cancer

In mammalian DNA, the landscape of DNA demethylation is catalyzed by TET dioxygenases mainly TET 1, 2, 3, which yields 5hmC as an oxidized intermediate in an alpha-ketoglutarate-dependent manner. The brain's central nervous system has the highest levels of 5hmC, which alter depending on the tissue. Unfortunately, it is unclear what 5hmC exactly does in cellular physiology. By using arrays or alignment assays, the localization of 5hmC within promoters and inside gene bodies was found to be prevalent [46]. *TET2* has mutations in several hematological malignancies. In the cancer genome, *TET2* mutations were associated with abnormal amounts of 5hmC and 5mC. Additionally, aberrant DNA methylation profiles have been correlated with *IDH1* mutations [47,48].

In cervical cancer patients, *TET1* and *TET3* are still expressed in undistinguishable amounts as normal cervical tissues, but 5hmC and *TET2* activity is reduced. This suggests that 5hmC could be a possible predictive marker and reduced expression of *TET2* might reduce 5hmC [49]. Hydroxymethylation of ACTG1, DNAJA3, SALL3, CDC14B, SERPINB6, and CALN1 has been identified as noteworthy gene-based biomarkers for the identification of cervical cancer. These emerging biomarkers exhibit significant potential in detecting cervical cancer through their hydroxymethylation patterns [50].

According to Li et al. (2017), both primary and malignant OC cells extremely lack 5hmC. Nuclear 5hmC markers in OC cells vary significantly depending on the histopathology. The highest amounts are found in healthy ovarian tissue, followed by benign ovarian adenomas, endodermal sinus tumors, serous papillary mucinous tumors, endometrioid tumors, and clear cell carcinoma [51]. OC frequently exhibits hypohydroxymethylation or decreased global and gene-restricted amounts of 5hmC. In (HGSOC) high-grade serous OC, reduced 5-hmC levels are an epigenetic marker for tumor development and malignancy. Furthermore, in patients with recently diagnosed HGSOC, globally, 5-hmC depletion is associated with reduced sensitivity to platinum-centered therapy, a quicker time until relapse, and a reduced overall life span [52].

Preliminary study of demethylation in uterine cancer as exemplified by Yang et al. (2019) showed that lower *TET1* activity led to hypermethylation in the promotor domain of the tumor suppressor *APC*. It is consistent with TET1's role as dioxygenase, which eliminates genome-wide DNA methylation signatures. The 3′-UTR of TET1 is directly targeted by miRNA-191 in EC cells as determined by high-

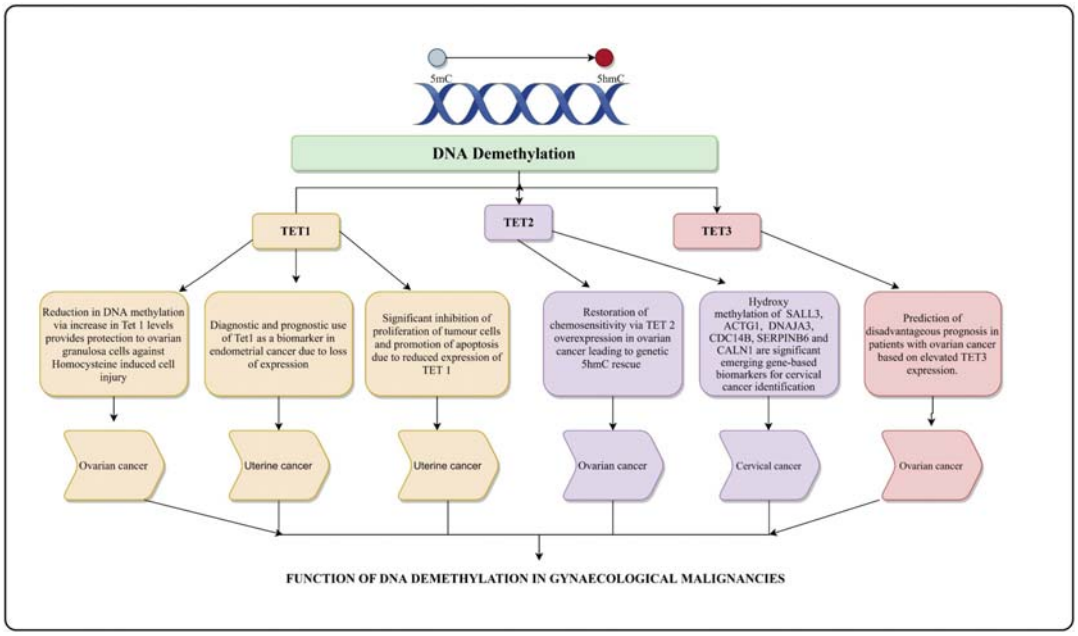

FIGURE 24.2

Overview of DNA demethylation mechanism—based preliminary studies in gynecological malignancies [52,54–57].

throughput sequencing technology. Noteworthy, miR-191 might have an oncogenic role in EC and could be a potential target for improved disease diagnosis and therapy [53]. The research on interrelating demethylation mechanism in vaginal and vulvar cancer is still unexplored. Fig. 24.2 represents the functional attribute of DNA hydroxymethylation in gynecological cancer [52,54–57].

24.5 Role of potent microRNAs and lncRNA signatures as diagnostic biomarker in women's reproductive system linked malignancies

Protein-coding regions make up only 2% of the human genome, yet more than 70% of the genome is translated into RNA without encoding the protein. However, the bulk of the most important biological processes, including cell growth, differentiation, and the cell cycle, as well as cancer and other disorders depend on this noncoding region of the genome [58]. A class of regulating small RNA fragments known as microRNAs has provided a fresh perspective to treating and identifying several disorders. Mature microRNAs (miRNAs) being noncoding, small, and endogenous RNAs comprising about 22 nucleotides degrade specific target mRNAs together with other protein partners to achieve translational repression in turn regulating gene expression at the posttranscriptional level. Alteration of miRNA expression often leads to health-related disorders including reproductive system malignancies [59].

A family of noncoding RNA molecules having transcripts usually more than 200 nucleotides is regarded as long noncoding RNAs (lncRNAs). Mechanistically, lncRNAs can directly bind to short miRNAs and lead to the weakening of miRNA–target gene interaction, which regulates gene expression [60]. Recent research on LncRNAs as epigenetic regulators in the female reproductive system has provided a new understanding of the causes of several reproductive diseases [61]. lncRNAs can function as a tumor suppressor gene and mediate cell signaling in EMT regulation resulting in the pathogenesis of cancer cells. Table 24.1 discusses the various roles of lncRNAs in gynecological malignancies.

According to ongoing studies, miRNAs and lncRNAs are active participants in regulating cellular function in gynecological cancers. EC, polycystic ovary syndrome (PCOS), and OC are prevalent reproductive system–linked malignancies in females; in each condition, miRNAs and lncRNAs appear to play essential roles or may serve as biomarkers of reproductive etiology. miRNAs have been shown as a distinguished biomarker for detecting polycystic ovaries in abnormally menstruating women. According to a study by Long et al. (2019), PCOS patients had significantly higher expression levels of miR-146a, miR-222, and miR-30c miRNAs than controls [62]. The lncRNAs PWRN2 and HCG26, expressed in cumulus cells and granulosa have also primarily been connected to PCOS [61]. In the pathogenesis of OC, the microRNA miR-200a-3p decreases PCDH9 expression by binding to the 3′UTR of its gene [63].

Studies have emphasized the role of miRNAs and lncRNAs in cervical cancer, particularly in relation to cell progression, apoptosis, prognosis, metastasis, and invasion. miR-574-5p upregulates matrix metallopeptidase 9 (MMP-9), cyclinD1, β-catenin, and the expression of N-cadherin and downregulates expression of E-cadherin in cervical cancer cells to promote their metastatic capability [64]. Shen et al. (2019) assessed the role of lncRNA CCAT-1 (colon cancer–associated transcript-1) and suggested that it suppresses miR-181a expression, resulting in increased expression of HB-EGF and MMP14 leading to proliferation, cancer cell colony formation, and invasion of cervical cancer cells [65].

Endometriosis is among the most prevalent and excruciating chronic inflammatory gynecological diseases that are distinguished by the presence and growth of ectopic external uterine endometrial tissue [58,62]. Furthermore, circulating miRNAs have been suggested as EC biomarkers. Additionally, it has been proposed that long noncoding RNAs may serve as EC biomarkers in the form of circulating miRNAs. It has been discovered that EC and endometriosis both share dysregulation of the lncRNAs MALAT1 and H19 [62].

Furthermore, other gynecological cancers such as vaginal cancer and vulvar cancer are also similarly associated with miRNAs and lncRNAs. UCA1 regulates WEE1 expression by sponging miR-103a and facilitates tumor growth and CDDP resistance in vulvar cancer cells [66]. Likewise, lncRNA ROCK1 was found to be associated with cell metastasis when studied by Akagi et al. (2014). Additionally, vulvar cancer tissues have higher levels of miRNA 4712-5p than healthy vulvar tissues, and this is linked to the downregulation of PTEN expression via the AKT/cyclin D1 signaling pathway [67]. Table 24.2 summarizes the upregulated and downregulated miRNAs in various gynecological cancers in Refs. [63,64,67–78].

24.6 Applications of DNA methylation–based biomarkers in clinics

DNA methylation has a lot of promise to be utilized as a nucleic acid–based biomarker for numerous applications. As the methyl group addition on the cytosines of DNA is stable and amplifiable, it can

Table 24.1 Long noncoding RNAs and their roles in gynecological cancers [65,66,79–95].

LncRNA	Cancer type	Role in malignancy	Mechanism of action	References
1. H19	Cervical cancer	■ Increased proliferation of cell and reduced cell apoptosis	↙ Downregulates miR-138-5p in turn upregulates sirtuin-1 (SIRT1)	[79]
2. Metastasis-linked lung adenocarcinoma transcript 1 (MALAT-1)	Cervical cancer	■ Decreased cell apoptosis and increased cell growth rate	↙ Downregulates miRNA-124	[80]
3. HOTAIR	Cervical cancer	■ Promotes cell invasion and migration	↙ Silences miR206 expression thus decreasing the expression level of MKL1	[81]
4. ZEB1-AS1	Cervical cancer	■ Promotes epithelial–mesenchymal transition and cell invasion	↙ Induces ZEB1-AS1 expression	[82]
5. HOTAIR	Cervical cancer	■ Promotes migration, proliferation, and invasion	↙ Competitively binds to miR-148a and modulates human leukocyte antigen-G (HLA-G) expression	[83]
6. Colon cancer–linked transcript-1 (CCAT-1)	Cervical cancer	■ Promotes proliferation of cell, colony formation, and invasion	↙ Suppress miR-181a expression, resulting in increased expression of HB-EGF and MMP14	[65]
7. Colon cancer linked transcript-1 (CCAT-1)	Cervical cancer	■ Stimulates proliferation of cell and inhibits their apoptosis	↙ Regulates the Wnt/β-catenin pathway	[84]
8. XIST	Cervical cancer	■ Promotes cell progression	↙ Binds with miR-200a and upregulates Fus	[85]
9. SPRY4-IT1	Cervical cancer	■ promotes epithelial–mesenchymal transition	↙ Directly binds to miR-101-3p and increases ZEB1 expression	[86]

10. Nuclear enriched abundant transcript 1 (NEAT1)	Endometrial carcinoma	■ Promotes cell migration, invasion, and proliferation	↙ Binds to miR-214-3p and regulate Wnt/β-catenin pathway	[87]
11. BANCR	Endometrial carcinoma	■ Enhances cell migration, proliferation, and cell invasion	↙ Activates MAPK/ERK signaling pathway increasing MMP1/MMP2 expression	[88]
12. Plasmacytoma variant translocation 1 (PVT1)	Endometrial carcinoma	■ Promotes cell proliferation, migration, and call invasion	↙ Binds to miR-195-5p and downregulates MAPK/Erk and AKT/PI3K pathways	[89]
13. ATB	Endometrial carcinoma	■ Promotes migration and invasion	↙ Binds miR-126 and induce epithelial–mesenchymal transition	[90]
14. LINC00504	Ovarian cancer	■ Stimulates aerobic glycolysis and promotes cell progression	↙ Inhibits miR-1244 and regulates HK2, PKM2, and PDK1 expression	[91]
15. Membrane-associated guanylate kinase inverted 1 (MAGI1) intronic transcript (MAGI1-IT1)	Ovarian cancer	■ Facilitates epithelial–mesenchymal transition	↙ Binds to 200a and upregulates ZEB1 and ZEB2	[92]
16. GAS5	Ovarian cancer	■ Causes drug sensitivity and progression of OC cells	↙ Regulates MAPK pathway	[93]
17. Ewing sarcoma–associated transcript 1 (EWSAT1)	Ovarian cancer	■ Promotes colony formation, cell proliferation, and invasion	↙ Suppresses miR-330-5p expression	[94]
18. ROCK1	Vulvar cancer	■ Promotes cell metastasis	–	[95]
19. UCA1	Vulvar cancer	■ Facilitates tumor growth and CDDP resistance	↙ Regulates WEE1 expression by sponging miR-103a	[66]

Table 24.2 MicroRNAs and their roles in gynecological cancers [63,64,67–78].

miRNA	Cancer type	Expression level	Mechanism of action	References
1. miR-93-5p	Cervical cancer	Upregulated	■ Increases matrix metalloproteinase-9 (MMP-9) and matrix metalloproteinase-2 (MMP-2) expression, whereas decreases extracellular matrix (ECM) expression	[68]
2. miR-574-5p	Cervical cancer	Upregulated	■ Upregulates matrix metallopeptidase 9 (MMP-9), cyclinD1, β-catenin, and N-cadherin and downregulates E-cadherin	[64]
3. miR-409	Cervical cancer	Upregulated	■ Downregulates Smad2 expression	[69]
4. miR-612	Cervical cancer	Downregulated	■ Regulates NOB1 expression	[70]
5. miRNA-1251-5p	Ovarian cancer	Upregulated	■ Downregulates TBCC expression	[71]
6. miR-200a-3p	Ovarian cancer	Upregulated	■ Decreases the expression of PCDH9	[63]
7. miR-96-5p	Ovarian cancer	Upregulated	■ Suppressing Caveolae1 expression	[72]
8. miR-204-5p	Ovarian cancer	Downregulated	■ Downregulates the expression of USP47 and RAB22A	[73]
9. miR-34	Ovarian cancer	Downregulated	■ Suppression of Notch 1 expression	[74]
10. miR-107-5p	Endometrial carcinoma	Upregulated	■ Downregulates estrogen receptor α (ERα) expression	[75]
11. miR-582-5p	Endometrial carcinoma	Upregulated	■ Downregulates AKT expression	[76]
12. miR-590-5p	Vulvar cancer	Upregulated	■ Downregulates TGFβRII expression	[77]
13. miRNA-4712-5p	Vulvar cancer	Upregulated	■ Downregulates PTEN expression through the AKT/cyclin D1 signaling pathway	[67]
14. miR-223-5p	Vulvar cancer	Upregulated	■ Downregulates TP63 expression	[78]

easily be converted from a research laboratory setting into routine diagnostics. A diagnostic tool that can be utilized to monitor people who might be at risk of developing cancer is necessary now more than ever. Researchers have been working in this direction for the past one or 2 decades, and they have been able to establish connections between epigenetic changes and cancer. One of the crucial necessities for a clinically effective biomarker is the ability to analyze the reliable biomarker in body fluids, such as blood, which can be procured via minimally invasive techniques. This enables scanning individual people at potential risk and examining therapy reactions and disease relapse states. DNA methylation is a preferred biomarker for cancer patients due to its sensitivity and specificity in distinguishing early tumor-specific marks via molecular techniques at distant sites. Therefore, cancer-associated or inducing changes ought to be beneficial as biomarkers. It is vital to note that neither single gene has been identified that is consistently methylated in a particular type of cancer. Therefore, panels of DNA methylation—built indicators would probably be used in clinical settings to achieve the necessary precision and sensitivity [22].

24.6.1 DNA methylation profiling for early diagnosis, prognosis, and therapeutic interventions of cervical cancer

Despite the creation of the HPV vaccine, cervical cancer continues to have a high incidence and predominantly affects younger people. Prompt detection and treatment of cervical cancer are therefore crucial and can lower mortality. Since every HPV infection seems to have a unique gene profile, many biomarkers may have clinical significance for various HPV strains. Each of the markers must have comparable degrees of sensitivity, selectivity, and methylation activity.

CADM1 has recently been demonstrated to be a possible cervical cancer biomarker for the detection of HPV-positive cervical carcinoma by Mersakova et al. (2018) and Rong et al. (2019). When assessing cervical cancer patients, a substantial difference in the promoter methylation of *CADM1* and its D-dimer was observed in plasma. Employing them together as the prognosticator of metastasis helps to achieve high specificity and sensitivity of 90.5% and 80.4%, respectively,. Mersakova and other researchers hypothesized that *CADM1* hypermethylation results in repressed Rb tumor suppressor signaling; however, the precise mechanism is still unknown [96,97].

The *DcR1* promoter is hypermethylated more frequently in enhanced-risk HPV-infected specimens than low-risk and non—HPV-infected samples. *DcR1* expression is repressed by high-risk HPV infection, which facilitates the development of cervical cancer [98].

ASCL1, ST6GALNAC5, and *LHX8* are three linked genes that showed 72.1%, 73.8%, and 55.7% sensitivity with (CIN3+) cervical intraepithelial neoplasia grade 3 or cancer, respectively. *ASCL1* and *LHX8* were associated effectively with CIN3+ (AUC 0.79 and 0.81, individually), and *ST6GALNAC5* was associated moderately with CIN3+ (AUC 0.71). In inference, *ASCL1* and *LHX8* are acceptable biomarkers for preliminary detection due to their improved performance and increased sensitivity [15].

An incredibly effective three-gene, viz., *ASCL1, LHX8,* and *ST6GALNAC5*, methylation predictor was described by Verlaat et al. (2018) to identify the early and late tumorigenic stages of cervical cancer. These genes are combined with increasing identification precision and sensitivity. It had outstanding clinical performance in the identification of CIN3. In hrHPV-positive lavage and brush self-samples, the sensitivity and specificity were 74%, 79%, 88%, and 81%, respectively. As a result, this can be utilized as a diagnostic approach [99].

The prospective biomarkers *CDH1, CDKN2A, RB1,* and *TP53* were identified by analysis of several data sets and cross-referencing; further research is required to determine the function of these genes in the diagnosis of cervical cancer [100].

The potential of *CADM1, MAL*, miR-124, *SOX1, ERT,* and *EPB41l3* as biomarkers has been described in prior research. Even during screening for cervical cancer, *DCC* was discovered to be a significant contender as a methylation biomarker. *DCC* is a component of cell cycle control including apoptosis and is present in a variety of malignancies, including OC, among others. Future research will need to verify whether methylated *DCC* may be used as a marker in cervical cancer assessments [14].

STK31 induces DNA hypomethylation, which was epigenetically stimulated by the HPV16 E7 and E6/E7 types of oncoproteins. Consequently, *STK31* is a promising target gene that may assist in the creation of new clinical methods for identifying and managing individuals with cervical cancer [18].

Triage is the process of prioritizing the care of patients based on their condition, complexity, prognosis, and available resources. Triage is used to identify patients who require rapid recovery, allocate patients to a specified area for patient care to prioritize their care, and start necessary diagnostic and therapeutic procedures. To further stratify those with positive primary screening values, a phase called triage test is allocated between screening and diagnosis. When a screening test is poorly specific, a diagnostic treatment is expensive, or both, triage is most valuable. An effective triage test should minimize unnecessary medical testing while maintaining disease detection sensitivity. It should also minimize overtreatment, patient stress and discomfort, and overall management expenses. However, the efficacy of the triage test is into doubt if the original screening test is extremely specific, the triage test is positive in the large majority of cases, or the triage test decreases sensitivity. For example, in cervical cancer. cytology is repeated, and colposcopy (visual method) and HPV DNA test are performed. The current biomarker panel proposed for triage testing in the early detection of cervical lesions consists of C13ORF18/EBP41L3/JAM3, C13ORF18/ANKRD18CP/JAM3, and ZSCAN1/SOX1. In cytologically abnormal women, the sensitivity of these biomarkers is reported to be 79%, 76%, and 72% respectively, while their specificities are 57%, 65%, and 68% respectively [101,102].

It is still unclear which biomarkers are helpful for cervical cancer prognosis. Chemotherapy, radiation therapy, resection, and medication therapy are the only options for cervical cancer treatment. But new targets must be discovered for prognosis. Fig. 24.3 illustrates preliminary studies interlinking methylation biomarkers with conventional therapies for cervical cancer treatment [18,103–107].

24.6.2 Diagnostic, prognostic, and predictive methylation allied biomarkers in ovarian cancer

Methylation-allied biomarkers are a potential method for the early diagnosis of OC because abnormal methylation is one of the first molecular changes underlying carcinogenesis.

Nevertheless, the methylation of a single gene might not be very useful in therapeutic settings. Currently, no single gene–based epigenetic biomarker can reliably identify primary OC in tissue or bodily fluids. It may be possible to create a technique for the biological categorization and prediction of OC that is more particular and sensitive by concurrently analyzing the methylation status of many genes in a fluid-based assay [108].

Active OC cases significantly affect the DNAme (DNA methylation) patterns in peripheral circulation, elucidated by genome-wide DNAme assessment of a large OC case–control population

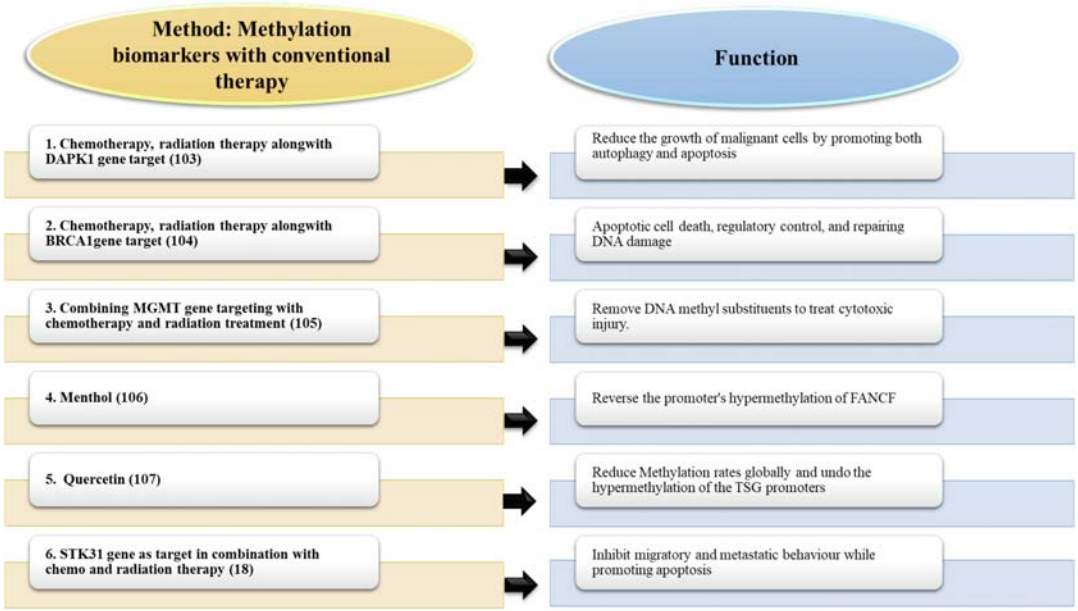

FIGURE 24.3

Illustration of recent methylation-based therapies for cervical cancer treatment [18,103–107].

[109]. Microarray studies concluded 112 methylation loci are predictive for nonmetastatic survival in women with advanced OC. According to the research, primary illness recurrence after chemotherapy is linked to a higher CpG island methylation variation [110].

In the latest report, the methylation level of seven potential genes (*RASSF1A, APC, CDH1, RUNX3, SFRP5, TFPI2,* and *OPCML*) in cell-free serum DNA was examined using multiplex methylation-specific PCR. The report's sensitivity and specificity were 85.3% and 90.5% in phase I EOC. When contrasted with a single CA125, which gave a sensitivity of 56.1% at a specificity of 64.15%, the prevalence rate was noticeably greater [111]. Another study used a 10-gene profile in plasma and showed substantial detection sensitivity and specificity [112]. Table 24.3 represents biomarker for early detection, diagnosis, prognosis, and predictive for OC [113–128].

24.6.3 Uterine cancer: Diagnostic and prognostic significance of methylation signatures

The discovery of methylation of CpG islands is one of the epigenetic alterations, which led to the broadening of horizons in the development of tools for better detection and personalized treatment of cancer [129]. Several reports have demonstrated that almost all body fluids such as serum/plasma, smears, vaginal fluid, and nipple fluid aspirate can be analyzed for the detection of methylation signatures. Hence, the identification of DNAme in the circulating cell-free DNA (cfDNA) found in body fluids proves to be an essential tool for uterine cancer, especially EC, diagnosis [130].

Table 24.3 Biomarkers for ovarian cancer management [113–128].

S. No.	Biomarker	Clinical application	References
Early detection			
1.	CA125	Useful indicator of tumor recurrence	[113]
2.	HE4	Detection and separation of histotypes	[114]
3.	Mesothelin	Initial screening	[115]
4.	ApoA1	Early-stage screening	[116]
5.	LDOC1	Early-stage screening	[117]
Diagnosis			
1.	PCDH17	Presence or absence of tumor	[118]
2.	EGFL7 and RASSF1	Detection and advancement	[119]
3.	CCBE1	Significant inverse correlation between metastasis	[120]
4.	TGFBI	Presence of tumor	[121]
Prognosis			
1.	EN2	Quick advancement-free survival	[122]
2.	MYLK3	Higher methylation level significantly predicted better overall survival with least residual disease	[123]
3.	GULP1	Disease persistence, decreased survival in patients, and reduced disease-specific survival	[124]
4.	ABCA1	Less overall survival rates	[125]
Predictive			
1.	MSX1	Sensitivity to the medication platinum	[126]
2.	TBX2	Sensitivity to the medication platinum	[127]
3.	MAL	Platinum resistance	[128]

EC, with an incidence rate of about 2.38%−4% and patients aged ≥50 years at a higher risk of endometrial malignancy, is ranked as the fourth most frequent cancer in women [131]. Therefore, the early diagnosis of EC can prove to be extremely valuable in the personalized treatment of cancer. The recent technological advancements in DNAme assay have shown potential toward enhanced identification of methylation markers via the analysis of thousands of gene loci at the same time. Technologies such as MeDIP [130], MeCAP, and reduced representation bisulfite sequencing (RRBS) [132] are used for DNAme profiling. Another platform for clinical DNAme assay is digital PCR, which is a highly specific and sensitive DNA methylation analysis method yielding absolute quantification of a DNA template with a high signal-to-noise ratio [130]. An example of the usage of DNAme assays is the experiment done by Fiegl et al. in which five genes—*RASSF1A, HSPA2, SOCS2, MLH1,* and *CDH13*—showed a sensitivity of 100% and specificity of 80% in the case of differential methylation [132].

As the importance of DNA methylation becomes significant in the role that it plays in the progression of cancer, the identification of new genes that are potentially regulated by aberrant methylation of CpG islands becomes indispensable. In this direction, the discovery of genes such as *ZNF154, DPP6, PCDHs,* and *TNXB* that might be regulated by DNAme in EC can prove to be contributors to the prognosis and targeted therapy of EC [133]. A separate study identified *PARVG, SYNE4,* and *CDO1* as DNA methylation−driven differential genes that can be viewed as predictors of poor prognosis. The abnormal DNA methylation pattern of these genes can be used to speculate the prognosis of individuals suffering from uterine corpus endometrial carcinoma (UCEC) as well as depict the tumor immune microenvironment, which can aid in the determination of prognosis [134].

24.6.4 Towards the domain of methylation signatures for vaginal and vulvar cancer

Vulvar cancer, generally a squamous cell carcinoma, includes the malignancies of vaginal openings, labia majora, labia minora, and clitoris. It progresses from vulvar intraepithelial neoplasia (VIN) albeit at a different rate in different individuals [135]. Not much about the methylation landscape of vulvar cancer is known; however, a recent study focused on discovering somatic mutations in vulvar cancer. Development in next-generation sequencing (NGS) techniques has made it possible for the exploration of different variants in a set of tumor samples and for the identification of various somatic mutations in the samples. This also gives hope for the emergence of potential methylation signatures for vulvar cancer as well as vaginal cancer, an unexplored area yet an area of interest for the researchers [136,137].

24.7 Liquid biopsy—based detection of gynecological cancers in clinical practice

In the year 2010, Pantel and Panabiéres used the word "liquid biopsy" to characterize the assessment of circulating tumor cells (CTCs) in patients' fluids. In oncology, the term is frequently employed to refer to the sample of tumor-originated material from various liquid-based biological entities such as

blood, urine, abscesses, saliva, and cerebral spinal fluid [138]. The presence of CTCs, circulating tumor DNA (ctDNA), miRNAs, circulating extracellular vesicles (cEVs), and tumor proteins (TPs) in bodily fluids are significant. Frequent therapeutic instances, notably cancer genotyping after diagnosis, detection of minimally persistent disease afterward surgery, tracing effectiveness of therapy, and the occurrence of early cancers, can be substantially enhanced by evaluating these circulating elements [139].

The significance of these elements lies in abnormalities, chromosomal rearrangements, copy number variations, and methylation signatures that are particular to the tumor type. For just several tumor loci, it is possible to isolate and enhance ctDNA following element size selection. The fact that it reflects tumor heterogeneity more accurately than tissue biopsies is one of its key benefits [140].

The three-gene DNA methylation−serum−marker group was able to distinguish high-grade serous OC patient's versus control in a recent trial involving more than 400 patients with the high specificity of 90.7% and still a low sensitivity of 41.4%. Although precision was reduced when the analysis was restricted to CA125-lacking individuals, it was still enhanced to specificity and sensitivity of 87.5% and 63.6%, respectively. Among 40 patients (primarily relapse cases) managed for high-grade serous OC, assessment of *TP53* mutations in cfDNA using digital PCR was employed to assess tumor volume and follow the treatment outcome [141]. On contrary to CA125, *TP53* atypical allele frequency was linked to tumor volume as revealed by CT scans and period of advancement. Measurement of CTCs may be regularly employed in the future for prognostic evaluation and follow-up. Due to their solid predictive significance and capacity to identify treatment resistance or advancement, standardized platform parameters are created. Similarly, novel functional research concerning CTC biology and metastatic dissemination may result from morphological, histopathological, and genetic variation studies. Patients with CTCs exhibiting ERCC1, a sound indicator of platinum tolerance, have a worse prognosis [142−144].

According to preceding research associating the dose of medication of ctDNA in patients with CC versus control subjects, plasma ctDNA gratification was considerably greater and correlated with lymph system metastases and infiltration complexity. Together in a separate, smaller study, Lee and colleagues isolated both ctDNA and CTC-DNA from the peripheral circulation of 20 patients with gynecological cancer (including four cases of CC) and evaluated by comparing the variants found for each pair of DNA samples, identifying *ERBB2, ESR1, FGFR4, BRCA2, STK11, PTCH1,* and *TSC2* as disclosed, intersecting genetic mutations in the entire cluster [145].

Regarding high-grade and *p53* mutant EC cancers, greater cfDNA levels were also reported, supporting their relevance as a prognostic marker [146]. In contrast to cfDNA surveillance, finding tumor-specific mutations in ctDNA during treatment has been linked to traditional risk markers such as higher grade, profound myometrial penetration, and FIGO progressed stages, along with a poor prognosis [147]. Because the findings of the postsurgery follow-up uncovered the presence of ctDNA in three patients with progressing disease, Casas-Arozamena et al. also proved the usefulness of ctDNA studies to ascertain the tumor load of EC patients who experienced a relapse following surgery [148]. Fig. 24.3 shows the pros and cons associated with liquid biopsy−based techniques [149,150] (Fig. 24.4).

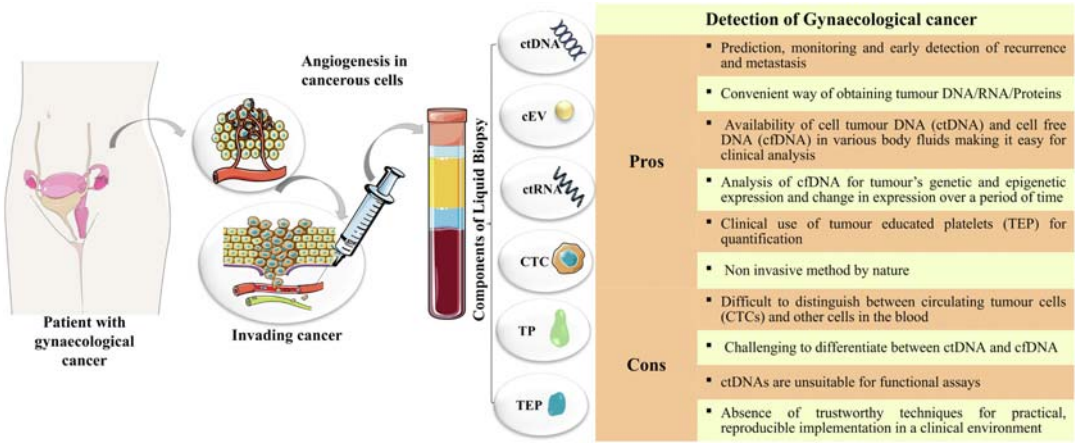

FIGURE 24.4

Pros and cons of liquid biopsy–based detection of gynecological cancer in clinical practice. *cEV,* circulating extracellular vesicles; *ctDNA*, Circulating tumor DNA; *ctRNA,* Circulating tumor RNA); *CTC*. circulating tumor cells; *TEP*, tumor-educated platelet; *TP*, tumor proteins [6,147,148].

24.8 Concluding remarks and future perspectives

Methylation-founded epigenetics has gained prominence over the past 10 years, evidenced by increasing publications in the domain. Many reports have included human DNA methylome, CpG island, shores, and individual CpGs coupled with carcinogenesis and its etiology. The discovery of *TET*s and the demethylation mechanism uncover the dynamic nature of the process. Currently, aberrant DNA methylation and demethylation are involved in gynecological cancer, but their interconnection in cancerous cells is still a sensible research question.

The use of DNA methylation–based biomarkers in clinical settings is currently quite restricted. The lack of the sensitivity and precision needed for a diagnostic test may be brought on by our partial understanding of the complexity of DNA methylation profiles, their heterogeneity, integrative studies, and CpGs in a gene of interest. The liquid biopsy–based analysis holds the enormous potential of many circulating markers for assessing tumor size, detecting minimal residual disease, evaluating therapy efficacy, and keeping track of cancer remissions. Using an integrative liquid biopsy–based approach, the single and multiplex methylation biomarkers hold great strength for early diagnosis, prognosis, and management of gynecological cancer.

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CHAPTER

Epigenetics in human reproduction and gynecologic diseases

25

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25.1 Introduction

Genomics is the study of the complex and dynamic processes of genomes. Previous studies have focused mainly on the effects of environmental factors on genetics or DNA mutations that are inherited and transmit abnormal phenotypes from parents to offspring. Demonstrations of genetic mutations in single genes or clusters of genes are the conventional procedures that are identified as causes of diseases or abnormal phenotypes [1]. Increasing evidence from clinical models has supported the role of epigenetics as the cause of many abnormal phenotypes. Moreover, epigenetic information is chiefly influenced by environmental factors. Human data have revealed that environmental exposure can increase the risk of some chronic illnesses, such as type 2 diabetes, cardiovascular disease, cancer, and behavioral disorders [1].

Presently, a vast amount of evidence has indicated that epigenetic markers can be conveyed from parents to their offspring through their gametes [1]. However, because of the intricate network of the reproductive system in the generation of gametes, fertilization, and development of a zygote, determination of the pattern of inheritance is laborious [2].

This chapter reviews the basics of epigenetics and its mechanism of modification, role in gametogenesis and embryogenesis, role in male and female infertility, role in assisted reproductive technology (ART), and, finally, role in gynecologic diseases and treatment prospectives.

25.2 Basic concepts of epigenetics

The concept of epigenetics emerged based on the hypothesis that a single identical genetic code could give rise to a diversity of phenotypes [3]. Thereafter, studies have focused on epigenetics and the consequences of epigenetic aberrations, which led to the current epigenetics definition. The epigenetics definition that is universally accepted was provided by Wolffe in 1992 and states, "Epigenetics is the study of heritable changes in gene expression that occur without a change in DNA sequence" [3]. In other words, the epigenome is a set of chemical compounds attached to the DNA sequence, which can be altered or modified to control gene expression without changing or altering the DNA sequence itself [4].

Epigenetics in Human Disease. https://doi.org/10.1016/B978-0-443-21863-7.00010-X
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The main mechanisms of epigenetic modifications include DNA methylation, histone modifications, noncoding RNA (ncRNA), and chromatin remodeling [1,2,4–7].

1. DNA methylation: DNA methylation is one of the most extensively explored mechanisms of epigenetic modification. DNA methylation exists in the 5′ position of the cytosine pyrimidine ring in CpG dinucleotides. CpG-enriched sites are associated with promoter regions called "CpG islands (CGIs)," which control gene expression or gene silencing [1,2]. DNA methylation is generated by DNA methyltransferase (DNMT), which is maintained during cell division. After DNA is methylated, variable proteins that have a methyl-binding domain attached to the DNA prompt gene silencing. On the other hand, DNA demethylation, either actively or passively, results in gene expression [2].
2. Histone modifications: Histones are proteins, and their basic function is to act as a core for DNA to wrap around and pack into the nucleosome. Histones have a tail of amino acids that can be modified by acetylation, methylation, phosphorylation, poly-ADP ribosylation, and ubiquitination [2]. These posttranslational modifications of histone proteins can influence the accessibility of transcription factors to DNA. Therefore, epigenetic modification of histone tails can determine transcriptional activity of specific DNA sequences. In other words, histone modifications can control gene expression [2].
3. ncRNAs: There are many types of ncRNAs, including microRNAs (miRNAs), small interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs) [1]. In animal studies, ncRNAs play a role in environmental epigenetic inheritance [1]. ncRNAs are transcribed from DNA but are not translated into proteins [2]. After being transcribed, ncRNAs are transferred into the cytoplasm and attach to the Argonaute (Argo) proteins, which create the RNA-induced silencing complex (RISC) [2]. RISC binds to mRNA to silence the translation process. However, some evidence has suggested that ncRNAs may play a role in enhancing the translation process, depending on different biological circumstances [2].
4. Chromatin remodeling: The particular exact position of nucleosomes can influence the accessibility of transcriptional factors and therefore the transcriptional process [2] (Fig. 25.1).

25.3 Gametogenesis

Epigenetic reprogramming begins during gametogenesis and early embryonic development. The gametogenesis process occurs during the development of primordial germ cells (PGCs), which are derived from progenitor cells in the embryo and have migrated to the developing gonads. The genetic code in DNA is considerably stable and secured. Contrarily, epigenetic modifications can be made easily with external stimuli [3]. Therefore, understanding the epigenetic modifications that occur during male and female gametogenesis, which can be inherited through gametes, can help define the factors involved in disease development in adulthood.

There are mainly two phases of epigenetic reprogramming [4]:

1. during gametogenesis and
2. during early embryo development.

Before fertilization, sperm and oocyte genomes are transcriptionally silent as a consequence of hypermethylation of their respective genomes, which ensures the repression of pluripotent markers.

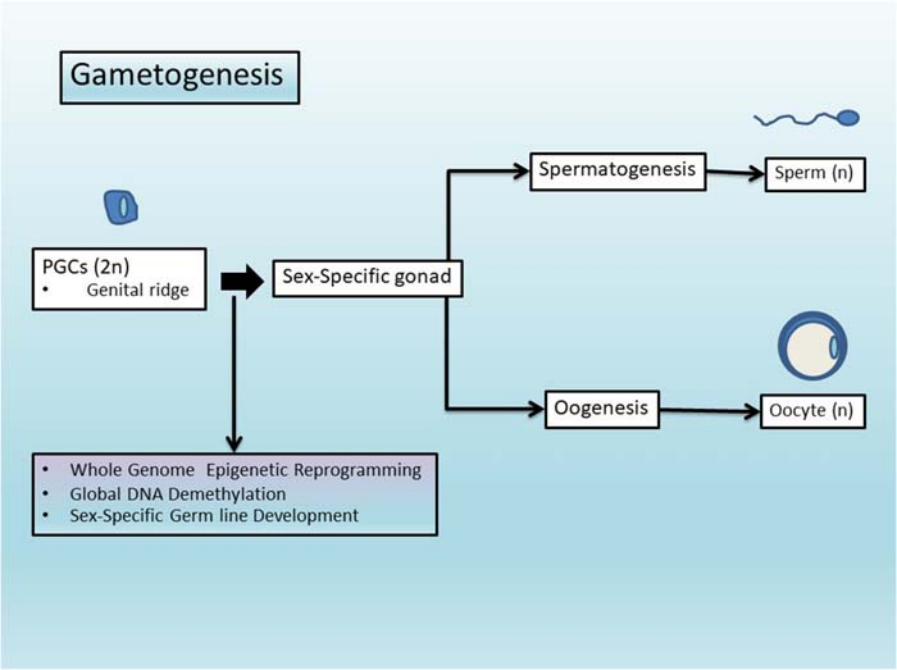

FIGURE 25.1

Primordial germ cells (PGCs) are the derivative source of both female and male germ cells. Reprogramming in the germline is essential to get sex-specific epigenetic information and germline development.

This mechanism of selective gene expression is called "genetic imprinting" [2,4,8]. Consequently, reprogramming in germ cells is a crucial event and gender-specific for sex-specific germline development (Fig. 25.2).

25.3.1 Spermatogenesis

Germ cell developments are both genetic and epigenetic operations. After global demethylation of PGCs, they develop into spermatogonial stem cells, which reside on the basement membrane of seminiferous tubules [8]. Spermatogenesis comprises three developmental processes:

1. self-renewal of spermatogonia by mitosis: incorporating histone or histone variants in chromatin structure;
2. development of spermatocytes into haploid spermatids by meiosis; and
3. spermiogenesis—the process of transformation of spermatids to spermatozoa, which involves maturation and differentiation of round spermatids into spherical and elongated spermatozoa.

The process of spermatogenesis comprises compaction of chromatin in sperm heads by means of histone-to-protamine transitions [2,8]. Histone variants play a key role in protamine transition and

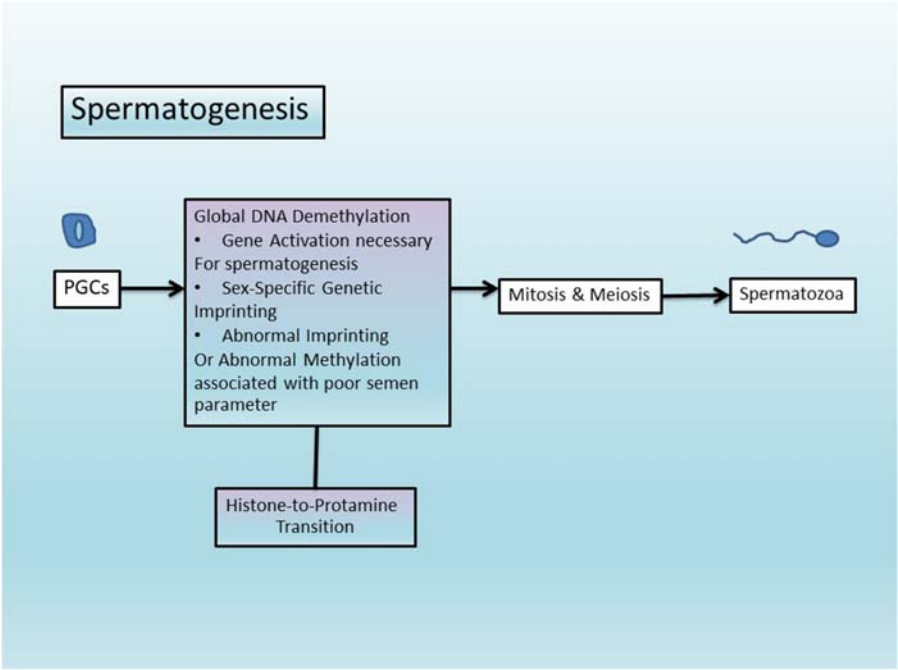

FIGURE 25.2

Male germ cells have compact nuclear DNA. Histones and their variants are substituted by transitional protein, and finally protamines, making sperms chromatin compacted inside sperms' head. *PGCs*, primordial germ cells.

chromatin reorganization during spermatogenesis, and variants of histones H1, H2A, H2B, and H3 expressed in male germ cells may support the preparation of the chromatin structure for histone-to-protamine transition [8]. Histones and their variants are substituted by transitional proteins and, finally, protamines. Protamines make the sperm's chromatin compacted inside the sperm's head, with the purpose of silencing their transcriptional activity [2,8]. Only 10%−15% of the sperm chromatin contains histone-bound nucleosomes. Moreover, there is evidence that suggests that protamines, histones, and histone variants may be involved in epigenetic regulation in early zygote development [8].

Global demethylation in PGCs causes active expression of many genes essential for gametogenesis, including deleted in azoospermia-like (Dazl), synaptonemal complex protein 3 (Sycp3), testis-expressed (Tex19.1, Tex19.2), and Mili (or Piwi-like2). In addition, miRNAs and small RNAs in spermatids may play a role in controlling spermatogenesis [8] (Fig. 25.3).

25.3.2 Oogenesis

After PGCs reach the developing gonads, they undergo global DNA demethylation. PGCs proliferate and give rise to seven million oogonia in humans [6]. Oogonia then undergo meiotic division and arrest

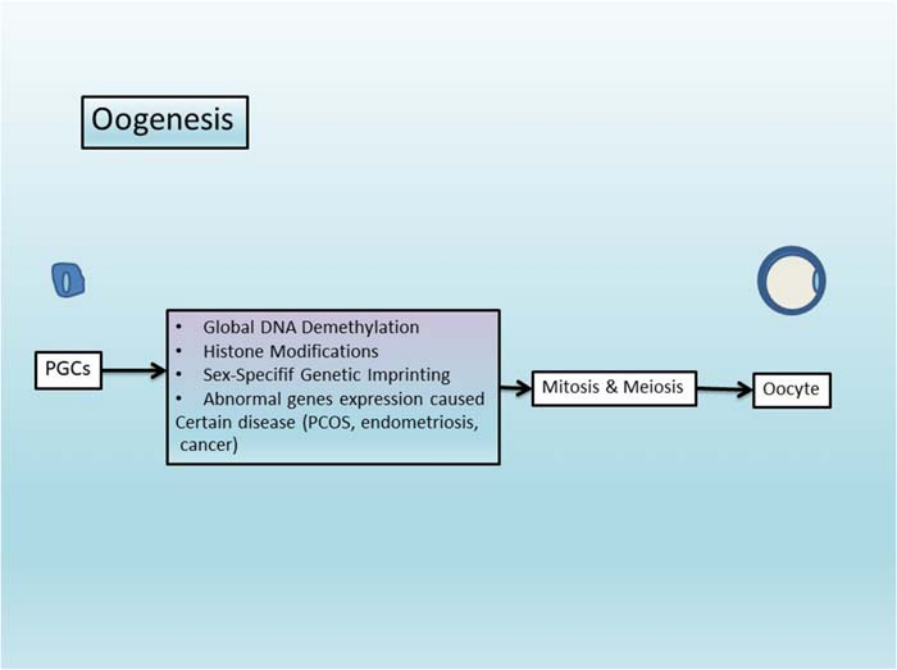

FIGURE 25.3

Maternal DNA in oocyte is bound by histones during oocyte growth. Histone methylations play important roles in the regulation of chromatin structure and gene expression during follicular maturation. *PCOS*, polycystic ovary syndrome; *PGCs*, primordial germ cells.

in prophase of meiosis I [2,6,8]. Female newborns have approximately 1,000,000 primordial follicles at birth [6]. The DNA stays unmethylated, as it remains in primordial follicles. During oocyte maturation, the DNA is gradually methylated to 15% by the end of maturation [6]. Oogenesis requires tight control of dynamic epigenetic changes to ensure normal development. Genetic imprinting in oocytes commences after birth and halts at the diplotene stage of meiotic prophase I [2].

During oogenesis, there is widespread transcriptional changes and de novo DNA methylation, allowing the oocyte to obtain fertilization and embryogenesis competency. A number of post-translational histone modifications or histone remodeling help direct de novo methylation events in the oocyte, independent of DNA methylation maintenance, between cell divisions [8]. Histone H3 lysine4 (H3K4) trimethylation (H3K4me3) and demethylation (H3K4me2) typically delineate sites of transcription initiation [8].

In addition to DNA methylation as stated above, DNA in oocytes is bound to histones and is compacted to form nucleosomes. Therefore, histone modifications, such as methylation or ubiquitination, also play a role in controlling gene expression in oocytes [2,8].

When puberty occurs, a gonadotropin surge triggers oocytes to resume their meiosis division, and they arrest at metaphase of meiosis II as secondary oocytes (MII) (Figs. 25.4 and 25.5).

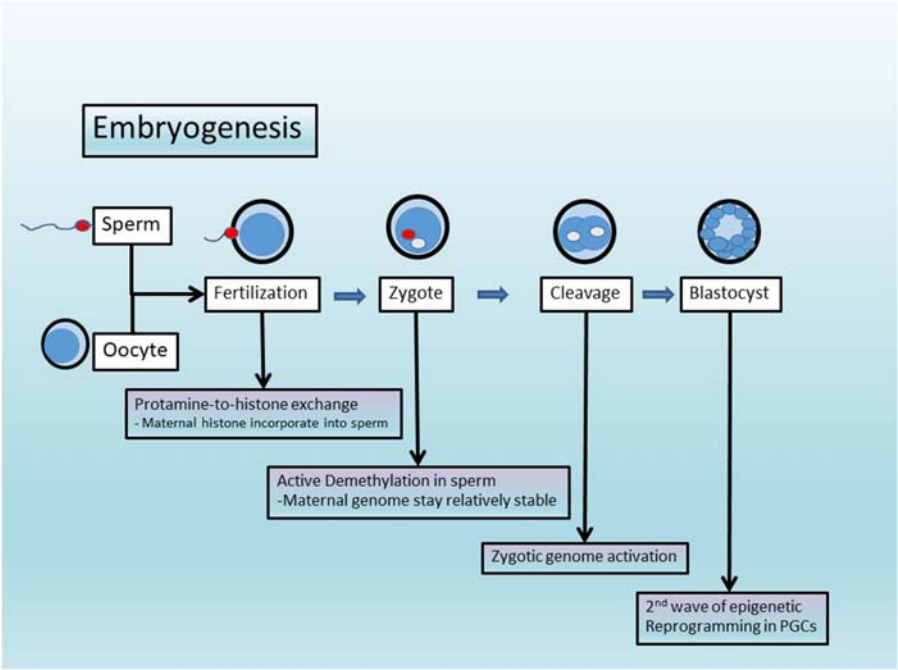

FIGURE 25.4

Embryonic development included an array of cell fate decisions to specify cell lineages that are established during embryo development. It is demonstrated that epigenetic modifier plays an important role to mark the information into cell-specific functional lineages. *PGCs*, primordial germ cells.

25.4 Embryogenesis

The second phase of epigenetic reprogramming occurs in the preimplantation embryo [2]. After fertilization, epigenetic play important role in early developmental events [9] both parental genomes undergo epigenetic or chromatin remodeling [2]. The two parental pronuclei have epigenetic asymmetry in terms of both DNA methylation and histone modifications.

DNA replication in the zygote has been demonstrated as one of the key processes for demethylation of DNA. Researchers showed that maternal Hira/H3.3 is upstream of DNA demethylation and is essential for DNA replication [8]. The epigenetic reconstruction, in terms of global DNA methylation, continues until the 6-cell stage of development [2]. In blastocysts, DNA methylation is generally removed; however, DNA remethylation resumes in most parts of the DNA, except in CGIs [2].

During embryonic development, epigenetics contributed significant role in lineage commitment and restriction of cell potential [10]. DNA methylation provides an epigenetic regulatory mechanism for the differentiation of cells toward their future lineages, while preventing their regression into an undifferentiated state. Epigenetic remodeling during early embryonic development is necessary to direct the zygote from being totipotent to becoming pluripotent stem cells and finally specific unipotent somatic cells. Nonetheless, the imprinted regions in DNA remain methylated to preserve specific epigenetic imprinting according to their parental origin.

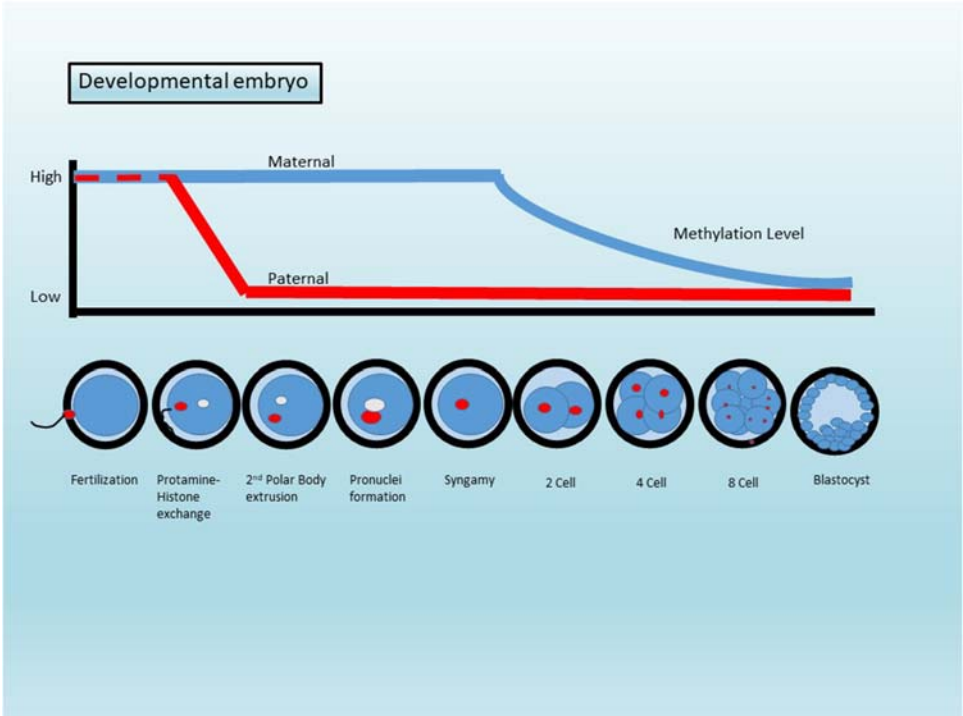

FIGURE 25.5

Developmental embryo and epigenetic modification. The paternal pronucleus undergoes active demethylation, but the maternal DNA is demethylated passively. This dramatic demethylation process is crucial to remove gamete-specific regulatory marks, which facilitated sperm function and allowed the laying down of marks competent to direct embryogenesis.

There are two main mechanisms of epigenetic remodeling in preimplantation embryos: first, histone modifications, which are flexible and transient, and second, DNA methylation and nucleosome repositioning, which are more permanent. Consequently, abiding by normal epigenetic remodeling will ensure normal embryo development, but abnormal epigenetic reconstruction may lead to abnormal development or failure of implantation of the embryo. This included X-inactivation [11], genomic imprinting [12,13], and zygotic genome activation [14]. Sperm DNA methylation alteration, such as insulin like growth factor-2-H19 differentially methylated region (IGF2-H19 DMR), intergenic differentially methylated region (IG-DMR), long interspersed nuclear element 1 (LINE1) promoter methylation, paternally expressed gene 3 (PEG3) and paternally expressed gene 10 (PEG10), might be associated with recurrent pregnancy loss [15].

25.5 Infertility

Infertility in both males and females is related to public health concerns because it can increase the burden on the economy and psychology of the couple. Aberrant genetics or epigenetics can give rise

to infertility or subfertility (Table 25.1). However, changes in epigenetics, without changes in genetic materials, induced by environmental factors can give rise to fertility problems. Therefore, focusing on epigenetics as a cause of infertility with potential heritability through generations is reasonable.

Table 25.1 Epigenetic modifications in infertility.

	Epigenetic modifications	References
Male infertility	• Hypermethylation at several maternal differentially methylated regions in oligozoospermia • Hypomethylation of H19 in oligozoospermia • Decrease methylation at all CpGs in oligoasthenozoospermia • Abnormal DNA methylation in imprinting disorder in oligozoospermia and oligoasthenozoospermia	[5,15]
Female infertility	• Differential methylation CpG site in peroxisome proliferator- activated receptor gamma 1 (PPARG 1) in polycystic ovary syndrome • Inconsistent result of epigenome-wide association studies • Hypermethylation of steroidogenic factor 1 (SF-1) in endometriosis • Hypomethylation at ER-β promoter in endometriosis hypermethylated at promoter of HOXA10 in endometriosis • Hypermethylated at promoter of PR-B in endometriosis	[19,20]

Table 25.1 Epigenetic modifications in infertility.—cont'd

	Epigenetic modifications	References
Assisted Reproductive Technology	• Hypermethylated of E-cadherin in endometriosis • Decline expression of certain miRNAs • Decrease DNA methylation CpGs • Increase risk of preterm and SGA • Frozen embryo transfer increases risk of LGA • Increase risk of Beckwith–Wiedemann syndrome • Conflicting results in Silver–Russell syndrome, Angelman syndrome, and Prader–Willi syndrome	[3,4,9,16,22]

LGA, *large for gestational age;* SGA, *small for gestational age.*

25.5.1 Male infertility

The causes of male infertility are related to many factors such as genetics, epigenetics, and the environment. Nevertheless, in 70% of male infertility cases, the underlying mechanism is unknown [5].

There is evidence supporting aberrant DNA methylation and imprinting disorders being associated with abnormal spermatogenesis and spontaneous abortions [2,5]. A reduced methylation load is found in men with asthenoteratozoospermia [5]. However, some evidence has suggested that hypermethylation is related to poor semen parameters [2]. Additionally, miRNAs play a crucial role in spermatogenesis and are associated with testicular dysgenesis [2].

More studies are needed to identify the causes of male infertility and their consequences.

25.5.2 Female infertility

Available evidence has indicated that epigenetic changes in oocytes are affected by factors such as maternal nutritional status and certain toxin exposures. The aberrations in epigenetics are linked with neonatal development and gestational defects [2]. Many conditions or diseases are associated with infertility or subfertility in females including polycystic ovary syndrome (PCOS), endometriosis, and

aging [2,19,20]. In PCOS, there is a wide range of clinical manifestations including menstrual irregularities, metabolic disturbances, hyperandrogenemia, and follicular cysts on ultrasonography [2]. Studies have indicated that exposure to hyperandrogenism prenatally may disturb epigenetic reprogramming in the reproductive system and cause PCOS [2]. Endometriosis is a hormonal disease with the involvement of immunological abnormalities. Endometriosis develops from a single endometriotic cell lineage, which proliferates and maintains the cellular and genetic identity. These cellular identities are believed to be heritable through epigenetic actions [20]. Advanced female age is associated with poor oocyte quality, reduced ovarian reserve, and decreased pregnancy rate, which may be induced by epigenetic changes [2,19]. More evidence has suggested that aging is related to genetic instability and impaired DNA repair [19], resulting in the inability to conceive. On the other hand, studies have revealed that aging is associated with global hypomethylation [19].

25.6 Role in assisted reproductive technologies

Gametogenesis, fertilization, implantation, and early embryo development are processes that are highly dependent on communication between cells. Research in the field of intercellular communication and epigenetics of reproduction demonstrated that extracellular vesicles and their contents, such as miRNAs and proteins, may contribute to oocyte and sperm maturation, fertilization, and embryo implantation. Successful implantation is dependent on coordination between the embryo and the endometrium, and extracellular vesicles may participate in this required interaction. Analysis of these extracellular vesicles revealed miRNAs and proteins expressed both by the trophectoderm of conceptus and by the endometrial epithelium, such as cathepsin L1 and prostaglandin synthase 2 [21]. In vitro fertilization (IVF), embryo culture, preimplantation genetic testing, frozen thaw embryo transfer are now widely use in contemporary medicine [9]. More than 5 million children, or approximately 1%−3% of yearly births, have been born through ART [2,4]. Data on imprinting disorders and ART are still controversial. It is problematic to distinguish the cause of epigenetic disorders between infertility itself and ART procedures (Table 25.1). Studies have shown that a singleton pregnancy conceived through an ART procedure has a slightly increased risk of preterm birth and babies being small for gestational age [4]. The recent use of Intracytoplasmic Sperm Injection (ICSI) to bypass conventional physiologic fertilization process has raised the concern to offspring (Fig. 25.6). The increased risk of adverse perinatal and long-term outcome might be related with epigenetic modification [16]. In addition, a fetus conceived through a frozen embryo transfer cycle has an increased risk for large-for-gestational-age status [4]. Furthermore, some studies have found that epigenetic disorders are more common in children conceived by ART. Because the epigenome is vulnerable to changes in the environment, especially during ART procedures, including ovarian stimulation, culture conditions, and duration, ART may play a role in triggering epigenetic abnormalities.

The prevalence of four known imprinting disorders is increasing in children conceived by ART; however, to date, data on these imprinting disorders are still in conflict [4].

A review in 2013 showed that the incidence of Beckwith−Wiedemann syndrome is increasing in ART, with a relative risk of 5.2 [4]. However, data on Silver−Russell syndrome are inconclusive. On the other hand, the samples in studies on Angelman syndrome, Prader−Willi syndrome, and retinoblastoma are too small to reach a final determination [4]. Therefore, more studies and data are needed to reach a conclusion.

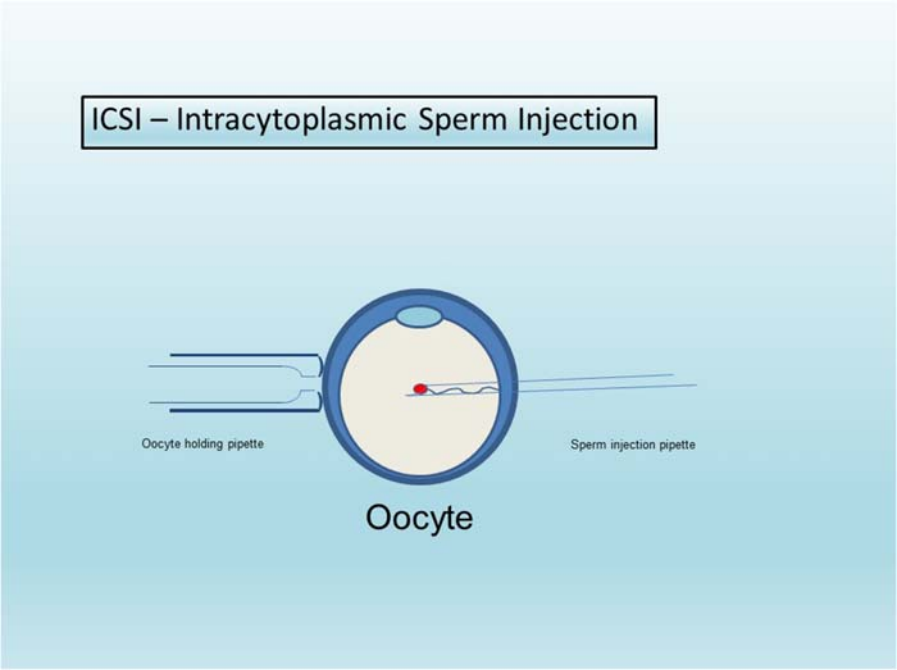

FIGURE 25.6

A breakthrough in modern Assisted Reproductive Technology by using Intracytoplasmic Sperm Injection (ICSI) to promote fertilization has caused concern in health consequences to offspring, both in early and adult life. DNA methylation and epigenetic modification in sperm cells during fertilization might increase risk of chromosomal alteration, congenital anomalies, epigenetic disorder, following ICSI.

A recent study showed that children born from intracytoplasmic sperm injection had a significantly decreased DNA methylation age at birth, lagging approximately half a week behind the controls. ART-susceptible CpGs were enriched in CGIs with low methylation values (0%−20%) and in imprinting control regions [22].

The role of epigenetics during Assisted Reproduction is yet to be elucidated to improve the treatment outcome [16]. The utilization of genome editing technologies to find new therapeutic option including control of epigenetic effectors, epigenome editing, noncoding RNA manipulation, epigenetic elements screening are new challenges [10]. The new technique called CNAMS, DNA methylation editing involves CRISP/Cas9 at system-based palindromic repeats and near-infrared upconversion-activated DNA, is an example for therapeutic strategy [10].

25.7 Transgenerational inheritance

Epigenomes are sensitive to environmentally induced alterations in gene expression. These epigenetic modifications can result in reproductive, behavioral, and metabolic disorders [7]. Apart from the direct

effect on individual epigenomes, the alteration in the germline can transmit the disorders through generations [16]. When the epigenetic changes can be transmitted through generations without direct exposure, this effect is termed epigenetic transgenerational inheritance [7]. The new term "mitotic gene bookmarking," a retention of sequence-specific transcriptional factors that sustains cellular identity following cell division, is used to define epigenetic mechanism that coordinated lineage commitment, cell growth, and cell identity [17]. Dysregulation of ribosomal DNA (rDNA) which is easily susceptible to damage and intra/inter chromosomal recombination may cause DNA instability. Extrinsic factors have been shown to influence the activity of gene expression process [18].

The growing evidence of the molecular mechanisms of epigenetic transgenerational inheritance across several generations in organisms ranging from plants to humans has led to the assumption of epigenetic transgenerational inheritance as an important role in molecular mechanisms of development [23]. The role of DNA methylation, histone modifications, and non-protein coding RNA in altering the epigenetic composition of individuals and producing stable epigenetics effects that were transmitted from parents to offspring. The possibility of heritable "epimutations" contributing to heritable phenotypic variation and thus to evolution. Many living organisms undergo one or more genome-wide reprogramming steps in development during which DNA methylation is erased before being established anew for the next generation [23].

Original examples were derived from data from Dutch and Chinese famines. Children conceived and born during the Dutch Hunger Winter suffered from a higher rate of heart disease, low birth weight, and hypomethylation of the imprinted insulin like growth factor 2 (IGF2) gene [7]. On the other hand, Chinese children born during the Great Chinese Famine had increased risk of developing diabetes, obesity, and schizophrenia. These studies emphasized that these environmental exposures can cause long-term health deterioration and can transmit across generations. The studies of family basis and twins showed that variations in epigenetic patterns, such as DNA methylation, are heritable. It has been estimated that 6.5% of the variance in methylation at the IGF2 locus in a study of five single nucleotide polymorphisms is heritable. The study of parent-of-origin effects on risk of common complex diseases has suggested a role of DNA methylation. Separating causal from noncausal associations will become a difficult task because epigenetic measures are phenotypic, not genotypic, and as with phenotypic measures in general, noncausal associations may be the rule rather than the exception. More research is needed to verify the link between epigenetic variation and common complex disease phenotypes other than cancer.

25.8 Epigenetics in gynecologic diseases

Disease-related environmental exposures are shown to be associated with epigenetic alterations. There is still a question remaining about epigenetic changes being a secondary outcome of either exposure or disease or the causal pathway linking the two. Researchers have demonstrated that epigenetic patterns can be altered by several environmental factors known to be associated with disease risk, such as the environment, diet, smoking, alcohol intake, and stress. If a cause−effect relationship cannot be demonstrated, interventions to prevent or treat common complex diseases based on epigenetic mechanisms will not be effective. Defining a prospective relationship between epigenetic alterations and phenotypic traits may have applications in diagnostics or in identifying high-risk cases for nonepigenetic-based interventions and treatment such as PCOS [24].

Previous studies from our labs have shown association of the long interspersed elements (LINE-1, L1s), a group of genetic elements that are found in the human genome with several gynecological diseases, such as PCOS [25], endometriosis [26], hydatidiform moles [27], cervical cancer [28], and epithelial ovarian cancer [29]. Our lab studies showed the association of LINE-1, controlling genes containing LINE-1 expression that translate into phenotypes or diseases. Intragenic LINE-1 RNA-controlling gene expression is regulated by transacting factors and several epigenetic changes at the LINE-1 promoter genes [30]. Intragenic LINE-1 produces RNAs that transcribe in the antisense direction to the pre-mRNA and mRNA level. Changes in gene expression control levels by intragenic LINE-1 have been found in embryonic development and many human diseases such as autoimmune, gynecologic diseases, and cancers [30].

25.8.1 Polycystic ovary syndrome

PCOS is a set of symptoms due to chronic anovulation and elevated androgens in women. Signs and symptoms of PCOS include irregular or missed periods, heavy periods, excess body and facial hair, acne, and infertility. Associated conditions include type 2 diabetes, obesity, heart disease, mood disorders, and endometrial cancer. PCOS is a common gynecologic endocrine disorder among women of reproductive age. It is found to be a combination of genetic and environmental factors [24]. Risk factors include obesity, not enough physical exercise, and a family history with this condition or diabetes. Diagnosis is based on two of the following three findings: no ovulation, high androgen levels, and ovarian cysts. Small, multiple cysts may be detectable by using ultrasound. In young adolescents, infrequent or absent menstruation may raise suspicion for the disease.

The exact etiology of PCOS is unknown. Early diagnosis and treatment along with lifestyle modification and weight loss may reduce the risk of long-term complications, such as type 2 diabetes, heart disease, and metabolic syndrome (Fig. 25.7). Recent study found that women with PCOS have a very high PPARGC1A promoter methylation ratio and lower mtDNA content compared with healthy women, even after controlling for confounding factors such as ATPIII metabolic syndrome risk factors or BMI [31]. A positive correlation was found between the PPARGC1A promoter methylation ratio and the free androgen index (FAI) in the PCOS group, but no correlation of mtDNA content and the FAI. In addition, PPARGC1A promoter methylation and mtDNA content had associations with ATPIII [+] PCOS [31]. These findings suggest that PPARGC1A promoter methylation ratios and mtDNA content may explain the association between PCOS and its metabolic syndrome.

Many studies have hypothesized the pathophysiological development of PCOS to be multifactorial in etiology. No single gene or cluster of genes has been identified as the cause of this syndrome. Many researchers believe that this syndrome involves several genes. Our study showed differences in the gene expression profiles in cumulus cells taken from PCOS patients [25]. The intrauterine environment might also contribute to the development of this syndrome, and fetal androgen exposure inside the uterus [32,33] has been shown to cause epigenetic changes leading to the development of PCOS.

There is growing evidence supporting the role of epigenetic modification as the predisposing cause of PCOS. Recently, LINE-1 DNA methylation levels were also shown to be associated with type 2 diabetes mellitus [34]. Hence, methylation modification of LINE-1 in PCOS might provide a clue to the pathophysiology and treatment of this disease.

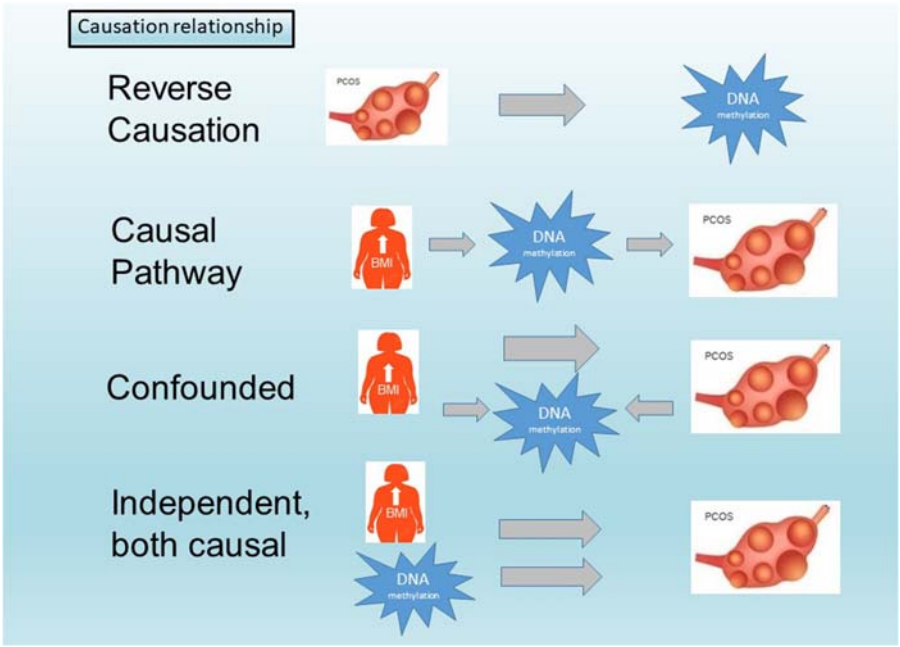

FIGURE 25.7

Possible causation relationships between Exposure, epigenetic patterns, and Diseases. In patient with PCOS, an observed association between BMI and DNA methylation, and DNA methylation and PCOS can help to reveal the causation and prevention. *BMI*, body mass index; *PCOS*, polycystic ovary syndrome.

25.8.2 Endometriosis

Endometriosis is a common gynecological disease defined by the growth of functional endometria outside the uterine cavity. The etiology of the disease remains unclear, and retrograde menstruation is the most widely accepted mechanism of peritoneal endometriotic implants. Pelvic pain, dyspareunia, and infertility are the common symptoms that mostly affect the quality of life of women with the disease. Several theories have tried to explain the possible mechanisms for the development of endometriosis. The possible mechanisms include coelomic metaplasia, immune system abnormalities, vascular and lymphatic spread, and transformation of embryonic rests. It is a benign disease, but it can manifest some malignant behaviors, such as invasion to surrounding tissues, metastasis to distant locations, and recurrence after treatment.

Researchers have found several predisposing factors of the disease, such as genetic traits, hormonal dependence, inflammation, and exposure to environmental toxins. Endometriosis is found more commonly among the relatives of patients with endometriosis compared with those of control subjects. Genetic factors associated with susceptibility to endometriosis were confirmed by several different studies. It is postulated that epigenetic mechanisms may also play a significant role in the origin and progression of endometriosis [20]. The number of gene mapping studies for this disease has increased as the role of genetic factors has become more widely accepted. Many deregulated genes have been

shown in endometriotic cells with a variety of functions, including apoptosis, DNA repair, neovascularization, cell cycle regulation, cell adhesion, encoding detoxification enzymes, and immune system regulation. Knowledge of epigenetics has helped us to understand other multifactorial diseases, such as cancer, which shares many common characteristics with endometriosis.

Deoxyribonucleic acid methyltransferases (DNMTs) comprise a family of enzymes that catalyze transfer of a methyl group to DNA. DNMT1 and DNMT3B are significantly increased in ectopic endometrium, but DNMT3A levels are decreased in eutopic and ectopic endometrium of women with adenomyosis. Promoter hypermethylation of progesterone receptor isoform B (PR-B), and silencing of PR-B expression, was shown in eutopic endometrium, resulting in progesterone resistance. DNA hypomethylation and increased expression of CCAAT/enhancer-binding protein β (CEBPB), a transcription factor regulating gene expression to control cellular differentiation, proliferation, and metabolism, may also be associated with the incidence of adenomyosis. In uterine myometrium, m6A RNA methylation-clustered regulators involved in cell adhesion, muscle contraction, and immune response also differed in women with and without adenomyosis. The pathogenesis of adenomyosis may be related to m6A RNA methylation regulators through aberrant expression and actions in both the uterine endometrium and myometrium [35].

Decidualization differentially enhanced miR-30a-5p expression in normal endometrial stromal cells and the enhanced miR-210 expression in endometriotic cyst stromal cells through independent downstream target molecules. The loss of miR-30a-5p-mediated mechanisms of decidualization and the acquisition of miR-210-mediated mechanisms of decidualization may be involved in the progesterone resistance in endometriosis [36].

Epigenetic alterations are linking intrinsic molecular changes detected in endometriosis to lifestyle and environmental influences. There is evidence suggesting that endometriosis is associated with epigenetic perturbation [20,37]. First, *HOXA10* is a transcription factor and is expressed at the highest level in the endometrium at the time of implantation. In endometriosis, the *HOXA10* promotor is hypermethylated, which may be the cause of subfertility in endometriosis patients. Second, PR-B is downregulated or hypermethylated in endometriosis. This supports the evidence of progesterone resistance in endometriosis. Third, *DNMT*, the enzyme involved in transferring a methyl group to DNA, is overexpressed in endometriosis. Moreover, evidence has shown abnormal expression in steroidogenic factor 1 (SF-1), estrogen receptor (ER)-beta, E-cadherin, and miRNAs. Several miRNAs, such as miR202-3p, miR424-5p, or miR556-3p, may contribute to a reduction in the angiogenic activity observed in ovarian endometriotic cyst or deep infiltrating nodules [37]. Researchers have shown that *DNMT1*, *DNMT3A*, and *DNMT3B* are overexpressed in the epithelial component of endometriotic implants compared with normal controls. A positive correlation among expression levels of *DNMT1*, *DNMT3A*, and *DNMT3B* has been shown. The upregulated expression of *DNMT*s in endometriotic tissue, which leads to hypermethylation, has been shown in women with endometriosis. Unchanged *DNMT* expression in response to in vitro decidualization in endometriotic cells may also confirm the aberrant epigenetic status that alters gene expression and contributes to the progesterone-resistant environment observed in endometriosis [20]. The silencing of progesterone and aromatase genes, which are important in the development of endometriosis, by promoter hypermethylation, may be a contributing factor in endometriosis. It has been shown that a single endometriotic lesion originates froms a single progenitor cell, which then forms a cellular lineage. This cellular lineage requires that cells transcribe, or it enables the transcription of specific genes, or regions of genes, at the same time it can suppress others. The gene expression program must be maintained through cell divisions in

a heritable manner through epigenetic processes to maintain cellular identity [20,37]. The monoclonal characteristic of endometriotic lesions suggests that they may carry neoplastic potentials.

The malignant transformation of endometriosis is a new research field in epigenetics. It has been found to be prevalent in approximately 1% of endometriosis cases [38]. Endometrioid and clear cell carcinoma are believed to originate from endometriotic implants [38,39]. Approximately 76% of all endometriosis-associated ovarian cancer cases are endometrioid and clear cell carcinoma. The most common site of malignant transformation is the ovaries. Endometriosis has gained attention from researchers as a possible initiating factor in ovarian carcinogenesis [39,40]. Ovarian cancer may originate from tissue implantation outside the ovaries. The oncogene demethylation and epigenetic silencing through the hypermethylation of tumor suppressor genes might initiate mechanisms such as uncontrolled cell division and the ability to infiltrate surrounding tissues, avoiding apoptosis or sustaining angiogenesis. The hypermethylation of the *MLH1* gene promoter, which is one of the most important mismatch repair genes, and the consequent decrease in gene expression have been shown to be associated with the malignant changes in endometriosis [37]. The runt-related transcription factor 3 gene (*RUNX3*) has been shown to be a tumor suppressor in a variety of cancers. The inactivation of the *RUNX3* gene by promoter hypermethylation may play a role in the malignant transformation of ovarian endometriosis and is closely related to estrogen metabolism [41]. The tumor suppressor gene, Ras-association domain family member 2 (*RASSF2*), is inactivated by hypermethylation of the promoter in several types of cancers [42]. A recent study confirmed that the epigenetic inactivation of *RASSF2* is associated with the malignant progression of ovarian endometriosis and that this epigenetic alteration may be an early event in ovarian tumors [42].

The study from our laboratory has confirmed that hypomethylation of long interspersed element 1 (LINE-1) may be an early molecular event involved in malignant progression of endometriosis [26]. Differentiating between the different subtypes of pathology may be useful for predicting treatment responsiveness and prognosis. Clinical gynecologic knowledge suggests that clear cell carcinoma is a potentially aggressive cancer type that is resistant to chemotherapy and has a higher recurrence rate after treatment. LINE-1 hypomethylation occurs in endometriotic ovarian cysts, and it may occur in endometriosis-associated ovarian carcinoma before its transformation into cancer. Our study found a significant difference in LINE-1 methylation between endometriotic ovarian cyst and endometriosis-associated ovarian carcinoma [26]. Because LINE-1 hypomethylation can promote carcinogenesis by several mechanisms, our data support the carcinogenic potential of epigenetics in endometriosis-associated ovarian carcinoma.

25.8.3 Epithelial ovarian cancer

Epithelial ovarian cancer is the most common type of ovarian cancer. It develops from the cells that cover the outer surface of the ovary. It is difficult to diagnose; most patients with epithelial ovarian cancer present in an advanced stage and may eventually die of disease persistence or recurrence a short period after relapse. Several prognostic factors, such as age, International Federation of Gynecology and Obstetrics (FIGO) stage, tumor grade, and residual tumor [43], are suspected to play a role in the progression of disease. Increasing evidence has been focused on the molecular and genetic aspects that occur in epithelial ovarian cancer [44,45].

Cancer cells are found to be associated with hypomethylation of the genome that largely affects the intergenic and intronic regions of DNA, particularly repeat sequences and transposable elements

[46,47]. Global hypomethylation, shown by the downregulation of genome-wide methylated CpG dinucleotides, is one of the most common molecular events in cancer [44–50]. The losses of genome-wide methylation in cancer may lead to carcinogenesis [51] and may facilitate chromosomal instability [52]. LINE-1 sequences represent genome-wide DNA because LINE-1s are highly repeated and widely interspersed human retrotransposon sequences. LINE-1s constitute approximately 17% of the human genome [53,54]. Most cancers show significantly increased levels of hypomethylation compared with their normal tissue. This epigenetic process is believed to result in chromosomal instability, increased mutation events, and altered gene expression [52]. The reverse transcriptase of LINE-1 was demonstrated to play a significant role in controlling cancer cell proliferation and differentiation, and the insertion of expression-modulating LINE-1 sequences into host gene introns was proposed to fine-tune gene expression. LINE-1 methylation may control this activity, and the consequences of LINE-1 demethylation may be locus-dependent. Therefore, the level of LINE-1 hypomethylation should be associated with the cellular functions and pathophysiology of malignancies [29]. The results of our study indicate that significant decreases in LINE-1 methylation levels occur during epithelial ovarian carcinogenesis [29]. These results are also consistent with recent data that demonstrated LINE-1 hypomethylation in other cancers and ovarian cancer [50].

25.8.4 Hydatidiform mole

Hydatidiform mole is a genetic imprinting disease, caused by abnormal fertilization, such as androgenetic (monospermic and dispermic) diploidy or biparental triploidy [55,56]. Molar pregnancies usually present with painless vaginal bleeding in the fourth to fifth month of pregnancy. Most hydatidiform mole patients show clinical improvement and remission after primary treatment. Approximately 8%–30% [54,57] of patients develop postmolar gestational trophoblastic neoplasms (GTNs). A high-risk hydatidiform mole is characterized by clinical criteria such as a human chorionic gonadotropin level (hCG) higher than 100,000 mIU/mL, theca lutein cysts that are more than 6 cm in diameter, and excessive uterine enlargement. These clinical features are only able to predict 40% of postmolar GTN [54,57]. There is still no appropriate method for predicting malignant changes in hydatidiform moles. Genetic factors may play a crucial role in the malignant transformation of hydatidiform moles. Investigation of molecular markers in hydatidiform moles may help in the early prediction of postmolar GTN [55,58].

Two common methylation changes associated with cancer are promoter hypermethylation and genome-wide hypomethylation at interspersed repetitive sequences (IRS) or transposon-derived sequences. The role of promoter hypermethylation is to inhibit tumor suppressor gene functions. Loss of IRS methylation leads to several consequences including genomic instability and genome-wide gene expression changes. A previous study from our lab showed the methylation statuses of IRS using combined bisulfite restriction analysis (COBRA). COBRA can differentiate IRS sequences into the following four methylation-status categories: hypermethylated, hypomethylated, and two forms of partially methylated loci. COBRA also provides information on the methylation levels [59]. These subclassifications improved the sensitivity of the test in early cancer detection, revealing only the overall methylation levels such as pyrosequencing. Recently, we reported that the LINE-1 hypomethylated loci distinguish tumor DNA more efficiently than the overall methylation levels [27]. The alteration in the percentage of the LINE-1 partially methylated loci may indicate early genome-wide hypomethylation in the multistep process of carcinogenesis.

Prophylaxis chemotherapy for high-risk hydatidiform moles showed improved outcomes [60,61]. Identifying patients at risk for developing postmolar GTNs is very important. However, it has been found that clinical indices were only limited to 40%−50% accuracy [60,61]. More than half of these patients may have experienced toxicity from unnecessary chemotherapy without any benefits. In one of our studies, we set up the ROC of the %mCuC and %uCmC to predict whether postmolar GTN would occur. By using a %mCuC level ≥40.9% and %uCmC level ≤10.7%, combined with a pretreatment βhCG level (considering the pretreatment hCG level ≥100,000 mIU/mL as positive), we show promising diagnostic power (sensitivity 60.0%, specificity 92.2%, PPV 77.4%, NPV 83.8%, and accuracy 82.3%) [25]. This diagnostic tool may allow for the early detection of postmolar GTN and improve the outcome of treatment.

In our study, a high level of %mCuC and a low level of %uCmC in LINE-1 were found in the postmolar GTN group [27]. These results can be found before the clinical manifestations of malignant transformation. The precise measurement of the LINE-1 methylation level may be a valuable marker in managing hydatidiform moles before progression to GTN.

25.8.5 Cervical cancer and human papillomavirus

Cervical cancer is a cancer arising from the cervix, due to the abnormal growth of cells that can invade or spread to other parts of the body. It is the third most common cancer in women [62]. The association of human papillomavirus (HPV) type 16/18 in cervical cancer cases is approximately 70% [63]. The HPV genome is circular and comprises several genes. It is found that the respective products (E6 and E7) of the oncogenic E6 and E7 genes have potent transforming capabilities, largely by targeting p53 and pRB, respectively, for degradation. In the integrated form, E6 and E7 become overexpressed due to E2 disruption [64−66]. In addition to p53 and pRB degradation, recent studies have indicated that there may be additional mechanisms by which E6 and E7 contribute to cancer development [67,68]. E7 may likely be involved in epigenetic alterations by augmenting the activity of *DNMT1*, resulting in a corresponding downregulation of E-cadherin that does not occur through promoter methylation [69,70]. In another study, E7 was shown to decrease tlr9 expression in cervical cancer, but this likely occurred through histone modifications. It is reported that HPV-positive cells show higher levels of promoter methylation than HPV-negative cells. We have previously reported that methylation of the *CCNA1* promoter was largely elevated in approximately 93% of cervical cancer cases, whereas the promoter remained unmethylated in normal controls [71]. Other studies suggested that the *CCNA1* promoter methylation status can be used as the optimal molecular marker for distinguishing between normal/low-grade squamous intraepithelial neoplasia and high-grade squamous intraepithelial neoplasia cancer lesions [72,73]. Our study also found a significant correlation between the integrated form of HPV infection and *CCNA1* promoter methylation [28]. However, the mechanism of virus-induced methylation was unknown. We explored the new possible mechanism using *CCNA1* as a gene model [28]. In addition to the induction of *CCNA1* promoter methylation, there are several other methylated genes also induced by HPV. Expression observation by mass spectrometry revealed evidence that E7 could upregulate and downregulate several genes in C33a cells transfected with HPV E7. Moreover, E7 can bind to several transcription factors, such as E2F, p300, and TBP [74,75]. Therefore, the proposed mechanism by which HPV induces methylation and suppresses expression of the genes is that the E7−*DNMT1* complex interacts with other transcription factors at gene promoters containing cis elements that respond to these transcription factors [76].

The study from our lab showed that E7 of HPV can induce *CCNA1* promoter methylation by forming a complex with *DNMT1*, resulting in decreasing expression of cyclin A1 mRNA. This finding provides a model by which E7 of HPV mediates the methylation of several gene promoters in HPV-associated cancers. This present observation could be very useful for drug discovery in treating cancers associated with HPV infection, as well as many other diseases in the future, by using an epigenetic treatment model.

25.8.6 Endometrial cancer

Endometrial cancer is the most common female genital cancer in the developing world, with adenocarcinoma of the endometrium being the most common type. Approximately 75% of women with endometrial cancer are postmenopausal. The most common symptom is postmenopausal bleeding. Aberrant DNA methylation is a feature of most cancers and can be an early event, together with histone modifications, chromatin remodeling, and ncRNA alterations, for endometrial carcinogenesis [77–79]. Most tumors show more aggregate CpG island (CGI) methylation than normal controls, suggesting that promoter methylation is more prevalent in endometrial cancer. Many variabilities in both the extent and patterns of methylation across and within cancer types and profiling methylation have increasingly become part of the molecular phenotyping for tumors. DNA methylation is an attractive biomarker with potential diagnostic, prognostic, and therapeutic applications [78,79].

25.8.7 Uterine leiomyoma

Leiomyomas of the uterus (or uterine fibroids) are benign tumors that arise from the overgrowth of smooth muscle and connective tissue in the uterus. They are the most common gynecologic tumors, occurring in nearly 70% of all reproductive-aged women, and are the leading indication for hysterectomy. Uterine leiomyomas involve a complex and heterogeneous association of hormones, growth factors, genetic, stem cells, and epigenetic abnormalities. Researchers found the important contribution of epigenetics in the pathogenesis of leiomyomas. Genome-wide methylation analysis demonstrated that a subset of ER response genes exhibits abnormal hypermethylation levels that are inversely correlated with its RNA expression [80]. Epigenetic mechanism has been shown to be related to uterine leiomyoma development, particularly by DNA methylation that regulates gene expression. Hypomethylation/upregulation of oncogenes (PRL, ATP8B4, CEMIP, ZPMS2-AS1, RIMS2, TFAP2C) and hypermethylation/downregulation of tumor suppressor genes (EFEMP1, FBLN2, ARHGAP10, HTATIP2) were identified, which are related to proliferation, invasion, altered metabolism, deposition of extracellular matrix, and Wnt/β-catenin pathway dysregulation. The findings suggested that DNA methylation inhibition may be a viable therapeutic target in uterine leiomyoma [81].

The important role of active DNA demethylation was recently identified with regard to the ten-eleven translocation protein 1–mediated and ten-eleven translocation protein 3–mediated elevated levels of 5-hydroxymethylcytosine in leiomyoma. There are several tumor suppressor genes, including Kruppel-like factor 11 (*KLF11*), keratin 19 (*KRT19*), deleted in lung and esophageal cancer 1 (*DLEC1*), and death-associated protein kinase 1 (*DAPK1*) that display higher hypermethylation levels in leiomyomas when compared with normal tissues. In addition, both histone deacetylase (*HDAC*) and histone methyltransferase are reported to be involved in the biology of leiomyomas [82]. In addition, the inhibition of HDAC by suberoylanilide hydroxamic acid (SAHA) increased expression of

hypoacetylated/downregulated genes in uterine leiomyoma cells, inhibits cell proliferation, cell cycle, extracellular matrix (ECM) formation, and TGF-β3 signaling in human uterine leiomyoma primary (HULP) cells [83]. Histone deacetylation reversion upregulates the expression of tumor suppressor genes in leiomyoma cells, suggesting targeting histone modifications as a therapeutic approach for leiomyoma. HDAC inhibitors may present another viable therapeutic option [84].

Deregulated miRNAs have been identified in leiomyomas, leading to an altered expression of their targets. The existence of side population (SP) cells with characteristics of tumor-initiating cells has been confirmed in leiomyomas. These SP cells exhibit a tumorigenic capacity in immunodeficient mice when exposed to 17β-estradiol and progesterone, giving rise to fibroid-like tissue in vivo. These new findings will likely emphasize our understanding of the important role for epigenetic contributions in the pathogenesis of uterine leiomyomas and pave the way for novel therapeutic options [80–82].

25.9 Epigenetics and the prospects for future treatment

Epigenomic modifiers are a promising prospect in epigenomic-based therapies [85]. However, this is only the case if epigenetic mechanisms can be confirmed in the disease pathogenesis. Many pharmacological industries are interested in epigenome, including *DNMT* inhibitors and histone deacetylase inhibitors (*HDAC*i), which have been used in clinical practice, largely for anticancer treatments [85,86]. Enzymes that regulate epigenetic alterations have been targets of research on pharmacological intervention in endometriosis. The target enzymes of epigenetic drugs include *DNMT*s, *HDAC*s, histone acetyltransferases, histone methyltransferases, and histone demethylases. However, these agents require further investigation to improve the specificity of their pleiotropic effects and evaluation of their safety and efficacy in noncancer treatment. Combination therapies involving *DNMT* inhibitors or *HDAC*s being used with other agents are actively being studied. miRNAs are also emerging as a promising technology in drug development after an increasing understanding of their molecular function and biogenesis. The association between miRNAs' expression and common complex diseases is emerging, providing greater confidence to pursue this useful treatment for the targeted modulation of gene regulation. However, their utility until now is still limited to disease diagnosis.

Reversibility is an important characteristic of epigenetic aberrations. This concept allows us to explore several epigenetic therapies that can potentially reverse the altered epigenetic patterns in affected tissues. In vitro studies have demonstrated possible results with pharmaceutical agents that disrupt the methylation cascade [86]. The selective inhibition of the PGE2 receptors, EP2 and EP4 has been shown to decrease the expression of *DNMT3a* and *DNMT3b* but does not modulate the expression of *DNMT1* [85,86]. It has been postulated that targeting EP2 and EP4 receptors may emerge as long-term nonsteroidal therapy for the treatment of active endometriotic lesions in women. Treatment with a demethylating agent (DMA) has also been shown to significantly increase ERβ mRNA levels in endometrial cells and may indicate a possible epigenetic therapeutic target. The treatment of endometrial stromal cells, which normally do not express aromatase, with a DMA markedly increased the aromatase mRNA expression. There are two classes of DNA DMAs: nucleoside and nonnucleoside *DNMT* inhibitors. They both inhibit *DNMT*s in the S phase of the cell cycle and consequently lower the overall DNA methylation in the target cells. In leiomyoma, the 5-aza-2′-deoxycitidine (5-aza-CdR) treatment inhibits cell proliferation, extracellular matrix formation, and Wnt/β-catenin signaling

pathway targets in HULP cells, suggesting that DNA methylation inhibition is a viable therapeutic target in uterine leiomyoma [87].

The use of synthetic biology approaches aim at controlling epigenetic modifications by sequence-specific artificial small molecules is one of the key strategies in developing personalized nucleic acid targeted therapy. The application of N-methylpyrrole (P) N-methylimidazole (I) synthetic polyamides (PIPs) in regulating sequence-specific gene expression has been reported, and these molecules are efficient enough to penetrate cell membranes even at nanomolar concentrations. SAHA-PIP was synthesized by conjugating a histone deacetylase inhibitor SAHA (suberoyl anilide hydroxamic acid) with PIPs. The application of SAHA-PIPs as epigenetically active artificial transcriptional regulators is under investigation [88].

There are other possible therapeutic options relating to epigenetic modification of the histone PTM landscape or of the miRNAs expression profile. These will specifically affect pathological features of endometriosis including pain and inflammation or cell proliferation. The new drugs may help in the future because there is an urgent need for nonhormonal treatments in endometriosis that ideally would reduce painful symptoms and preserve fertility. Recently, the FDA approved *HDAC*i for treating patients with cutaneous T-cell lymphoma [89]. Drugs with acceptable side effects in cancer treatment may appear applicable in some cases of endometriosis in the future.

Practical safety considerations arise that influence the utilization of DMA in current therapeutic strategies of benign gynecologic disease such as endometriosis. The administration of these drugs results in a long-lasting demethylating effect, even after a short period of treatment. Because endometriosis is a benign disease, prolonged cytopenia and gastrointestinal system toxicity are considered serious adverse effects in otherwise healthy patients. Moreover, many of the women with endometriosis may need repeated treatments during their reproductive lifespan; therefore, safety issues should be the primary concern of future studies.

25.10 Conclusion

The understanding of epigenetics and epigenetic modifications is necessary because not only genetic mutations but also epigenetic modifications can induce wide ranges of disorders such as reproductive, metabolic, and behavioral disorders. Genome-wide association studies and other approaches may confirm associations between genetic variants and epigenetic patterns, possibilities for elucidating causal pathways and predicting the effect of manipulation are considerable, through environmental and lifestyle modification, or pharmacotherapeutic means. Moreover, epigenetic changes not only cause abnormalities in individuals who are exposed to certain environments but can also transmit the changes through generations. Through examining the role of environmental factors in causing variations in epigenetic patterns (exposure/epigenotype) and ultimately exploring the causal impact of epigenotype on disease outcomes (epigenotype/disease) using genetical epigenomics and other methods, progress toward epigenetic interventions can be made (Fig. 25.7). Epigenetic markers may become targets for modification and biomarkers for exposure and predicting disease risk. A novel technique such as gene editing needs more understanding in tissue-specific and disease-specific risk and benefit before application. Better knowledge can also provide preventive methods and therapeutic options for epigenetic alterations in the future.

Chapter key points

- The understanding of Epigenetic in Human Reproduction leads the way to new research in assessing the epigenetic risks of assisted reproductive technology and finding the novel method to prevent them.
- In vitro fertilization (IVF), Intracytoplasmic Sperm Injection (ICSI), Embryo culture, Preimplantation genetic testing, Frozen thaw human embryos, changing the external environment of human embryo during development could cause epigenetic alterations and possible health consequences to future generation.
- Recurrent pregnancy loss might be associated with sperm DNA methylation alteration of several imprinted genes, such as insulin like growth factor-2-H19 differentially methylated region (IGF2-H19 DMR), intergenic differentially methylated region (IG-DMR), paternally expressed gene 3 (PEG3), and paternally expressed gene 10 (PEG10).
- The research in Epigenetic and Gynecologic Diseases including metabolic and cancer such as PCOS, Endometriosis, Leiomyoma, Endometrial cancer, Ovarian cancer. Most investigators rely heavily on deregulated genes including apoptosis, cell cycle regulation, encoding detoxification enzymes, and DNA repair. However, safety and efficacy issues should be the primary concern of future studies.

Human reproduction epigenetics case study

Recurrent pregnancy loss (RPL) and DNA methylation deregulation.

Objective

To identify the role of DNA methylation of imprinted genes of recurrent pregnancy loss (RPL) couples.

Scope

RPL is defined as loss of two or more pregnancies. There are several maternal and paternal etiological factors contributing to RPL. The research should focus on idiopathic group where there is no obvious pathology observed. The abnormal DNA methylation of imprinted genes, placenta-specific genes, immune-related genes, and sperm DNA may, directly or indirectly, affect embryo implantation, growth and development, leading to the occurrence of RPL should be study.

Audience

RPL affecting 1%−2% of couples of reproductive age women. It is one of the most frustrating and difficult areas in reproductive medicine. Doctors would find this information useful for counseling and treatment.

Rational

Recurrent pregnancy loss (RPL) is a common and severe pathological pregnancy, whose pathogenesis is not fully understood. Half of the RPL cases remain idiopathic. There is a need to identify putative parental factors that could be contributing toward pregnancy loss in cases of idiopathic RPL and may lead to prevention and treatment.

Expected results and deliverables

The paternal genetic and epigenetic factors and the regulation of the maternal–fetal immune microenvironment that could be associated with RPL especially DNA methylation of selected imprinted genes involved in embryo development could be demonstrated.

Safety considerations

None.

The process, workflow, and action taken

This is a case–control study. Couples undergoing RPL with no identifiable cause will be recruited for study. The control group comprised of healthy fertile couples with no history of infertility or miscarriage. The imprinted genes, placental/decidual genes, sperm DNA, and immune-related genes could be target of study.

Tools and materials used

The paternal genetic and epigenetic factors that could be associated with RPL will be analyzed by pyrosequencing. This included DNA methylation of selected imprinted genes implicated in embryo development, such as long interspersed nuclear element 1 (LINE1) promoter methylation, IGF2-H19 DMR, IG-DMR, MEST, ZAC, KvDMR, MTHFR (C677T/A1298C) polymorphism, PEG3, PEG10, and small nuclear ribonucleoprotein polypeptide N (SNRPN) study in spermatozoa of men whose partners present RPL.

Results

A significant decrease in the global sperm 5 mC levels and significant decrease in DNA methylation at three CpG sites in LINE1 promoter, IGF2-H19 DMR and IG-DMR, a significant decrease in sperm DNA methylation at specific CpG sites may be observed in RPL group. For maternally imprinted genes like MEST, ZAC, KvDMR, PEG3, and PEG10 hypermethylation. Polymorphism studies for MTHFR (C677T/A1298C), IGF2-H19 DMR and IGF2 may reveal significant differences in the genotypic frequencies in males.

Challenges and solutions

Detection of methylation changes occurring at the genome-wide level may reveal further candidate genes having a better distinction between the control and study groups. The supplementation of "folic acid" may compensate for the methylation defects in MTHFR-related RPL patients.

Learning and knowledge outcomes

The study of paternal genetic and epigenetic factors could be useful in identification of possible causes of idiopathic RPL. The understanding of complex association network between different regulation methods and their mechanism acting in fertilization, early embryo development, embryo implantation,

and the maternal−fetal interface is crucial. This may lead to screening couples prior to pregnancy for the risk of miscarriage and point to the potential of therapies based on epigenetics in the prevention of RPL.

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PART

8 Development, aging, and transgenerational effects

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CHAPTER

Epigenetics of transgenerational inheritance of disease

26

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26.1 Introduction

It is becoming clear that the environment we experience during our lifetime can influence the health and development of our descendants. Although environmental stressors might lead to beneficial or disease-causing genetic mutations, the formation and inheritance of these mutations is rare, making it difficult for adaptation to keep pace with relatively rapid fluctuations in the environment. Furthermore, there are several noncommunicable diseases (e.g., type 2 diabetes and cardiovascular disease) that cannot be explained by a monogenic disorder. Instead, polygenic effects and/or gene−environment interactions are likely central to the cause. Indeed, an alternative to genomic inheritance of disease is called "epigenetic inheritance," which involves passing of altered epigenetic information from gamete to early embryo, sparked by an adverse environment. The transfer of such epigenetic "messages" about the environment from generation to generation implicates either the faithful replication of epigenetic information or meaningful reconstruction of an abnormal epigenetic state despite normal rounds of epigenetic reprogramming. Several candidate epigenetic factors are responsive to environmental conditions, are able to escape epigenetic reprogramming, and can be passed directly from germ cells to the zygote at fertilization (the generational boundary) to regulate the eventual transcription of the offspring genome. This chapter explores the mechanisms of germline epigenetic inheritance that influence phenotype in the offspring over several generations—so-called "transgenerational epigenetic inheritance" (TEI).

Although researchers have observed increased disease risk in human populations after exposure of a parent or grandparent to environmental stressors, the incidence and underlying mechanism(s) of epigenetic inheritance from parent-to-offspring or over multiple generations remain uncertain. Identifying a mechanism for epigenetic inheritance in humans is challenging for several reasons. Humans live highly heterogenous lifestyles over many decades and have long generation times, which together make drawing connections between one environmental exposure and a single disease risk in descendants problematic. Furthermore, few human studies involve epigenetic analysis because of a historical lack of candidate molecules or uncertainty about the genomic regions on which to focus. Other challenges include teasing apart genetic influences and the effects of parental physiology (e.g., uterine environment) from germline inherited epigenetic factors. As a result, researchers have turned to an increasing number of exciting plant and animal models of epigenetic inheritance that control for

Epigenetics in Human Disease. https://doi.org/10.1016/B978-0-443-21863-7.00011-1
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confounding factors. These include rodent models of true TEI in which phenotypes persist over several generations. While the field is young with many outstanding questions, a greater mechanistic understanding of TEI will impact how disease prevention and prediction is approached in the clinic.

26.2 Defining epigenetic inheritance

Epigenetic inheritance is the transmission of molecular information between generations in a manner peripheral to the DNA base sequence [1,2]. It is usually triggered by an environmental insult (e.g., deficiency or overabundance of nutrients, presence of toxicants, psychological trauma, etc.) that alters germline epigenetic "information." When transmitted to the offspring, this "information" leads to increased disease risk even when the stressor is no longer present [3]. Patterns of DNA methylation and histone modifications, chromatin architecture (e.g., nucleosome composition and spacing), and small noncoding RNA (sncRNA) content are postulated as germline epigenetic vectors of information that can mediate epigenetic inheritance [2] since they are capable of altering the transcriptome in the early embryo of the offspring, escape epigenetic reprogramming, and are responsive to environmental cues [3]. Disruption of epigenetic regulators or mitochondrial function might also play a role.

In this complex field, it is important to distinguish between direct epigenetic inheritance (or intergenerational epigenetic inheritance) and TEI. Intergenerational epigenetic inheritance occurs when the parent (i.e., the F0 generation) is exposed to an environmental stimulus that causes a disease state in the immediate offspring (i.e., the first filial (F1) generation). While this short-term inheritance of phenotype might have an epigenetic mechanism, it does not necessarily involve sustained germline transmission of epigenetic factors. Instead, the epigenetic change might be present in the parent (e.g., somatic tissue of gonad, uterine environment), which then manifests as phenotype in F1 offspring and is subsequently lost in the following generation [3] (Fig. 26.1). However, in humans and other mammals, it is also difficult to exclude direct stressor exposure of the second filial (F2) offspring via the maternal lineage since the prospective F2 generation is present as a fetal germ cell in the F1 offspring during gestation (Fig. 26.1A). The inheritance of epigenetic change is deemed transgenerational when the phenotype that results is present in a generation that was not directly subjected to the initiating stressor. This includes up to and beyond the F2 generation in the paternal lineage and the F3 generation in the maternal lineage [2,4] (Fig. 26.1A and B). There are few true examples of TEI, and for that reason, its existence is controversial. It is unclear how many generations phenotypic inheritance persists in cases of TEI, but it might depend upon the type and severity of the initial stressor and the underlying genetic background of the model system or human population.

To better understand mechanisms of TEI, it will be vital in the first instance to appreciate how epigenetic information is passed directly from parent to child. That is, by identifying environmentally sensitive epigenetic factors in the sperm or oocyte that are inherited by the early embryo at fertilization to cause disease, we can then explore whether similar epigenetic mechanisms are responsible for disease inheritance over subsequent generations. Researchers tend to use mammalian models that focus on paternal inheritance. This is because father-to-child inheritance allows for the exclusion of confounding factors that occur during maternal inheritance, such as the effects of the in utero environment and maternal care of the offspring (reviewed in Ref. [5]). Indeed, sperm only pass genetic and epigenetic material to the offspring. Yet, additional male influences need to be appreciated, including seminal fluid composition and microbiome [6] and mate choice [7], which together may affect offspring health. While not as well studied, epigenetic inheritance of disease does occur through the

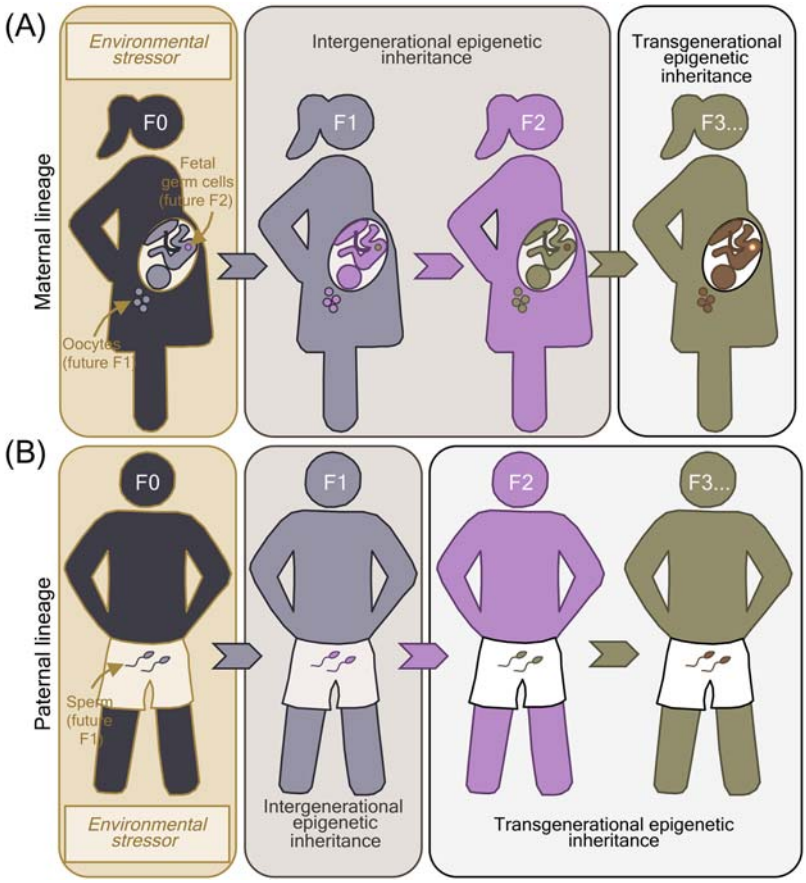


FIGURE 26.1

Intergenerational and transgenerational epigenetic inheritance. Exposure of the parental (F0) generation only to an environmental stressor (tan *background color*; e.g., protein restriction, high-fat diet, vinclozolin, psychological trauma, etc.) leads to epigenetic changes, and increased phenotypic risk in the offspring is called epigenetic inheritance. The generational during of phenotypic inheritance provides mechanistic insight. Intergenerational epigenetic inheritance (*light brown background color*) involves the appearance of phenotypes and/or epimutations in generations in which direct exposure of the stressor cannot be ruled out as a cause. Transgenerational epigenetic inheritance (TEI; *light gray background color*) involves the appearance of phenotypes and/or epimutations in generations not directly exposed to the environmental stressor. The number of generations that are required to pass before inheritance is considered to be TEI depends on whether inheritance is through the (A) maternal lineage or (B) paternal lineage. *F0*, parental generation (*dark gray*); *F1*, first filial generation (*gray*); *F2*, second filial generation (*pink*); *F3*, third filial generation (*green*); F4, fourth filial generation (*yellow-orange*).

maternal lineage [8,9], but fewer experimental models are available for investigation. Furthermore, an affected sperm might initiate TEI in a manner different from an affected oocyte [10], which might be explained by differences in their cellular contribution to the zygote.

26.3 Multigenerational inheritance of human disease

Evidence for epigenetic inheritance in human populations is often correlative and limited due to genetic diversity within and between populations as well as the lack of tissue samples or stringent information when obtaining the data. Few human studies conclusively show persistence of disease phenotypes over multiple generations. However, the study of parent-to-child transmission of disease risk after parental exposure to an environmental stressor is useful for teasing apart direct physiological effects from epigenetic inheritance effects. Human cohorts that support epigenetic inheritance focus on the effects of environmental stressors, such as poor nutrition (e.g., famine or excess nutrients), environmental pollutants (e.g., smoking, toxicant exposure), or psychological insults (e.g., trauma). While the molecules behind such findings are unclear, a flurry of studies in recent years has explored potential epigenetic mechanisms as genome-wide sequencing technologies have become more accessible and affordable. Prominent findings of human multigenerational inheritance of disease are summarized in the following sections.

26.4 Environmental stressors potentially leading to epigenetic inheritance in humans

26.4.1 Nutrition

Worldwide obesity has tripled since 1975 to become a public health crisis. According to the World Health Organization (WHO), most of the world's population lives in countries where overweight and obesity kills more people than underweight, with the consequences of increased risk for cardiovascular diseases, diabetes, musculoskeletal disorders, and some cancers [11]. Given the public health consequences of obesity, it is imperative to investigate the pathogenesis of the disease [12]. Obesity is traditionally attributed to overnutrition and a sedentary lifestyle, but more recently, it has become clear that other factors are involved [12]. Several genome-wide association studies (GWAS) have identified that few genetic variants associate with obesity [12]. This highlights a "missing heritability problem" [12] that shifts us away from genetic mechanisms and allows us to propose epigenetic mechanisms.

Along these lines, an environmental exposure, such as dietary changes, while an individual is in utero or in early life is known to change metabolism. Developed in 1990 by Barker and colleagues, the theory of Developmental Origins of Health and Disease (DOHaD) correlates in utero experience with increased risk for cardiovascular disease, obesity, and diabetes later in life (Box 26.1) [13–16]. When the concept of "developmental programming" was developed, an epigenetic mechanism was not considered since the field of epigenetics was only rudimentary at the time. However, several studies have since shown an epigenetic basis for the mechanisms behind DOHaD [17,18].

Initial investigations into the role of maternal nutrition in developmental programming in humans mainly focused on the Dutch Hunger Winter, which was 8 months of famine that occurred during World War II. Food rations with low nutritional value allocated to the population, including pregnant women, resulted in sharp drop in infant birth weights [19,20], and remarkably, an increased risk for

Box 26.1 Developmental origins of health and disease

The phenomenon of Developmental Origins of Health and Disease (DOHaD), also known as the "Barker hypothesis," emerged from epidemiological studies comparing infant birth parameters with adult disease onset and mortality. The hypothesis was proposed by David Barker and colleagues in 1990 based on studies revealing that intrauterine growth restriction, low birth weight, and premature birth might lead to the origins of adult-onset hypertension, heart disease, and diabetes [13,165,166]. Therefore, undernutrition during gestation might "developmentally program" an individual such that the body's structure, function, and metabolism are permanently altered. Individuals exposed in utero to famine in early gestation had the highest cumulative incidence of coronary artery disease [21], predisposition to diabetes [21], increased female body weight, BMI, waist circumference [22], and breast cancer [27]. Altogether, these studies suggest how the environment influences early development with consequences later in life. The mechanism through which DOHaD occurs is likely epigenetic-based, whereby environmental stressors cause abnormal establishment of stable epigenetic marks during gestation to alter gene expression, developmental pathways, and physiology. However, more research is required to mechanistically understand developmental programming, particularly when these disease risks are inherited by subsequent generations as in TEI.

cardiovascular disease and metabolic syndrome later in adult life [21–23]. More recently, similar metabolic disease risk was observed after prenatal exposure to the Great Chinese Famine (1959–61) and other famines [24–26]. Interestingly, disease risk changes depending on the timing of in utero exposure to famine (i.e., first trimester vs. third trimester). For example, the prevalence of type 2 diabetes in Ukraine [25] or Austria [26] was highest when these individuals were exposed to famine as fetuses during the first half of pregnancy compared with the second half. The first trimester of pregnancy might be the most sensitive period for fetal programming, at least in the case of famine exposure [21,22,27], because this is when most epigenetic patterns throughout the genome are being reestablished as fetal and placental stem cell lineages are specified and differentiation is initiated [28]. Thus, the human fetal epigenome may be sensitive to environmental stressors with the potential for lifelong consequences to health.

There is circumstantial evidence that increased disease risk associated with in utero exposure to the Dutch famine in human populations might also be transgenerationally inherited. For example, women who were exposed to famine during their own in utero development had first born children with increased birth weights [29] and grandchildren with increased neonatal adiposity [23] (Fig. 26.2A). In support of this finding, in utero exposure of the F1 generation to the Chinese famine leads to increased risk of hyperglycemia in the F2 generation [24]. These multigenerational effects of undernutrition might also be sexually dimorphic. For instance, the granddaughters of women exposed to famine in utero had higher mean ponderal indices (relationship between weight and length) at birth compared with their grandsons [23] (Fig. 26.2A). The consequences of ancestral famine on adult health of the grandchildren are unclear in these studies. However, given that these neonatal phenotypes appear in a generation that was not directly exposed to famine as a fetus or germ cell, TEI can be hypothesized.

The diet of men is also linked multigenerational disease inheritance. In a human population in Overkalix, Sweden [30], detailed historical records of food supply of boys during their slow-growth period (the time before the onset of puberty) were linked to sex-specific causes of death of their grandchildren [31,32] (Fig. 26.2B). For instance, a good harvest year during a paternal grandfather's slow-growth period resulted in increased risk of diabetes and reduced survival of his grandchildren [32] (Fig. 26.2B). Additionally, paternal grandmothers in a similar situation had granddaughters with

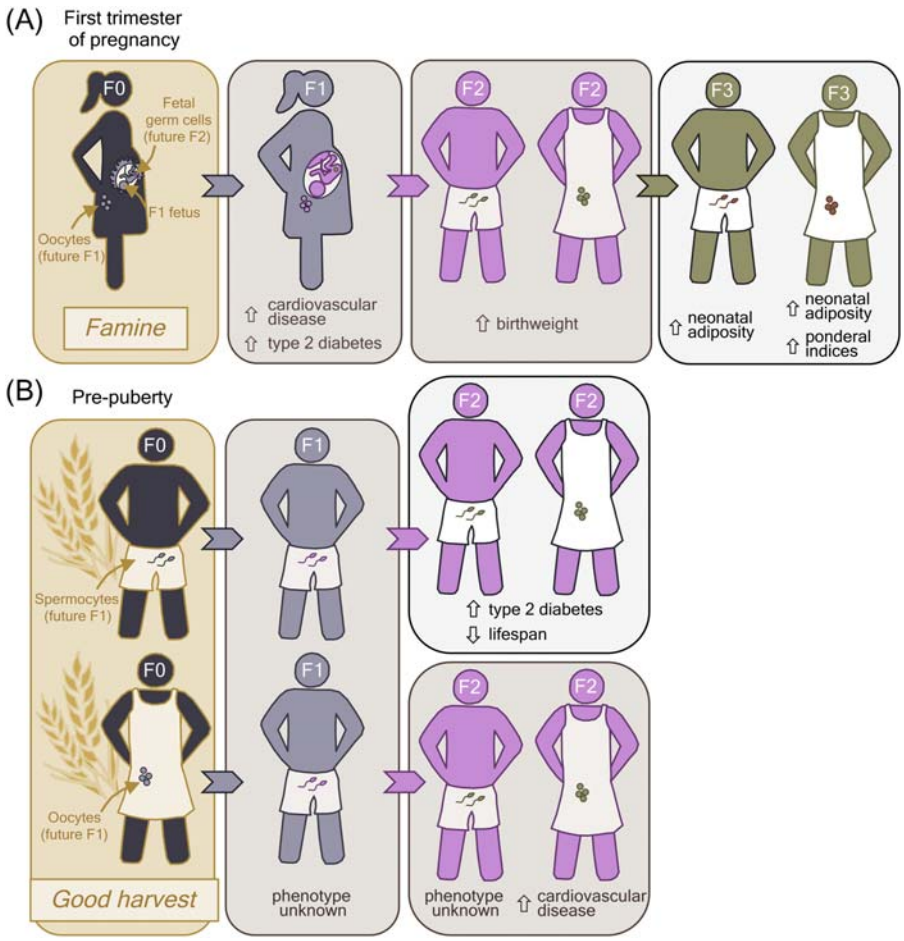


FIGURE 26.2

Multigenerational effects caused by nutrition in human populations. (A) Famine during the first trimester of pregnancy leads to intergenerational (*light brown background*) and transgenerational inheritance (*light gray background*) of disease risk and phenotypes in offspring [21–23,25–27,29]. (B) A good harvest during the slow-growth period (prepuberty) results in the inheritance of increased disease risk in a sexually dimorphic manner [30–32].

an increased risk of cardiovascular disease [30] (Fig. 26.2B). These studies identify another environmental exposure-sensitive period during development (i.e., the slow-growth period) that has implications for the germ cell epigenome in the prepubescent period. Multigenerational epigenetic analysis was not possible due to the retrospective nature of these historical famine studies. While the effects of modern day famines should be sensitively analyzed, it is important to appreciate that psychological stress caused by famine might be a confounding factor to multigenerational inheritance (see Section 26.4.3), particularly in famine associated with warfare.

26.4.2 Toxicant exposure

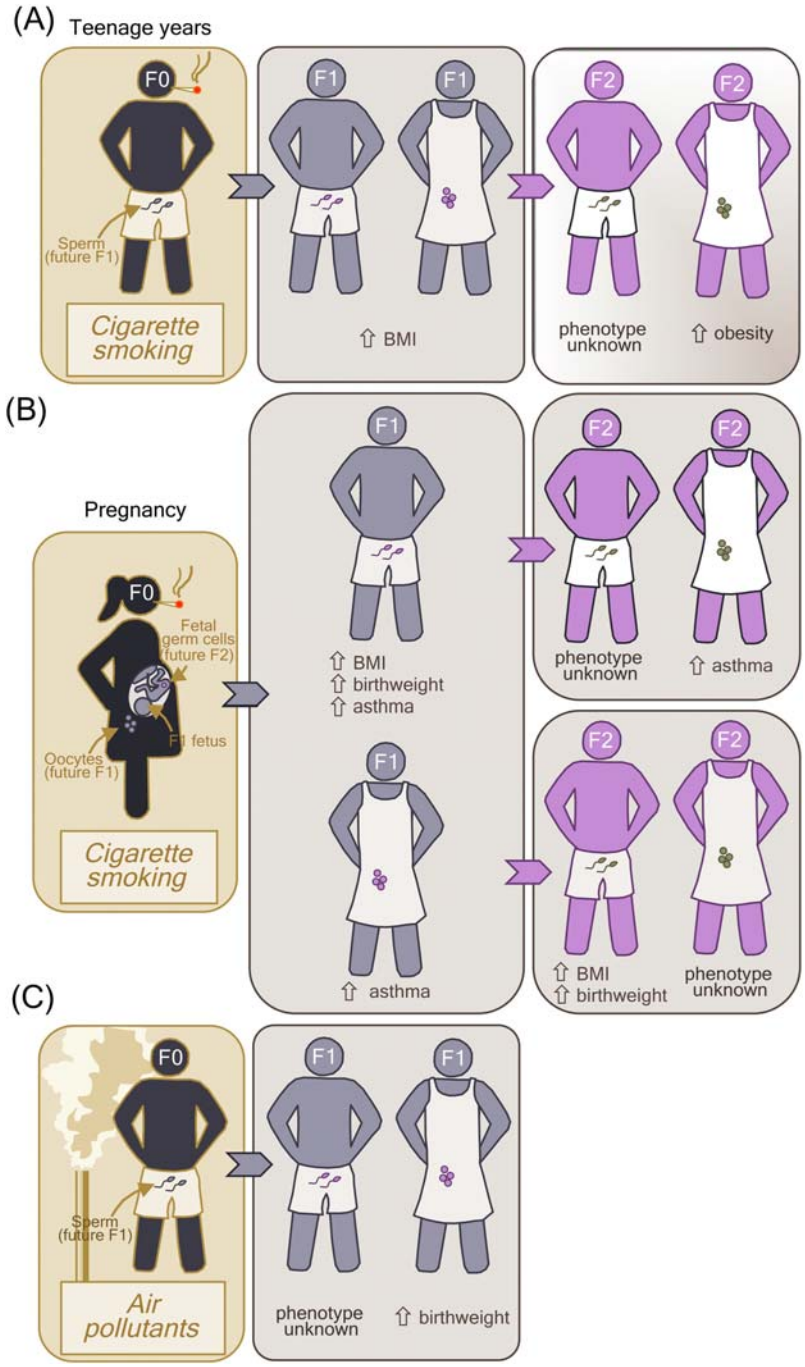
There are a wide range of toxicants in our environment including agricultural fungicides (e.g., vinclozolin), plastic compounds (e.g., bisphenol-A [BPA]), insect repellants (e.g., diethyltoluamide [DEET]), and pesticides (e.g., dichlorodiphenyltrichloroethane [DDT]). Recent studies show that specific toxicant exposure might alter the human epigenome leading to increased risk of asthma, cancer, and other diseases over multiple generations [3]. For instance, the Avon Longitudinal Study of Parents and Children concluded that cigarette smoking in teenage boys was associated with increased body mass index (BMI) in their daughters and sons at age 9 [30,33] and obesity in their granddaughters by age 12 [34] (Fig. 26.3A). Additionally, nonsmoking women, who were exposed in utero to maternal smoking and had increased risk of asthma [35,36], had sons (but not daughters) with increased birth weights and BMI [37] (Fig. 26.3B). If the paternal grandmother smoked, granddaughters had a greater risk of developing asthma [38] (Fig. 26.3B). Collectively, these studies emphasize sexually dimorphic effects of cigarette smoke that result during parent-to-child transmission of phenotype and intergenerational inheritance of phenotype. Whether these phenotypes are caused solely by germline epigenetic inheritance is unclear because the effects of maternal physiology (including high BMI and asthma in F1 mothers) were not mechanistically excluded in these analyses (Fig. 26.3A). Assessing the F3 generation in each lineage might reveal whether TEI occurs in this scenario.

Beyond cigarette smoking, the effects of air pollution on offspring health have come to the forefront because 99% of the global population breathes air that exceeds WHO guideline limits for pollutants, with low- and middle-income countries suffering from the highest exposures [40]. So far, no studies have explored the true transgenerational effects of air pollutants on health risk in humans. Yet, when males (and their germ cells) were exposed to specific air pollutants, increased birthweight was evident in their daughters [39] (Fig. 26.3C). This study is important because it indicates air pollutants might alter the sperm epigenome, potentially affecting the development and health of the next generation. However, one must consider that the children of exposed parents might be born into a similarly polluted environment making the inherited mechanism of disease difficult to separate from direct postnatal exposure. More experimentation is required to more fully understand the transgenerational effects of air pollutants (and other environmental toxicants) on the germ cell epigenome. To do this, animal models are required.

Furthermore, the potential mutagenic nature of some toxicants should be considered when exploring their inherited epigenetic effects (see Section 26.13).

26.4.3 Psychological trauma

Several recent studies in humans have explored the germline-inherited effects of psychological traumatic stress including genocide, war, torture, or paternal death (reviewed in Ref. [41]). For instance, studies focusing on the children to survivors of the Holocaust reveal that these children have increased anxiety and guilt [42] (Fig. 26.4A). Furthermore, parental posttraumatic stress disorder (PTSD) is correlated with the occurrence of PTSD in their children, who were not involved in the Holocaust, themselves [43]. These disease traits might be attributed to lower plasma cortisol levels in adult children of Holocaust survivors, particularly with maternal PTSD [44], which highlights a sexually dimorphic effect (Fig. 26.4A). However, the mother's own mental state and the quality of maternal care must be fully appreciated to separate genetic and epigenetic influences from additional environmental causes. While it is difficult to isolate the impact of parental behavior on human


(A)
Teenage years
F0
Sperm (future F1)
Cigarette smoking
F1
F1
⇧ BMI
F2
F2
phenotype unknown
⇧ obesity
(B)
Pregnancy
F0
Fetal germ cells (future F2)
F1 fetus
Oocytes (future F1)
Cigarette smoking
F1
⇧ BMI
⇧ birthweight
⇧ asthma
F2
F2
phenotype unknown
⇧ asthma
F1
⇧ asthma
F2
F2
⇧ BMI
⇧ birthweight
phenotype unknown
(C)
F0
Sperm (future F1)
Air pollutants
F1
F1
phenotype unknown
⇧ birthweight

offspring from germline inherited effects, researchers have turned to animal models of psychological stress where cross-fostering of offspring to control mothers can mitigate this problem [46]. One example of a mouse model of psychological stress involves the separation of F0 mothers from their newborn pups (F1) in combination with unpredictable maternal stress exposure during the first 2 weeks of F1 postnatal life [45] (Fig. 26.4B). This experimental program leads to transgenerational inheritance of depressive-like behaviors in the F2 and F3 generations. We might conclude that this behavioral phenotype was independent of poor maternal care since newborn F2 and F3 pups were cross-fostered to control lactating mothers [45]. Their results allowed the researchers to more confidently focus on germline epigenetic effects [47] as the mechanism of phenotype inheritance.

26.5 Potential mechanisms of epigenetic inheritance in humans

The majority of information collected from human studies of environmental stress leading to multigenerational inheritance of disease comes from detailed historical records, death certificates, questionnaires, and interviews. Limited molecular or epigenetic analysis has been conducted, though there are studies that have investigated epigenetic biomarkers that provide insight into epigenetic mechanisms. To date, this largely comprises of directed assessment of locus-specific DNA methylation patterns in offspring of stressor-exposed ancestors. For example, in utero famine exposure correlates with decreased DNA methylation in the regulatory control region of the imprinted gene *IGF2* (involved in growth regulation) in blood, a change that was apparently maintained for over 6 decades [48]. This result was in comparison to *IGF2* methylation in same-sex siblings that had normal nutrition during gestation [48]. Others showed that maternal smoking associates with increased DNA methylation at the *IGF2* gene in their offspring compared with offspring of nonsmoking mothers [49]. Interestingly, this smoking-related methylation increase was most pronounced in male offspring [49].

Whether these alterations in DNA methylation have a functional relevance, such as by altering gene expression leading to a disease state, is still to be determined. Furthermore, while assessment of locus-specific DNA methylation can be useful for predicting change in the broader methylome, it is not ideal for uncovering the mechanistic cause of phenotype inheritance. This type of directed analysis potentially misses important methylation alterations throughout the entire genome, including in repetitive regions or genic regions important for the initiation of the phenotype. Therefore, greater focus should be placed on whole-genome analysis for more complete understanding of epigenetic inheritance. For example, genome-wide DNA methylation analysis using an Illumina 450K array of whole blood from individuals exposed to famine in utero found a small increase (0.7%−2.7%) in methylation

FIGURE 26.3

Sexually dimorphic multigenerational effects of toxicant exposure in humans. Cigarette smoking (tan *colored background*) in an F0 (A) male or (B) pregnant female leads to sexually dimorphic effects on the health of their F1 children and F2 grandchildren [30,33–38]. While transgenerational inheritance of phenotype (*white background*) is hypothesized when the effect occurs through the paternal lineage, the confounding effects of the uterine environment cannot be eliminated when the phenotypes are inherited via the maternal lineage. As a result, an intergenerational inheritance is proposed (*light brown background*). (C) Direct effects of paternal air pollutant exposure on birthweight of their daughters [39]. *F0*, parental generation (*dark gray*); *F1*, first filial generation (*gray*); *F2*, second filial generation (*pink*).

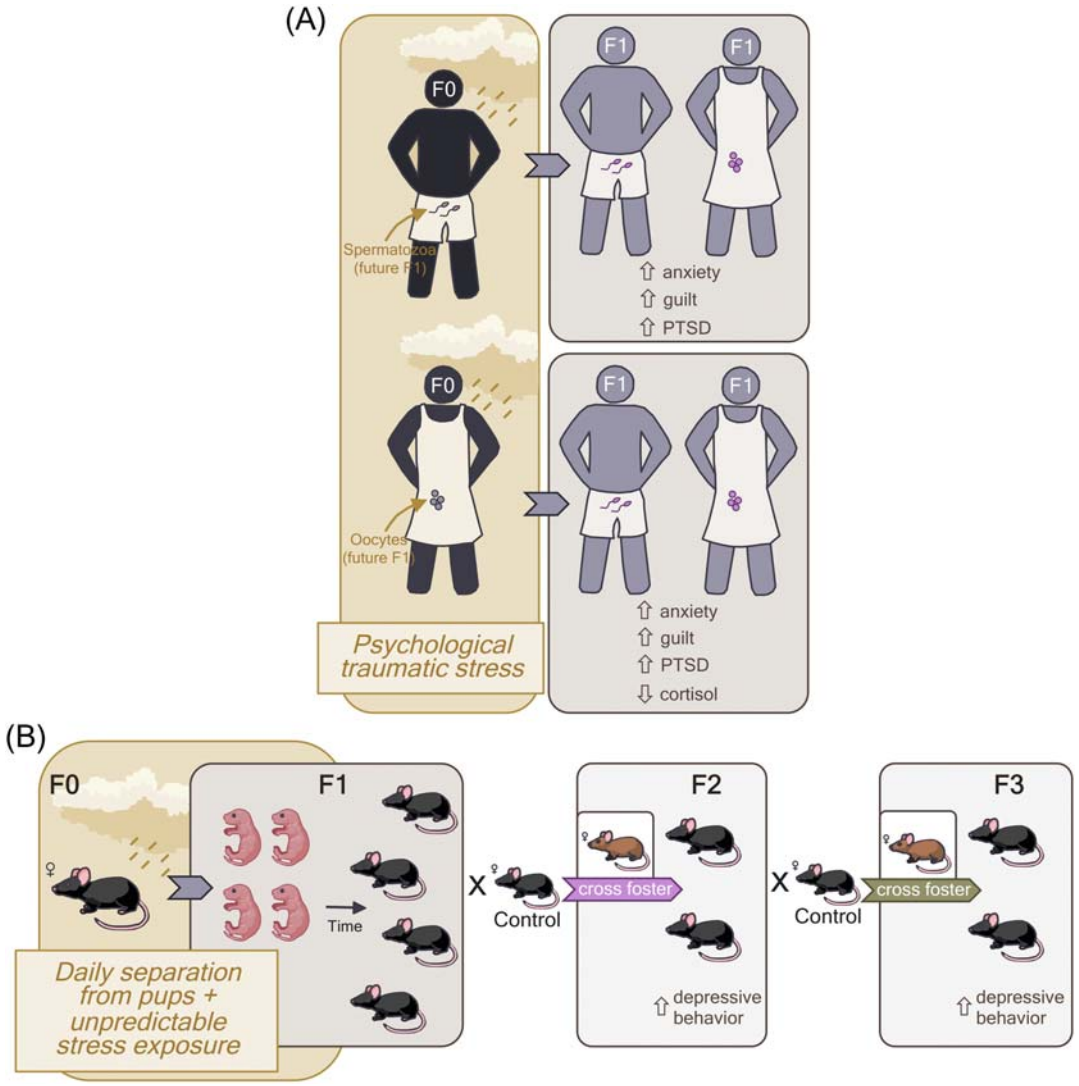


FIGURE 26.4

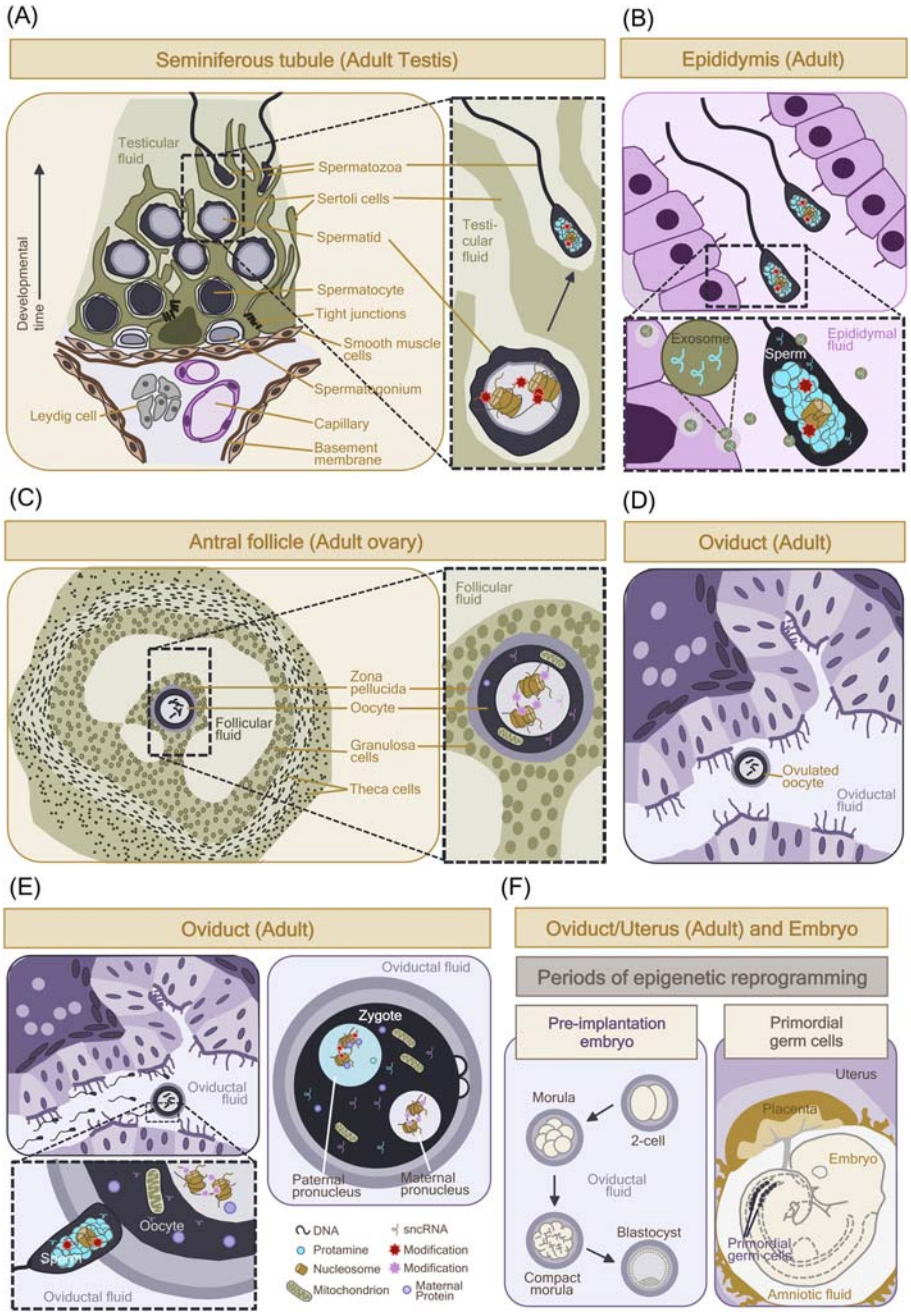
Psychological traumatic stress leads to inheritance of disease risk. (A) Psychological traumatic stress, such as war and genocide experienced during the holocaust, in the parental (F0) generation (tan *colored background*) increases the risk of anxiety, guilt, and PTSD in the first filial (F1) generation offspring through direct parent-to-offspring inheritance [42–44]. (B) A mouse model of psychological traumatic stress (daily maternal separation and unpredictable stress exposure) leading to transgenerational inheritance of depressive behavior risk. Maternal care confounders were eliminated by cross-fostering the second filial (F2) and third filial (F3) generations to control females [45].

at specific CpG sites associated with genes involved in growth, development, and metabolism (e.g., *FAM150B*, *SLC38A2*, *PPAP2C*, etc.) [50]. Only individuals exposed to famine during the first 10 weeks of gestation showed these specific methylation changes potentially reinforcing that epigenetic programming during the first trimester is particularly sensitive to in utero undernutrition [50]. These and other sequencing technologies are now more affordable and provide increased coverage of the genome for higher-resolution data. Once candidate loci are identified using whole-genome approaches, it will be important to confirm the functional impact of changed epigenetic status of these specific loci in relation to disease state. Exploring whether different human populations around the world show similar or different responses to the same environmental cue will help us to validate the findings on a larger scale or to appreciate genetic background effects.

26.6 Developing a mechanistic understanding of epigenetic inheritance

Currently, our understanding of the underlying epigenetic processes of TEI remains in its infancy. Human research showing phenotypic evidence of TEI is encouraging, yet mechanistic exploration of the molecules behind this inheritance phenomenon will only be achieved using experimental models, such as plants and animals. This is because generation times in these models are shorter than humans and access to material, such as germ cells and embryonic tissue, for molecular analysis has fewer ethical barriers. Using animal models to explore epigenetic mechanisms of TEI requires careful consideration of experimental design to meticulously control for confounding factors. Researchers should be mindful of the duration and timing of the environmental exposure (e.g., diet), and whether to use in vitro fertilization, embryo transfer protocols, and/or cross-fostering of litters to control females to negate the effects of seminal fluid and/or so-called "maternal effects" [46]. Other important experimental considerations are as follows: (1) How is epigenetic change measured over generations? For example, what generations, sex, tissue types, and epigenetic marks will be selected for analysis? Will the analysis include single-cell or whole tissue methods, or locus-specific or genome-wide methods? (2) Do the candidate inherited epigenetic changes have functional implications? Alterations of the epigenome (sometimes referred to as "epimutations") have the potential to affect gene expression or chromatin stability. If epimutations occur in germ cells, it has implications for zygotic genome activation and transcription thereafter, leading to increased risk of a phenotype or disease in the offspring [51]. (3) Lastly, certain environmental stressors, such as chemicals (e.g., cigarette smoke) or dietary deficiencies (e.g., deficiency in one-carbon metabolites), might cause genetic mutations leading to disease risk. Indeed, genetic stability is intricately linked to epigenetic effects since epimutations might be precursors to genetic mutations [3,52] that can then be passed on to offspring through classical genetic inheritance. Thus, genetic mutations might be potential confounders or contributing factors in models of TEI of disease (see Section 26.13) and should be considered when generating data sets and during data analysis [10].

It is essential to consider the normal periods of epigenetic reprogramming that occur in the preimplantation embryo and in the primordial germ cells that, if done incorrectly, might enable inheritance of an abnormal epigenetic factor (Fig. 26.5F; Box 26.2). Additionally, attention should be paid to the milieu in which germ cells form and mature since these microenvironments are sensitive to environmental stressors (e.g., protein restricted diet [54]) and play a role in shaping the germline


(A)
Seminiferous tubule (Adult Testis)
Testicular fluid
Spermatozoa
Sertoli cells
Spermatid
Spermatocyte
Tight junctions
Smooth muscle cells
Spermatogonium
Capillary
Basement membrane
Leydig cell
Developmental time
Testi-cular fluid
(B)
Epididymis (Adult)
Exosome
Sperm
Epididymal fluid
(C)
Antral follicle (Adult ovary)
Follicular fluid
Zona pellucida
Oocyte
Granulosa cells
Theca cells
(D)
Oviduct (Adult)
Ovulated oocyte
Oviductal fluid
(E)
Oviduct (Adult)
Oviductal fluid
Oocyte
Sperm
Zygote
Paternal pronucleus
Maternal pronucleus
DNA
Protamine
Nucleosome
Mitochondrion
sncRNA
Modification
Modification
Maternal Protein
(F)
Oviduct/Uterus (Adult) and Embryo
Periods of epigenetic reprogramming
Pre-implantation embryo
Morula
2-cell
Oviductal fluid
Blastocyst
Compact morula
Primordial germ cells
Uterus
Placenta
Embryo
Primordial germ cells
Amniotic fluid

Box 26.2 Epigenetic reprogramming

The dynamics of epigenetic reprogramming is best studied in mouse, though similar processes occur in humans as well. In humans and rodents, there are two waves of dynamic epigenetic reprogramming that occur between generations. The first wave is in the developing primordial germ cells, and second wave is in the preimplantation embryo [61] (Fig. 26.5). As primordial germ cells migrate to the genital ridge of the developing embryo, the majority of DNA methylation is removed throughout the genome [167] and histone marks are remodeled [168]. Prior to birth, DNA methylation patterns are reestablished in the germ cells, including at imprinted genes [167]. In sperm, the majority of histones are replaced by protamines to increase DNA compaction within the nucleus [82] (Fig. 26.5). Up to 85% and 99% of histones are replaced in human and mouse sperm, respectively [82]. Some histones are retained in sperm at two distinct sites: promoters of developmentally regulated genes [169] and gene-poor repeat regions [170]. Nucleosomes containing histones are retained genome wide in the mouse oocyte [169]. During the second wave of reprogramming in postfertilization zygotes, most DNA methylation is erased, although imprinted regions and some repetitive loci are protected and therefore, maintained [65]. Shortly after fertilization, protamines are replaced by histones on the DNA of the paternal pronucleus (Fig. 26.5), and appropriate histone modifications (e.g., methylation, acetylation, etc.) are acquired [171].

epigenome (Fig. 26.5A–E). Indeed, the niche in which the germ cells form, develop, and mature might act as a mediating factor promoting epigenetic inheritance.

How an environmental stressor leads to germline epimutations that are stable over multiple generations is complex given epigenetic reprogramming events that occur between each generation. Currently, there are two key paradigms of epigenetic inheritance: replication and reconstruction [51].

26.6.1 Replicating the abnormal epigenome between generations

Replicative inheritance requires epigenetic marks along with DNA to be directly copied between cell divisions and generations [51] (Fig. 26.6A). This theory predicts that the same epigenetic change should be evident in each generation. However, in normal development, the majority of epigenetic marks are removed and replaced between generations during epigenetic reprogramming (Box 26.2).

FIGURE 26.5

Tissue environments in which development and epigenetic reprogramming of germ cells and the preimplantation embryo occur. These niches include (A) seminiferous tubule of the testis where spermogenic cells develop with direct and/or hormonal support from Sertoli cells and Leydig cells [53]. Upon differentiation, spermatozoa are released into seminiferous tubule lumen in fluid secreted by the Sertoli cells. (B) Spermatozoa mature in the lumen of the epididymis where they are bathed in epididymal fluid and take up exosomes secreted by epididymal epithelium that contain small noncoding RNAs (sncRNAs) [54,55]. (C) Oocytes develop in ovarian follicles and are in direct contact with granulosa cells and follicular fluid [56,57]. (D) Ovulated oocytes are exposed to fluid secreted by oviduct cells (fallopian tube in humans) [58]. (E) Upon fertilization, spermatozoa and the oocyte are exposed to oviductal fluid. Note: Spermatozoa are also ejaculated with seminal plasma [59] into the vagina and are exposed to the cervical secretions and uterine fluid on their path to reach the oocyte (not shown). Fertilization involves the fusion of an oocyte and spermatozoon, and the deposition of sperm epigenetic factors, such as modifications bound to DNA and histones/protamines and sncRNAs obtained in the epididymis, into the oocyte. (F) There are two periods of epigenetic reprogramming (see Box 26.2): Within the preimplantation embryo when it is exposed to the oviductal and uterine fluids, and within the primordial germ cells, which form within the genital ridge of the postimplantation embryo.

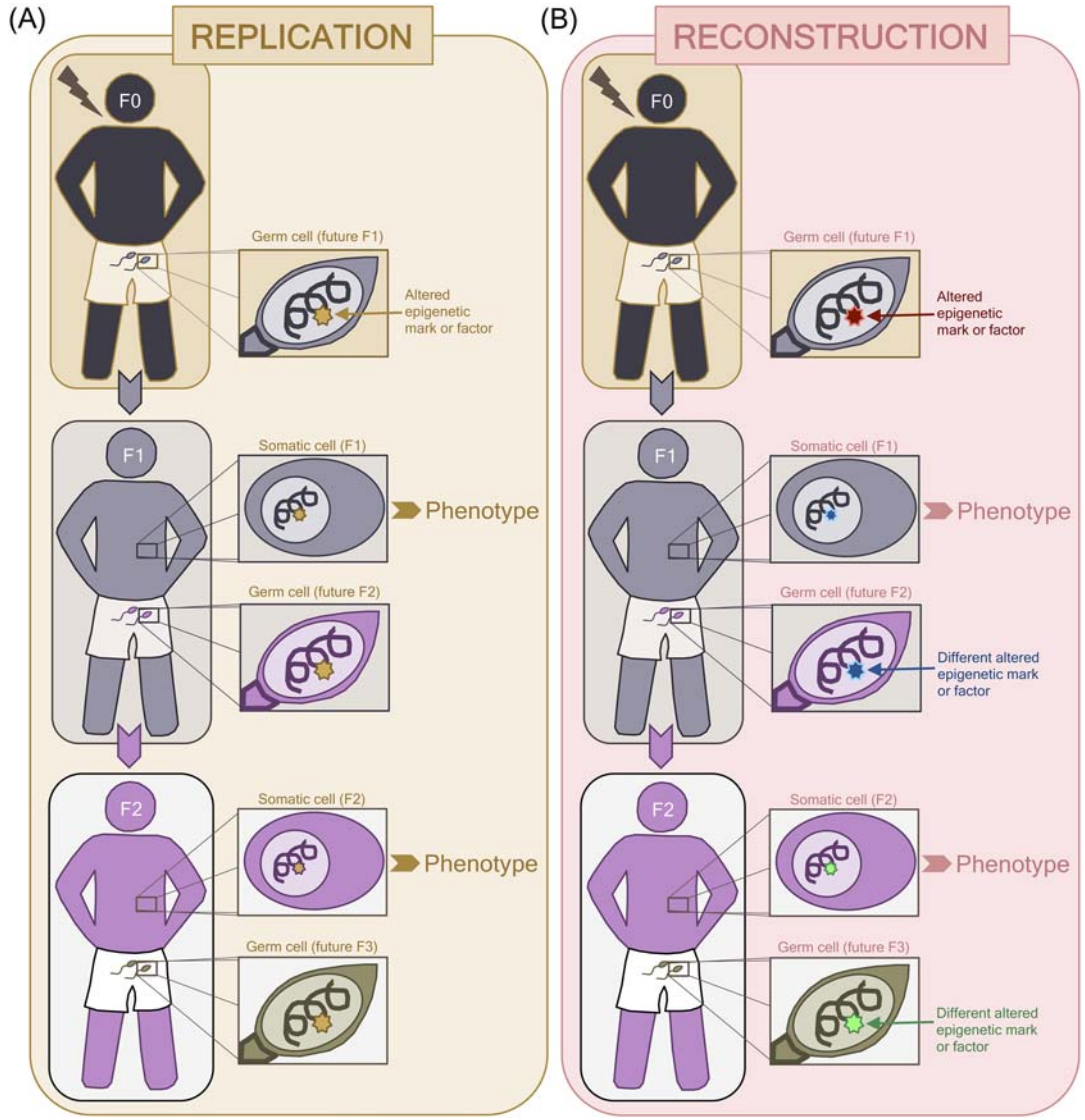


FIGURE 26.6

Replication versus reconstruction models of transgenerational epigenetic inheritance. Two potential models of epigenetic inheritance include the replicative and reconstruction models of inheritance. (A) Replicative inheritance involves the exposure of the F0 generation to an environmental insult resulting in the direct inheritance of an epimutation(s) (denoted by *yellow star*) via the germline. For this to happen, epimutations must be stable and escape two waves of epigenetic reprogramming that occur in each generation, allowing them to persist in subsequent generations (i.e., F1, F2, etc.) and lead to a phenotype or disease state. (B) Reconstructive inheritance, hypothesized by Eva Jablonka, is an alternative explanation for epigenetic inheritance [60]. This model suggests that epimutations (*red star*) caused by an environmental insult resulting in a phenotype or disease state in the F0 generation undergo epigenetic reprogramming. However, these epimutations are "reconstructed" (*blue star*) in the F1 generation based on traces (e.g., small noncoding RNA) of the parental epigenetic landscape. These traces are sufficient to direct the formation abnormal epigenetic marks in the next generation with the potential to cause a similar phenotype or disease state. A similar event between the F1 and F2 generations results in successive programming of the phenotype over multiple generations.

This allows for the epigenome to be reset and to reestablish totipotency. Epigenetic reprogramming is also meant to remove epigenomic alterations that represent the history of environmental exposures experienced by the parental generation [61]. However, replicative inheritance potentially allows for the inheritance of select epigenetic alterations so that the offspring might predict the environment into which they are born (see Section 26.16). Studies in human primordial germ cells in normal conditions showed that there are regions of the epigenome that are resistant to DNA demethylation during epigenetic reprogramming [62]. Reprogramming "escapees" are largely retrotransposable elements, but other genomic regions were identified, including over 7000 repeat-poor regions (with less than 10% sequence overlap with repeat sequences) and single-copy genes involved in human neural and metabolic disorders [63]. Some of the repeat-poor escapees remain partially methylated in the early human preimplantation embryo suggesting these regions escape both waves of epigenetic reprogramming in the germ cell and the preimplantation embryo [63]. Resistant regions show enrichment for the histone methylation mark H3K9me3 and binding sites for Krüppel-associated box zinc finger proteins (KRAB-ZFPs) that induce heterochromatin formation [63]. Reprogramming resistant loci were also identified in mouse primordial germ cells [64] and blastocysts [65], which lends mechanistic relevance to mouse as an animal model of epigenetic inheritance. Replicative inheritance of epigenetic marks might occur if exposure to an environmental stressor drastically alters the epigenomic regions that are more resistant to normal reprogramming than other parts of the epigenome. In this case, an abnormal epigenetic mark in the germline would be replicated in the early embryo, allowing for the inheritance of environmental information. TEI would require this event to similarly occur over several generations (Fig. 26.6A). It is currently unclear whether a genomic region that is typically epigenetically reprogrammed can gain reprogramming resistance after environmental stress for replicative inheritance.

26.6.2 Reconstructing the abnormal epigenome between generations

Eva Jablonka postulated an alternative theory to replicative inheritance called the "reconstruction" model (Fig. 26.6B). This model avoids the challenges presented by epigenetic reprogramming [60] by suggesting that epigenetic marks responsible for a phenotype undergo normal reprogramming in the germline and preimplantation embryo but are then reconstructed in each successive generation [60]. Traces of the parental epigenetic landscape may be sufficient to reconstitute the parental epigenetic state in the offspring and thus bring about a phenotype [60]. For example, a well-known human epimutation characterized by DNA hypermethylation of the *MLH1* gene promoter [66] lends support to reconstructive inheritance in humans. The *MLH1* epimutation occurs in all somatic tissues in association with loss-of-expression of same allele and increased risk for Lynch syndrome (early-onset cancer in several tissue types) [66,67]. The *MLH1* epimutation most often forms de novo and in the absence of genetic variants [68], though very rare instances of epigenetic inheritance were reported [69]. One such case involves apparent mother-to-son inheritance of the *MLH1* epimutation, yet there was clear evidence of germline epigenetic reprogramming of this locus in the mother's oocytes that gave rise to unaffected offspring and in the affected son's spermatozoa [69]. Mechanistically, this type of phenotype inheritance might be driven by non−DNA-associated factors passed on from the germ cells to the zygote, such as sncRNA [47,54,55,70] or maternally derived proteins or organelles [8,10,71,72] (Fig. 26.5). These inherited factors might cause the reconstruction of similar (but not necessarily identical) abnormal DNA-associated epigenetic marks after reprogramming is concluded. This mechanism may lead to serial reconstruction of one or more induced phenotypes over multiple generations, such that an abnormal phenotype in the F0 generation is epigenetically reconstructed in the F1 generation, and so on (Fig. 26.6B) [73].

Despite their seemingly contradictory messages, the replication and reconstruction models need not be juxtaposed. In reality, there may be an element of direct inheritance of some epigenetic marks (replication) and reconstruction of additional marks linked to an ancestral environmental exposure.

26.7 Mechanistic candidates of transgenerational epigenetic inheritance

Mediators of epigenetic inheritance must be responsive to the environment. Key mechanistic candidates that are postulated to pass heritable information between generations via the germline include DNA methylation, histone modifications, and sncRNA. Inherited proteins or organelles might also play a mechanistic role. Each of these mediators of epigenetic inheritance will be considered in the key models in which they have been investigated.

26.7.1 DNA methylation

In humans and other mammals, methylated cytosine in form of 5-methylcytosine (5mC) is the predominant form of DNA methylation. In other organisms, such as *Caenorhabditis* (*C.*) *elegans* and *Drosophila melanogaster*, 5mC is either absent or detected at extremely low levels. However, methylation of adenine to generate N6-methyladenine (6mA) is present in these species [74] (and human cells at very low levels [75]) and may play a similar role. DNA methylation in the control regions of genes is generally associated with gene repression, although its exact role in gene regulation is likely locus-dependent [76]. Genomic patterns of DNA methylation are typically tissue-specific signatures and are largely established as a cell differentiates. Beyond regulation of gene expression, a key role of DNA methylation is to silence repetitive DNA [77].

DNA methylation has many properties that make it an attractive mechanistic candidate for epigenetic inheritance: (1) it can be environmentally modulated; (2) machinery exists to replicate methylation patterns onto newly synthesized DNA and thus it is mitotically heritable; and (3) methylated loci resistant to epigenetic reprogramming in the zygote and germline have been identified. In the context of epigenetic inheritance, exploring how DNA methylation status of germ cells and somatic cells associates with gene expression and phenotype remains challenging. Indeed, researchers are only beginning to understand how DNA methylation and transcriptomic information overlap. For example, in utero undernutrition of mice causes intergenerational epigenetic inheritance of a metabolic phenotype and yet DNA methylation patterns are either largely unchanged in the F1 and F2 generations despite gene misexpression or are changed but associated with normal gene expression [78]. Further examples of DNA methylation in models of epigenetic inheritance are mechanistically explored in the following (see Section 26.8).

26.7.2 Histone modifications

Chromatin packaging and nucleosome positioning on DNA are regulated, in part, by chemical modifications (e.g., methylation, acetylation, etc.) that are applied to histone proteins around which DNA is packaged. These modifications serve to regulate gene expression through the recruitment of protein complexes and by controlling the accessibility of the DNA to the transcription machinery [79]. Histone modifications can also be environmentally modulated and propagated onto newly assembled

chromatin by complex regulatory machinery [80]. While present in oocytes [81], most histones are removed during spermatogenesis and replaced with protamines [82]. Protamine modifications are similar to histone modifications and are present in sperm [83], though their functional importance remains uncertain, particularly during epigenetic inheritance. The retention of some histone modifications in the germline and zygote during epigenetic reprogramming makes them another possible vector of inheritance across generations [82]. The role of histones in epigenetic inheritance has largely been studied in *C. elegans*, though their role in a mammalian context has recently been explored (see Section 26.15).

26.7.3 Short noncoding RNA

How environmental cues experienced by adult somatic cells influence the establishment and/or maintenance of DNA methylation and histone modifications in gametes is unclear. Yet, there is evidence to support somatic-to-germline transmission of epigenetic information by RNA. Germ cells have extensive RNA profiles making RNA another mechanistic candidate for epigenetic inheritance. These germ cell RNAs have diverse roles from direct regulation of gene expression to localizing other epigenetic pathways, such as piwi-interacting RNA (piRNA)−mediated transposon silencing [84]. Although once thought to lack undegraded RNA, sperm contains both long and short ncRNAs (sncRNAs), including microRNAs (miRNAs), piRNAs, and transfer RNAs (tRNAs) (reviewed in Ref. [85]). Compared with sperm, significantly more RNA is found in oocytes as they contain the RNAs required for the first few days of embryonic development before zygotic genome activation is truly established [86]. The complexity of germ cell RNA profiles is increased by chemical modification of RNAs, such as methylation [87], which provides an additional layer of epigenetic information that is transmitted to the offspring. How epigenetic messages transmitted by RNA are perpetuated over multiple cell divisions and generations is not well understood. However, a unique aspect to RNA-mediated inheritance is that sncRNAs might be taken up by germ cells from the surrounding somatic tissues [88,89]. For example, maturing sperm receive sncRNAs (e.g., tRNA fragments) from small extracellular vesicles called exosomes that are released from the epididymal epithelium [89] (Fig. 26.5B). The deposition of sncRNA content of sperm into the oocyte at fertilization can influence gene expression in the early embryo [54] (Fig. 26.5E). It is unclear whether the ovulated oocyte interacts with oviductal exosomes in a similar manner (Fig. 26.5D). Regardless, the theoretically impassable wall between somatic cells and the germline (the so-called Weismann barrier) may be circumvented and penetrated by RNAs (see Section 26.14).

26.7.4 Epigenetic regulatory machinery: The "readers" and "writers"

For epigenetic information to be created, understood, and enacted upon, epigenetic regulatory machinery within the cell is required [51]. These molecular complexes are the so-called epigenetic "readers" and "writers." For example, a family of DNA methyltransferase (DNMT) enzymes enables the establishment and maintenance of DNA methylation. DNMT1 ensures that methylation is recapitulated on the daughter strand of DNA after replication has occurred and DNMT3A and DNMT3B establish de novo DNA methylation patterns in germ cells [90]. DNMT2 is required for RNA methylation [91]. Other regulator proteins, namely the ten−eleven translocases (TETs), remove methylation from 5mC via a 5-hydroxymethylcytosine (5hmC) intermediate [92]. Of note, 5hmC may have a functional role independent of 5mC demethylation [93]. Histone modifications must also be

replicated on nascent histones on the daughter strand. An army of histone-modifying enzymes, including methyltransferases and acetyltransferases, work in large complexes with other proteins to add and remove histone marks [80]. How histone modifications are replicated still remains somewhat ambiguous [85]. Elaborate machinery is also associated with the activity of sncRNAs, such as the piRNA complexes that include the argonaute superfamily cofactors [94].

Epigenetic machinery might play an important role in the reconstruction model of TEI (Fig. 26.6B). Individuals with deleterious mutations in this machinery may be more susceptible to epigenetic inheritance of phenotypes [95]. While functionally defective DNMTs and TETs are yet to be associated with TEI, histone methylation machinery is vital for the process of epigenetic inheritance in *C. elegans* and to some extent in mice. Paternal deficiencies in the H3K4me3 chromatin modifiers ASH2, WDR5, or SET2 in *C. elegans* lead to TEI of increased longevity for up to three wild-type generations [96]. Furthermore, overexpression of lysine (K)-specific histone demethylase KDM1A during spermatogenesis in mice caused specific changes in H3K4me3 enrichment in sperm and the paternal allele of the F1 preimplantation embryo in association with a transgenerationally inherited phenotype [97,98]. H3K4me3 methylation, which is normally required for transcriptional activation, can act as a metabolic sensor that connects paternal diet with offspring phenotype [99]. Similarly, germline deletion of the *Kdm6a* gene in F0 male mice resulted in increased tumor incidence in the F1 and F2 wild-type generations (note that tumor risk is normalized by the F3 generation) [100]. In this context, tumor risk was associated with changes in H3K27me3 and DNA methylation patterns in somatic tissue [100]. Together, these studies suggest that mutations in epigenetic regulatory machinery might cause inheritance of broad epigenetic instability that influences disease risk over at least two wild-type generations. Though, it is currently unclear whether sperm-inherited histone methylation patterns that escape reprogramming are able to influence transcription in the early embryo [97].

26.7.5 Mitochondria

As mentioned in Section 26.6, the majority of the cellular material that comprises the offspring zygote is maternally inherited from the oocyte (Fig. 26.5). This includes stored nutrients, metabolites, and organelles, such as mitochondria, the energy-producing compartment. The mitochondrial genome encodes for some of the proteins required for its biogenesis and function [101]. In most cells, mitochondria can fuse and divide and form extensive networks so that they can respond to changes in energy demand. The oocyte has the greatest copy number of mitochondrial DNA (mtDNA) compared with any other cell type, and this number drops as epigenetic reprogramming occurs in the preimplantation embryo, likely due to cell division and the partitioning of mitochondria to daughter cells [71]. In cases of TEI via the maternal lineage, particular attention should be paid to mitochondria as an inherited factor. This is because mitochondrial respiration is sensitive to changes in maternal diet, and, at least in *Drosophila*, this can alter methionine production required for cellular methylation (i.e., methylation of DNA and RNA) in the offspring [102]. Whether a similar mechanism involving mitochondria occurs in maternal epigenetic inheritance in mammals remains unknown.

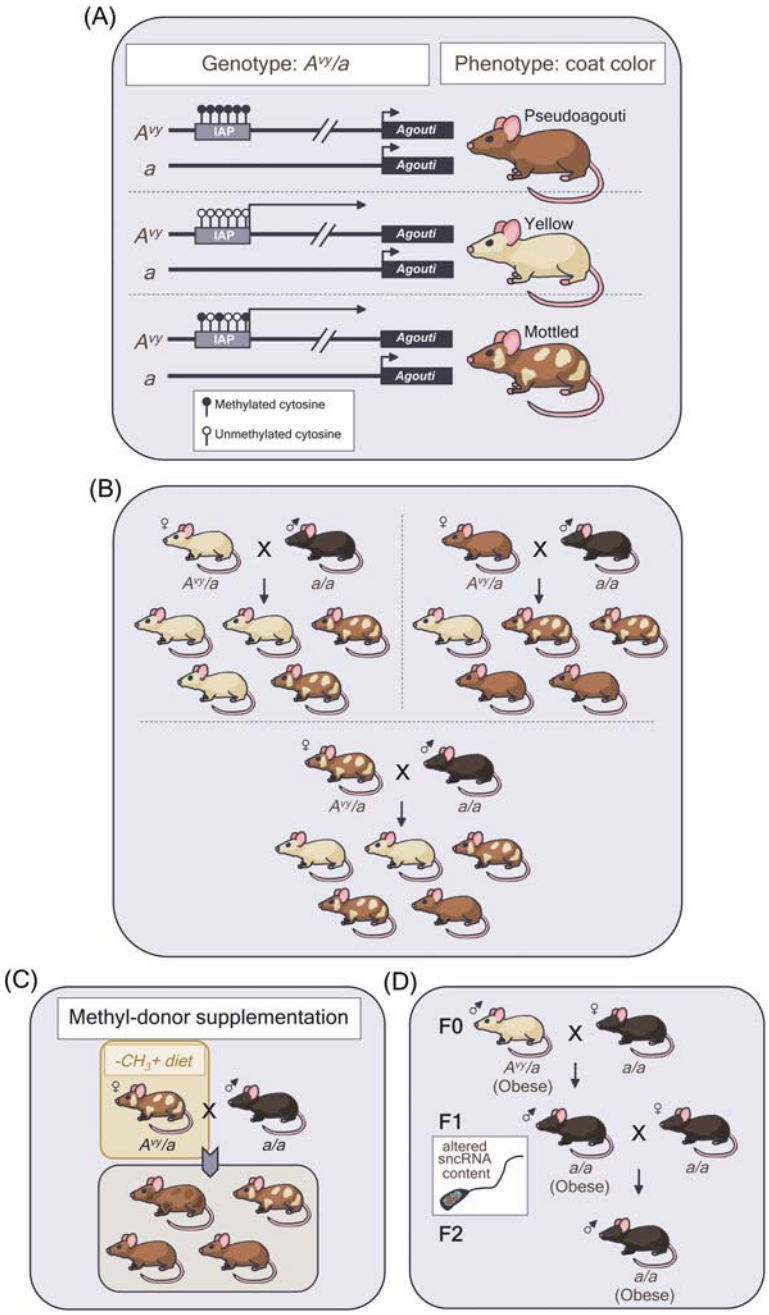
26.8 The classic mammalian example: Agouti viable yellow

The classic mammalian model of epigenetic inheritance is the A^{vy} mouse model, from which much of our mechanistic understanding has derived [103]. The *agouti* gene (*a* allele) is involved in establishing

mouse coat color. An insertional mutation of an intracisternal A-particle (IAP) retrotransposon upstream of the *agouti* gene (A^{vy} metastable epiallele) was found to regulate *agouti* gene expression in a DNA methylation–dependent manner [103,104] (Fig. 26.7A). Variable methylation of the IAP element leads to variable levels of *agouti* gene expression and coat color (from yellow to mottled to pseudoagouti) in isogenic A^{vy}/a mice [103] (Fig. 26.7A). Beyond coat color changes, A^{vy}/a mice also have obesity and a diabetic-like phenotype, owing to disruption of their satiety signaling pathways [104,107]. Importantly, the methylation status of the A^{vy} epiallele can be inherited via the maternal lineage, along with coat and metabolic phenotypes [103]. For example, offspring of yellow mice have an increased propensity to have yellow offspring [103] (Fig. 26.7B). The A^{vy} mouse line has been comprehensively assessed in the field of environmental epigenetics [108] since the methylation status of the IAP is influenced by a range of early-life exposures to environmental factors, including nutritional interventions [105,109]. For instance, feeding A^{vy} mothers a methyl-donor rich diet (e.g., folate, methionine, choline, betaine) leads to a shift in offspring coat color from yellow to pseudoagouti [105,109] (Fig. 26.7C). Even so, due to conflicting reports, it is unclear whether the DNA methylation pattern at the IAP element changes in response to increased dietary methyl groups [105,109]. Epigenetic reprogramming during early embryogenesis removes IAP methylation in the A^{vy} locus following maternal transmission of the allele [110]. However, artificially editing DNA methylation at the A^{vy} locus in individual oocytes followed by in vitro fertilization was sufficient to inform offspring IAP methylation and coat color [111]. Therefore, inheritance of DNA methylation likely requires an additional epigenetic factor(s) (e.g., germ cell sncRNA, histone modification patterns, maternal-derived epigenetic regulatory machinery, etc.) to reconstruct the oocyte pattern in the offspring.

Several studies have tried to probe the mechanisms of phenotype inheritance in the A^{vy} model (reviewed in Refs. [2,108]). While potentially informative for understanding direct epigenetic inheritance, these studies are often limited because (1) only parent-to-offspring transmission is considered meaning that TEI is not usually studied; and (2) a locus-specific approach for assessing DNA methylation is performed rather than genome-wide approaches [9,10]. Even though IAP methylation in A^{vy} mice is inherited via the maternal lineage, we might glean mechanistic insight into the inherited epigenetic effects of the A^{vy} allele in sperm. Yellow A^{vy} mice overeat and become obese because A^{vy} allele expression interferes with hypothalamic melanocortin receptor–mediated satiety signaling [112]. Mating F0 A^{vy} obese males with control (*a/a*) lean females results in an intergenerationally inherited obesity phenotype in a/a offspring through the paternal lineage (note: IAP methylation of the A^{vy} allele was not assessed in this study) [107]. Obesity was associated with abnormal sncRNA content in sperm of F1 *a/a* males including miRNAs and tRNA fragments [107] (Fig. 26.7D) in a manner similar parent-to-offspring inheritance of phenotype in other contexts [47,55]. Therefore, one hypothesis is that disruption of RNA-based mechanisms might occur in germ cells of A^{vy} males, and by extension A^{vy} females, to contribute to the reconstruction of DNA methylation profiles at the offspring IAP element. Further studies are required to support this hypothesis.

Along with the A^{vy} model, other metastable epialleles, namely $Axin^{Fu}$ and $Cabp^{IAP}$, are described as epigenetic biosensors of environmental perturbations associated with phenotypic change [109,113]. A recent genome-wide screen identified a set of variably methylated IAPs (VM-IAPs) that displayed characteristics similar to A^{vy} [114,115]. However, maternal exposure to BPA, an obesogenic diet, or a methyl donor–rich diet (e.g., methionine) did not have a profound effect on VM-IAP methylation levels [116], which contrasts the previous studies on classical metastable epialleles. Indeed, metastable


(A)
Genotype: A^{vy}/a
Phenotype: coat color
A^{vy}
IAP
Agouti
a
Agouti
Pseudoagouti
Yellow
Mottled
Methylated cytosine
Unmethylated cytosine
(B)
A^{vy}/a
a/a
(C)
Methyl-donor supplementation
-CH_3+ diet
(D)
F0
F1
F2
altered sncRNA content
(Obese)

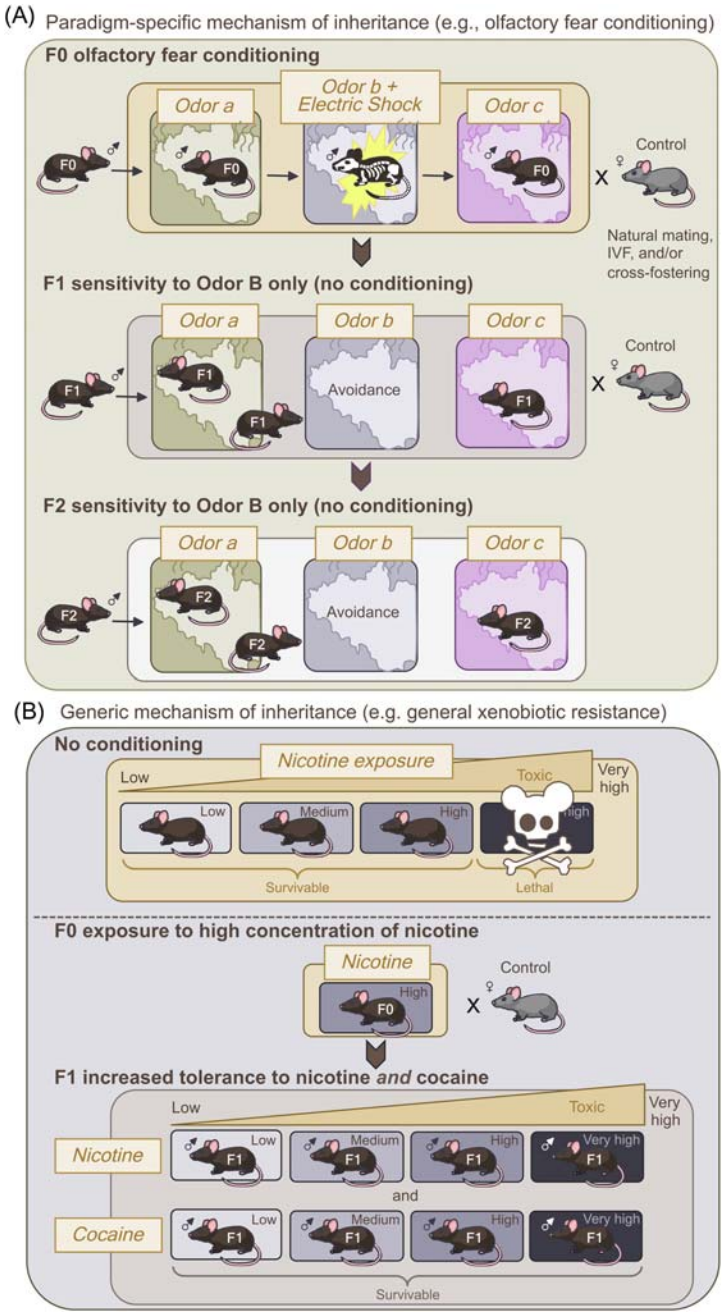
epialleles may be inadequate biosensors of environmental compromise [116], but this does not preclude the use of A^{vy}, $Axin^{Fu}$, $Cabp^{IAP}$ mouse models to investigate mechanisms behind TEI, as these models might be the exception rather than the rule.

26.9 The bandwidth of inheritance

A key question in the field of epigenetic inheritance is whether paradigm-specific information is transmitted to the next generation or whether a generic signal of suboptimal conditions is inherited. This is described in terms of the extent or "bandwidth" of information that can be communicated [85]. An example of a paradigm-specific effect is a multigenerational study assessing olfactory fear conditioning in mice [117]. In this study, F0 males were conditioned to associate a specific odor with a foot shock [117] (Fig. 26.8A). The F1 and F2 generations derived from conditioned F0 males had increased behavioral sensitivity to the same odor even though the offspring had not been conditioned themselves [117] (Fig. 26.8A). Importantly, inherited sensitivity to other odors did not occur suggesting a specialized effect. Social transmission of odor sensitivity was excluded by techniques, such as in vitro fertilization and cross-fostering [117]. While this specific odor sensitivity correlated with DNA hypomethylation of an odorant receptor gene, *Olfr151*, in sperm of the F0 and F1 generations [117], it is unclear if the methylation change was the cause of the inherited phenotype. Alternatively, recent evidence for inheritance of a generic message comes from exposing F0 mice to high levels of nicotine [118]. This nicotine exposure primes the male F1 offspring resulting in reduced sensitivity (and thus increased survival) when exposed to toxic levels of nicotine or to toxic levels of cocaine, the latter of which the F0 male was never exposed [118] (Fig. 26.8B). Therefore, the F1 mice display a generic inherited mechanism of enhanced xenobiotic resistance. However, whether a generic inherited message exists between different stressor types (e.g., fear conditioning, xenobiotic resistance, and metabolic disease) is yet-to-be determined.

FIGURE 26.7

Agouti viable yellow (A^{vy}): A classic example of epigenetic inheritance. (A) An intracisternal A-particle (IAP) upstream of the A^{vy} allele regulates *agouti* gene expression in a DNA methylation–dependent manner [103]. In A^{vy}/a heterozygous mice, different degrees of DNA methylation at the IAP element result in different coat colors. When fully methylated (upper panel), *agouti* expression is driven from the nascent promoter resulting in mice with a brown (pseudoagouti) coat color. When the IAP element is unmethylated (center panel), an ectopic promoter in the IAP element drives ectopic agouti expression, leading to a yellow coat color and a metabolic phenotype. Partial methylation at the IAP element (lower panel) leads to a mottled coat color. (B) The methylation status of the agouti allele, and therefore the coat color and metabolic phenotypes, is heritable over multiple generations via the maternal line [103]. When mated to an *a/a* male mouse, a yellow A^{vy}/a female mouse has an increased propensity for yellow offspring (top left) compared with a brown A^{vy}/a female (top right) due to epigenetic inheritance at the A^{vy} allele methylation patterns [103]. Mottled A^{vy}/a females mated to *a/a* males (bottom) result in an intermediate propensity for yellow offspring compared with the other matings. In the schematic, only A^{vy}/a offspring are depicted, and *a/a* progenies have been excluded for simplification. (C) A methyl-rich diet fed to a mottled colored A^{vy}/a female reduces the number of yellow offspring in her litters compared with unsupplemented controls (shown in bottom of panel B) [105]. Images based on [106] and [103]. (D) Intergenerational inheritance of obesity initiated by A^{vy} allele in F0 males was associated with altered short noncoding RNA (sncRNA) content in F1 *a/a* males [107].


(A) Paradigm-specific mechanism of inheritance (e.g., olfactory fear conditioning)
F0 olfactory fear conditioning
Odor a
Odor b + Electric Shock
Odor c
F0
Control
Natural mating, IVF, and/or cross-fostering
F1 sensitivity to Odor B only (no conditioning)
Odor a
Odor b
Odor c
F1
Avoidance
Control
F2 sensitivity to Odor B only (no conditioning)
Odor a
Odor b
Odor c
F2
Avoidance
(B) Generic mechanism of inheritance (e.g. general xenobiotic resistance)
No conditioning
Nicotine exposure
Low
Toxic
Very high
Low
Medium
High
Survivable
Lethal
F0 exposure to high concentration of nicotine
Nicotine
High
F0
Control
F1 increased tolerance to nicotine and cocaine
Low
Toxic
Very high
Nicotine
Low
Medium
High
Very high
F1
and
Cocaine
Survivable

The degree of epigenetic change should also be considered in terms of whether it is sufficient to cause disease. For instance, the DNA methylation status at any given cytosine residue is binary meaning that it can be methylated or unmethylated. However, a moderate change in CpG methylation in a given subpopulation of cells (e.g., <10% of cells) can profoundly alter gene expression patterns associated with complex disease phenotypes [119], particularly in context of TEI. For example, midgestation exposure of pregnant rats to the toxic endocrine disruptor p,p′-dichlorodiphenyldichloroethylene (DDE; metabolite of the insecticide DDT) leads to a transgenerational reduction in sperm quality and infertility in males up to the F3 generation [120]. These phenotypes are associated with a 10%–50% reduction in methylation within DMR2 of the *Igf2/H19* locus in sperm collected from the F1–F3 generations and significant changes in sperm *Igf2* and *H19* RNA expression [120]. Yet, whether misexpression of these specific genes leads to infertility or whether it is indicative of more widespread epigenetic change is unclear. In general, the biological relevance of the degree of DNA methylation change (or change in other epigenetic factors) remains under appreciated in many TEI contexts and should not be ignored.

26.10 The importance of the repetitive genome

A large part of most genomes are made up of repetitive elements, such as transposons and retrovirus-derived sequences that require silencing to prevent mutation by their transposition into other regions of the genome [121]. Epigenetic mechanisms are vital to their suppression. Epigenetic marks at repetitive elements are more likely to be resistant to reprogramming than unique loci, thus providing scope of heritability [63–65]. As discussed in Section 26.8, the IAP retrotransposon in the A^{vy} mouse model is a notable example of a metastable epiallele in TEI [103], even though methylation of IAPs more broadly throughout the genome is not responsive to the environment and is unlikely to play a role in multigenerational inheritance of disease [116]. The importance of the repetitive genome in TEI has been demonstrated in *C. elegans*. In this context, histone modifications and their regulatory enzymes appear to be vital. For example, *C. elegans* were genetically modified with a multicopy array of a transgene containing a fluorescent reporter gene under the control of a heat shock promoter to mimic endogenous repetitive loci [122]. After exposure to high temperature, these worms showed elevated reporter gene expression, which was heritable for up to 14 generations even in the absence of heat [122]. In contrast, when only a single copy of the transgene was present, heritability of elevated reporter gene expression occurred for only seven generations [122]. It was proposed that the activity of SET-25, an H3K9me3 methyltransferase, is reduced at high temperatures [122]. Therefore, heat treatment may derepress the transgene (and other loci) by altering histone methylation and

FIGURE 26.8

Examples of a paradigm-specific mechanism of inheritance versus a generic mechanism of inheritance in mice. (A) A paradigm-specific mechanism of epigenetic inheritance involving olfactory fear conditioning in F0 generation male mice [117]. Electric shock of F0 mice in combination with exposure to a single odor "b" leads to increased sensitivity of the F1 and F2 generations to odor "b." Odors "a" and "c" did not lead to a similar behavioral effect suggesting that the mechanism of epigenetic inheritance was paradigm-specific. (B) An example of a generic mechanism of epigenetic inheritance involving exposure of the F0 generation to a high concentration of nicotine resulting in epigenetic inheritance of xenobiotic resistance in the F1 offspring. That is, the F1 offspring showed an increased tolerance for toxic (i.e., lethal) levels of more than one chemical substance (e.g., nicotine *and* cocaine) rather than a specific response to nicotine only [118].

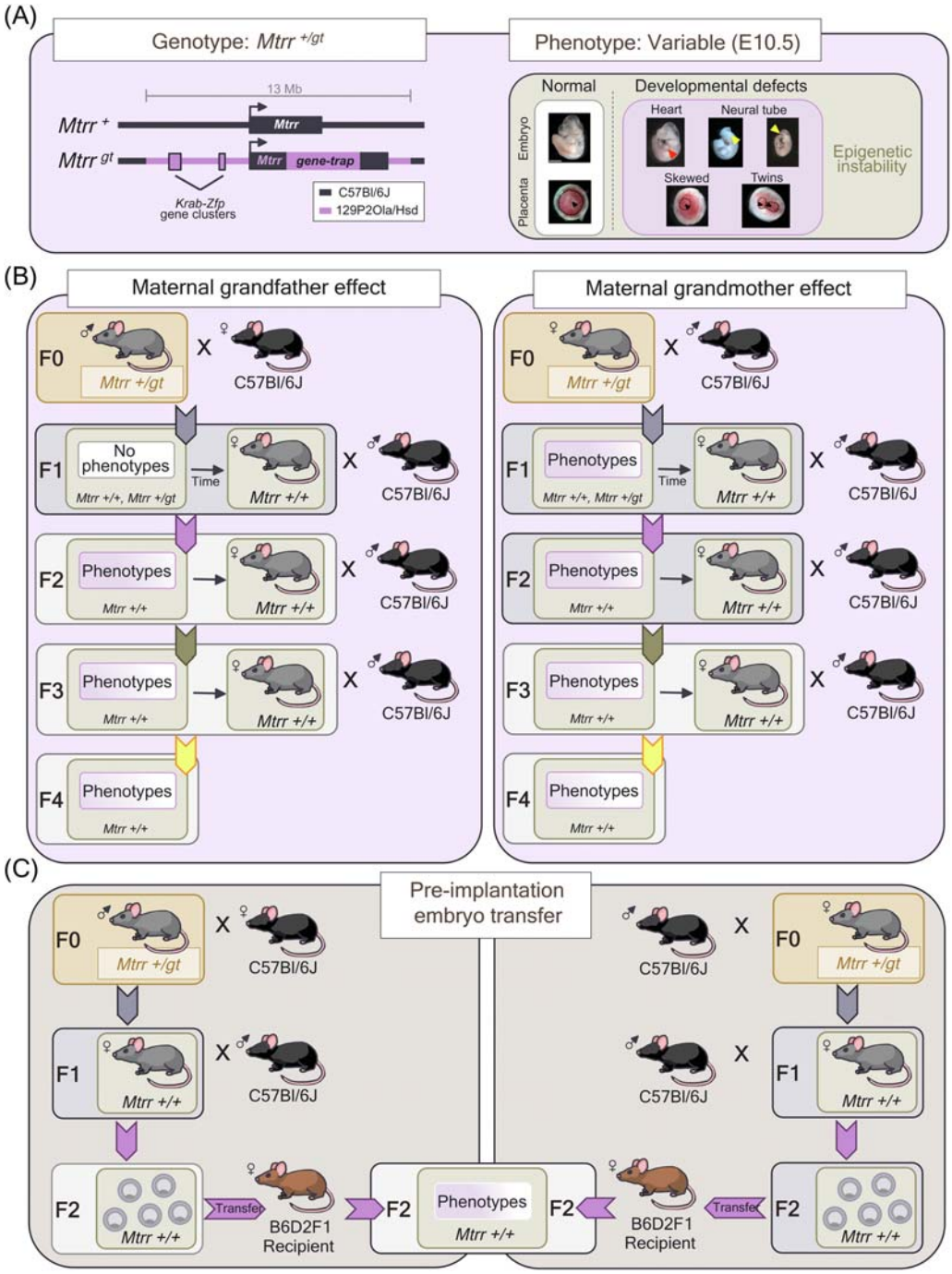
heterochromatin structure [122]. In fact, normalization of temperature allows for the restoration of SET-25 activity, but it takes many generations of worms to reestablish proper histone methylation [122]. The extent to which repetitive elements contain functionally important inherited epigenetic marks is yet-to-be fully revealed. Encouragingly, sequencing technology and bioinformatics analysis now better allows for assessment of repetitive elements, which will give researchers better insight into this question.

26.11 Tracking phenotypes and epimutations over multiple generations

A challenge of the field is to trace epigenetic changes and associated phenotypes between many generations to mechanistically understand epigenetic inheritance. Long generation times in mammalian models often limit the number of generations assessed (frequently only up to the F2 generation), which constrains our ability to rule out physiological and genetic effects and to determine how many generations a phenotype or epigenetic change might persist. Studies have reported that phenotypes caused by an ancestral environmental stressor diminish over time with a complete loss after several generations. For example, the metabolic phenotype observed in offspring derived from obese A^{vy} males is attenuated by the F3 generation [107]. Because epigenetic inheritance potentially provides adaptive benefits to the environment, phenotypic attenuation is logical as to promote responsiveness to the current environment.

Another mouse model of true TEI can be used to explore the functional relationship between inherited epigenetic factors, alterations to the offspring epigenome and transcriptome, and phenotype. The hypomorphic mutation in the mouse *Mtrr* gene (denoted $Mtrr^{gt}$) disrupts one-carbon metabolism and causes TEI of epigenetic instability and a wide range of congenital malformations for at least up to the F4 wild-type generation (Fig. 26.9A and B) [9,10,123]. In this model, TEI occurred through the maternal grandparental lineage [9]. That is, when an F0 male or female is a carrier for the $Mtrr^{gt}$ mutation, their genetically wild-type F2, F3, and F4 grand-offspring display a wide range of phenotypes but only via a wild-type F1 female [9] (Fig. 26.9B). Embryo transfer showed that phenotype inheritance was independent of the F1 peri-/postimplantation uterine environment (Fig. 26.9C), thus implicating germline epigenetic inheritance as the mechanism of phenotypic inheritance [9]. The point of phenotype attenuation has not yet been explored. Since one-carbon metabolism (includes the folate and methionine cycles) is essential for the transmission of methyl groups for all methylation reactions in the cell, its disruption by the $Mtrr^{gt}$ mutation has a profound effect on the germline and somatic epigenomes [9,10]. Importantly, the degree of methylation change in the F2 and F3 generations correlated with the level of transcriptional dysregulation and phenotype severity [9,10]. This inherited epigenetic instability occurred even though the specific alterations in sperm DNA methylation in F0 males were not present in the somatic tissue of F1, F2, and F3 generation, thus ruling out a replication mechanism of TEI [10].

Others have attempted to track specific differentially methylated regions (DMRs) over multiple generations (as in replicative inheritance, Fig. 26.6A) in dietary models [78]. Animal models whereby diet is manipulated (i.e., high fat, low protein, "western diet," or undernutrition) display metabolic disease in the offspring. However, only a few studies have successfully identified epigenetic changes in the F0 generation that persist to the F1 generation and beyond. For example, a methylome-wide analysis of prediabetic male mice with impaired fasting glucose and insulin resistance revealed that specific DMRs at insulin signaling genes in the F0 sperm were also present in pancreatic islet cells of


(A)
Genotype: Mtrr +/gt
Phenotype: Variable (E10.5)
13 Mb
Mtrr +
Mtrr
Mtrr gt
Mtrr gene-trap
Krab-Zfp gene clusters
C57Bl/6J
129P2Ola/Hsd
Normal
Developmental defects
Heart
Neural tube
Skewed
Twins
Embryo
Placenta
Epigenetic instability
(B)
Maternal grandfather effect
Maternal grandmother effect
F0
Mtrr +/gt
C57Bl/6J
F1
No phenotypes
Phenotypes
Mtrr +/+, Mtrr +/gt
Time
Mtrr +/+
F2
F3
F4
(C)
Pre-implantation embryo transfer
Transfer
B6D2F1 Recipient

F2 males [124]. Yet, in a model of maternal undernutrition, which leads to a robust metabolic phenotype and reduced birthweight in the F1 and F2 generations, specific altered DNA methylation patterns could not be tracked across generations [78,125]. Several sperm DMRs were identified in F1 males that were undernourished in utero [78], yet these epimutations did not persist in the F2 fetal brain and liver even though gene expression at associated loci was altered and the phenotype persisted [78]. This potential transcriptional memory of F0 sperm DMRs was also observed in F2 and F3 generations of the *Mtrr*^{gt} mouse model [10], thus implicating additional inherited epigenetic mechanisms beyond DNA methylation and/or a reconstruction mechanism of inheritance whereby each generation assessed shows a different epigenomic profile (Fig. 26.6B). While different models of epigenetic inheritance might undergo distinct mechanisms, the tissue selected for analysis might be a defining factor when it comes to generational comparisons.

26.12 Allowing for phenotypic and epigenetic variability

Under normal circumstances, considerable epigenetic variation exists between individuals [126]. The degree of epigenetic variability likely affects data collection from models of epigenetic inheritance, particularly when samples are pooled for analysis. Remarkably, one model of diet manipulation revealed less than natural epigenetic variation between individuals [126], but this might reflect the lack of variation in phenotype or epigenomic hotspots. Alternatively, the wide spectrum of phenotypes the *Mtrr*^{gt} model [9,127–129] suggests a variable interindividual response to abnormal one-carbon metabolism resulting in stochastic effects on the epigenome of each germ cell. Single-cell sequencing of DNA and histone methylomes and the transcriptome will permit us to better understand epigenetic heterogeneity [130] and sensitivity to specific environmental cues.

26.13 Considering genetic effects

Epigenetic instability may promote genetic instability and confound (or even contribute to) our understanding of the mechanisms of TEI. Analyzing the underlying genetic sequence of epimutations to

FIGURE 26.9

The *Mtrr*^{gt} mouse model of transgenerational epigenetic inheritance. (A) The *Mtrr*^{gt} mutation results from a gene-trap (gt) insertion in intron 9 of the *Mtrr* gene in mice. The gene-trap insertion and cross-over frequency resulted in a 13 Mb section of the genome around the *Mtrr* gene to be 129P2Ola/Hsd genome sequence (*pink*) rather than the C57Bl/6J genome (*black*) of the rest of the *Mtrr*^{gt} mouse line [9,10,116,123]. The 129P2Ola/Hsd sequence contains two *Krab-Zfp* gene clusters not present in the C57Bl/6J genome [116]. The *Mtrr*^{gt} mutation is hypomorphic and disrupts one-carbon metabolism, leading to a wide spectrum of developmental phenotypes and epigenetic instability that are transgenerationally inherited [9,123]. (B) Transgenerational epigenetic inheritance in the *Mtrr*^{gt} mouse line occurs through the maternal grandparental lineage reveals phenotypic inheritance in the wild-type offspring at least until the F4 generation [9]. This phenomenon is likely differently initiated in the F0 sperm compared with F0 female oocytes based on phenotypic patterns of inheritance [10]. (C) Transfer of F2 generation wild-type preimplantation embryos into a control uterine environment indicates that the mechanism occurs epigenetic inheritance via the germline and rules out a uterine effect of the F1 wild-type mother [9].

rule out potential genetic effects will improve reliability and understanding of the data. DNA methylation is a known mutagen because spontaneous deamination of 5mC forms thymine, and thus generates a C → T transition mutation [131,132]. Regional loss of DNA methylation may also lead to activation of transposable elements that could transpose into new genomic locations to generate genetic mutations [133]. Alternatively, changes in the number of tandem repeats can affect the level of DNA methylation detected. For example, there is a higher level of variation in DNA methylation at ribosomal DNA (rDNA) repeats that correspond to rDNA copy number rather than epigenetic changes induced by diet [126]. Therefore, in some cases, genetic variation may be the underlying cause of epigenetic variation. Telomeres, which are epigenetically regulated regions of repetitive DNA sequences at the end of chromosomes [134], were shorter in F2 generation females derived from F0 mice fed a low-protein diet [135]. This reduction in telomere length associated with reproductive tracts that were prematurely aged and perceived chromosome instability. Therefore, epigenetic regulation of telomere length might provide another mechanistic interface between genome and epigenome during epigenetic inheritance.

The literature is awash with examples of how toxicant exposure, from endocrine disruptors to jet fuel, can influence our epigenomes and the health of our offspring [136]. Toxicants may be mutagenic themselves, and if so we would expect to see a broad spectrum of phenotypes due to an increased rate of de novo mutations. Conversely, it is often observed that a specific toxicant is associated with inheritance of a specific phenotype or disease. Although mutagenic effects are unlikely to be a common cause for disease inheritance associated with toxicant exposure, this does not eliminate a genetic effect. For example, in utero exposure of rats to the endocrine disruptor vinclozolin at a time critical for sex determination results in a range of adult-onset diseases, such as infertility and kidney defects in the unexposed offspring for up to four generations [137]. These phenotypes were associated with widespread changes in DNA methylation in sperm of the F3 generation [138]. However, others have been unable to recapitulate this result [139], perhaps due to differences in methodology. Importantly, the effects of vinclozolin exposure are associated with an increase of repeat element copy number variations in the genome [140] indicating a genetic effect. This observation highlights the importance of examining the genome and epigenome in parallel.

Genetic background effects might also influence the appearance of phenotypes and/or epigenetic changes associated with specific environmental stressors [138]. Few TEI studies have taken potential genetic background effects into account. For example, sequencing the genomes of two A^{vy} littermates, one pseudoagouti and one yellow, to assess genetic differences revealed no difference in the germline mutation rates, and genetic changes were deemed unlikely to cause the coat color phenotype [141]. Similarly, whole-genome sequencing of the $Mtrr^{gt}$ mouse line showed a normal de novo mutation rate [10]. However, the region surrounding the *Mtrr* locus where the mutation was generated by a gene-trap insertion was shown to contain a genetic background (i.e., 129P2Ola/Hsd mouse strain) that was different from the rest of the $Mtrr^{gt}$ mouse genome (i.e., C57Bl/6J mouse strain) even though substantial backcrossing occurred (Fig. 26.9A) [10,116]. Notably, the inserted 129P2Ola/Hsd genome sequence contained a KRAB-ZFP gene cluster that is not normally present in the C57Bl/6J strain (Fig. 26.9A). KRAB-ZFP transcription factors mediate the repression of transposable elements [142], and the gene cluster inherited with the $Mtrr^{gt}$ allele was linked to altered DNA methylation at several VM-IAPs in homozygous $Mtrr^{gt/gt}$ mice [116]. Notably, the KRAB-ZFP cluster is likely unimportant for phenotype establishment and/or inheritance in the $Mtrr^{gt}$ mouse model because (1) F0 heterozygous $Mtrr^{+/gt}$ mice contain the KRAB-ZFP gene cluster but display a normal range of VM-IAP

methylation [116]; (2) the subsequent wild-type generations displayed phenotypes but lacked the KRAB-ZFP gene cluster; (3) paternal dietary folate deficiency in mice also leads to epigenetic inheritance of developmental phenotypes [143] in the absence of the KRAB-ZFP gene cluster; and (4) the distinct phenotypes that form in the $Mtrr^{gt}$ model are similar to clinical disorders that arise in cases of human folate deficiency (e.g., hyperhomocysteinemia, macrocytic anemia, neural tube closure defects, intrauterine growth restriction, etc.) [9,10,123,127,128]. Ultimately, these data highlight the importance of understanding genetic factors that might contribute to TEI mechanisms.

26.14 RNA-mediated inheritance of phenotype

The mechanistic focus in the field has expanded from the inheritance of DNA methylation and chromatin modifications to inheritance of germ cell sncRNAs, such as miRNAs and tRNA fragments that have the potential to influence epigenetic marks and gene expression in the zygote. So far, most of our knowledge has come from *C. elegans*, *D. melanogaster*, and plants whereby germ cell sncRNA content is influenced by changes in the environment and TEI mediated by RNA (reviewed by Ref. [144]). These organisms have RNA polymerases that replicate an RNA signal, which might allow for more robust RNA-mediated inheritance compared with mammals that lack this RNA replicative mechanism [85]. In *C. elegans*, RNA obtained from the environment can mediate heritable gene silencing. This phenomenon known as RNA interference (RNAi) is extensively used to experimentally generate gene knockdowns [145]. Although the mechanism is complex, it is thought that piRNAs, which are typically involved in transposon silencing in the germline, and RNAi converge on a common pathway involving RNA and chromatin regulatory complexes [94]. It was demonstrated that a mechanism similar to those involved in exogenous RNAi plays a role in inheritance at endogenous loci. For example, starvation-induced developmental arrest in *C. elegans* causes the TEI of sncRNAs and an increased life span over multiple generations [146].

There is evidence for mammalian sncRNAs in epigenetic inheritance of offspring phenotypes [47,54,55]. For instance, in a mouse model of traumatic stress involving maternal separation in early postnatal life of the F1 generation (see Fig. 26.4B), severe behavioral phenotypes were observed up to the F2 generation and were associated with altered miRNA profiles in the F1 sperm [47]. Microinjection of sperm miRNAs from stressed F1 males into fertilized oocytes mimicked the behavioral phenotypes [47]. Furthermore, changes in DNA methylation at the glucocorticoid receptor gene (implicated in neuroendocrine stress response) in sperm from F1 males and brains of F2 offspring correlated with gene misexpression [147], though it is uncertain if the miRNAs in question were the causative factor. Remarkably, environmental enrichment of the F1 stressed males when they reached adulthood rescued the behavioral phenotype and DNA methylation patterns further demonstrating the plasticity of the epigenome [147], a result that has clinical implications. Other examples involve fragmented tRNAs, which are 28–40 nucleotides in length and derived mainly from the 5′ end of tRNAs [54]. Poor diet (e.g., protein restricted or high-fat diet) in male mice is sufficient to modify sperm tRNA fragment content [54,55], cause adult onset metabolic disease in the offspring [55], and/or repress the expression of early embryonic genes in offspring with murine endogenous retrovirus-like (MERVL) elements in their control regions [54]. These genes are hypothesized to influence fetoplacental development [54], the dysregulation of which might lead to adult-onset metabolic disease. A similar effect on sperm RNA content by these two separate dietary modulations might indicate that

metabolic disruption causes a generic mechanism for inheritance rather than a paradigm-specific effect. This mechanistic question might be resolved by assessing other models of metabolic disruption for sperm sncRNA content (e.g., the *Mtrr*^{gt} mouse model).

RNA modifications (e.g., methylation) are currently underappreciated in the TEI field and may play a key role in functional RNA inheritance. For instance, microinjection of synthetic tRNA fragments without methylation was unable to recapitulate the metabolic phenotype caused by high-fat diet in mice [55]. Furthermore, mice that are deficient for the RNA methyltransferase, DNMT2, abolished sperm sncRNA-mediated transmission high-fat diet-mediated metabolic disease to offspring [148]. RNA modifications might also be important in paramutation effects [149]. A paramutation involves a mutant allele (i.e., the paramutagenic allele) that alters the expression of the homologous wild-type allele (i.e., the paramutant allele) in trans via an RNA-mediated mechanism [150,151]. Remarkably, a meiotically stable paramutant allele can be transgenerationally inherited in absence of a paramutagenic allele [150,151]. For example, multigenerational transmission of a mouse paramutant "white-tail-tip" phenotype results after mutational transgene insertions into the *Kit* locus, such as *Kit*^{lacZ} and *Kit*^{copGFP} [151–153]. DNMT2 is also required for the transmission of the *kit* paramutation [154] suggesting that RNA modifications are more broadly implicated in RNA-mediated inheritance.

sncRNA-mediated mechanisms of inheritance in rodents have only been studied in a parent-to-offspring context [47,54,55,155] and not a true TEI framework. Indeed, microinjection of miRNAs into oocytes can initiate phenotype inheritance over multiple generations, but an increase in expression of the same miRNA is not observed beyond the injected generation [153]. This suggests that RNA interacts with other epigenetic mechanisms to promote phenotype inheritance. For this reason, RNA-mediated inheritance might fit into the reconstruction model of TEI (Fig. 26.6B) by directing the reestablishment of altered DNA methylation or histone marks in each generation.

26.15 Interactions between epigenetic mechanisms

Within the individual cells, DNA methylation, chromatin modifications, and sncRNAs are interconnected and interdependent. In the context of epigenetic inheritance, these molecules likely act in a coordinated and collaborative manner to cause disease phenotypes. For example, DNA methylation or histone modification patterns might regulate sncRNA expression. This, in turn, may direct DNA/histone methylation patterns to ultimately establish an interactive feedback system [51]. Collaborative, multifaceted, and often costly approaches to assess all epigenetic pathways in a single model are required to fully explore epigenetic mechanisms of inheritance.

There are studies demonstrating interdependence between epigenetic mechanisms. For instance, an initiating mutation in the *C. elegans spr-5* gene that encodes an H3K4 histone demethylase causes a transgenerational decline in fertility, even when all subsequent generations are wild-type for *spr-5* [96,156]. This transgenerational effect correlates with an accumulation of H3K4me2 and an increase in 6mA levels in each generation [96,156]. Loss of a putative 6mA DNMT leads to a partial rescue of the *spr-5* infertility phenotype [96] demonstrating a mechanistic interaction between histone modifications and DNA methylation. In mice, ectopic expression of an *spr-5* ortholog, *Kdm1a*, during spermatogenesis leads to developmental abnormalities for up to three wild-type generations [98]. However, the sperm of the F1 generation, whose offspring displayed developmental abnormalities, had normal H3K4me2 and DNA methylation profiles [98]. This finding suggests that while the disruption

of the histone methylation machinery can instigate the TEI of developmental abnormalities, additional epigenetic mechanisms are at work [98].

26.16 Potential adaptive benefits of transgenerational epigenetic inheritance

TEI might be viewed as memory of an adverse environment so that subsequent generations are better prepared to cope with similar hostile conditions [157]. Disease phenotypes might occur when the environment experienced by an individual does not match the one they are "primed" to experience. For example, an undernourished ancestor might prime offspring for famine conditions. However, if the offspring have abundant access to nutrition instead, the risk for metabolic disease and obesity might be increased [158]. Researchers have used a challenge paradigm whereby offspring born following an ancestral exposure to a stressor are then challenged with that stressor to determine their ability to cope. For example, prenatal exposure to nicotine primes the offspring for increased survival when exposed postnatally to toxic levels of nicotine [118] (Fig. 26.8B). The adaptive response over further generations has not been investigated. However, prolonged exposure to a stressor might be required for TEI effects to be obvious. As discussed previously (see Section 26.10), heat exposure of *C. elegans* containing a multicopy fluorescent reporter array under the control of a heat shock promoter leads to elevated transgene expression over many generations in the absence of heat [122]. Heat exposure for a single generation led to elevated transgene expression for only 7 generations, whereas exposure to high temperatures for 5 successive generations led to elevated transgene expression for 14 generations [122]. This type of transgenerational effect could be viewed as an adaptive response and might ensure that inheritance of a phenotype only occurs when the environment experienced by the offspring is almost certain to be the same in subsequent generations. Ultimately, this would make it less likely that a potential disease-causing mismatch between the epigenotype and the environment is created.

26.17 Mechanistic extrapolation to human populations

Animal models may be particularly useful for investigating diseases where a so-called "missing heritability problem" is present identified (e.g., autism, asthma, type 2 diabetes) [159], and there is a need to explore environmental effects in a true physiological context that is not currently possible in in vitro settings. Genome-wide association studies in humans have identified a range of genetic loci that may increase susceptibility to these conditions, but thus far, they do not account for the complete heritable risk of an individual [159]. This implicates gene−environment interactions and thus an epigenetic component. Beyond dietary intervention, animal disease models are being developed to explore environmental influences in complex diseases. For instance, mice treated with valproic acid leads to an autism-like phenotype and leads to TEI of these behaviors up to the F3 generation [160]. Similarly, maternal exposure to air pollutants showed an increased likelihood of allergic asthma in the F2 and F3 generation that may be related to DNA methylation changes in dendritic cells [161]. While epigenetic mechanisms are not yet fully explored in these disease models, they hold promise for predicting disease origin and inheritance.

Given that humans and animal models have epigenetic factors with similar regulation and responses to environmental exposures, the mechanistic understanding gleaned from animal models holds

relevance to human populations [162]. Nonmammalian models, including *D. melanogaster* and *C. elegans*, also provide us with highly tractable experimental systems to test fundamental principles of epigenetic inheritance over many generations. Despite sharing epigenetic similarities with mammalian systems, such as homologous histone-modifying machinery, there are major distinctions that need to be taken into account when extrapolating data to human populations. For instance, invertebrate models lack of cytosine methylation and the presence of RNA polymerases. Mammalian models (e.g., rodents) have fewer fundamental epigenomic differences, which allow for mechanistic insights to be more easily applied to human populations. Rather than focusing on the inheritance of shared disease phenotypes, researchers might instead explore the human genome for epigenetically labile sites, such as the mouse A^{vy} allele. This might help to ascertain common mechanisms involved in epigenetic inheritance. Humans lack IAP elements, which are fundamental to our best-understood mammalian model of TEI. However, humans do have equivalent repetitive genomic sequences, such as LINE1 elements, which are regulated by similar epigenetic mechanisms and should be explored in the context of epigenetic inheritance. It is difficult to separate genetic effects from epigenetic effects in humans because of genetic diversity. Therefore, animal models, which are usually inbred and isogenic, are experimentally valuable because potential confounding genetic effects can be avoided. However, testing these same mechanisms in genetically outbred models should give researchers further insight into potential overlap with humans. Additionally, more histones are retained in human sperm than in rodent sperm (15% retention in human vs. 1% retention in mouse [82]) offering more opportunities for histone-mediated inheritance in human populations than in rodent models. Although there is little overlap between the loci that escape reprogramming in the mouse versus in humans [64], this may not be important if reconstruction paradigms are correct. For these models that represent human disease to be useful, care should be taken to accurately mimic not only the disease phenotype but also the parameters and severity of environmental exposure (e.g., amount of toxicant exposure, type of diet, etc.) to make it similar to what would be experienced by human populations.

26.18 Conclusions

Although our understanding of TEI of disease has come a long way in recent years, there are still many unanswered mechanistic questions. To fully understand the implications of how the environment influences the epigenome, genome-wide next-generation sequencing techniques should be performed to correlate DNA and histone modifications with gene and sncRNA expression in germ cells and somatic cells over multiple generations. These findings will allow us to determine whether even minor epigenetic changes can increase heritability of disease risk. These studies require a large number of animals or patients and therefore will be a costly when considering individual sample sequencing, bioinformatic analyses, and molecular validation. As a result, this undertaking will likely require collaboration across research groups. The importance of epigenetic regulation of repetitive regions of the genome has become increasingly apparent as epigenome profiling tools become better at mapping of the repetitive genome. Disruption of epigenetic control of repetitive regions has implications for genome-wide epigenetic instability. Inherited epigenetic instability that occurs in a different pattern in each generation increases complexity for data interpretation. Separately, new technologies allowing for the manipulation of the epigenome by altering locus-specific DNA methylation or individual

histone modifications using CRISPR-cas9–based acetyltransferases [163] and TALE-TET1 fusions [164] will enable us to explore whether the epigenetic changes we identify are capable of causing disease phenotypes over multiple generations.

Among other modes of adaptation, a flexible epigenome likely improves the chances of survival of our descendants by allowing them to prepare for the environmental conditions into which they are born. By further exploring the mechanisms involved in epigenetic inheritance, we will begin to better understand the causes and inheritance of noncommunicable diseases in the human population. Only then successful predictive and preventative measures can be implemented in the clinic, though it might take many generations to fully appreciate the outcome.

List of abbreviations

5hmC	5-Hydroxymethylcytosine
5mC	5-Methylcytosine
6mA	N6-methyladenosine
a	Agouti
A^{vy}	Agouti viable yellow
BMI	Body mass index
BPA	Bisphenol-A
C. elegans	*Caenorhabditis elegans* nematode
CpG	Cytosine nucleotide followed by a guanine nucleotide in the $5' \rightarrow 3'$ direction
CRISPR	Clustered regularly interspaced short palindromic repeats
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DEET	Diethyltoluamide
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DNMT	Deoxyribonucleic acid (DNA) methyltransferase
DOHaD	Developmental Origins of Health and Disease
F	Filial generation
H3K4me3	Histone 3 lysine 4 trimethylation
IAP	Intracisternal A-particle
IGF2	Insulin-like growth factor 2
KDM1A	Lysine (K)-specific histone demethylase 1A
KRAB-ZFP	Kruppel associated box-zinc finger protein
LINE1	Long interspersed nuclear element-1
MERVL element	Murine endogenous retrovirus-like element
miRNA	Microribonucleic acid
MTRR	Methionine synthase reductase
piRNA	Piwi-interacting ribonucleic acid
PTSD	Posttraumatic stress disorder
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
sncRNA	Small noncoding ribonucleic acid

TALE-TET1	Transcription activator-like effector (TALE) repeat arrays and the TET1 hydroxylase catalytic domain
TEI	Transgenerational epigenetic inheritance
TET	Ten−eleven translocation
tRNA	Transfer ribonucleic acid
VM-IAP	Variably methylated intracisternal A-particle
WHO	World Health Organization

Glossary

Body mass index (BMI) An approximate quantification of the amount of tissue mass in an individual to categorize that person as normal weight, underweight, overweight, or obese. It is calculated by dividing the body mass by the square of the body height (kg/m^2).

Developmental Origins of Health and Disease (DOHaD) A theory that examines how environmental factors during fetal development interact with genotypic variation to change the capacity of the organism to cope with its environment in later life. For example, intrauterine growth restriction is associated with increased risk of adult-onset cardiovascular disease and diabetes (see Box 26.1).

Differentially methylated region (DMR) Genomic regions with different methylation status across different cells/tissues from the same or different individuals. Usually refers to methylation at CpG sites in DNA.

Environmental stressor An external stimulus, such as a chemical or biological agent (e.g., tobacco, pollution, alcohol), environmental condition (e.g., temperature, poor diet), or an event (e.g., social stressor, psychological stress) that causes stress in an organism. The stressor may lead to physical, mental, or epigenetic responses in an individual.

Epigenetic inheritance A type of nonconventional inheritance that is independent of the DNA base sequence and involves the inheritance of an epigenetic factor from one generation to the next.

Epigenetic reprogramming Erasure and reestablishment of epigenetic marks during development, first during gametogenesis, and again in early embryogenesis. This allows the formation of totipotent cells in each new generation.

Epigenome Chemical changes to DNA and histones or changes in ncRNA expression that can be inherited and are involved in regulating gene expression and suppression of transposable elements. The epigenome is dynamic and can be altered by environmental conditions.

Epimutation Alterations in the pattern of normal epigenetic mark that has the potential of being inherited from one generation to the next.

Filial generation Pertaining to the sequence of generations following the parental generation. Each generation is designated by an "F" followed by a number indicating the generation (e.g., F1 is the first filial generation).

Germ cell A totipotent, haploid cell that combines with a germ cell from the opposite sex to form a new individual. Also known as a gamete.

Intergenerational epigenetic inheritance The transmission of non-DNA base sequence (i.e., epigenetic) information between generations via the germline. It affects offspring phenotype and/or epigenetic marks, which is not inherited past the F1 generation through the paternal lineage and the F2 generation through the maternal lineage (see Fig. 26.1). In this type of epigenetic inheritance, direct exposure of the offspring to the causative environmental stressor or to parental physiological effects cannot be mechanistically ruled out.

Metastable epiallele An allele that is variably expressed in genetically identical individuals due to epigenetic modifications and is thought to be vulnerable to the environment.

Paramutation Interaction between two alleles at a single genetic locus whereby one mutant allele induces a heritable epigenetic change in the other wild-type allele.

Reconstruction model In epigenetic inheritance, this mechanistic model suggests that epigenetic marks responsible for a phenotype undergo normal reprogramming in the germline and zygote but are then "reconstructed" or recreated by each successive generation (see Fig. 26.6B).

Repetitive DNA Patterns of nucleic acids that occur in multiple copies throughout the genome. A significant fraction of the genome is repetitive. Repeats that occur in tandem repeat sequences or dispersed throughout the genome as transposable elements (e.g., transposons, retrotransposons).

Replication model In epigenetic inheritance, this mechanistic model requires epigenetic marks (e.g., DNA methylation, histone modifications) to be directly copied between cell divisions and generations (see Fig. 26.6A).

Slow-growth period The period before the onset of puberty, usually between ages 9 and 12 in humans.

Transgenerational epigenetic inheritance The transmission of non-DNA base sequence information between generations via the germline. It affects offspring phenotype and/or epigenetic marks, which must be inherited at least to the F2 generation through the paternal lineage and to the F3 generation through the maternal lineage (see Fig. 26.1) to exclude direct effects of these individuals to the initiating environmental stressor.

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CHAPTER

Epigenomic mechanisms and episignature biomarkers in rare diseases

27

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27.1 Introduction

Embryonic and fetal development encompasses complex biological processes that integrate regulation and execution of gene expression. The temporal and spatial restrictions placed on these patterns of cell- and tissue-specific gene expression produce a viable organism that has potential to survive and propagate. Hence, development is not the consequence of isolated decisions at the level of individual genes; instead, developmental gene regulatory networks coordinate cell-type−specific expression (at the level of transcription factors) that initiate and maintain differentiation, activate or repress morphogenetic function, interpret transient internal signals, and react and respond to external environmental signals [1].

Integrated within these gene regulatory networks, and central to their successful activity, are genes encoding proteins that comprise the so-called epigenetic machinery. Although it is an evolving term, "epigenetics" refers to the mechanistic regulation of heritable gene expression patterns and associated phenotypes persisting through mitosis (and sometimes meiosis) without a change in the primary DNA sequence [2,3]. Several studies have provided evidence that differing epigenetic states contribute to cellular heterogeneity despite identical or nearly identical genetic sequence [4]. Epigenetic regulation of gene expression occurs at the level of chromatin and through protein complexes that permit efficient packaging of a cell's genetic material in a manner conducive to transcription, replication, DNA repair, and mitosis [5,6]. A primary role for the epigenetic machinery, from a developmental perspective, is to act (and react) at an interface between the genome and the external environment. Reversible gene-specific and temporally regulated epigenetic processes permit cellular differentiation and nuanced responses to environmental stimuli through dynamic and reversible changes in chromatin structure and gene expression. This is especially important for an embryo/fetus that can be vulnerable to gestational variations in maternal nutrition or to chemical exposures that may challenge embryonic differentiation to stay on its "proper," predestined course [7,8]. At the cellular level, these epigenetic processes include gene-specific DNA methylation changes, developmentally regulated modulation of chromatin-specific histone modifications, the transcription of noncoding RNAs (ncRNAs), and the elaborate expression and repression of proteins that write, read, and erase the epigenetic patterns in cells [9]. As a result, these factors establish and maintain chromatin organization, while ensuring that

Epigenetics in Human Disease. https://doi.org/10.1016/B978-0-443-21863-7.00012-3
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transcriptional regulators have temporally and spatially relevant accessibility to gene regulatory regions that initiate (and restrict) developmental cascades [10]. Changes in DNA methylation, for example, the most extensively studied epigenetic mark, are well characterized in mammalian development [11,12]. Global demethylation occurs first in the primordial germ cells to eliminate cell lineage−specific marks, followed by the establishment of sex-specific methylation patterns as well as imprinted regions. A second wave of demethylation occurs after fertilization to allow for the targeted re-methylation that allows for totipotency in the embryo [11]. Errors in this process, often due to a mutation in an epigenetic machinery gene, can result in neurodevelopmental disorders, highlighting the importance of epigenetic regulation in development [12,13].

Given the roles for epigenetic processes in embryonic, fetal, and postnatal development, it is not surprising that inherited and de novo DNA sequence errors affecting chromatin structure, maintenance, and transcriptional accessibility would directly impact the carefully coordinated developmental process. In this chapter, we address epigenomic mechanisms in the etiology of developmental disorders. We provide a brief overview of the reversible DNA and histone modifications that contribute to and enable gene expression and genomic order and plasticity. We discuss imprinting disorders that result from errors in selective, parent-of-origin−specific gene expression, and a group of classical epigenetic diseases caused by DNA trinucleotide repeat expansion associated with aberrant site-specific DNA methylation. Next, we describe Mendelian disorders of the epigenetic machinery [14,15], where mutations in epigenetic writers, readers, erasers, and remodelers (WRERs) interfere with the context-appropriate gene expression patterns that are essential to the execution of the developmental cascade. We also discuss the interactions between genetic variation, environment, and the epigenome. The diagnostic challenges of Mendelian neurodevelopmental disorders will be explored, as well as the identification and evolution of disorder-specific DNA methylation patterns, called episignatures, as biomarkers before concluding with an overview of the clinical utility of epigenetic testing as well as advantages and limitations of its incorporation into the clinical setting. These new epigenome-based technologies can be cost-effectively integrated with microarray-based technologies to screen for a wide range of complex, clinically overlapping congenital and developmental phenotypes. These technologies also shed light on pathological mechanisms of these diseases, with the promise of insights into potential novel therapeutic and diagnostic approaches to conditions.

27.2 DNA and chromatin modifications

Reversible modifications of the DNA and histone proteins that comprise chromatin are central to the processes of epigenetic regulation during development [16]. DNA methylation involves a covalent modification occurring primarily at the 5-position carbon of cytosines within cytosine-guanine site (CpG) dinucleotides, resulting in a 5-methylcytosine [17]. In general, CpGs are underrepresented in the genome and are disproportionately located in CpG "islands" at the 5′ ends of genes that encompass potential regulatory/promoter regions [18]. The majority of CpG dinucleotides are methylated across different cell lineages [19], however promoter CpG islands are mostly unmethylated [20,21]. The most common theory as to why CpGs are underrepresented in the genome is the frequent deamination of methylated cytosines, ultimately converting cytosine to thymine after replication, with de novo mutation rates demonstrating an increase up to 18-fold compared to other sites [22]. Given the majority of CpG islands are unmethylated, the cytosine to thymine substitution rate is approximately seven times

lower [22]. CpG islands in regulatory regions of housekeeping genes are usually unmethylated, allowing for a transcriptionally permissive chromatin state; however, cytosine methylation (mCG) at these CpG-rich regions contributes to the condensation of chromatin and transcriptional repression and permits tissue-specific, context-dependent accessibility to the DNA template. CpG islands in promoters of germline-specific genes are methylated after differentiation to ensure proper cell lineage commitment [23,24]. This life-long maintained DNA methylation also occurs in imprinted genes and X-inactivation, which will be discussed later in this chapter, indicating that methylation of CpG islands in somatic tissues may represent a way to ensure gene silencing important to development [12,23–26]. Furthermore, CpG island promoters are often dynamically silenced through histone methylation by the Polycomb repressive complex 2; this epigenetic mark offers more plasticity than DNA methylation, allowing for re-initiation of gene expression when developmentally appropriate or in response to environmental factors [12].

Enhancers, which are long range cis-regulatory elements that enhance transcription through transcription factor recruitment, demonstrate low amounts of CpG dinucleotides when compared to other genomic regions; however, CpG-rich enhancers have been identified [27–29]. mCG levels at enhancers are correlated to transcription factor binding [20,30], with increased methylation levels correlated with less chromatin accessibility at 20% of sites and less transcription factor binding at 70% of sites [31]. This is in line with a study that surveyed more than 500 human transcription factors and found that 22% demonstrated decreased binding to their motifs when methylated [32]. It is also worth noting that DNA methylation can also recruit specific transcription factors to increase expression of certain genes, many of which are involved in cell differentiation [12]. Less is known about the role of mCG outside of regulatory elements, particularly within gene bodies. Studies have indicated that, at least in certain tissue types, intragenic mCG correlates to more actively transcribed genes [18,19,33,34]. Two hypotheses for how intragenic mCG can increase transcription have been proposed: (1) DNA methylation in gene bodies impacts the RNA polymerase processivity and splicing [35–39], and (2) intragenic mCG inhibits intragenic promoters [12,18,40,41]. In these cases, transcriptional activation of genes with intragenic mCG could be due to a combination of both hypotheses, with further work required to better understand the function of DNA methylation in the gene body context.

DNA methylation reactions are catalyzed by DNA methyltransferase (DNMT) enzymes that transfer the methyl group from S-adenosylmethionine (SAM) to cytosine [42]. DNMT1, DNMT3A, and 3B are the major DNA methyltransferases with distinct and well-characterized functions [43]. DNMT1 is a maintenance methyltransferase that executes its main function of copying DNA methylation patterns after replication through its close association with the DNA replication machinery and enhanced affinity for hemi-methylated DNA. In contrast, DNMT3A and DNMT3B are essential de novo methyltransferases with no specific preference for unmethylated versus hemi-methylated CpG sites. Complete loss of DNMT1 in mice, as well as human embryonic stem cells, results in embryonic lethality and cell death [44,45], highlighting the importance of DNA methylation maintenance throughout development.

Patterns of mCG are remarkably plastic. On the one hand, site-specific DNA methylation can be maintained throughout the life of an individual [46]. In contrast, the individual CpGs that comprise the "DNA methylome" can be precisely manipulated, with active enzymatic mechanisms able to quickly reverse and reset mCG at specific DNA sequences. The precise regulation of DNA methylation within CpG islands ensures that housekeeping genes are free of methylation and transcriptionally accessible,

while at the same time, tissue-specific gene expression can be inactivated through processes that include DNA methylation. Thus, DNA methylation provides cells with an extra layer of gene regulation in addition to other methods of pre- and posttranslational control of gene expression.

DNA methylation also plays a role in the regulation of imprinting [47], and X-chromosome inactivation in females [48]. These processes ensure gene dosage compensation, as well as the repression of potentially unstable genomic elements such as transposable and retroviral elements. Thus, the maintenance of proper DNA methylation marks during development and tissue differentiation contributes to chromosome stability and the appropriate spatial and temporal control of gene expression that is critical during embryonic/fetal development. Impairment of these epigenetic signals—for example through mutations within genes encoding components of the epigenetic machinery—can lead to clinically significant developmental errors [14,15,49].

Another class of modifications that directly target CpG sites is DNA hydroxymethylation [50]. 5-hydroxymethylcytosine (5 hmC) is catalyzed by the enzymes of the ten-eleven translocation (TET) family, by converting 5-methylcytosine to 5 hmC. These enzymes, particularly TET1, are abundantly found in embryonic stem cells, where ChIP-Seq analyses have shown that it preferentially binds to CpG-rich regions in promoters. This observation has led to the postulation that 5 hmC contributes to maintaining the CpG sites in a hypomethylated state to avoid aberrant de novo methylation during epigenetic reprogramming [50]. 5 hmC is enriched in gene bodies and promoters, as well as brain-specific enhancers [51], however, the exact role of 5 hmC in gene expression is complex given the TET1-bound genes can be both up- and downregulated [52]. During neural development, neural progenitor cells show an increase in 5 hmC as they develop into mature neurons [51]. TET enzymes are essential to development as loss of all three TET enzymes results to early embryonic lethality in mice [53]. In developed mammals, 5 hmC is abundant in the central nervous system, and unlike DNA methylation, it shows significant heterogeneity in its abundance in different cell lines [50,54,55], with highest expression in neurons [56]. In adult neural progenitor cells, the TET enzymes have differing roles in proliferation and differentiation that are context specific [51]. TET3 is the most abundant TET enzyme in the brain overall, with highest levels in the cerebellum, cortex, and hippocampus when compared to TET1 and 2 [56]. 5 hmC accumulates over the course of life and is enriched at neurogenesis genes, and this epigenetic mark also has roles in post-mitotic neurons through the ability to adjust gene expression in response to neuronal activity [51]. 5 hmC, and therefore the TET enzymes, has implications in disease as well, and it is widely accepted that disease can occur when abundance and distribution of this epigenetic mark is altered, as well as learning deficits [57,58]. Other types of CpG modifications can also occur following the oxidization of 5 hmC, resulting in 5-formylcytosine (5 fC) and 5-carboxylcytosine (5caC) [59]; a process called active demethylation occurs when 5 fC and 5caC are removed by DNA glycosylases and the base excision repair pathway [51].

DNA modifications could also occur at non-CpG sites (CpA, CpT, and CpC), although the function of this type of modification is not clearly understood. There have been efforts to relate non-CpG methylation to tissue specificity, gene expression, or serving as a by-product of hyperactivity of nonspecific de novo methylation of CpG sites [60–64]. Although it is rare to find non-CpG methylation in most differentiated cell types, researchers have reported its presence in embryonic stem cells, germ cells, and various cells of the brain [59]. Along with modifications at the nucleotide level, a complex and varied array of posttranscriptional modifications to histone proteins have also been well characterized over the past 2 decades. Histones organize the DNA molecule and regulate its

accessibility to replication and transcriptional machinery, thereby providing an additional level of gene- and context-specific chromatin regulation. Histone protein modifications are coordinated by a variety of enzymes that can catalyze relatively well-defined histone acetylation, ADP-ribosylation, methylation, phosphorylation, sumoylation, and ubiquitination [65–67], and less well-understood modifications such as butyrylation, crotonylation, and propionylation [68,69]. As a result, combinations of distinct histone modifications can act sequentially or in combination to form a "histone code" that can be interpreted by proteins of the epigenetic machinery to initiate distinct downstream events [66–69]. Histone acetylation and methylation are the most studied and best-characterized histone modifications, with distinct functions related to the specific histone on which they occur, whether it be a residue within the histone tail or body, as well as type of modification. Dynamic, reversible histone acetylation on lysine residues, for example, neutralizes lysine's positive charge and weakens the DNA/histone interaction. This modification is highly regulated by the opposing actions of histone acetylases and deacetylases (HDACs). Additionally, histone methylation occurs primarily on arginine and lysine residues and can occur in mono-, di-, or trimethylated states. These modifications are initiated by a diverse family of arginine methyltransferases and by specific lysine methyltransferases (KMTs) that can methylate N-terminal lysines via conserved enzymatic Su(var)3–9, Enhancer-of-zeste, Trithorax domains [66,69].

The modes of action of these various histone tail and core modifications influence the overall structure of chromatin compaction over short or long distances and can have positive or negative influences on the ability of effector molecules to bind to DNA [66–69]. Both euchromatin and heterochromatin domains are marked and enriched (or depleted) by specific characteristic histone modifications that are dynamically responsive to intrinsic and extrinsic regulatory signals. The most common modifications include the methylation and acetylation of lysine residues in the N-terminal tails of histones H3 and H4. The changes that are associated with active gene expression are monomethylation at H3K9 and H3K27, trimethylation of H3K4, and acetylation at H3K9 and H3K14. On the other hand, demethylation of H3K4 and trimethylation of H3K9 and H3K27 exert a repressive effect on gene expression and are associated with a closed chromatin state. These histone marks also label the regulatory elements in the genome. For instance, regions flanking transcription factor binding sites and active enhancers are commonly marked with nucleosomes with acetylation of H3K27 and monomethylation of H3K4, whereas active promoters often contain trimethylation of H3K4 [70]. The organizational complexity of these histone modifications and the specificity of their functional roles in regulating transcription and chromatin organization are further complicated by the inherent cross talk between histone and DNA-modifying enzymes (including those involved in DNA methylation), as well as the context-dependent chromatin modifications they are responsible for establishing, maintaining, and removing [71,72]. Different combinations of DNA modifications (such as DNA methylation or hydroxymethylation) and histone modifications, as well as the genomic sites of these modifications, result in different impacts to gene expression [73,74]. For example, DNMT3B1 recruitment to transcribed genes requires methylation of lysine 36 on histone H3, with ablation of H3K36me3 disrupting DNMT3B1 binding and de novo methylation at these sites [75]. Also, studies have found that DNMT1, DNMT3A, and DNMT3B interact with HDACs, supporting the linkage of DNA methylation and histone modification [76] to other chromatin regulatory proteins [73,74]. Efforts are underway to integrate specific individual and combinatorial epigenetic signatures with the disease- and trait-associated genetic variants, to better understand gene regulation, cellular differentiation, and the molecular basis of human disease [77,78].

Histone modifications are essential throughout development [79]. Proper histone marks are required to ensure gene expression is coordinated for cell pluripotency, as well as cell differentiation, body patterning and ultimately organogenesis. Much like DNA methylation, early embryos show unique histone methylation patterns for lineage-specific gene expression for a given tissue [79]. Histone methylation defects during development can lead to improper development of specific organs, with conditional knockout analysis of specific histone modifiers identifying tissue-specific targets [79]. Several inherited genetic disorders involve histone methylation regulators for example, with patients often exhibiting skeletal and craniofacial abnormalities, as well as intellectual disability [80]. Depending on which histone methylation regulator is impacted, those affected can exhibit overgrowth or reduced body size and microcephaly [15,79,80]. [15,79].

27.3 Epigenetically regulated monoallelic gene expression: imprinting diseases

Genomic imprinting refers to nonrandom monoallelic gene expression, in which the choice of the allele to be expressed is determined by parental origin. Imprinting involves the introduction of epigenetic marks in the germline of one parent, but not the other, resulting in monoallelic expression of a single autosomal gene or of multiple genes within an imprinted region [81–84]. The key molecular mechanism involved in the establishment and maintenance of genomic imprinting is allele-specific DNA methylation at imprinting control regions.

As previously discussed, de novo methylation in gametes is established by DNMTs, including DNMT1, DNMT3A, DNMT3B, and accessory DNA methyltransferase 3–like (DNMT3L) proteins in both gametes before fertilization. Some of these enzymes act in a germ cell and sex-specific manner. This methylation primarily targets transposons, repeat elements, and imprinted loci. This implies that the imprinted loci are inherited with differential methylations in the egg and sperm. Therefore, the allele from one parent is unmethylated and transcriptionally active, whereas the allele from the other parent is methylated and transcriptionally repressed. This imprinted state persists postnatally, through cell division, to some or all differentiated tissues. The maintenance of parent-of-origin–specific gene expression is facilitated by DNMT1 and accessory proteins [81,85].

To date, over 120 imprinted genes have been identified with many other genes predicted to be imprinted under investigation [86–88], most of which are grouped in clusters or at loci containing at least one imprinting control region [89,90]. One important feature of genomic imprinting is that some imprinted genes show monoallelic expression *throughout* the body, whereas others demonstrate only *tissue-specific* imprinting, for example, the *UBE3A* gene on chromosome 15 is imprinted in the human brain but expressed from both alleles in other tissues [85,90]. Another example is the tissue-specific imprinting of the *GNAS* locus on chromosome 20q13, which is maternally expressed in the renal tubules but expressed biallelically in most other tissues [90].

Imprinting disorders can occur during the establishment of genomic imprinting or cellular division, and they are responsible for a broad range of disorders affecting growth, development, and metabolism [84,91,92]. Imprinting disorders may be caused by either epigenetic changes or genetic mutations, and expression of the disease phenotype depends on parent of origin, whether the mutant allele is inherited from the father or the mother [84,93]. Among the best-studied examples of congenital genomic imprinting disorders are Angelman syndrome (AS), Prader–Willi syndrome (PWS), Beckwith–

Wiedemann syndrome (BWS), and Silver–Russell syndrome (SRS) [84,93]. Recent evidence has also revealed multi-locus methylation defects in patients with clinically diagnosed imprinting disorders [93]. In these cases, aberrant methylation does not only affect the disease-specific imprinted loci (for example, 11p15 in BWS) but also involves changes in other genomic regions. Studies have also shown that patients with opposite phenotypes, such as BWS (overgrowth) and SRS (growth retardation), can share some aberrant methylation patterns in lymphocytes [94], suggesting a potential overlap in the molecular etiology and functional impact of these imprinting disorders [93,94].

AS is characterized by severe intellectual disability and speech impairment, gait ataxia, tremulousness of the limbs, frequent laughter, excitability, and seizures [95]. Genetic and epigenetic defects in the same genomic region on chromosome 15 (15q11.2-q13) cause PWS, which is characterized by severe hypotonia and feeding difficulties in infancy, followed by hyperphagia and gradual development of obesity, cognitive impairment, and hypogonadism [96]. The AS/PWS critical region on chromosome 15 contains multiple paternally expressed genes (including small nuclear ribonucleoprotein N [*SNRPN*]), and a single maternally expressed gene (*UBE3A*) [97]. PWS results from hypermethylation of the 15q11.2-q13 critical region and the associated loss of the paternally expressed genes (including *SNRPN*), whereas AS results from a loss of imprinting (hypomethylation) and/or loss of the maternally expressed *UBE3A* gene [97]. The causal molecular mechanisms in both PWS and AS include microdeletions, point mutations, translocations, uniparental disomy (UPD), and imprinting defects. Apart from point mutations in the maternally expressed *UBE3A* gene, which may cause AS in ~11% of the cases [98], all other known mechanisms that cause PWS and AS affect DNA methylation at the imprinting control region on chromosome 15. SNRPN upstream reading frame (*SNURF)/SNRPN* is one of the paternally expressed loci in this region, and its parent-of-origin–specific methylation has long been recognized as a diagnostic indicator of PWS and AS [99]. In addition to SNRPN/SNURF, loss of *MAGEL2* gene expression is proposed to contribute to the features of PWS. *MAGEL2* is also located in the PWS critical region, and mutations in this gene have been associated with the PWS-like phenotype and autism spectrum disorder (ASD) [100]. About 5% of AS and PWS are caused by imprinting defects. These can occur secondary to small deletions at the imprinting control region leading to errors in the mechanism for resetting and maintaining the genomic imprinting or directly as a result of an abnormal imprint (epimutation) [101,102].

BWS and SRS are imprinting disorders associated with overgrowth and growth restriction, respectively. Both syndromes are caused by dysregulation of imprinted genes associated with cell cycle and growth control that are clustered in the 11p15.5 chromosome region [82,103]. Two important imprinting control regions (ICR) have been identified in this region: the H19/IGF2 domain (ICR1), which is methylated on the paternal allele, and the KCNQ1OT1/CDKN1C domain (ICR2), which is methylated on the maternal allele. ICR1-mediated methylation extends to the *H19* promoter, thereby silencing paternal *H19* expression while permitting expression of *IGF2* (which, in contrast, is repressed on the maternal allele) [104]. Genetic alterations that result in biparental *IGF2* expression cause BWS, an overgrowth syndrome characterized by the presence of macrosomia, macroglossia, and omphalocele. Most cases of BWS are sporadic and result from de novo epimutations at the ICR within 11p15.5 [105]. However, a range of molecular alterations—including point mutations acting in *cis*, frameshift mutation acting in *trans*, and UPD11—are associated with familial BWS [106]. A subgroup of BWS patients also show hypomethylation at other imprinted loci [107], suggesting that genes outside of the 11p15.5 ICR are involved in the pathogenesis of this syndrome. Conversely, paternal loss of methylation at *H19* usually causes SRS, which is characterized by severe prenatal and postnatal

growth compromise, developmental delay, and a variable combination of facial dysmorphic features. Molecular mechanisms for SRS are complex and involve structural chromosomal anomalies and maternal UPD11 [82]. In addition, 10% of SRS patients display locus heterogeneity and are caused by maternal UPD7, including the known imprinted loci *GRB10*, *SGCE/PEG10*, and *PEG/MEST* [108].

The number of genes that are consistently imprinted in humans is not limited to the above and is estimated at over 120 genes, an additional 110 are predicted to be imprinted or contain provisional data to indicate they may be imprinted (www.geneimprint.com). One theory to explain the evolution of imprinting suggests the parental genome conflict related to fetal growth. The maternal genome is programmed to preserve resources and thus restricts the growth, whereas the parental genome encourages the growth of the fetus irrespective of the maternal resources available for future pregnancies [109,110]. Consistent with this theory, numerous of imprinted genes, including the ones discussed above, are involved in the regulation of growth and many of the related syndromes present with overgrowth or growth retardation as part of the clinical phenotype. For instance, CDKN1C (relevant to BWS) is a strong inhibitor of several G1 cyclin/Cdk complexes and a negative regulator of cell proliferation, and IGF2 (relevant to SRS) is an insulin growth factor with growth-promoting activity [103].

27.4 Epigenetically regulated monoallelic gene expression: X-linked diseases

In contrast to the nonrandom monoallelic regulation of genomic imprinting, X-chromosome inactivation is a result of the *random* monoallelic inactivation of one of the two X chromosomes in the female genome. X-chromosome inactivation is a mechanism of dosage compensation that results in epigenetic silencing of most genes in one of the two X chromosomes in females, resulting in balanced expression of most X-linked genes in males and females. In normal cells, the choice of which X chromosome becomes inactivated is random and results in mosaicism of X-linked gene expression in females.

X-inactivation occurs early in female embryonic development and is under control of the X-inactivation center, a locus that contains the key regulatory gene *XIST* (X-inactive−specific transcript). *XIST* encodes a long ncRNA that coats the X chromosome and establishes an inactive state [111]. Overall, the process of X-chromosome inactivation can be categorized into four stages: counting, choice, inactivation of one X chromosome, and maintenance of the expression state of the X chromosome. DNA methylation, along with histone modifications, participates in the maintenance of the inactive state of the silenced X chromosome [112].

X-chromosome inactivation is indispensable for female embryogenesis and development. It is estimated that defects in ∼60% of X-linked genes in mice can cause growth retardation or developmental defects [111]. Female heterozygote carriers of X-linked conditions can show variable disease presentation (ranging from complete absence of the disease phenotype to a presentation comparable to that seen in affected males), which is largely dependent on the pattern of X-inactivation (also referred to as X-chromosome lyonization), and thus the proportion of cells with expressed mutant allele. In Duchenne muscular dystrophy (DMD, caused by mutations in the X-linked dystrophin gene), for example, female mutation carriers are typically asymptomatic, and yet ∼8% exhibit muscle features [113]. Females DMD carriers with moderate/severe muscle involvement exhibit skewed

X-chromosome inactivation (i.e., the mutant allele is found preferentially on the active X chromosome), whereas carriers with mild muscle involvement show random X-chromosome inactivation [113].

X-chromosome inactivation in females, however, does not occur in the entire chromosome and a small fraction of the genes escape this inactivation. This ratio of the genes on inactive X chromosome that can be expressed has been estimated to range between 3% and 15% in various cell lines and tissues [114]. A large portion of these genes have homologous genes on the Y chromosome, also known as pseudoautosomal genes [114]. This incomplete X-inactivation explains the aberrant gene dosage in Turner (XO) or Klinefelter (XXY) syndromes despite the fact that X-chromosome inactivation will keep one X chromosome activated in either of the conditions. The defects in genes that escape X-chromosome inactivation represent conditions with autosomal effect. An example includes Claes-Jensen syndrome, which is caused by loss-of-function mutations in *KDM5C* [115–118]. Even though this gene is located on X chromosome, it does not undergo X-inactivation and the disease is represented with an inheritance pattern more similar to autosomal recessive conditions [115–117].

27.5 Epigenetic involvements in trinucleotide repeat diseases

Epigenetic regulation is a key mechanism involved in a number of genetic disorders that are caused by trinucleotide repeat expansion (TRE) of DNA. TRE disorders include Fragile X syndrome (FXS), Huntington disease, and Friedrich ataxia and are characterized by an unstable expansion of a segment of DNA containing repeating units in tandem of three nucleotides. Genes associated with these diseases contain a variable number of repeat units in the normal population; however, as the gene is passed across subsequent generations, the number of repeats can increase, leading to abnormal gene expression and function. Although TRE disorders can result in pathology through various mechanisms, altered DNA methylation has emerged as an important cause in some of these conditions.

One well-studied example of this epigenetic mechanism is the cytosine-guanine-guanine (CGG) TRE in the 5′ untranslated region (5′ UTR) of the fragile X mental retardation 1 gene (*FMR1*) (Xq27.3), which causes FXS [119]. FXS is the most common inherited cause of ID in males, with clinical features including facial dysmorphism (long ears, long face, prominent jaw) and macro-orchidism. Based on the number of CGG repeats present, four clinically significant alleles are defined [120]: Normal ($\leq$44 repeats); premutation alleles (55–200 repeats); and full mutation (>200 repeats), with alleles containing 45–54 repeats commonly referred to as being in a "gray zone" and are precursors for premutation alleles (i.e., they have increased susceptibility to expansion into a premutation or full mutation allele). FXS is caused by expansion to the full mutation allele, which results in DNA hypermethylation of the CpG island at the *FMR1* promoter region, detectable through DNA methylation array screening [121], and results in consequent gene silencing [121]. The *FMR1* promoter is upstream of the CGG repeat sequence, which exists in the 5′ UTR, separating the *FMR1* promoter and start codon [122]. Upstream of the *FMR1* promoter and CCG repeat DNA is methylated, however there is a 5′ boundary that prevents methylation of the CpG island within the promoter and the CGG repeat sequence [122]. CGG repeat expansion to a full mutation disrupts the boundary resulting in expansion of DNA methylation to the promoter CpG island and transcriptional silencing of *FMR1* [122]. This silencing occurs during development, at 11 weeks of gestation, resulting in chromatin structure changes that includes repressive histone modifications [122]. There also exists a 3′ DNA methylation boundary of the *FMR1* promotor, which also becomes compromised with expansion of the

CGG repeat [122]. As expected, for an X-linked disorder, full mutations are penetrant in all males, whereas in females heterozygous for the full mutation, the typical physical and behavioral features show low penetrance and variable expressivity [123]. The lower penetrance observed in females is explained by the presence of a second, normal copy of the *FMR1* gene, and nonrandom X-inactivation [124].

The fragile X mental retardation protein (FMRP) is an RNA-binding protein that regulates synthesis of proteins required for the formation of synapses during neuronal development, with a number of functional roles, including the repression of mRNA translation through association with polyribosomes, and via interactions between FMRP and microRNAs and Argonaute proteins, which are part of the RNA-induced silencing complex [125]. Disruption of these processes is likely to underlie the intellectual disability seen in FXS patients.

Premutation alleles are not hypermethylated and do not result in FXS-related features, but in some patients can cause the fragile X-associated tremor/ataxia syndrome, which is characterized by late-onset progressive cerebellar ataxia and intention tremor [126]. The premutation is associated with increased levels of *FMR1* mRNA, resulting in a toxic gain-of-function of FMRP, which may interfere with nuclear function and lead to neurodegenerative symptoms. Female premutation carriers are also at risk of primary ovarian insufficiency, defined as cessation of menses before age 40 years, which is seen in ~20% of carriers [127]. Also, female premutation carriers may transmit a full mutation allele to their children because the expansion of the premutation to full mutation allele occurs during maternal, but not paternal, transmission of the X chromosome [128].

It has been proposed that certain trinucleotide repeat expansions located in noncoding regions of the gene, such as those in FXS, induce abnormal DNA or DNA/RNA hybrid structures such as hairpin formations, which in turn can result in DNA methylation and gene silencing [129,130]. Evidence suggests that the tendency of DNA to form hybrid structures of this type is dependent on the repeat number and that DNA methylation is likely to be a direct consequence of hairpin formation [131,132]. In the example of FXS, hairpins are suggested to provide the structural basis for the major molecular phenomena, including site-specific fragility, repeat amplification, and hypermethylation of the CpG island adjacent to *FMR1* [131].

27.6 Diseases of the epigenomic machinery defects

An intricate and essential network of proteins establishes, maintains, and localizes epigenetic modifications to chromatin, and these signals can be stably retained to "shape" the genome, while at the same time remaining flexible and reversible in their response to environmental stimuli. These proteins function as *writers* that establish the epigenetic marks, *readers* that interpret them, and *erasers* that remove the epigenetic marks, and *remodelers* that can reposition nucleosomes and establish insulator functions that define boundaries between epigenetic domains [14,15,49,133,134]. Together, these writers, readers, erasers, and remodelers (or *WRERs*) serve as interpreters of the epigenetic state while allowing cross talk and synchronization of epigenetic signals across the genome [14,15,49,133,134].

Several recent papers have reviewed the scope, diversity, function, and integration of the various protein classes comprising the epigenetic WRERs [14,15,49,134,135]. Briefly, *writers* include the aforementioned DNA and histone methyltransferases (DMNTs [136–139] and HMTs, such as *MLL/KMT2A*, respectively [140–142]), as well as histone acetyltransferases (HATs) [67,143]; *readers*

possess intrinsic functional domains (such as MBD methyl-binding domains) that can recognize, interpret, and relay specific modifications (e.g., MeCP2) [144,145]; and *erasers*, including histone deacetylases (HDACs), can, as their name implies, remove acetyl groups from histones [66,146]. In many cases, the contextual accuracy and reversible nature of these epigenetic modifications are ensured by counteracting activities, such as in the case of HATs and HDACs that, respectively, write and erase histone acetylation. Finally, four families of chromatin-remodeling complexes (including the SWItch/Sucrose Non-Fermentable (SWI/SNF) complex) [147] function to move nucleosomes along DNA and eject or exchange nucleosomes as a means to enable DNA accessibility [133,134,147]. Together, when properly integrated, these highly coordinated and interactive WRER proteins act within multiprotein, multifunctional complexes on chromatin to ensure the tissue- and lineage-specific transcriptional activation, and suppression that is essential to embryonic and fetal development.

Mutations in the genes encoding WRER proteins can compromise the functional complexity and integration of the epigenetic machinery and lead to errors in the epigenetic signatures on chromatin. Such mutations are now implicated in a broad range of neurodevelopmental genetic disorders [14,15,49]. This model also suggests that some imprinting disorders, as well as a group of multiple congenital anomalies and developmental disability and intellectual disability syndromes that exhibit phenotypic overlap, result from mutations in genes encoding components of the epigenetic machinery that impact development. Overlap in shared molecular targets may be the basis for phenotypic overlap [15,148,149]. For example, RUNX2 (a transcription factor involved in bone and cartilage development) is dysregulated in Kleefstra [150] and chromosome 2p37 deletion (formerly brachydactyly-mental retardation) [151] syndromes, both of which feature brachydactyly (shortened fingers and toes). Kleefstra syndrome is caused by haploinsufficiency of EHMT1 [150], a histone methyltransferase, and haploinsufficiency of histone deacetylase 4 occurs in chromosome 2p37 deletion syndrome [152]. Therefore, mutations in two different WRER proteins result in the dysregulation of the same transcriptional pathway through differing mechanisms, resulting in overlapping phenotype. Another example is seen in the shared phenotype of disordered sleep in intellectual developmental disorder, autosomal dominant 1 (MRD1), Smith−Magenis, and BDMR syndromes, all three of which are characterized by abnormalities of the mTOR and circadian rhythm pathways [15,49,148]. Mutations in reader Methyl CpG binding protein 2 (*MeCP2*) result in Rett syndrome, a neurodevelopmental disorder that impacts females through progressive encephalopathy [153]. *MeCP2* expression increases postnatally in neurons and targets methylated DNA at CpG islands recruiting histone deacetylases, ultimately repressing transcription [154]. *MeCP2* is most highly expressed in the brain, where it has a crucial role in neuronal function; indeed, a mouse model with MeCP2 loss only in the brain demonstrated the same phenotypes as a whole-body knockout mouse model, solidifying its importance in the brain [155]. Rett syndrome results in severe intellectual disability, autism, gait apraxia, hypotonia, seizures, and deceleration of head growth after 7−18 months of age. Mutations in *MeCP2* and Rett syndrome demonstrate another example of how epigenetic regulation of chromatin is essential, even in postnatal development.

A recent review systematically examined epigenetic machinery with a documented domain with one of the WRER functions to identify 82 conditions with corresponding mutations in 70 epigenetic machinery genes [15]. Through assessment of these epigenetic machinery disorders, common phenotypic changes were observed, with most (83%) of those affected presenting with intellectual disability. This further highlights the importance of epigenetic regulation in brain development [14,15,49]. Additionally, abnormal growth was observed in 71% of epigenetic machinery gene

disorders, with abnormal growth occurring in 87% of disorders presenting with intellectual disability. Epigenetic machinery genes are also expressed in a non-tissue specific manner [156], much like housekeeping genes, and as such there are many different tissues involved in the clinical presentation of those with epigenetic machinery disorders. Importantly, epigenetic machinery as a gene class is very intolerant to loss of function mutations [156]. When compared to dosage sensitive transcription factors, with epigenetic machinery often recruited by transcription factors, epigenetic machinery displayed a higher sensitivity to haploinsufficiency [156], indicating the importance of epigenetic machinery in gene expression regulation. Epigenetic machinery with dual function, meaning those with enzymatic (are able to write, erase, or remodel) and reader functions, are the most dosage sensitive. Furthermore, co-expression of these epigenetic machinery genes across tissues supports the notion that a delicate balance of epigenetic states is required for proper development and function [15,156]. Co-expression seems to be most important in neurons; this is in line with the predominant involvement of the central nervous system in the majority of epigenetic machinery disorders.

Inherent to this model is the concept that variations in the normal balance of open and closed chromatin states would lead to aberrant expression of downstream disease-relevant target genes [14,157–161]. For example, mouse models of Sotos syndrome, which is caused by haploinsufficiency of writer histone methyltransferase NSD1, exhibit a similar neuronal phenotype as mice lacking APC2, a crucial protein involved in neuronal migration and axon guidance in neurodevelopment [158]. The defects observed in the *NSD1* knockdown mice were then rescued by forced expression of APC2, indicating that APC2 is a downstream target of NSD1 and providing a pathogenetic mechanism for the intellectual disability associated with Sotos syndrome. Another example of epigenetic regulator involvement in disorder pathology is the requirement of the chromatin remodeling BAF complex subunit switching for proper cell cycle control during neural development [162]. Several BAF subunits are implicated in neurodevelopmental syndromes that exhibit intellectual disability, autism spectrum disorder and speech disorders. Braun et al. demonstrated the importance of the BAF complex in the developing brain by directly controlling accessibility to target genes involved in neural stem cell proliferation and differentiation [162]. Finally, mutations that do not result in complete loss of epigenetic machinery can also contribute to disease. Specific mutation in the DNMT3A reader domain does not render the enzyme non-functional but instead resulted in inappropriate DNA methylation [163,164]. This impacts expression of *Hox* genes, and others, resulting in premature differentiation and overall decrease in mouse brain and overall size. This mechanism was demonstrated in mice and corresponds to patient phenotypes with relevant gain-of-function mutations in *DNMT3A* who present with microcephalic dwarfism [163,164]. These described mechanisms above provide examples of connections between loss or change in function of an epigenetic regulator and the observed changes in gene expression that contribute to specific patient phenotypes.

27.7 Interaction of genetic and epigenetic variations in human disease

The interplay between the genome and epigenome largely determines the intraindividual variation observed in different developmental conditions. Variable expressivity and penetrance are fundamental characteristics of Mendelian conditions, while multifactorial and complex human traits show a lack of full concordance in monozygotic twins. These two observations suggest a role for molecular factors beyond the genetic code, which interact with the primary causal genetic defect, through accumulative

and interactive effects, to determine the phenotype [165]. While epigenomic changes can alter the phenotypic outcome of a genetic variant, so too can genetic variations, directly and indirectly, influence epigenomic profiles. The latter phenomenon results from two structural and functional mechanisms as described in the following paragraphs.

Genetic variants—including point mutations, copy number variants, and common SNPs—can impact genomic regions in which epigenetic variation is found, potentially leading to a direct mechanistic effect on the DNA methylation pattern at those loci. Depletion/generation of CpG sites by single-nucleotide variants at gene promoters has been the focus of research in both Mendelian and multifactorial conditions [166]. In theory, such polymorphisms can change the methylation status of the surrounding CpG sites, influencing the overall methylation density of the region [167]. An SNP in the 5′ UTR of the *GDF5* gene (rs143383C > T), for example, has repeatedly been shown to be associated with multiple musculoskeletal conditions, including height, congenital hip dysplasia, intervertebral disk displacement, and osteoarthritis at multiple sites [168]. The variant is located at a CpG site, where the risk allele (T) eliminates the CpG dinucleotide in the promoter of the gene and results in an imbalance between the *GDF5* promoter DNA methylation and subsequently altered gene expression between the carriers of C and T alleles, as observed in both cultured chondrocytes and human cartilage [169]. Another example is an SNP situated in a CpG island within the regulatory region of *DYX1C1* (dyslexia susceptibility 1 candidate 1), which disrupts the CpG island, resulting in *DYX1C1* methylation and transcriptional inactivation. Carriers of this SNP (rs3743205) have an increased risk of dyslexia [170]. Several candidate susceptibility genes have been identified, and dyslexia can co-occur with other neurodevelopmental disorders such as ADHD, though the molecular mechanism of dyslexia has not been defined [171,172]. Dyslexia a complex condition and is highly polygenic in nature; therefore, the role of epigenetic regulation of *DYX1C1* may partially contribute to the observed phenotype in some patients [171,172]. Such mechanisms of genetic/epigenetic interaction, where the alternative alleles change the methylation mark in the same loci by directly changing the structure of a methylation site (e.g., SNP or Indel), are known as allele-specific DNA methylation [173].

A second, more common mechanism of epigenetic interaction involves genetic variation in distal elements, occurring as a result of alterations in the sequential chain of regulatory events involved in the establishment of epigenetic marks. It is well established that the epigenomic landscape can be predicted by modeling the cumulative effect of these types of genetic variants across the genome, indicating that the epigenome is at least partly encoded in the genome [174]. These genetic variants are referred to as methylation quantitative trait loci (meQTL) and may exert their effect on DNA methylation in both *cis* and *trans* mechanisms, although they are primarily *cis*-acting and located in the vicinity of their target sites [175]. The effect of a *cis*-meQTL on methylation of adjacent CpGs can extend up to 3 kb [176]. The mechanism through which meQTLs interact with the epigenome is poorly understood, but one hypothesis proposes that they impose a change in affinity of the DNA-binding sites for alternative regulatory factors involved in controlling the chain of events leading to epigenetic mark establishment or chromatin modeling in downstream loci [177]. These sites are also frequently associated with various properties of gene regulation beyond DNA methylation, including histone modifications, DNase I accessibility, and chromatin accessibility. A recent study identified more than 11 million meQTLs enriched for functionally relevant characteristics, including shared chromatin state (meaning, chromatin with histone modifications that correspond to a functional region) and high-throughput chromosome confirmation interaction [175]. *Trans* acting loci are enriched for

transcription factors and other interacting proteins [175,178] that likely impact DNA methylation through alterations to recruitment of DNA methyltransferases or TET proteins, as well as physically blocking accessibility to the CpG site.

The majority of data available for the involvement of meQTLs in human diseases have been obtained from the study of multifactorial and complex conditions, where the etiology is understood to comprise accumulative and interactive effects of multiple genetic and environmental factors. Over the past decade, genome-wide association studies (GWASs) have identified hundreds of SNP associations with complex conditions. Given that the vast majority of the reported variants are located in noncoding regions and at a significant distance from genes with any known biological relevance, their interpretation remains a challenge. Assuming a functional property to the identified SNPs, recent studies have suggested a genome−epigenome interaction to explain the function of these variants. Consistent with this interpretation, many GWAS-identified SNPs have subsequently been found to contain meQTL properties [179−181]. For example, a reevaluation of 52 top-ranked obesity-associated GWAS-identified SNPs found 28 of them to be strongly associated with DNA methylation changes at proximal promoters and enhancers [182]. Furthermore, a recent study identified nuclear regulatory pathways through molecular interaction and colocalization experiments that link sequence variation to phenotypic variation in BMI, rheumatoid arthritis, blood pressure and white cell blood counts [175].

In other cases, meQTLs have been employed to explain the heterogeneity in effect sizes of genetic associations across multiple GWAS [183]. For example, a large number of meQTLs are specific to human brain tissue and are associated with neuropsychiatric conditions such as bipolar disorder and schizophrenia [184]. Many of these meQTLs reside in regulatory domains that affect fetal brain development and are enriched among the loci identified by GWAS for schizophrenia [185]. Similarly, an enrichment of cerebellum-specific *cis*-meQTLs has been found among the top susceptibility variants from a GWAS of bipolar disorder [186]. An important finding of the same study is that the meQTLs rarely overlap with expression quantitative loci (eQTLs; a locus that contributes to the variance of gene expression related to a phenotype), indicating a differential mechanism of involvement for meQTLs and eQTLs in human disease. A comprehensive study of cord blood, peripheral blood, and fetal brain tissue also revealed significant enrichment of autism spectrum disorder GWAS SNPs in meQTLs of both blood and brain tissues [187]. Nicotine dependence and obsessive-compulsive disorders are among the other neuropsychiatric conditions where the involvement of meQTLs is also reported [188,189].

Understanding the effect of epigenetic variation on the phenotypic outcome of genetic variants has been mainly elucidated from the study of Mendelian conditions, where the primary etiology is a single genetic defect. For example, in proximal spinal muscular atrophy, an autosomal recessive neuromuscular disorder caused by mutations in *SMN1*, several differentially methylated candidate genes have been found to be associated with disease severity [190]. In facioscapulohumeral muscular dystrophy 1 (an autosomal dominant myopathy), contractions of the microsatellite *D4Z4* repeat lead to upregulation of the downstream *DUX4* gene, and methylation levels at the *D4Z4* repeat correlate with penetrance, permitting the discrimination of affected individuals from asymptomatic mutation carriers [191]. In monogenic intellectual disability disorders, human-specific clusters of CpG islands within candidate genes have also been identified [192]. These CpG clusters represent regions of potential modulation of expression of adjacent genes by DNA methylation, contributing to the etiology of intellectual disability disorders by acting as a modifier of the phenotype.

Other mechanisms of genetic/epigenetic interaction in the same locus occur through chromosomal aberrations such as copy number variants, including deletions and duplications, as well as inversions, and translocations. These changes can induce inappropriate local rearrangements of the chromatin structure, affecting the epigenetic regulation of the overlapping genes [193,194]. A study using CNV and methylation data from 1000 genomes identified genome-wide associations, with CNVs being associated with multiple CpGs as well as CpGs being associated with multiple CNVs [195]. Additionally, these CNV-associated methylation variations were correlated with gene expression changes and were enriched at regulatory regions and transcription factor-binding sites [195]. These regulatory regions and transcription factor-binding sites make long range interactions with the CNV-associated CpGs, providing another mechanism of how genetic variation, in this case CNVs, can impact methylation changes. Trinucleotide repeat expansions can also cause structural modifications to the DNA helix, which in turn induce excessive DNA methylation of the overlapping genes and can lead to gene silencing (discussed in the example of Fragile X syndrome earlier in this chapter).

27.8 Epigenomic involvement in multifactorial neurodevelopmental conditions

Multifactorial disorders result from cumulative effects of genetic variants and environmental factors. More recently, epigenetic factors, including DNA methylation, have been associated with various multifactorial disorders, including obesity, diabetes, neurodevelopmental disorders, and congenital birth defects [196,197]. The following section provides an overview of multifactorial neurodevelopmental conditions with demonstrated epigenetic involvement.

The development of the nervous system and normal brain function is influenced by prenatal and postnatal environmental exposures, such as maternal stress, malnutrition, toxins, and infection [196,198–202]. Such factors are shown to be related to an elevated risk of neurodevelopmental conditions, such as mood disorders, schizophrenia, and autism spectrum disorders, the underlying pathophysiology of which is postulated to begin during embryonic development and continues throughout the entirety of childhood [196]. Epigenetic regulation, including DNA methylation, constitutes a potential mechanism through which prenatal environmental exposures can interfere with the development of the nervous system. During epigenetic reprogramming at prenatal development, DNA methylation is highly sensitive to environmental exposures, which in mice have been shown to result in long-term effects on the status of DNA methylation and gene expression in the brain [196]. Such DNA methylation changes can be maintained throughout cell division into adulthood and influence development of neurodevelopmental diseases later in life.

Autism spectrum disorder is a neurodevelopmental disorder characterized by impairment in communication and reciprocal interaction, and stereotypical patterns of behaviors [203], which has been associated with maternal obesity, diabetes, and stress [200,204]. These environmental factors have been reported to be associated with developmental perturbation of epigenetic mechanisms regulating glial function, growth, and neural development [203,205–207]. In particular, changes in global DNA methylation patterns, including multiple imprinting regulatory regions, both in placental tissues and in the newborn, have been associated with maternal obesity and diabetes [208,209]. Also, DNA hypermethylation of the autism susceptibility gene *SHANK3* was observed in postmortem brain tissue from autism spectrum disorder individuals. SHANK3 acts as a scaffold protein in the brain and plays a role in neuronal synapses, which has also found to be down-regulated in ASD [210–212].

Teratogenic exposures during pregnancy can also impact the genome and epigenome and lead to severe neurodevelopmental impairments. Prenatal alcohol exposure can result in developmental consequences to a fetus, with fetal alcohol spectrum disorder (FASD) representing the variable clinical presentation in patients exposed to alcohol in utero that may include physiological, neurological and behavioral abnormalities [213,214]. Fetal alcohol syndrome, which is characterized by growth retardation, distinctive facial dysmorphology and central nervous system involvement, is the most severe outcome of chronic exposure to high amounts of alcohol during development [215], should the exposure not result in loss of the fetus [216]. The variation in effect of prenatal alcohol exposure are due to timing and amount of alcohol exposure, however several biological factors also contribute to outcomes including maternal age, nutrition of the mother, genetic background of the mother and fetus, as well as exposure to other drugs [217–219]. Several studies have identified changes to the transcriptome of both humans exposed to alcohol prenatally as well as mouse models. Alternations to histone modification levels have also been observed, and changes to overall DNA methylation levels have been documented in FASD mouse models and available human data [217–221]. Both increases and decreases in DNA methylation levels have been documented as studies vary in exposure timing, length, and specific tissue and model studied [217–219]. One study did demonstrate DNA methylation is decreased to a similar level in neural cells treated with alcohol or a potent methylation inhibitor [222]. Another group aimed to define specific changes in DNA methylation in patient buccal epithelial cells and found alterations in novel genes and confirmed changes in genes previously associated with FASD, providing a potential methylation episignature that requires further validation [223,224]. In contrast, Sharp et al. assessed genome-wide methylation in the cord-blood of newborns through a meta-analysis of data from six population-based birth cohorts and found no consistent evidence between prenatal alcohol exposure and single site methylation [225]. This group did, however, identify 19 larger regions in the genome that were differentially methylated in pregnancies with sustained exposure, though when validating with a different region-based analysis, these results were not replicated and therefore not considered strong evidence [225]. A rat model of FASD assessed DNA methylation changes in the brain and in leukocytes to identify differentially methylated regions as well as potential biomarkers [226]. The differentially methylated regions were functionally related to FASD phenotypes, such as neurological function and immune response [226]. This model identified concordance between methylation changes in the hypothalamus and in the blood; providing preliminary evidence for the potential of relevant blood-based biomarkers for FASD [226]. Indeed, our group has established a distinct DNA methylation pattern in fetal alcohol syndrome patients in peripheral blood (manuscript in preparation) through analysis of whole genome methylation data. One suggested mechanism to explain how alcohol may be influencing DNA methylation during development involves an alcohol-induced reduction in folate, B6 or B12 vitamins that are important to the methionine cycle. This decreases overall availability of S-adenosylmethionine (SAM), an essential donor of methyl groups for both DNA and histone methylation [217,219,227–229].

The evidence is accumulating to propose an epigenetically mediated role for another teratogenic exposure, cigarette smoking, in a number of neurodevelopmental conditions. Brain-derived neurotrophic factor (*BDNF*) gene plays a key role in neurodevelopment and synaptic plasticity [230,231], and is linked to a number of neurologic disorders, including central hypoventilation syndrome, bipolar disorder, major depressive disorder, schizophrenia, anorexia nervosa, and Alzheimer's disease [232–234]. Prenatal exposure to maternal smoking is shown to be associated with *BDNF* promoter hypomethylation [235]. Cigarette smoking during pregnancy also has been shown to be related to

downregulation of miR-146a in the placenta, which is associated with the quality of movement and attention scores in newborns [236]. Additionally, children of mothers who smoke during pregnancy have a lower birth weight and are also impacted by neuropsychological issues later in life [237]. Maternal smoking has repeatedly been reported to associate with the development of attention-deficit hyperactivity disorder (ADHD), Tourette syndrome comorbid with ADHD, and chronic tic disorders [238]. A large-scale study combined data across 13 cohorts from the US and Europe, generated through the same technique (DNA methylation array analysis) from newborn blood samples, to assess reproducible effects on the epigenome of children born to mothers who smoke cigarettes [239]. This study found DNA methylation changes at 3932 CpG sites after blood cell proportion analysis was considered, and found that the genes associated with these CpGs are involved in anatomical development, neurodevelopment, as well as cell communication processes [239]. Of the findings that were novel to this study, genes implicated in the DNA methylation changes included those that have been implicated in orofacial clefts, with some of these genes also playing roles in lung development and colorectal cancer risk later in life [239]. One other additional interesting finding discussed in the study is that *PRDM8*, a histone methyltransferase, demonstrated the largest number of CpGs with differential methylation associated with maternal smoking [239]. The authors highlight that *PRDM8* expression is tightly regulated in a spatiotemporal manner during neurodevelopment as it regulates the morphological transition in the neocortex and contributes to neural circuit assembly through methylation of lysine 9 within histone 3; a mark that is repressive in the context of transcription [239]. This provides a functional example of environmental exposure impacting expression of epigenetic machinery through DNA methylation changes to associated CpGs from environmental exposures, resulting in impacts to development.

Prenatal exposures to plastic driven chemicals are found to be associated with higher anxiety, hyperactivity, and depression scale scores, aggressive behavior and inattention symptoms related to ADHD or conduct disorders, as well as poor psychomotor development, cognitive, language, and motor disabilities [240]. These agents are reported to increase miR-146a expression in placental cell lines. High concentrations of phthalates in urine are found to be associated with a lower expression of miR-185 in placenta. Exposure to these chemicals may also increase the expression of *MECP2*. These factors are regulated during the neurodevelopmental period and have important roles in neuronal plasticity and formation of dendritic spine [241]. Heavy metal exposures during pregnancy, such as lead and manganese, are also known to negatively affect neurodevelopment and are associated with lower IQ and ADHD postnatally [242]. In umbilical cord blood, maternal exposure to lead is associated with hypomethylation of LINE-1 and Alu repetitive elements [243], providing a potential mechanism through which they exert an epigenetic dysregulation in neural development.

Finally, exposure to essential medically prescribed drugs can also affect fetal development, as such is the case with fetal valproate exposure. Valproate is an anti-seizure medication that is used to treat a variety of seizure disorders and is used as a first-line anti-epileptic drug in generalized seizures [244]. It can also be used as a mood stabilizer for psychiatric disorders. Valproate is a known teratogen and histone deacetylase inhibitor that acts on chromatin to impact gene expression during development [245–248]. Due to the impacts to the fetus, it is strongly recommended to be avoided during but there is a slow release version of valproate that can be used when absolutely necessary [249]. Increased risk of spina bifida after valproate exposure during pregnancy originally flagged concerns around the drug and shortly after fetal valproate syndrome was defined [250]. It is characterized by malformations, facials dysmorphisms, impaired development with high risk of neural tube defects, congenital heart

defects, as well as ophthalmological and genitourinary abnormalities [249]. Clinical presentation of these patients overlaps with several epigenetic machinery disorders, making diagnosis challenging [249]. Several studies investigating gene expression changes in fetal valproate syndrome models have identified differential expression of some of the genes responsible for the epigenetic machinery disorders with overlapping phenotypes. For example, histone methyltransferase EHMT1 (Kleefstra syndrome), histone demethylase KDM6A (Kabuki syndrome), and DNA methyltransferase DNMT3B (Immunodeficiency-centromeric instability-facial anomalies syndrome) show aberrant expression when mice are exposed to valproate in utero [251]. These findings highlight potential overlap in pathological mechanisms between environmental exposures and disorders that involve the epigenetic machinery, as valproate hinders histone acetylation and also impacts the expression of other epigenetic regulators, further disrupting the balance of epigenetic marks throughout development.

Taken together, these findings suggest that DNA methylation is an important mechanism in fetal growth and neurodevelopment, as well as in metabolic programming, and that disruption of this mechanism by environmental stressors can induce the development of neurodevelopmental and metabolic diseases later in life.

27.9 Identification of episignatures in neurodevelopmental disorders

As discussed in previous sections of this chapter, development requires a delicate balance of epigenetic states. Genetic alterations that impact this balance can cause epigenetic changes that can influence chromatin organization and gene expression, resulting in disease phenotypes [14,15,49]. This impact can be observed through analysis to changes in epigenetic markers, such as DNA methylation. An episignatures is defined as a recurring and reproducible DNA methylation pattern associated with a common genetic or environmental etiology in a disorder specific population.

Some of the first episignatures that were defined form our laboratory include patients that have confirmed *KMT2D* mutations (a histone methyltransferase) [252], as well as X-linked alpha-thalassemia/mental retardation (ATRX) syndrome (caused by mutations in chromatin remodeler *ATRX*) [253], Floating-Harbor syndrome associated with mutations in the chromatin-remodeling protein SRCAP [254], and in Genitopatellar and Ohdo syndromes, both caused by mutations in histone acetyltransferase *KAT6B* [255]. Aberrant episignatures, in contrast to the methylomes observed in unaffected control individuals, may represent developmental remnants of those upstream mutations within the epigenetic machinery. The previously mentioned *NSD1* gene is involved in methylation of H3K36, which is involved in de novo methylation of DNA. Loss-of-function of this gene in Sotos syndrome generates a DNA hypomethylation profile in the blood, which can also be detected in other tissues of the patients such as fibroblasts [256]. In Kabuki syndrome, on the other hand, the loss-of-function of *KMT2D*, which has a role in blocking DNA methylation, generates a DNA methylation signature predominantly composed of hypermethylation signals [252]. Other groups have identified episignatures, such as Butcher et al. [257] identifying DNA methylation signatures for truncating mutations in *CHD7* (associated with CHARGE) and *KMT2D*, providing evidence that these syndromes result from epigenetic dysregulation of key genes involved in embryonic development that are expressed in a tissue-specific manner. Furthermore, these signatures, while syndrome-specific, provide evidence that CHD7 and KMT2D regulate common biological pathways, likely reflected in the phenotypic overlap between CHARGE and Kabuki syndromes [257]. Thus, these "epigenetic echoe,"

defined as the downstream epigenetic changes following the initial defects in an epigenomic machinery gene, offer a surrogate signature of the gene within which the initial mutation occurred, and as such, highlight the potential common molecular basis for clinical effects across seemingly unrelated clinical disorders, which nonetheless share underlying developmental pathways.

27.10 Mapping episignatures and multiclass classifier development

Episignatures are considered a functional consequence of pathogenic variants in a disorder-specific gene. These DNA methylation patterns are genome wide and selecting the most specific and sensitive methylation changes for a given disorder is key when developing an episignature. Currently, the most commonly used methylation array for whole genome methylation assessment is the Infinium Methylation EPIC (850K) array. The array replaced the Human Methylation 450 (450K) array and nearly doubled the number of probes to increase genome coverage of regulatory regions, though both arrays use the same technology to measure DNA methylation changes [258]. The 850K array covers more than 90% of the probes found on the previous 450K array, with the majority of probes removed due to poor performance [258]. Importantly, studies found high correlation between the two arrays [258–260]. This array has over 850,000 CpG probes and covers 96% of RefSeq genes, 95% of CpG islands, along with FANTOM5 enhancers, Encyclopedia of DNA Elements (ENCODE) transcription factor binding sites, non-CpG methylated sites identified in human stem cells, DNase hypersensitivity sites, and differentially methylated sites in cancer. Similar to the current genomic sequencing or CNV microarray analyses, which enable comprehensive screening of 100–1000s of conditions, genomic DNA methylation analysis enables genome-wide assessment of DNA methylation profiles across a spectrum of disorders. Our group has identified 57 episignatures associated with 65 disorders [261] and currently many initiatives are underway to assess more neurodevelopmental disorders for global DNA methylation changes. The majority of these episignatures are found in disorders where the causative gene is part of the epigenetic machinery, however enzymes without chromatin regulatory mechanisms have also demonstrated specific methylation changes, including ubiquitin-conjugating enzyme E2 A (UBE2A) and spermine synthase (SMS), both implicated in X-linked syndromic forms of intellectual disability [262]. Another X-linked gene, histone lysine demethylase *KDM5C,* associated with X-linked intellectual disability demonstrates a unique episignature in affected males, but carrier females show a less pronounced version of the episignature that is also distinct from controls [117]. *KDM5C* is able to escape X-chromosome inactivation [118], demonstrating a unique instance where two distinct, but related, DNA methylation profiles reflect affected and carrier status.

Germline inheritance of these neurodevelopmental disorders allows these episignatures to be detected in peripheral blood, which is a readily available clinical sample. A tissue-specific whole genome methylation database, such as the EpiSign Knowledge Database (EKD) which contains DNA methylation data from thousands of peripheral blood samples, is essential for mapping and developing sensitive and specific epigenetic biomarkers. Our group has developed and trademarked a test called EpiSign that uses a proprietary bioinformatic pipeline that works in conjunction with the EKD to assess new disorders for distinct episignatures [255,261–263]. Briefly, the objective of defining a new episignature is to determine whether a set of selected CpGs across the genome can differentiate patients with a given disorder from unaffected individuals and from others with a confirmed diagnosis of another neurodevelopmental disorder. To do this, case samples must first be matched with control

samples within the EKD by age and sex to control age and sex related methylation differences. Using whole genome methylation data generated from Infinium Methylation EPIC arrays, where each probe represents CpG site where methylation is measured, approximately 100−500 probes are selected that most differentiate case samples from controls through a series of statistical tests. Using analytical tools created by Houseman et al. [264], cell proportions are estimated and considered as covariates when comparing cases and unaffected controls to ensure DNA methylation changes are not due to cell heterogeneity between the two groups. Results of unsupervised clustering, through hierarchical clustering and multidimensional scaling plots based on probes selected, can then be assessed. Fig. 27.1A and B shows an example of unsupervised clustering indicative of a robust episignature. To ensure each sample in our case cohort would be appropriately categorized should it not be part of the case samples used to select probes, a series of leave-one-out cross validation experiments are completed to ensure the left-out case still matches the other case samples. In this case, one sample is left out and probes are reselected using remaining samples. The left-out sample is then treated as a "test" sample to ensure it clusters with cases and not controls. Finally, a supervised algorithm is trained so that it considers the methylation patterns of all disorders with a defined episignature simultaneous to provide a probability score, called methylation variant pathogenicity (MVP) score, as to whether a sample matches a given disorder episignature. MVP scores close to 1.0 indicate concordance with a given episignature. This support vector machine (SVM) is a machine learning algorithm that is trained using all defined episignatures to prevent misclassification in closely related syndromes that share similar methylation changes [261,262]. Indeed, assessment of similarity of episignatures for neurodevelopmental disorders shows overlap [262], and this is also reflected when all significant methylation changes across the genome are considered [265]. Many differentially methylated regions and probes are shared across syndromes, highlighting the importance of using both supervised and unsupervised methods in the computational assessment of episignatures to ensure proper case classification is achieved and impact of overlap in DNA methylation changes across disorders is minimized. This overlap also requires that new episignatures for existing disorders continue to be defined and added to the SVM classifier; this allows for further specificity in episignature detection as the SVM considers all methylation patterns when assessing a new sample [261,262]. It is possible that samples that contain a mutation in a gene that is upstream or downstream in the pathway of a current episignature gene, or that interact with a given episignature gene, may demonstrate a partial match due to DNA methylation changes that are similar. This again highlights the importance of A) considering both supervised and unsupervised methods and B) continuing work to define new episignatures so that the SVM can be better trained to differentiate disorder with overlapping pathology.

This episignature overlap is especially observed in genes that are part of the same protein complex, such as the BAF chromatin remodeling complex. A study from our group reported overlapping DNA methylation changes in cases with various subtypes of Coffin-Siris syndrome (caused by haploinsufficiency in *ARID1B, SMARCB1* or *SMARCA4*) and Nicolaides-Baraitser syndrome (caused by mutations in *SMARCA2*), resulting in a combined episignature for all genes [266]. The similarity in episignatures between some Coffin-Siris subtypes and Nicolaides-Baraitser syndrome was even greater than within Coffin-Siris cases. This provides functional evidence that supports previous publications that argued these disorders represent a spectrum of disease, known as BAFopathies, instead of two distinct conditions with differing pathogenic mechanisms [266]. A follow-up study added to these findings through episignature analysis of *SMARCA2* cases where the recorded variants are outside the typically mutated domain in Nicolaides-Baraitser syndrome. A distinct methylation

pattern was identified for these patients and through episignature detection, as well as other functional and reverse-phenotypic analysis, a new distinct disorder was classified as Blepharophimosis Intellectual disability SMARCA2 syndrome [267].

Though closely related disorders present at challenge when defining sensitive and specific episignature, this relatedness can be overcome through adjustments in approach. A recent publication from our group discussed the challenges of identifying episignatures that are able to distinguish between paralogous lysine acetyltransferases KAT6A and KAT6B [261]. Loss of function mutations of either gene result in a neurodevelopmental disorder [268,269]. These proteins provide the same functionality in a complex and therefore loss of either will likely result in similar downstream effects to histone acetylation and overall chromatin states. Therefore, in order to define gene specific episignatures our group included samples from the KAT6B cohort as controls when selecting probes for a KAT6A episignature, and vice versa for KAT6B episignature analysis [261]. This strategy allowed for a probe set to be selected that can differentiate between KAT6A and KAT6B cases. The same method was applied to split Rubinstein-Taybi syndromes 1 and 2 into two distinct episignatures; this syndrome is also caused by mutations in one of two closely related paralogous lysine acetyltransferases, CREBBP and EP300 [261].

While identifying gene specific episignatures in closely related conditions can be difficult, variants in different regions of the same causative gene can present with distinct episignatures. For example, two domain-specific partially opposing episignatures were identified in Helsmoortel-van der Aa syndrome associated with dominant negative truncating mutations in the chromatin regulating activity-dependent neuroprotector homeobox gene (*ADNP*) [270]. Mutations in the N- and C- terminus resulted in a hypomethylated episignature, while alterations in the nuclear localization sequence caused a predominately hypermethylated episignature. Another study then assessed the phenotypic correlations with mutation location and found modest differences in patient phenotypes. A domain-specific signature was classified in Menke-Hennekam syndromes 1 and 2 [261], caused by pathogenic variants in exons 30 and 31 of *CREBBP* and in the homologous region of *EP300* [271,272]. These patients show a phenotype distinct from previously mentioned Rubinstein-Taybi syndromes, which is caused by variants in the same genes. This episignature distinguished samples with variants in the intrinsically disordered linker domain from control samples, as well as all other Menke-Hennekam syndrome samples with mutations in other domains [261]. Interestingly, no overall episignature has been identified for all Menke-Hennekam syndrome cases, indicating that there may be different downstream consequences depending on how protein is altered.

Episignatures can be even more specific, with specific genetic regions, and even single nucleotide variations, resulting in distinct DNA methylation patterns. Coffin-Siris syndrome 1 and 2, caused by mutations in paralogs *ARID1A* and *ARID1B*, respectively, are part of the previously mentioned BAF chromatin remodeling complex. Our group recently identified a region within these two genes, surrounding nucleotide 6200, that when mutated results in a strong episignature [261]. Samples with variants surrounding this key region did not match this new missense variant episignature, or the previously defined BAFopathy episignature. This region of interest occurs outside of the ARID reader domain, responsible for DNA binding, and occur in the C-terminal domain of unknown function [273,274]. In this case, episignature discovery indicates functional relevance, at least in terms of consequences to global DNA methylation changes, for a domain with unknown function. Another BAFopathhy gene, *SMARCA4*, responsible for Coffin-Siris syndrome 4, demonstrates an extreme example of sub-signature specificity. A specific mutation in this gene demonstrates a unique

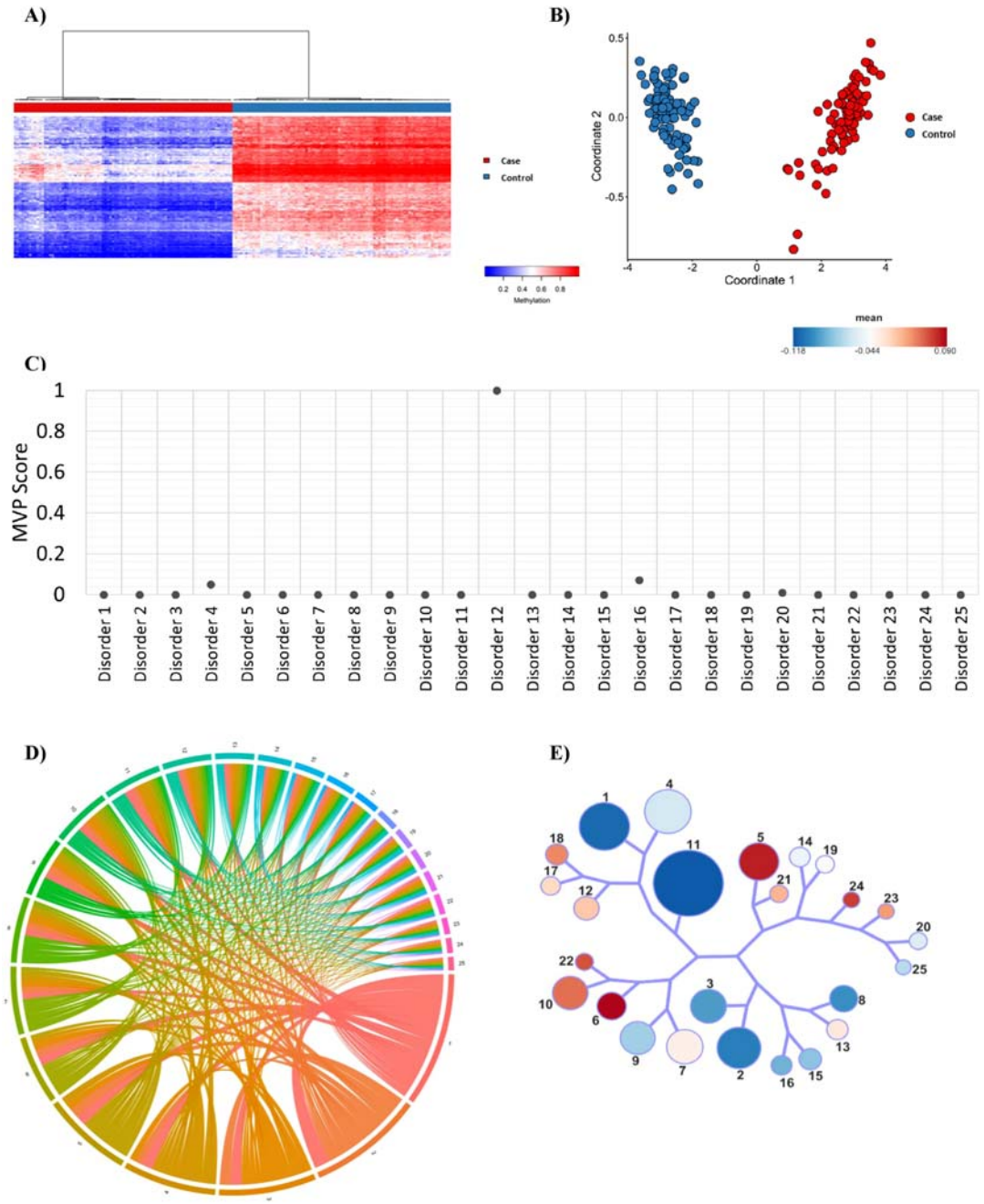

FIGURE 27.1 DNA methylation as functional evidence for neurodevelopmental disorders.

(A and B) represent output of unsupervised clustering of case and control samples after probe selection in episignature discovery pipeline. This output represents an example strong episignature. (A) Hierarchical

episignature that is difference from cases with other variants in the same genes, and even the same domain, as well as from all other BAFopathy samples [261].

These domain, region, and nucleotide-specific sub-signatures highlight the complexity of downstream effects to DNA methylation, and ultimately chromatin, in neurodevelopmental disorders. It is likely that many other epigenetic machinery disorders exhibit unique changes to the DNA methylation profile depending on variant location and further analysis into candidate disorders that harbor non-truncating mutations is warranted. Furthermore, there is potential to correlate sub-signature with differing phenotypes within a given disorder to understand whether the differing DNA methylation patterns may be impacting clinical presentation. The current EpiSign SVM classier demonstrates a high level of accuracy. Only 3.4% of all disorder samples demonstrated an elevated MVP score for a secondary episignature and most of these cases could be ruled out through unsupervised clustering assessment (sample did not cluster with secondary episignature cases in MDS or hierarchal clustering plots) or overlapping biological pathways that were previously known [261]. Similarly, only 5 of the 1200 unaffected control samples demonstrated MVP scores above 0.1 and none scored higher than 0.25 [261]. This level accuracy provides the foundation for episignature analysis to be used in the clinic as disorder biomarkers, in conjunction with standard genetic testing to improve the diagnostic yield in neurodevelopmental disorder patients.

27.11 Episignatures as a diagnostic biomarker in neurodevelopmental disorders

As described throughout this chapter, Mendelian neurodevelopmental disorders that present with developmental delay, intellectual disability, dysmorphic features and/or congenital anomalies can exhibit variable, complex, and often overlapping clinical features. This can make diagnosis challenges

clustering heatmap demonstrating methylation changes at selected probes for an example episignature. Each row is a selected probe and each column is a case or control sample, with blue representing controls and red representing cases. The colors within the heatmap represent the spectrum methylation levels at each probe, with red representing most hypermethylated and blue representing most hypomethylated. (B) Multi-dimensional scaling plot demonstrating unsupervised clustering of case and control samples by similarity in methylation pattern at selected probes. (C) Methylation Variant Pathogenicity (MVP) scores for a new testing sample being assessed against 25 disorder episignatures concurrently. Each dot is the same sample's MVP score for a different disorder in each column between 0 and 1, with 1 representing the highest probability of the sample matching the specific disorder episignature, in this case disorder 12. (D) Tree and leaf diagram showing relatedness of all significantly differentially methylated probes for a disorder, not just those probes selected for an episignature. Each branch represents disorders that are most closely related, represented by circles that are numbered. The size of the circle related to number of probes that exhibit significant changes to methylation, and color of circle represented mean methylation of all differentially methylated probes, with red representing hypermethylation and blue indicating hypomethylation. (E) Circos plot demonstrating relatedness of all significantly differentially methylated probes for a disorder, not just those probes selected for an episignature. Each colored, numbered section of the circle represents a disorder with the emerging colored lines representing differentially methylated probes that overlap with another disorder. Where the emerging lines connect to another section of the circle indicated which other disorder the probe is shared with.

and is why genetic assessment is so relevant in this patient population. Chromosomal microarray is the first-tier test for patients with a suspected genetic disorder [275], and provides a diagnosis in 15%−20% of patients [276−279]. Whole exome and whole genome sequencing have improved this diagnostic yield to 25%−36%, leaving patients undiagnosed in nearly two-thirds of cases [280−283]. Furthermore, a large proportion of the genetic changes identified through genetic testing in a potentially relevant gene are not clinically interpretable, resulting in variants of unknown clinical significance (VUS). This results in a diagnostic odyssey in these patients ranging from 1 to 8 years [275,284−291]. Receiving a molecular diagnosis also has implications to patient care management, especially for those with genetically heterogenous disease or atypical presentation; a quicker diagnosis results in access to treatment and services during the critical window of development that may improve patient outcomes [285,288]. With time to diagnosis taking years for many patients through standard genetic testing and sequencing approaches, a cost-effective screening test for multiple disorders involved in a differential diagnosis would provide another method to guide diagnosis. Such a test would need to look outside of genetic sequence at a functional biomarker.

In 2019 the first clinical epigenomic test, called EpiSign, became available. EpiSign uses proprietary bioinformatic algorithms and EpiSign Knowledge Database, described earlier in this chapter, to assess patient DNA methylation patterns to determine if it matches an established episignature. The current version of EpiSign can detect over 60 disorders associated with more than 80 genes, including imprinting disorders and fragile X syndrome. This test is currently used in clinical practice in the US and parts of Europe [292], with centers participating in clinical validation of new episignatures as they are discovered. These new episignatures are then added to new versions of the EpiSign test should they pass clinical validation. Clinical validation was performed on 211 samples, and included both samples with confirmed diagnosis of a disorder associated with a defined episignature (positive control) and negative controls that were blinded and submitted for episignature analysis [292]. Clinical validation resulted in 98% concordance, with all negative controls concordant and 4 of the positive controls discordant [292]. This test can be used as a first-tier screening tool for patients with complex or ambiguous clinical presentation, or as a reflex test for patients with a VUS or no genetic variants identified. Fig. 27.1C demonstrates an example of an MVP score that is indicative of a match to a given episignatures after EpiSign concurrent assessment.

An early study provided evidence of the potential of episignatures as a functional biomarker through concurrent assessment of ambiguous cases presenting with VUSs for 14 disorders with defined episignatures [255]. A follow up study with an expanded list of defined episignatures assessed 67 individuals with non-specific clinical features or who had a VUS for an episignature [263]. 31% of these patients demonstrated a methylation profile that matched one of the available episignatures, some of which had no causative variants identified [263]. Next, a larger cohort of 965 unresolved patients with a standard clinical and genetic work up identified 9 patients with DNA methylation patterns that matched an episignature [262]. Most of these individuals were not available for follow up but further genetic testing confirmed episignature assessment in 2 individuals, and clinical presentation matching the episignature disorder detected was documented for those unavailable [262].

Assessment of diagnostic utility of episignatures was recently demonstrated in a cohort of prospective patients where follow up and further genetic testing was possible [292]. Overall, 27.6% of total patients were positive for an episignature with the targeted cohort demonstrating a positivity rate of 35.3% including reclassification of several VUSs and 11.3% of the screening cohort (without previous genetic findings) displaying an episignature. A similar study focused on the diagnostic utility

of episignature analysis in disorders of the epigenetic machinery [293], demonstrating diagnostic utility for episignature testing. Several other case studies have demonstrated the utility of DNA methylation profiling [294–298], with ongoing episignature discoveries expected to continue this trend. Episignature analysis has shown the most utility in VUS reclassification. It is important to note that presence of an episignature provides functional evidence for a specific genetic etiology, however lack of a defined episignature is not sufficient to reclassify a VUS as benign. Episignatures are based on a set of samples with distinct variants and ultimately changes or losses to specific proteins. For example, a given disorder may include variants that are complete loss of function or those that result in decreased function or detrimental gain of function for the associated protein, resulting a phenotypic spectrum. Depending on what variants and etiologies are captured in the episignature samples, certain variants may or may not display complete matches the defined methylation pattern. This does not mean the patient does not have the associated disorder, but rather that their DNA methylation changes are not a concordant match. This highlights the importance of continued refinement of existing episignatures as more is learned about the associated syndromes; this include the discovery of sub-signatures, as described above, to better capture the complexity of observed DNA methylation changes.

Additionally, the assessment of episignatures in Mendelian disorders can provide another avenue to diagnosis for newly defined disorders with limited variant data and a non-specific phenotype. For example, an episignature was defined in Beck-Fahrner syndrome, which is caused by mutations in TET Methylcytosine Dioxygenase 3 (*TET3*) [299]. This enzyme is involved in DNA demethylation and the disorder itself was only characterized in 2020. Development of an episignature for known variants allowed for classification of variants of unknown significance and diagnose patients who were not previously suspected to have this disorder [299]. This represents an example where DNA methylation signatures are a very important tool in the diagnostic pathway of Mendelian disorders with ambiguous or incompletely penetrant phenotypes. Episignature definition in a Mendelian disorder can also shed light on its pathology. A recent publication from our group described detection of an episignature in Phelan-McDermid syndrome, a disorder caused by deletions of chromosome 22. Originally it was thought that SH3 and multiple ankyrin repeat domains 3 gene (*SHANK3*) contributed to the observed phenotype as deletions impacted the gene and genetic variants alone have exhibited a range, but not all, of the phenotypic features in Phelan-McDermid syndrome [300]. The episignature analysis identified a critical region of deletion, meaning that only samples with an overlapping region of deletion shared an episignature [300]. This region did not include *SHANK3* but instead included epigenetic machinery gene bromodomain-containing protein 1 gene (*BRD1*) [300]. This gene is a part of a histone acetyltransferase complex and interacts with several chromatin remodeling genes. Further work is required to confirm the involvement of BRD1 in this disorder's pathology, however, metabolic data from these patients reflected the episignature results in that those patients with deletions covering the critical region exhibiting very different metabolic profiles form those with deletions outside this region [300]. In this case, episignature analysis provided new insights into potential pathological mechanisms.

In addition to diagnostic clinical utility, genome wide DNA methylation profiling can be used to identify epigenetically deregulated molecular pathways [265]. Differentially methylated regions may involve functional genomic elements involved in transcriptional regulation. Additionally, the relatedness of epigenetic machinery disorders can be assessed through comparison of impacted CpG sites, or probes. Fig. 27.1D and E provides two examples of how this data can provide an overview of probe overlap between disorders of interest, including a circos plot (Fig. 27.1E) and a tree and leaf plot (Fig. 27.1E). The circos plot shows the overlap in differentially methylated probes across disorders,

supporting overlapping epigenetic changes across the genome and possibly overlapping pathological mechanisms. The tree and leaf diagram demonstrates relatedness of each disorder, with those on the same branch showing more similarities.

27.12 Clinical utilization of epigenomic technologies and the advantages of episignatures

Analysis of DNA methylation testing has been performed in clinical laboratory settings over the past 3 decades. Some of the targeted DNA methylation experiments in clinical laboratories include screening for imprinting disorders (PWS, AS, BWS, and SRS) [99], FXS [121], X-inactivation analysis, and somatic cancer—related testing, including *RB1*, *MLH1*, and *MGMT1* promoter DNA methylation analysis as part of retinoblastoma, colon cancer, and brain cancer diagnostic assessment [301]. The majority of these assays use targeted, gene-specific, and often complex molecular techniques. Unlike other areas of genomic medicine where targeted genetic analysis (single-gene sequencing, PCR analysis, genotyping) is augmented or in some cases completely replaced by genome-wide approaches (e.g., cytogenetic copy number variation [CNV] microarray analysis, exome or whole-genome sequencing), clinical epigenomics is still in its infancy.

Targeted DNA methylation analysis involves classical, well-established techniques, including bisulfite-sequencing, or various forms of bisulfite-sensitive PCR and/or restriction enzyme assays, all of which can be designed to generate accurate, reproducible data amenable for clinical laboratory setting, albeit limited to a single analyte. Also, targeted analyses are linked with clearly defined clinical utility and analytical/interpretative ability.

In contrast, technical requirements for epigenome-wide analysis through whole genome bisulfite-sequencing are much more complicated. The development of analytical and experimental tools for bisulfite genome sequencing and decrease in sequencing costs will promote broader clinical utilization of these approaches, however currently the Infinium Methylation EPIC array provides a low-cost, robust and reproducible way to assess the epigenome. Additionally, these methylation arrays can be used to assess imprinting disorders and Fragile X [99,121], as well as episignatures. As described in the above section, the diagnostic utility of episignatures has been demonstrated by several studies, with the EpiSign test providing a multiclass classifier that is sensitive and specific to 65 genetic disorders. One major advantage of an epigenome-wide approach for screening patient populations is that it addresses the issue of phenotypic overlaps in patient populations. Many of the conditions discussed, particularly in the pediatric ID and developmental delay spectrum, often have similar clinical presentations [14,15,49]. Genotype-first or in this case epigenotype-first approach to clinical diagnosis in these patient populations, as it has been demonstrated in clinical genomics, may enable a more rapid and efficient approach to diagnosis. Furthermore, the Illumina Methylation EPIC array uses similar chemistry and equipment as chromosomal microarrays, the current first-tier test for a patient with a suspected neurodevelopmental disorder [275]. Additionally, episignatures provide another layer of functional evidence for a given disorder outside the causative genetic variant. While the use of whole exome and genome sequencing has improved diagnostic yield, the advancement in the technology's ability to read the DNA sequence is coupled with a lack of ability to interpret variants, as shown by the prevalence of VUSs [302,303]; episignatures provide functional evidence to assess pathogenicity of a VUS. One major requirement for successful episignature

analysis is continuing the expansion of large reference databases of whole genome methylation data sets, such as EpiSign Knowledge Database. Another level of complexity is that studying DNA methylation patterns, and using the corresponding databases, is the need to consider tissue-specific and age-related variability, along with ethnic/demographic differences. Finally, the systematic assessment of the ability of episignature assessment in the analytical sensitivity in detecting genetic mosaicism, which is defined as the presence of two or more distinct genotypes observed in a single organism that occurred post-zygotically, is required. While mosaic variants have been detected by episignature analysis, as described in the case study at the end of this chapter, a better understanding of the lower limit of detectability would be useful as it would provide another use case where DNA methylation profiling in the clinic.

Although it is important to recognize the limitations in our current ability to fully utilize genomic information from such analyses, these technological advances have enabled simultaneous assessment of 57 episignatures associated to 65 genetic syndromes, in addition to imprinting and Fragile X syndrome assessment, and the list of disorders with known DNA methylation patterns is ever-growing. We are approaching the point where epigenomic approaches may start to replace the targeted DNA methylation analysis in clinical laboratories. Along with genomic sequence-based analyses, epigenomic analysis has a potential to be used as a tier 1 testing or first-tier modality, meaning as a standardized assessment provided at first visit with a geneticist, in the primary diagnostic workup in both constitutional and oncology conditions. Indeed episignature analysis is now being adapted for testing in clinical laboratories [292] and a large-scale national trial assessing the health system impact of this technology is underway (https://genomecanada.ca/project/beyond-genomics-assessing-improvement-diagnosis-rare-diseases-using-clinical-epigenomics-canada/). Similar to the way clinical utilization resulted in rapid advances in clinical genomics in the past decade, generation of large epigenomic, tissue-specific databases in clinical laboratories linked to clinical phenotypic information in individual patients will enable discoveries of clinical and epigenetic associations and continue to expand the utility of epigenetic testing.

Furthermore, these underlying epigenetic defects create the potential to reassign existing targeted epigenetic (and combinatorial) therapies [15,304] to treat individuals with complex DD/ID syndromes across seemingly unrelated clinical disorders, based on the syndrome-specific episignatures by methylome screening. Harnessing the epigenome for diagnosis of neurodevelopmental disorders further highlights the importance and relevance of epigenetic changes in disease. Understanding of genome wide methylation changes may also provide avenues to better understand disease pathogenesis and identify potential therapeutic targets.

Chapter key points

- Epigenetic processes are key in development and gene expression.
- Disruption of the epigenetic machinery can lead to neurodevelopmental disorders.
- Multifactorial diseases also exhibit epigenomic involvement.
- Prenatal teratogenic exposures also impact the epigenome.
- Genome wide patterns of DNA methylation, an epigenetic mark, represent a functional consequence of genetic or environmental etiology in a disorder specific population. These patterns are called episignatures.

- Episignature establishment requires a large tissue-specific database to determine if there is a set of CpG sites that can differentiate disorder samples from unaffected controls.
- Episignature analysis has diagnostic utility in neurodevelopmental disorders.

Case study

Episignature analysis as functional evidence for mosaic variant pathogenicity

The case discussed in this case report is described in detail in a case report published by Montano et al. in the American Journal of Medical Genetics (https://doi.org/10.1002/ajmg.a.62754) [294].

Objective

Constitutional disorders that present with developmental delay, intellectual disability, or ***neurodevelopmental disorders***, with or without congenital anomalies are associated with variable, complex, and overlapping clinical features, making a definitive diagnosis challenging. These disorders are termed ***rare***, which primarily reflects the low population frequency of a given condition, however it is estimated that 6%–8% of the population are affected by a rare disorder [305]. Current methodologies for the screening and diagnosis of rare neurodevelopmental syndromes with or without congenital anomalies involve clinical microarray testing [275,306], along with targeted gene panel and exome sequencing, based on phenotypic presentation [307]. This observed phenotypic overlap is in part due to the overrepresentation of ***epigenetic machinery genes*** involved in these disorders, which control similar pathways that may contribute to the proper balance of epigenetic marks on the chromatin [14,15]. Diagnosis is further complicated by the increasing gap in our ability to read the genetic sequence and our ability to interpret and classify genetic variants [308]. These challenges result in nearly two-third of patients left without a diagnosis after extensive genetic testing [276–279]. Furthermore, ***genetic mosaicism*** adds another layer of difficulty in understanding how detected variant contributes to phenotype. ***Episignature*** analysis, which examines a patient's whole genome DNA methylation pattern to determine if it matches an established disorder specific pattern, has presented itself as additional functional evidence to support the pathogenicity of a detected variant, especially those with ***a variant of uncertain significance*** [252,255,262,263,292–298].

The goal of this case study was to confirm a genetic diagnostic using episignature detection as functional evidence caused by the detected variant. In this case, the patient presented with a low-level mosaic de novo pathogenic variant in *KMT2D* associated with Kabuki syndrome 1 [294]. The physicians in the patient's circle of care determined that use of DNA methylation analysis through episignature analysis could provide confirmation of pathogenicity the detected mosaic variant as the patient presented with an ambiguous phenotype, with some overlap with Kabuki syndrome 1.

Scope

The patient in this study received standard clinical testing to determine the molecular cause of their genetic disorder, with the use of exome sequencing allowing the detection of the low-level mosaic variant associated with Kabuki syndrome. The patient presented with some clinical features that are present in Kabuki syndrome, but it was unclear whether the detected mosaic variant was the molecular cause. The physicians then looked to the ***epigenome***. Kabuki syndrome has an established episignature

that is both sensitive and specific for Kabuki syndrome 1 and 2 [252]. *KMT2D* is a histone methyltransferase and loss of function this gene impacts chromatin accessibility. This has downstream effects on epigenetic marks, such as DNA methylation. Therefore, the physicians wanted to determine if their patient matched the Kabuki episignature; if so, this would provide further evidence of the identified variant contributing to the observed phenotype. Indeed, ***this patient's DNA methylation profile did match the Kabuki episignature***.

One limitation of this study is that episignature analysis and whole exome sequencing were both performed using whole blood. Therefore, the episignature is confirming the variant is present in blood only, however, the presence of the episignature is confirming broader chromatin accessibility changes that are likely contributing to the phenotype. Additionally, the patient presented with a heart defect and the authors note that peripheral blood and cardiac tissue are both derived from the mesoderm, indicating that it is likely the variant is also present in heart tissue. Unfortunately, no tissue was from the heart was available to assess for the detected variant's presence.

Audience

This research is useful for both ***clinical geneticists***, ***genetic counselors***, as well as any ***physician involved in the circle of care of patient with rare genetic disorders***. Episignature detection of a low-level mosaic variant is an important clinical finding and provides another use case for episignature analysis in the clinic. Additionally, this research is also ***relevant to those studying rare genetic disorders*** as it provides insight into the pathogenic changes that occur at the molecular level as a consequence of a genetic variant. This case study included physicians, who documented the phenotypic findings, previous genetic findings and medical history, as well as order all diagnostic tests, and research scientists who developed the episignature analysis and completed episignature analysis for this patient.

Rationale

The main issues that this case study aimed to address were 1) ***obtain further evidence to support the contribution of the low-level mosaic variant to the patient's phenotype*** and 2) ***determine whether episignature analysis can detect the low-level mosaic variant***. Systematic assessment of episignature detection in mosaic variants has not been completed to date and this case study provides evidence that the DNA methylation changes are detectable in a low-level mosaic case and provide rationale to explore the utility of episignature analysis in mosaics further.

Expected results and deliverables

This study aimed to assess ***the clinical utility of episignature analysis in a patient with a low-level mosaic variant*** with some clinical features of the associated rare disorder. Mosaic variants provide a challenge when determining pathogenicity of a variant and this study assessed whether episignature assessment could provide functional evidence regarding how this variant may be impacting patient phenotype.

Tools and materials used

EpiSign analysis was completed as previously described [292]. Briefly, ***Illumina Infinium methylation EPIC arrays*** are used to generate whole-genome DNA methylation data across 850,000 CpG sites. This array covers 96% of RefSeq genes, 95% of CpG islands, along with FANTOM5

enhancers, Encyclopedia of DNA Elements (ENCODE) transcription factor binding sites, non-CpG methylated sites identified in human stem cells, DNase hypersensitivity sites, and differentially methylated sites in cancer. The patient's specific DNA methylation is then compared to established episignatures using the ***EpiSign Knowledge Database***. The EpiSign Knowledge Database contains thousands of unaffected controls as well as patients impacted by a neurodevelopmental disorder, and these reference samples are used to determine similarity to the patient's DNA methylation profile thorough unsupervised and supervised bioinformatic analysis. Each established episignature contains a set of 100−500 probes, which represent key CpG sites across the genome, where DNA methylation changes are observed in the disorder population. The patient is assessed for similar DNA methylation changes at the defined probes for each episignature to determine which they are most similar to, if any. In this case, the patient matched the Kabuki 1 episignature, confirming the pathogenicity of the observed variant.

It is worth noting that if a patient ***does not match a defined episignature, it does not mean that their DNA methylation pattern is "normal."*** Instead this means that they do not match the established episignatures, but they could still demonstrate DNA methylation changes that relate to an episignature that has not been discovered yet. Furthermore, if a patient contains a variant of unknown significance in a disease gene with an associated episignature and their DNA methylation pattern does not match, it does not mean the variant can be reclassified as benign. Episignatures are constantly evolving as more disease variants are assessed and as sub-signatures, which may be domain, region or nucleotide-specific, present themselves. A non-match simply means that the patient's variant does not result in the same methylation changes observed in the episignature.

Results

Trio exome sequencing performed using peripheral blood uncovered a de novo pathogenic variant in KMT2D present in 11.2% of the 223 sequencing reads [294]. This ***mosaic variant presented a challenge as it is difficult to determine which tissues may be impacted by this variant***, as well as the variant proportions. Determining when this variant presented during development is challenging and therefore understanding how the variant may be contributing to the broader phenotype is also difficult. The use of episignature analysis assesses genome-wide methylation changes to determine if they are concordant with established methylation profiles, or episignatures, associated with a given disorder. Episignature analysis through a clinical molecular diagnostics laboratory in US in peripheral blood found a match with Kabuki syndrome one, providing further evidence that the variant is causing the global methylation changes expected in Kabuki syndrome.

Challenges and solutions

This mosaic variant presented a challenge as it is difficult to determine which tissues may be impacted by this variant, as well as the variant proportions. ***Determining when this variant occurred during development requires assessment of DNA from many tissue sources, an approach that is not clinically feasible due accessibility to tissue, which has cost and ethics implications for the patient***, and therefore understanding how the variant may be contributing to the broader phenotype is also difficult. To determine if this variant is impacting patient phenotype, physicians looked to assess the patient for functional evidence to support the impact this mosaic variant may be having on the patient. Episignatures analysis found a match with the Kabuki syndrome 1 episignature. This genomic and epigenomic approach confirmed the diagnosis in this patient.

Learning and knowledge outcomes

This case study demonstrates the use of episignature analysis when assessing the pathogenicity of a low-level mosaic variant. It provides rationale for ***episignature analysis use as a reflex test***, but if episignature analysis was performed as a first-tier test, targeted sequencing of the Kabuki genes may have saved both time and cost of analysis for this patient. ***Episignature detection in this case provided the functional evidence required to firmly establish the pathogenicity of the detected variant***, and ultimately provide a diagnosis for the patient.

Systematic assessment of episignature detection in patients with varying levels of mosaicism is required to establish a case for episignature analysis for all mosaic cases. We currently do not know the lower range of mosaicism detection by episignature analysis; this analysis would provide better insight into which mosaic patients may benefit from this kind of testing.

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CHAPTER

Therapeutic approaches to imprinting diseases

28

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28.1 Introduction

Unlike most autosomal genes, which are expressed biallelically, a certain number of genes located on genomic imprinting regions are defined by a specific parental contribution with a monoallelic gene expression. These regions are of essential roles in early development and are finely regulated (Fig. 28.1) [1]. About 100 imprinted genes have been identified in humans [2–5]. Disruption of genetic or epigenetic mechanisms in these regions causes aberrant dosages of imprinted genes leading to their expression's dysregulation. The main epigenetic processes involved in genes' regulation are DNA methylation, histone modification, and chromatin remodeling. Methylation marks' alterations in imprinting control centers (IC) are the main epigenetic mechanisms silencing or overexpressing imprinted genes. Some rare alterations may occur such as genetic mutations in imprinted genes, duplications, deletions, and uniparental disomy (UPD) [6–8]. Genomic imprinting disorders are congenital orphan diseases encompassing several distinct syndromes such as Prader–Willi syndrome, Angelman syndrome, Beckwith–Wiedemann syndrome, and Silver–Russel syndrome [7]. This chapter brings together studies that focused on genomic imprinting disorders and their mechanisms after a brief overview of the organization and the control of these imprinted regions. Next, we will manage to focus on advances in therapeutic approaches to possibly overcome these diseases.

28.2 Chromatin organization

Chromatin is composed of deoxyribonucleic acid (DNA) and DNA-binding proteins, mainly histones, allowing accessibility to highly regulated and specific processes, such as transcription, replication, and repair. Nucleosomes, first level of chromatin compaction, are composed of two copies each of H2A, H2B, H3, and H4, binding to DNA [9,10]. The histone amino and carboxy-termini are target to posttranslational modifications (PTMs) on multiple residues which are acetylation, methylation, phosphorylation, sumoylation, and ubiquitinylation. These modifications can directly affect the degree of chromatin local compaction leading to decondensation or condensation [11]. DNase I

Epigenetics in Human Disease. https://doi.org/10.1016/B978-0-443-21863-7.00013-5
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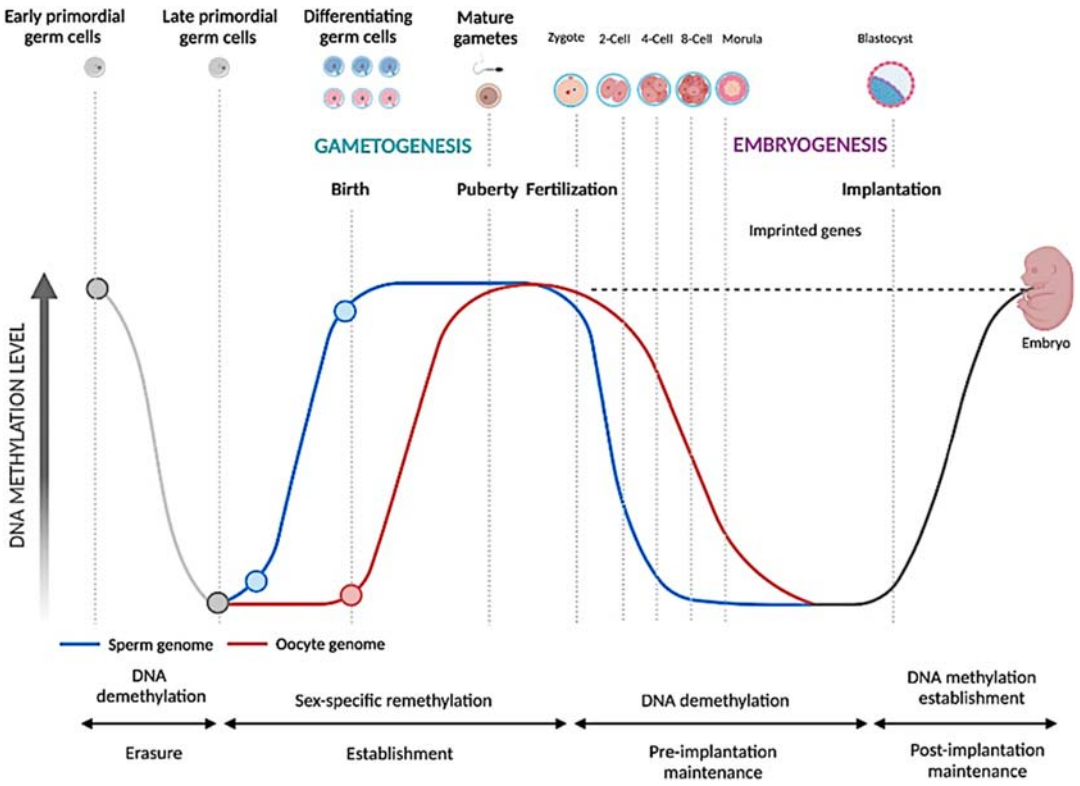


FIGURE 28.1 DNA Methylation Reprogramming During Human Development: The Imprinting Cycle

The erasure step consists of a slow imprinting center demethylation. It is followed by the establishment step where do novo imprinting center methylation occurs prenatally in male germ cells. For female germ cells, it is initiated prenatally and completed in germinal vesicles. After fertilization of mature gametes, the zygote is targeted by a rapid demethylation of paternal genome and a slower demethylation in maternal one. During the post-implantation maintenance, the chromatin conformation and the transcription regulator patterns aim to maintain these germline differentially methylated regions. The specific methylation is established depending on the differentiation stages and the tissue-specificity. The subcortical maternal complex proteins and ZFP57 (zinc finger protein 57) protect imprinting center from demethylation.

hypersensitive sites are high local accessible regions of DNA and are markers of cis-regulatory elements including promoters, enhancers, insulators, silencers, and locus control regions [12].

Research in the field of chromatin biology has moved from the chromosome conformation capture (3C) technology to the 4C, 5C, and Hi-C methods (3C coupled to Next-Generation Sequencing (NGS) technology) to detect genome-wide chromatin interactions at scales of 10–100 Kb [13–17]. Chromosomes are organized into large compartments (megabase scale) of either active (A-compartments) or inactive chromatin (B-compartments). Cell types express different genes combination driven by distinct regulatory elements. Thus, the positions of A- and B-compartments change according to cell types. Recently, 5C and Hi-C methods have demonstrated that these A and B compartments are

partitioned into smaller regions called topologically associating domains (TAD) (Fig. 28.2). Loci within the same TAD interact more frequently with each other than with loci located in neighbors' domains and intra-TAD contacts change depending on cell types and differentiation state [18]. TAD's boundary positions are highly conserved among species and are enriched in active transcription factors, transcriptional repressors CTCF, and cohesion proteins [18–20]. TAD boundaries' disruption lead to aberrant gene expression [21]. Most long-range interactions in mammals across regions are mediating regulatory enhancer-promoter interactions within TADs [19,22]. Efforts were made in the imprinting region 11p15.5 to represent alternative chromatin loops on the maternal and paternal chromosomes that facilitate the differential activation of *H19* and *IGF2* genes by common enhancers (Fig. 28.2) [23–25].

28.3 Epigenome editors and genomic basis of imprinting regions

28.3.1 Epigenome editors

The epigenetic mechanisms are shaped by groups of enzymes known as writers, erasers, and readers.

DNA methylation has a repressive effect with remodeling chromatin condensation by the recruitment of the writer, the DNA methyltransferases (DNMTs) that catalyze the addition of a methyl group to cytosine [26]. According to their roles, some DNMTs ensure the methylation marks maintaining after the replication as DNMT1 and some DNMTs are responsible for de novo DNA methylation with the establishment of imprinting regions or gene silencing during the embryonic development such as DNMT3A, DNMT3B, and DNMT3L [27,28]. These DNA methylation marks are perceived by readers like MeCP2 (methyl CpG binding protein 2) and MBD1–6 (Methyl-CpG-binding domain protein) [29]. The demethylation may occur as a passive replication-dependent manner or as an active replication-independent process through oxidation or deamination erasers enzymes [30]. Major ones are ten-eleven translocation family (TET1, TET2, and TET3) that oxidize methylated cytosine (5 mC) producing 5-hydroxymethylcytosine (5hmC) which is oxidized to 5-formyl-cytosine (5 fC) and 5-carboxyl-cytosine (5caC) [31]. The deamination pathway requires the activation-induced deaminase (AID) and ApoliPOprotéins B mRNA Editing enzyme, Catalytic polypeptide-like (APOBEC) to change 5 mC or 5hmC into 5-hydroxymethyluracil (5hmU) that is further processed by uracil DNA glycosylase [32].

Histone modification is a regulatory process through PTMs with writers histone methyltransferase (HMT) and histone acetyltransferase (HAT), and erasers histone demethylase (HDM) and histone deacetylase (HDAC). These effectors ensure histone marks characteristics of condensed chromatin if it is linked to transcriptionally repressed genes (H3K9me2, H3K9me3, H4K20me3, and H3K27me) and decondensed chromatin leading to gene activation (H3K4me2, H3K4me3, H3K36me, H3K9ac, and H3K27ac) [33–35]. The histone methylation marks are recognized by readers like HP1 and the acetylation ones by the BRD4 proteins [29].

28.3.2 Genomic basis of imprinting centers

Functionally independent domains, called imprinting centers (IC), aim to control the transcription of imprinted genes organized in clusters, by long-range cis-acting germline differentially methylated regions (DMRs) and regulatory elements such as long non-coding RNAs (lncRNAs). Two DMR

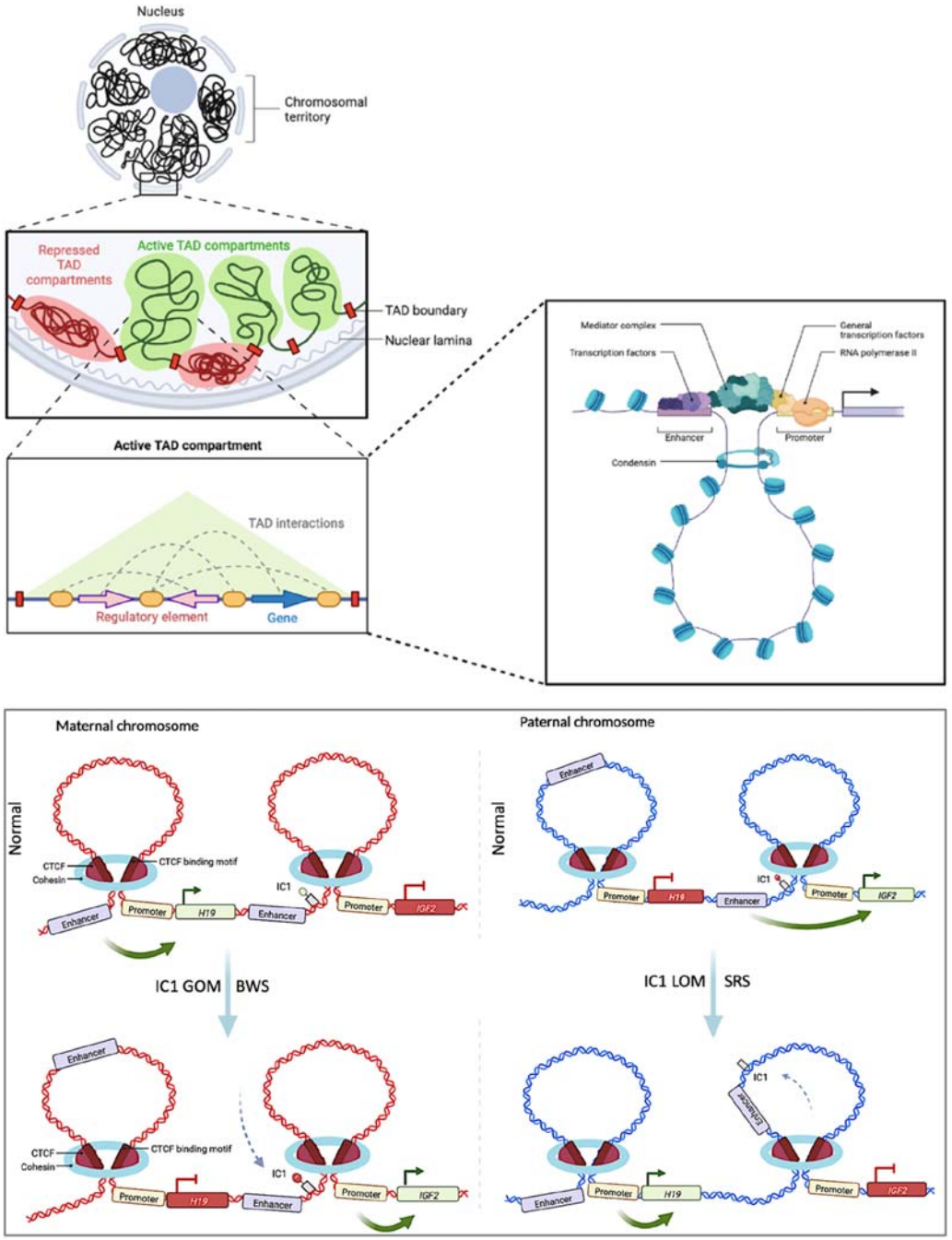


FIGURE 28.2 Chromosome Organization in Nucleus: Topological Associating Domains and Chromatin Loop Adapted for Two Mechanisms in the Chromosomal Region 11p15.5-11p15.4

BWS, Beckwith–Wiedemann syndrome; *IC*1, Imprinting center 1. *IC1-GOM*, Gain of methylation in imprinting center 1; *IC1-LOM*, Loss of methylation in imprinting center 1; *SRS*, Silver Russell syndrome; *TAD*, Topological associating domain; , CH_3 methylation; , CTCF.

classes were described: primary or germline DMRs, about 35 gDMRs, the imprint control regions (IC), and secondary or somatic DMRs where methylation is acquired after fertilization (Fig. 28.1). The maternal and paternal alleles are differentially methylated regions following the finely regulated imprinting cycle that reprogram the DNA methylation during the human development (Fig. 28.1) [36,37]. Maternally methylated gDMRs are most intragenic compared to those on paternal chromosomes that are intergenic [1,36].

Main mechanisms of imprinting disorders are loss of methylation (LOM) and gain of methylation (GOM) of the imprinting center through alternative chromatin loops (Fig. 28.2) and epigenetic mechanisms. Additional imprinted DMRs could be affected leading to a situation called multi-locus imprinting disturbance, reported in patients with Beckwith−Wiedemann syndrome presenting the highest and most severe cases [38].

The 11p15 imprinted region comprises two imprinting centers (IC1/IC2). *H19* and *IGF2* in the telomeric domain controlled by IC1, and *CDKN1C*, *KCNQ1,* and *KCNQ1OT1* genes in the centromeric domain controlled by IC2 with a maternal expression of the *H19, KCNQ1,* and *CDKN1C* genes and a paternal expression of the *IGF2* and *KCNQ1OT1* genes (Fig. 28.3) [39,40].

The chromosomal imprinting region 15q11.2−q13 encompasses a cluster of imprinted genes under the control of a bipartite IC located upstream the small nuclear ribonucleoprotein-associated protein N (SNRPN) region (Fig. 28.4). The 15q11.2−q13 region is relevant for two distinct neurogenetic disorders, Prader−Willi syndrome, caused by loss of function of paternally expressed genes (*MKRN3, MAGEL2, NDN*, and the bicistronic *SNURF-SNRPN* gene), and Angelman syndrome related to loss of function of the maternally expressed *UBE3A* gene that encodes an E3 ubiquitin ligase [41−46].

28.4 Gene therapies: Additive gene therapy and CRISPR/Cas9 epi-edition

The epigenetic marks could be changed by promising epigenetic drugs targeting writers and erasers, but they lack locus specificity in terms of imprinting disorders. The development of new tools for gene therapy by gene editing has experienced renewed interest in the recent years. Additive gene therapy aims to introduce a healthy recombinant nucleic acid sequence of the genetic defect at the origin of the pathology, into the genome of a target cell whose expression product has a therapeutic effect. The modification can be in-vivo or ex-vivo in the cells by direct injection of the viral vector (integrative viral vector, γ-retroviral vector, and lentivectors). Despite the improvement of retroviral vectors by the use of internal promoter in "Self-inactivating (SIN) lentiviral vectors," additive gene therapy still has major drawbacks: the risk of insertional mutagenesis entailing clonal expansions, splicing defects due to the particles integrating into intronic regions with a nonnull oncogenic potential, and the possibility of modifying the transcription of neighboring genes [47−51]. The mastery of this process would get rid of several technical locks by the development of endonucleases that induce specific double-strand breaks at genome's specific sites, the understanding of the DNA-strand breaks repair pathways and the overcoming of the insertional mutagenesis' risk by the use of non-integrative vectorization methods to preserve the physiological regulation offered by the endogenous locus. Gene editing has registered four generations of specific endonucleases: meganucleases, zinc finger nucleases (ZFNs), Transcription Activator Like Effectors Nuclease (TALENs) and finally the revolutionary Clustered Regularly Interspaced Short Palindromic Repeats CRISPR-associated protein system (CRISPR-Cas) [52].

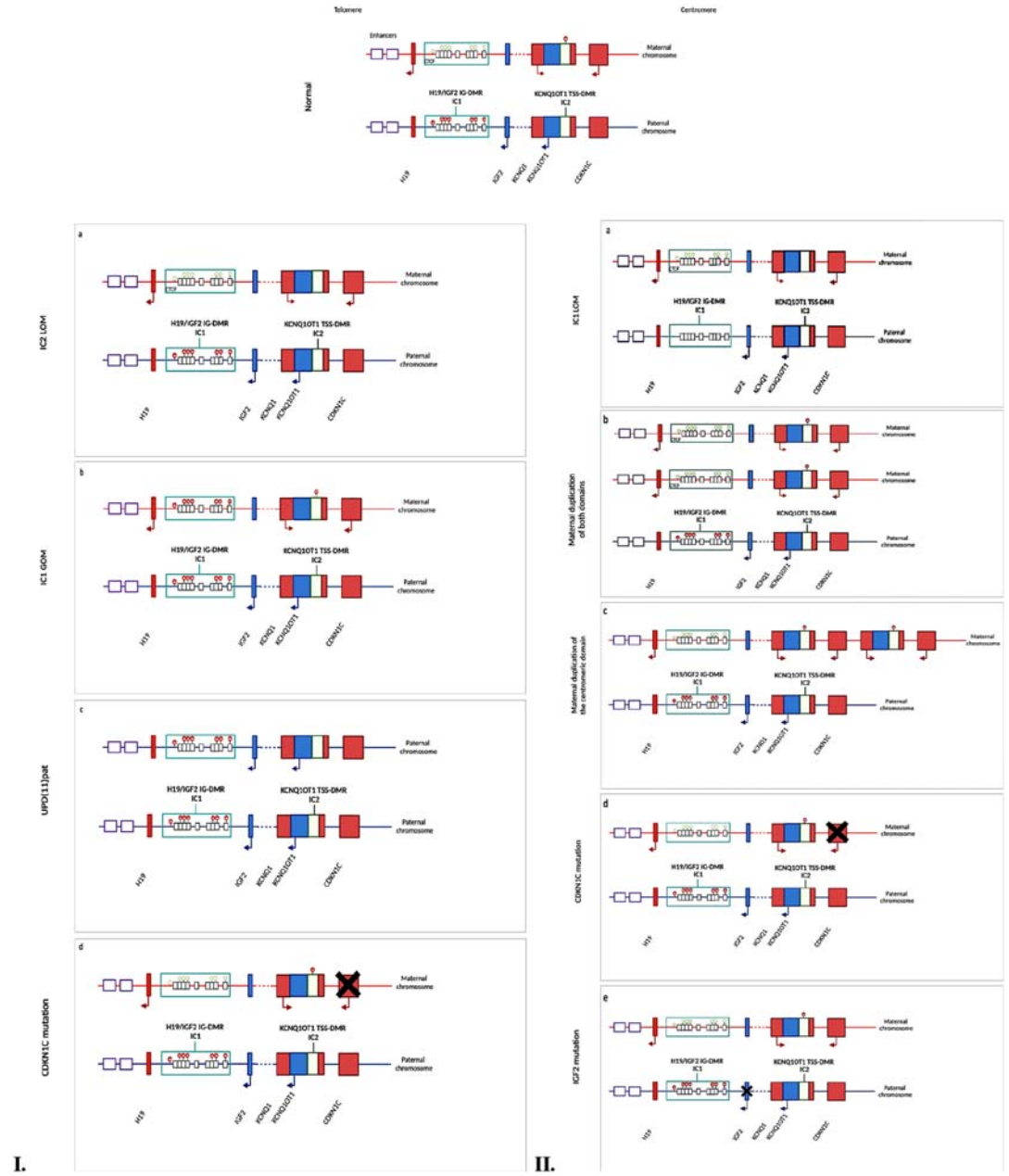


FIGURE 28.3 Schematic Overview of the Chromosomal Region 11p15.5-11p15.4

The 11p15 region comprises two imprinting centers (IC1/IC2). *H19* and *IGF2* in the telomeric domain, and *CDKN1C*, *KCNQ1* and *KCNQ1OT1* genes in the centromeric domain are controlled by IC1 and IC2, respectively. (I) refers to the main mechanisms related to Beckwith—Wiedemann syndrome. (II) refers to the main mechanisms related to Silver—Russell syndrome. *IC1-GOM: gain of methylation in the imprinting center 1; IC1-LOM: loss of methylation in the imprinting center 1; IC2-LOM: loss of methylation in the imprinting center 2; UPD(11)pat: Paternal uniparental disomy of chromosome 11. CDKN1C mutations occurs in 5% of sporadic cases and 40% of familial cases of Beckwith—Wiedemann syndrome.* , *CH_3 methylation;* , *CTCF.*

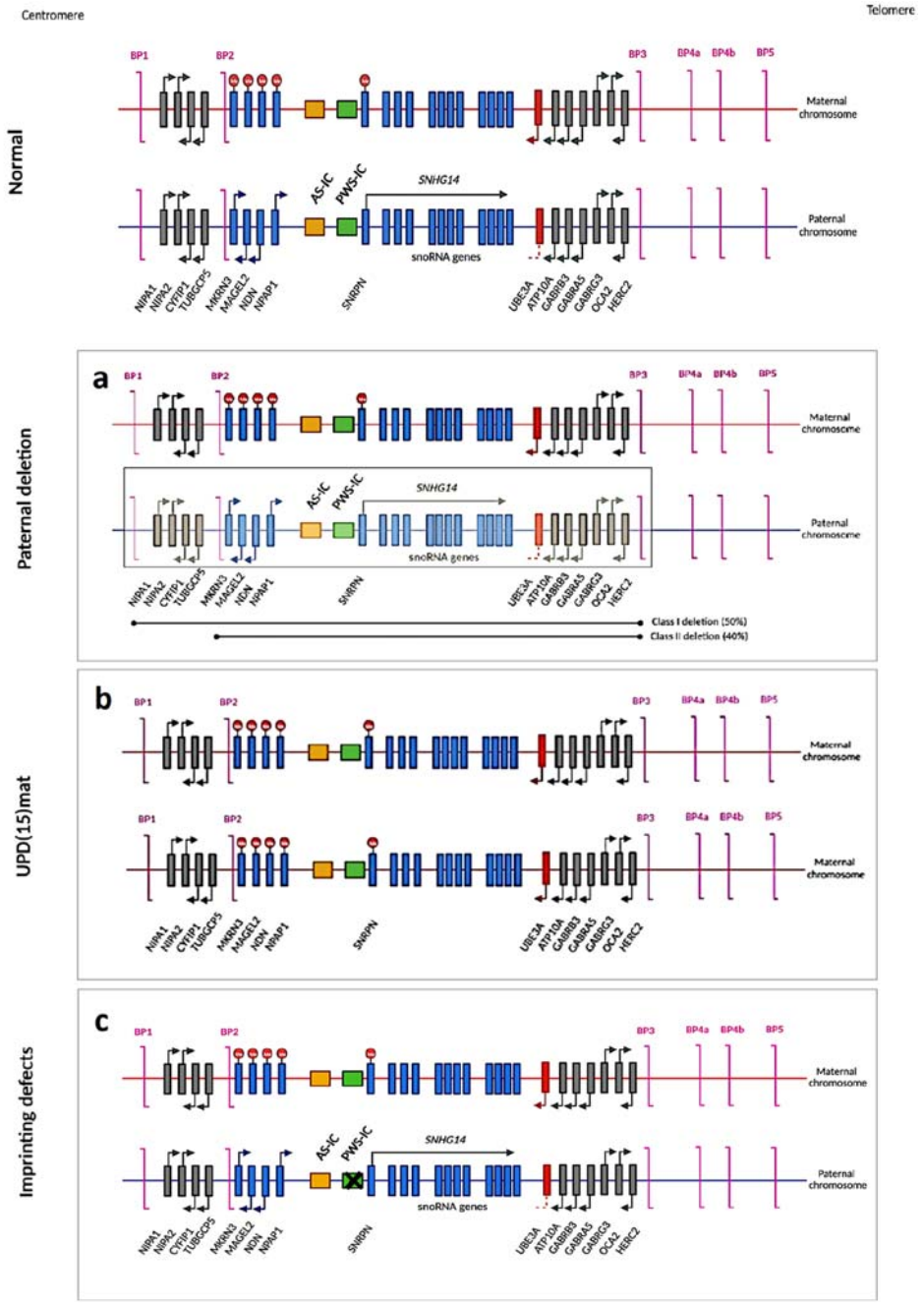
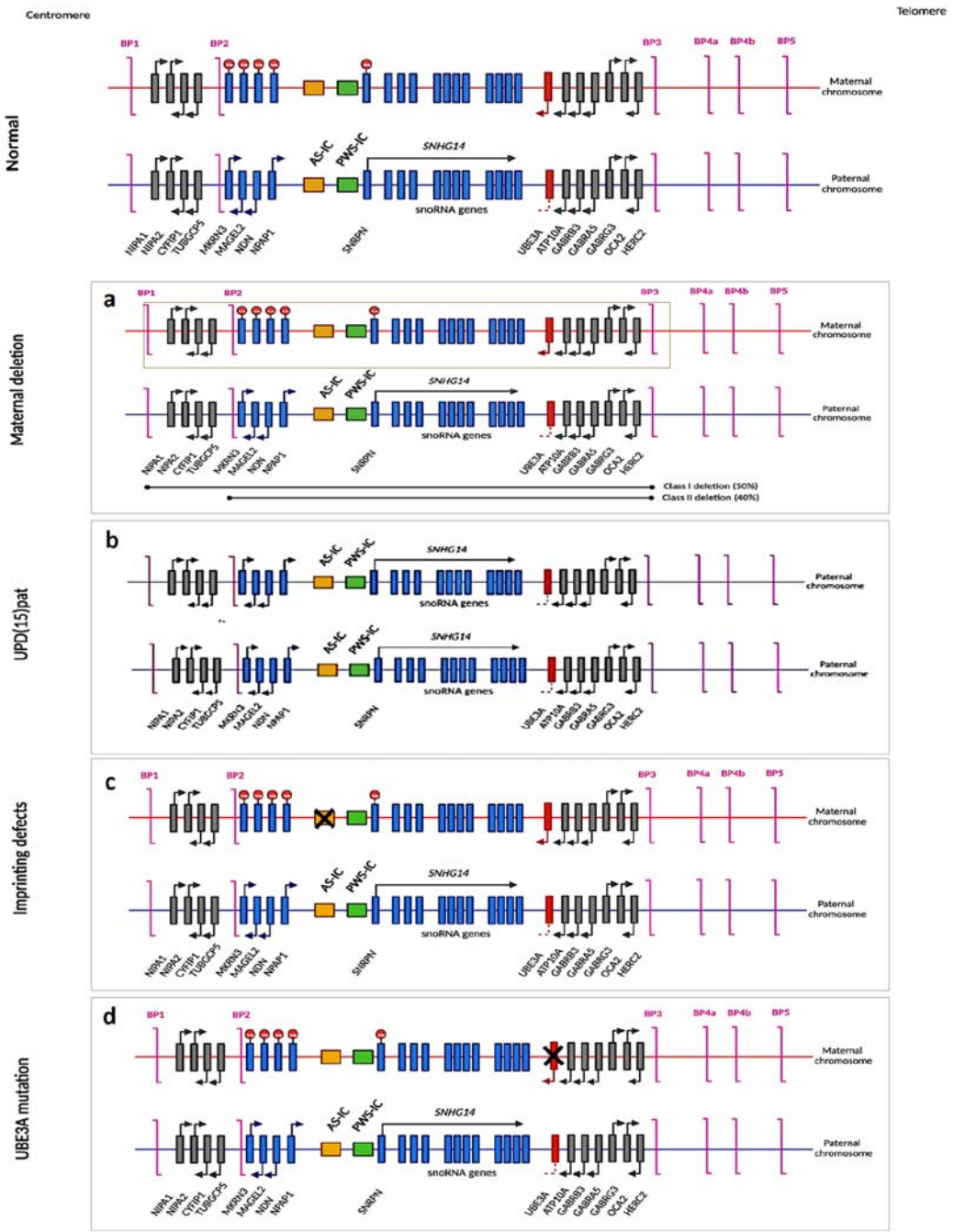


FIGURE 28.4 Schematic Overview of the Chromosomal Region 15q11.2–q13

Blue, red, and gray boxes refer, respectively, to paternally expressed genes, maternally expressed genes, and genes that are expressed from both parental alleles. Arrows indicate the transcription's orientation. (I) refers to the main mechanisms related to Prader–Willi syndrome. (II) refers to the main mechanisms related to Angelman syndrome. *AS-IC: Angelman syndrome imprinting center; BP: break point cluster region;* Me, *CH_3 methylation;* , *CTCF; PWS-IC: Prader–Willi syndrome imprinting center; snoRNA, small nucleolar RNA; SNRPN, small nuclear ribonucleoprotein-associated protein N; UBE3A, ubiquitin–protein ligase E3A.*



II.

FIGURE 28.4 Cont'd

The CRISPR-Cas system consists of a guide RNA (gRNA), a short RNA molecule targeting a substrate DNA sequence and Cas protein with a site-specific nuclease activity. The more detailed understanding of the CRISPR-Cas system led Makarova et al. to propose a functional classification, based on the Cas proteins, with two distinct classes (Table 28.1). Classes are subdivided into five types according to the Cas genes "signature" with a novel type VI dedicated to RNA-targeting CRISPR-Cas systems. For gene editing, class 2 systems are of particular interest since their endonuclease activity provided by a single effector module coupled to interfering CRISPR RNA (crRNA), in contract to class 1 that are defined by the presence of a multi-subunit crRNA-effector complex [53–55].

The most widely used CRISPR-Cas9 system is part of the class 2 type II subtype A [56]. It leads to the RNP formation capable of inducing a specific double-strand break (DSB) (Fig. 28.5). The Cas gene encodes ubiquitous Cas genes (cas1/cas2) and the csn2 gene (subtype A specific). The specificity is attributed to the cas9 signature gene, encoding a large multidomain effector protein [54–57]. Nuclease activity is executed by two Cas9 cleavage domains mediating DSB: RuvC and HNH (Fig. 28.5) [57,58]. Major modified Cas9 proteins were generated to gene regulation editing and to overcome epigenetic dysregulations: The Cas9 nickase harbors an inactivating mutation in one of the DNA cleavage domains that induces single-strand breaks (SSB) with lower off-target effects and shift the cell toward the repair pathways. The dead Cas9 (dCas9), a catalytically inactive Cas9, has mutations within RuvC and HNH domains [59–62].

CRISPR epigenome editors target the effectors catalytic domains of DNMTs, mostly DNMT3A and TETs enzymes, that are attached to dCas9 protein and result in DNA methylation or demethylation, respectively [63,64] (Fig. 28.6A). The effectors' attachment to RNA aptamers or repetitive peptide epitopes through binding proteins improves the methylation modification by multiplying the connection to several effectors rather than the attachment to a linker (Fig. 28.6D and E) [63–66]. Besides, the synergic combination of the engineered transcriptional repressors at the targeted locus ensures the repressive epigenetic memory state at the long-term. The human genome encodes more than 350 Krüppel-associated box (KRAB) domains, composed of 75 amino acid residues within human zinc finger protein-based transcription factors acting as transcriptional repressor domains [67]. KRAB repressors fused to a dCas9 were also adopted to CRISPR interference (CRISPRi) (Fig. 28.6B). It was demonstrated that the bipartite construction of methyl-CpG binding protein 2 (MeCP2) fused to the first functionally characterized KOX1 KRAB showed higher repressor performances of this KRAB–MeCP2–dCas9 system [67–69]. A recent study tested 57 KRAB domains and established that the ZIM3 KRAB–dCas9 fusion is an exceptionally efficient CRISPRi repressor [70]. As for the CRISPR epigenome editors inducing the demethylation, an effective promoter demethylation was established through fusion of the TET catalytic domains to the dCas9 in vitro as well as in vivo to reduce renal fibrosis [64,71]. Shaping histone epigenetic landscape was demonstrated through fusing dCas9 to catalytic domains of histone enzymes: SET domain of PRDM9 (PR domain zinc finger protein 9, HMT), Lysine-Specific Demethylase 1 (LSD1, HDM), p300 (HAT), or HDAC3 (Fig. 28.6A) [72–75].

In the field of CRISPR-mediated activation (CRISPRa) process, there are three major "second-generation" activator approaches with the use of gRNAs within the promoter of interest or the synergistic activation mediator (SAM) or the SunTag system fused to dCas9 (Fig. 28.6D–E) [66,76]. No approach appeared superior and all of them showed levels of activation within an approximatively same order of magnitude [76].

Therapeutics based on epigenome editing require an efficient and tissue-specific delivery with a maintained expression or repression and no adverse effects. The delivery systems are yet to be

Table 28.1 Functional classification of cas proteins [53–55].

		Expression	Interference					Adaptation			Ancillary	
Class	Type	Pre-crRNA processing	Effector module (crRNA and target binding)				Target cleavage	Spacer insertion			Regulation	Helper
1	**I**	Cas6	Cas7	Cas5	Small subunit	Cas8	Cas3	Cas1	Cas2	Cas4	Cas4	
	III	Cas6	Cas7	Cas5	Small subunit	Cas10	Cas10	Cas1	Cas2		CARF	
	IV		Cas7	Cas5	Small subunit	Csf1						DinG
2	**II**	RNase III	Cas9	Cas9	Cas9	Cas9	Cas9	Cas9/ Cas1	Cas2	Cas4	Cas4	Csn2
	V		Cpf1	Cpf1	Cpf1	Cpf1	Cpf1	Cas1	Cas2	Cas4	Cas4	

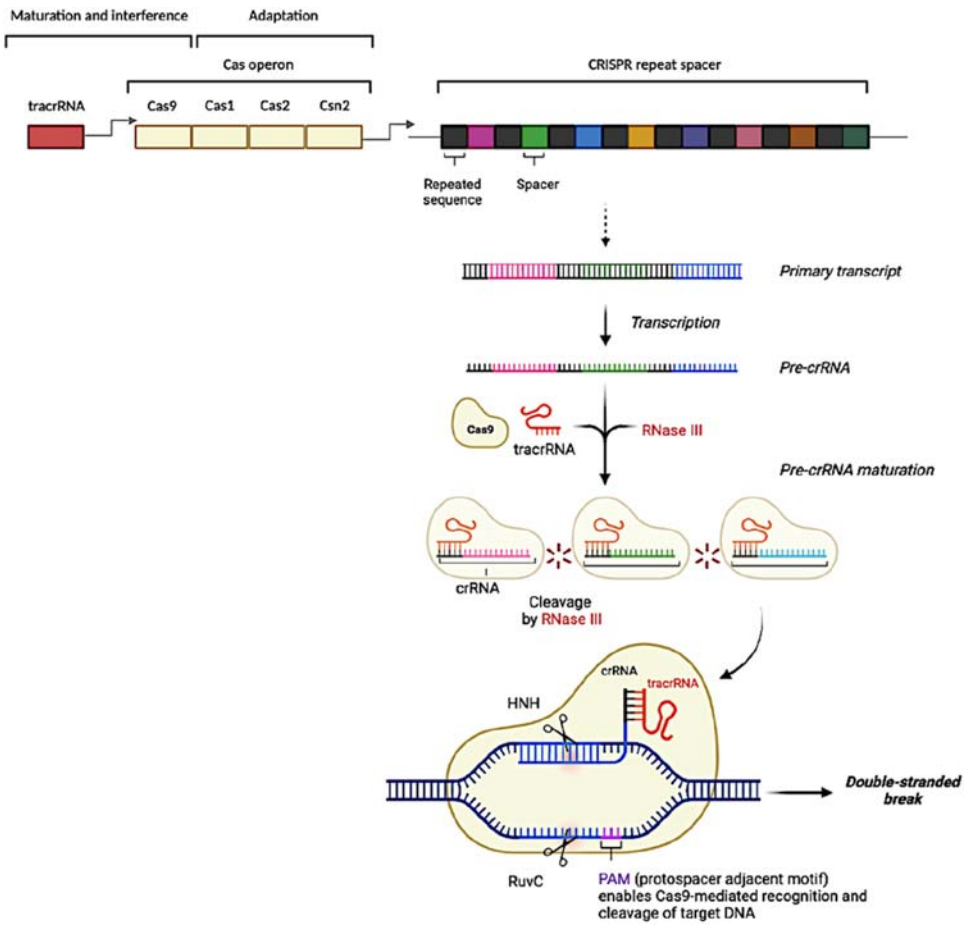


FIGURE 28.5 CRISPR Cas9 System

The pre-crRNA is composed of repeats (black) and spacers (colored). The tracRNA (transactivating CRISPR RNA) with its secondary structures are shown in red. Cas9 and RNAse III are required for the maturation step. The tracrRNAs recognize crRNA sequences and target Cas9 enzymes to the crRNA. CRISPR–Cas9, targeting and editing DNA, was simplified by the use of a synthetic single-guide RNA (sgRNA) mimicking tracrRNA and crRNA structures. Type II CRISPR systems employ a single DNA endonuclease, Cas9, to recognize double strand DNA to cleave with a different nuclease HNH or RuvC.

overcome. All these potentially promising results with DNA or histone epi-editors could be applied on imprinting disorders although we still lack of the full regulation epigenome knowledge.

28.5 Insights into rare imprinting diseases: Clinical management, novel therapies, and clinical trials

Many imprinted DMRs are well characterized and are responsible for the developmental disorders (Table 28.2). We provide a summary of some syndromes (Figs. 28.3 and 28.4).

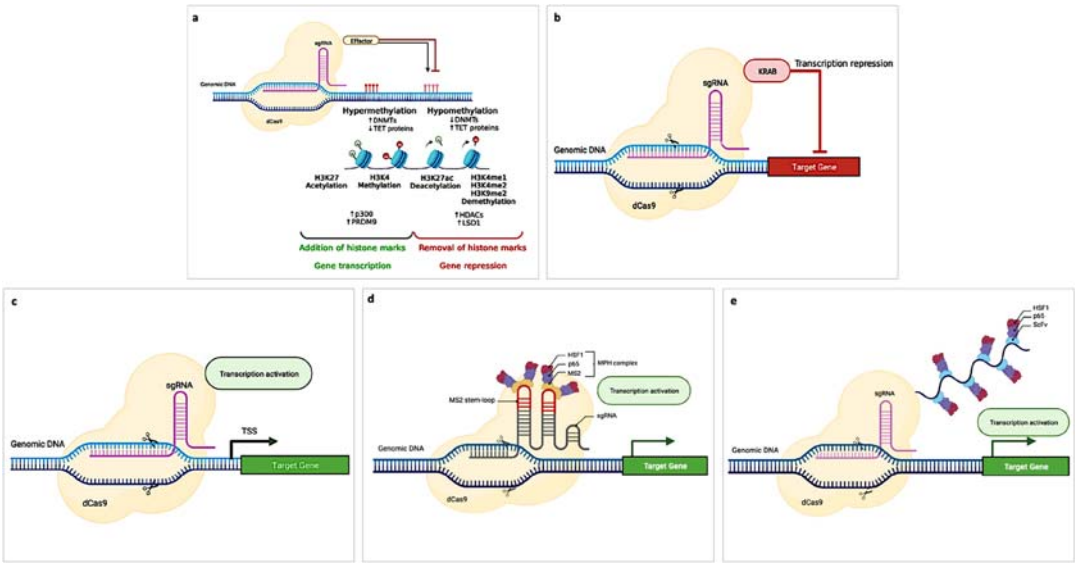


FIGURE 28.6 CRISPR Edition

(A) Modifying epigenetic landscape through fusing dCas9 to effector like catalytic domains of histone enzymes: SET domain of PRDM9 (PR domain zinc finger protein 9, HMT), Lysine-specific demethylase 1 (LSD1, HDM), p300 (HAT) and HDAC3 that mediates, respectively, the addition of CH3 groups to H3K4 inducing H3K4me3, the removal of the CH_3 group from H3K4me1/2 and H3K9me2, the addition of acetyl groups on H3K27 and the removal of acetyl group on H3K27ac; (B) dCas9 KRAB (Krüppel-associated box (KRAB) CRISPR transcriptional repression system; (C) gRNAs within the promoter of the gene of interest as dCas9 CRISPRa; (D) Synergistic activation mediator (SAM) system as dCas9 CRISPRa, consisting on MPH complexes composed of MS2 aptamers bounded to functional proteins: p65 and heat shock factor 1 (HSF1); (E) SunTag system as dCas9 CRISPRa composed of repetitive peptide, the trans-activation proteins p65 and HSF1 and single-chain variable Fragment (ScFv) antibodies.

28.5.1 Prader–Willi syndrome

Prader–Willi syndrome (PWS) (OMIM #176270) was first described by Prader et al. in 1956 and the consensus diagnostic criteria were established in 1993 [99,100]. It is characterized by severe hypotonia at birth and during the neonatal period, thick saliva, increased head/chest circumference ratio, feeding difficulties, and hypogonadism in both sexes. The phenotype is more specific in children with short stature, obesity, developmental delay, sleep disruption, and dysmorphic features with a narrow bifrontal diameter, almond-shaped palpebral fissures, a thin upper lip with a downturned mouth and small hands and feet [101].

The three main genetic mechanisms are linked to the locus 15q11–q13: 5–6 Mb paternal 15q11-q13 deletion within breakpoints (type I and II), maternal uniparental disomy (UPD) (15) and imprinting defects (Fig. 28.4) [43,79,102–104]. The PWS critical region encompasses five single copy snoRNA genes (*SNORD64, SNORD107, SNORD108, SNORD109A,* and *SNORD109B*) and two snoRNA gene clusters (*SNORD115* and *SNORD116* with 48 and 29 gene copies, respectively) [105].

Table 28.2 Some human imprinting syndromes.

Disease	Incidence	OMIM	Region	Main clinical features	Main genetic mechanisms
Prader–Willi syndrome [77–80]	1 in 25,000 neonates	#176270	15q11–q13	Developmental delay Hyperphagia and insatiable appetite Obesity Sleep disruption Hypotonia Hypogonadism Dysmorphic features Cognitive impairment Characteristic behavioral profile Psychiatric comorbidities	70% paternal 15q11–q13 deletion 25% UPD(15)mat Rare imprinting defects
Angelman syndrome [80–82]	1 in 20,000 neonates	#105830	15q11–q13	Developmental delay Microcephaly Severe intellectual disability Absent or limited speech Tongue protrusion Inappropriate happy grimacing Gait ataxia Sleep disturbance Seizures Characteristic EEG Scoliosis	75% deletion of the maternal region 15q11.2–q13 5%–10% *UBE3A* mutations 1%–2% UPD(15)pat 2% imprinting defects
Beckwith–Wiedemann syndrome [40,83]	1 in 13,700 neonates	#130650	11p15.5	Prenatal and/or postnatal Macrosomia Hydramnios Facial naevus simplex Macroglossia Excessive lateralized growth Abdominal wall defects Hyperinsulinism Organomegaly Placental overgrowth Predisposition to embryonal tumors	50% IC2 loss of methylation 20% UPD(11)pat 4% IC1 gain of methylation 2%–4% DUP(11p15)pat *CDKN1C* mutations (5% sporadic; familial 25%–50%)

Continued

Table 28.2 Some human imprinting syndromes.—cont'd

Disease	Incidence	OMIM	Region	Main clinical features	Main genetic mechanisms
Silver–Russell syndrome [84,85]	1 in 30,000–100,000 neonates	#180860	Up to 65% in 11p15.5, 10% chromosome 7	Prenatal and postnatal growth retardation Neonatal relative macrocephaly Feeding difficulties Body asymmetry Protruding forehead	Up to 65% in 11p15.5 region - 40% IC1 LOM loss of methylation - ≤1% UPD(11p15) mat Maternal 11p15 duplications - UPD(11p15)mat - *CDKN1C* maternal gain-of-function mutation - *IGF2* paternal loss-of-function mutation 10% UPD(7)mat
Transient Neonatal Diabetes Mellitus [86,87]	1 in 400,000 neonates	#601410	6q24	IUGR Neonatal insulin-requiring hyperglycemia without ketoacidosis Transient diabetes mellitus Macroglossia Abdominal wall defects	41% UPD(6)pat 33% paternal 6q24 duplication 26% PLAGL1:alt-TSS-DMR loss of methylation
Kagami–Ogata syndrome [88–90]	Less than 1 in 1 million neonates	#608149	14q32.2	IUGR Polyhydramnios Developmental delay Full cheeks and a protruding philtrum Skeletal abnormalities Small bell-shaped thorax Coat hanger ribs Respiratory distress Abdominal wall defects	70% UPD(14)pat 20% del(14q32)Mat 10% MEG3/DLK1: IG-DMR gain of methylation
Temple syndrome [88,91–95]	Unknown Fewer than 100 cases	#616222	14q32.2	Prenatal and postnatal growth Facial dysmorphism Feeding problems Hypotonia Motor delay Truncal obesity Early puberty Small hands and feet.	70% UPD(14)mat 10% del(14q32)pat 20% MEG3/DLK1: IG-DMR-Loss of methylation

Pseudo hypoparathyroidism Ib [96–98]	Unknown	#603233	20q13.3	Macrosomia Early onset obesity Hypocalcemia and hyperphosphatemia due to PTH resistance Occasional resistance to TSH Brachydactyly Advanced bone age Ectopic ossification Cerebral calcifications	>60% GNAS DMRs loss of methylation 2%–20% UPD(20) pat Rare del(20q13)mat *GNAS* point mutations

IUGR, intrauterine growth retardation.

The most one that could play a key role in the neuroendocrine phenotype is *SNORD116*, a snoRNA noncanonical cluster expressed from the host transcript 116HG. *SNORD116* has no ribosomal RNA target and encodes small nuclear RNAs that methylate ribosomal RNAs [106−110].

Growth hormone (GH) therapy is commonly used in the management of PWS. It has improved oxygenation and cardiovascular function, 6 months after the initiation of GH in the field of the sleep disruption and the sleep-disordered breathing as well as in the field of short stature. PWS patients have a mean height without GH of 148 cm in girls and 155 cm in boys with low serum GH and IGF-1 levels and are able to achieve normal adult height with GH therapy [79,111−116]. Multidisciplinary approach improves PWS patients care with the symptom-based therapies and a checklist at every life stage [117].

Current therapeutic approaches remain symptomatic and partially satisfactory. *SNORD116* being the main candidate in the neuroendocrine phenotype, a suitable gene therapy would consist in reactivating this paternally expressed genes by countering the repression mechanism of the *SNORD116* cluster and 116HG on the maternal allele. In the human embryonic stem cells, ZNF274, a Kruppel-associated box-A-domain zinc finger protein (KRAB), were mapped in the 116HG cluster of the PWS region (ENCODE). It had been demonstrated on induced pluripotent stem cells (iPSCs) derived from AS and PWS patients that ZNF274 were capable of recruiting the HMT SETDB1 (SET Domain Bifurcated Histone Lysine Methyltransferase 1) that specifically trimethylates Lys-9 of histone H3 (H3K9me3), increases the H3K9me3 deposition in the *SNORD116* cluster, and leads to repress specifically the maternal cluster [118]. Knockdown of SETDB1 in PWS iPSCs using a lentiviral vector expressing a small hairpin RNA against SETDB1 mRNA and ChIP assay decreased the repressive mark at this locus, increased the H3K4me2 active histone mark, and partially restored the maternal expression of 116HG RNA. But it lacked specificity at this locus since this HMT had several off-targets and affected the DNA methylation by TET binding to the PWS-IC [119]. This approach was improved by CRISPR/Cas9-mediated inactivation of ZNF274 binding sites at the specific *SNORD116* locus. The normally silent maternal *SNORD116* was expressed in neurons derived from PWS iPSCs without affecting the DNA methylation at the PWS-IC [120]. These studies were conducted on iPSCs which reflect the conditions during early development. A proposed CRISPR-dCas9 systems linked to LSD1, a H3K9 demethylase, would specifically reduce the repressive histone marks and allow the maternal *SNORD116* expression with no assumed adverse effects [106].

Small molecules to activate PWS candidate genes have been carried out in mouse embryonic fibroblast (MEFs), a mouse model carrying paternal deletions of *SNRPN* and *SNORD116*. UNC0642 and UNC0638, two selective histone methyltransferase inhibitors, activated the expression of *SNORD116*, reduced H3K9me3 marks with no alteration of the DNA methylation status at the PWS-IC [121]. All these promising studies would make thereby potential therapeutic strategies to propose to PWS patients. Current clinical trials in PWS are summarized in Table 28.3.

28.5.2 Angelman syndrome

Angelman syndrome (AS) (OMIM #105830) is a rare neurogenetic disorder, with an estimated prevalence from 1/20,000 to 1/12,000 [81]. It was first described by the pediatrician Harry Angelman in 1965 and consensus diagnostic criteria were updated in 2006 [122,123]. The main clinical characteristics are microcephaly, tongue protrusion, an inappropriate happy grimacing, severe intellectual disability, severe speech impairment, seizures at 1−3 years of age, characteristic delta EEG pattern

Table 28.3 Leading clinical trials for improving the management of imprinting diseases.

Disease	Study NCT number	Country	Phase	Action	Therapy	Cohort	Age	Start	End	Objectives	State
Prader−Willi syndrome	NA	USA	Preclinical	shRNA	**Knockdown of SETDB1**	NA	NA	NA	NA	NA	Preclinical
	NA	USA	Preclinical	Selective inhibitors of histone lysine N-methyltransferase-2 (known as G9a)	**UNC0642 UNC0638**	NA	NA	NA	NA	NA	Preclinical
	DYSMOT (NCT05298085)	France	3	Hormone	**Oxytocin nasal spray**	36	2−17 Years	April 5th, 2022	January 24th, 2023	Efficacy on Oropharyngo-esophageal dysmotility	Recruiting
	OTBB3-FU (NCT05032326)	France	3 Open-label	Hormone	**Oxytocin**	80	12−36 Months	September 7th, 2021	April 1st, 2025	Efficacy and safety	Recruiting
	NCT05322096	USA	2	MCHR1 antagonist drug	**RGH-706**	176	≥17 Years	September 22nd, 2022	April 2024	Safety, tolerability and efficacy on hyperphagia	Recruiting
	NCT03848481	USA	2	Homolog of the phytocannabinoid cannabidiol (CBD)	**Cannabidivarin (CBDV)**	26	5−30 Years	November 23rd, 2020	December 2023	Efficacy and safety	Recruiting
	NCT05504395	USA	1 Open-label	Triple monoamine reuptake inhibitor (TRI)	**CSTI-500**	14	13−50 years	November 14th, 2022	January 15th, 2023	Pharmacokinetics and safety	Recruiting
	NCT05153434	USA	2 Open-label	Target of extraoral bitter taste receptors (TAS2R)	**ARD-101**	12	17−65 Years	May 27th, 2022	March 31st, 2023	Efficacy on hyperphagia	Recruiting
	NCT03714373	UK USA	3 Open-label	ATP-dependent potassium channel agonist	**Diaoxide choline controlled release (DCCR)**	115	≥4 Years	October 1st, 2018	June 2023	Efficacy and safety on hyperphagia	Recruiting
	NCT04257929	USA	2	Histamine H3 receptor antagonist	**Pitolisant**	60	6−65 Years	December 9th, 2020	August 17th, 2022	Efficacy on excessive daytime sleepiness	Recruiting
Angelman syndrome	ELARA (NCT03882918)	USA	3 Open-label	Extrasynaptic GABA receptor agonist	**OV101 (gaboxadol)**	125	13−49 Years	January 31st, 2019	June 30th, 2021	Long-term safety, tolerability and efficacy	Terminated failed to prove benefit over placebo

Continued

Table 28.3 Leading clinical trials for improving the management of imprinting diseases.—cont'd

Disease	Study NCT number	Country	Phase	Action	Therapy	Cohort	Age	Start	End	Objectives	State
	A-MANECE (NCT02056665)	Spain	2 Open label	Tetracycline antibiotic	**Minocycline**	32	6−30 Years	January 2014	November 2014	Efficacy and safety	Terminated failed to prove benefit over placebo
	HALOS (NCT05127226)	USA	1/2 Open label	RNA interference (paternal unsilencing) (ASOs)	**ION582**	44	2−49 Years	December 22nd, 2021	December 2023	Safety, tolerability, pharmacokinetics, and pharmacodynamics	Recruiting
	KIK-AS (NCT04259281)	Canada USA UK	1/2 Open label	RNA interference (paternal unsilencing) (ASOs)	**GTX-102**	20	4−17 Years	February 6th, 2020	October 24th, 2022	Safety and tolerability	Recruiting
	(NCT04428281)	Italy, Netherland,s Spain USA	1 Open label	RNA interference (paternal unsilencing) (ASOs)	**RO7248824**	74	1−12 Years	June 11th, 2020	December 31st, 2023	Safety, tolerability, pharmacokinetics, and pharmacodynamics	Recruiting
	NA	USA	Preclinical	shRNA-551	**OV882**	NA	NA	NA	NA	NA	Preclinical
	AS-001 (NCT05011851)	Australia	2 open label	Compound chemical structure similar to the cyclic glycine proline	**NNZ-2591**	20	3−17 Years	August 18th, 2021	May 18th, 2023	Safety, tolerability, and pharmacokinetics	Recruiting
Beckwith−Wiedemann syndrome	NCT00945009	Australia, Canada, Israel, New Zealand, Puerto Rico, USA	3	Kidney preservation after preoperative chemotherapy for Wilms tumor	**Combination Chemotherapy and Surgery**	NA	Up to 29 years	July 13th, 2009	November 26th, 2023	Event-free survival (EFS)	Active, not recruiting
Silver−Russell syndrome	NCT01520467	France	NA	Aromatase inhibitor	**Anastrozole**	NA	5−12 Years	April 2012	August 24th, 2016	Efficacy and tolerance	NA

ASOs, *Antisense oligonucleotides;* NA, *not available;* shRNA, *short hairpin RNA.*

with high-amplitude slow waves diffusely distributed associated to the sharp and slow waves, ataxia and/or tremulousness of the limbs and scoliosis and gastroesophageal reflux in adulthood [122,124–129].

The 5–7 Mb interstitial deletion of the maternal chromosomal region 15q11.2–q13 (75%) causes the most severe symptoms. It is the result of non-homologous recombination events within breakpoint cluster regions BP1–BP5 containing repetitive sequences (Fig. 28.3). This deletion includes a cluster of three GABA receptor genes *(GABRB3, GABRA5,* and *GABRG3)* [130–132]. The genetic mechanisms associated with a high risk of recurrence are *UBE3A* mutations (5%–10%) and IC defects (2%) (Fig. 28.3) [133–138]. *UBE3A* encodes an ubiquitin–protein ligase E3A targeting proteins ECT2, p27, HR23A, Arc, and ephexin-5. Disruption of UBE3A function in neurons enhances expression of ephexin-5 and Arc, resulting in reduced synapse formation and impaired excitatory synaptic transmission [139–144]. AS patients have symptomatic treatment with effective results on seizures by valproate, clonazepam, lamotrigine, topiramate, and levetiracetam. A close follow-up with a pedopsychiatrist is advocated for the speech therapy and the behavioral modification strategies [145–147].

The introduction of a functional copy of *UBE3A* gene was realized by injection of an adeno-associated virus (AAV) carrying *UBE3A* into the hippocampus of *UBE3A*-deficient mice. It improved only the cognitive deficits [148]. Preclinical *UBE3A* gene replacements are ongoing (TSHA-106, PTC-AS, and STRX-220).

Small nucleolar RNA host gene 14 (*SNHG14*) is a non-coding antisense transcript initiated at the promoter of paternal *SNRPN,* regulating *UBE3A* expression [149–152]. Appropriate gene therapy for AS patients would be the reactivation of the silent copy of *UBE3A* on the paternal allele to compensate for the loss of function of the maternal copy by the knockdown of the antisense transcript SNHG14.

Some therapies are expected to reactivate the paternal *UBE3A* allele by suppressing *UBE3A* antisense transcript. A clinical trial (NCT00348933) with pro-methylation rich dietary to silence paternal *SNRPN* promoter with betaine and folic acid was unsuccessful [153]. The administration of topoisomerase inhibitor (topotecan), an anticancer therapy, to mice was promising but safety profile was not achieved and adverse interferences were reported in the expression of long transcripts [154,155]. Antisense oligonucleotides (ASOs) against SNHG14, an RNA interference, could lead to degradation of its transcript. Mouse models of AS showed promising results to a phosphorothioate modified chimeric 2′-O-methoxyethyl DNA ASO [156]. ASO efficacy is related to its target's affinity preventing rapid degradation and reducing toxicity. ION582, the latest RNA-targeted therapy administered by intrathecal injection, is being evaluated in the current open-label phase 1/2 HALOS Study (NCT05127226), in up to approximately 44 participants with AS. Current clinical trials in AS are summarized in Table 28.3.

Other engineered DNA-binding proteins are considered as tools to reactivate the paternal allele in AS such as zinc finger-based artificial transcription factors (Zinc-ATF), designed against a site upstream of *SNRPN*, delivered with subcutaneous and intraperitoneal injections to AS mice in order to upregulate UBE3A expression [157,158].

Two recent studies disrupted the antisense transcript by the CRISPR/Cas9 system with a guide RNA specific of the region within *SNORD115*, leading to UBE3A upregulation, encouraging results in the field of the behavioral and the anatomical features such as the body weight and the brain size [159,160].

Besides, small molecule drugs gained interest in the treatment of Angelman syndrome. The phase 3 NEPTUNE study (NCT04106557) enrolled 97 AS patients for safety assessments of OV101, a small molecule extra-synaptic GABA receptor agonist, also known as gaboxadol. It triggers the activation of specific receptors on the surface of the nerve cells that bind the neurotransmitter GABA (gamma-aminobutyric acid) to enhance the tonic inhibition [161]. The study was discontinued since the primary goals were not reached.

Preclinical studies are ongoing for small molecules in mice AS models: PP2A inhibitor or LB-100 reduces PP2A activity, a protein phosphatase 2A, rescues the reduced miniature excitatory post-synaptic circuits, and improves the motor behavior [162]. NNZ-2591, an important molecule for the development of nerve cells is tested in the study (NCT05011851) to restore the normal communication and the signaling between the brain cells.

These promising strategies are attractive but their administration during early development is important to counter the phenomenon of cerebral plasticity, applicable to all imprinting disorder with neurological manifestations and a surrogate of response treatment.

28.5.3 Beckwith–Wiedemann syndrome

Beckwith–Wiedemann syndrome (BWS; OMIM #130650) was first described in 1963 [163,164]. The international consensus of 2018 established a clinical score consisting in cardinal and suggestive features that introduced a new terminology "Beckwith–Wiedemann spectrum" [40]. It is an overgrowth syndrome with an assortment of clinical manifestations which may include hyperinsulinism, neonatal hypoglycemia, macroglossia, abdominal wall defects, hydramnios, excessive lateralized growth, macrosomia, facial naevus simplex, nephromegaly, and hepatomegaly [40,165,166].

BWS is a cancer predisposition syndrome with typical tumors such as neuroblastoma, rhabdomyosarcoma, Wilms tumor, hepatoblastoma (HB), adrenocortical carcinoma, and phaeochromocytoma [40,167–172]. The excessive lateralized growth and nephromegaly have been correlated with a higher risk of developing tumors in BWS [171].

BWS is linked to genetic mechanisms within the 11p15 imprinted region. The most frequent one is the loss of IC2 methylation on the maternal allele (50%) (Fig. 28.3) [40].

The overall tumor risk depends on the underlying mechanism in BWS, very low (2.6%) in IC2-LOM, and higher in UPD(11)pat (16%) and IC1-GOM (28.1%) [40,171,173] (Table 28.4). Abdominal ultrasound surveillance is recommended until the age of 7 years for Wilms tumors in case of IC1 GOM and UPD(11)pat and neuroblastoma if confirmed *CDKN1C* mutation [40].

The follow-up and the management of BWS patients is multidisciplinary to overcome the complications: lateralized overgrowth could lead to leg length discrepancy and is the most frequent feature with segmental UPD(11)pat considering epiphysiodesis for lower limbs if the discrepancy >2 cm [171,174,175]. About 40% of children with BWS undergo a surgical tongue reduction for macroglossia, most commonly an anterior wedge resection [176]. Hyperinsulinism lead to hypoglycemia which is often transient within few days in BWS and only 20% of neonates require medical treatments [170,171,174,177]. The management of congenital heart defects is required in 6.5% cases and is similar to the sporadic cases [178]. Nephrological anomalies in BWSp are observed in 28%–61% patients, mainly with UPD(11), but clinical considerations remain to hypercalciuria, nephrocalcinosis, and nephromegaly which might have an increased risk of Wilms tumor [40,179].

Table 28.4 Tumor types in different molecular subgroups of Beckwith–Wiedemann syndrome in worldwide cohorts.

Studies	Country	Total cohort	Tumors in UPD(11) pat	Tumor type in UPD(11) pat	Tumors in IC2-LOM	Tumor type in IC2-LOM	Tumors in IC1-GOM	Tumor type in IC1-GOM	Tumors in *CDKN1C* mutation	Tumor type in *CDKN1 in 41C* mutation
Weksberg et al. (2001) [83]	Canada	125	6/21	H (1); W (5)	5/35	H (2); G (1); R (2)	1/3	W (1)	0/5	–
Gaston et al. (2001) [183]	France	97	6/11	GG (1); Ph (1); W (4)	1/45	T (1)	5/11	GG (1); W (4)	1/2	N (1)
Hertel et al. (2003) [184]	Denmark	1	–	–	1/1	A (1)	–	–	–	–
Bliek et al. (2004) [185]	Netherlands	66	9/13	A (1); H (1); L (1); N (1); Ph (1); W (4)	2/27	H (1); T (1)	6/9	W (6)	Not studied	Not studied
Sasaki et al. (2007) [186]	Japan	47	2/7	H (2)	1/15	R (1)	0	–	1/4	C (1)
Alsultan et al. (2008) [187]	USA	1	–	–	1/1	A (1)	–	–	–	–
Wijnen et al. (2012) [188]	Netherlands	2	–	–	2/2	A (2)	–	–	–	–
Brioude et al. (2013) [168]	France	407	17/81	A (2); H (2); L (1); N (1); R (1); W (10)	8/257	H (2); M (1); N (2); R (1); S (1); T (1)	10/35	W (10)	3/34	L (1); GG (1); N (1);
Ibrahim et al. (2014) [174]	UK	637	8/16	A (1); H (5); W (2)	3/288	H (1); R (1); W (1)	3/28	W (3)	Not studied	Not studied
Mussa et al. (2016) [170]	Italy	318	13/87	A (1); H (5); Hg (1); N (2); P (1); W (3)	4/190	N (2); R (1); g (1)	8/31	P (1); W (7)	0/10	–
H'mida et al. (2015) [189]	Tunisia	1	–	–	1/1	Ab (1)	–	–	–	–

Continued

Table 28.4 Tumor types in different molecular subgroups of Beckwith−Wiedemann syndrome in worldwide cohorts.—cont'd

Studies	Country	Total cohort	Tumors in UPD(11) pat	Tumor type in UPD(11) pat	Tumors in IC2-LOM	Tumor type in IC2-LOM	Tumors in IC1-GOM	Tumor type in IC1-GOM	Tumors in *CDKN1C* mutation	Tumor type in *CDKN1 in 41C* mutation
Maas et al. (2016) [171]	Netherlands	229	6/44	H (1); My (1); Ph (1); W (3)	3/114	H (1); W (2)	6/19	W (6)	Not studied	Not studied
Kim et al. (2019) [190]	Korea	1	1/1	A (1)	–	–	–	–	–	–
Cöktü et al. (2020) [172]	Germany	321	10/64	A (1); H (5); N (1); Np (3)	3/208	As (1); H (1); W (1)	0/31	–	0/6	–
Eltan et al. (2020) [191]	Turkey	1	–	–	1/1	A (1)	–	–	–	–
Sassi et al. (2021) [192]	Tunisia	2	1/1	A (1)	–	–	–	–	–	–
Duffy et al. (2021) [193]	USA	215	27/73	A (1); H (9); N (1); NET (3); Ph (1); P (1); W (11)	6/112	H (2); N (1); NET (1); W (2)	16/30	W (16)	Not studied	Not studied
Total	–	2471	106/419	A (9); GG (1); H (31); Hg (1); L (2); My (1); N (6); NET (3); Np (3); Ph (4); P (2); R (1); W (42)	42/1297	A (5); Ab (1); As (1); G (1); g (1); H (10); M (1); N (5); NET (1); R (6); S (1); T (3); W (6)	55/197	GG (1); P (1); W (53)	5/61	C (1); GG (1); L (1); N (2)

A, *adrenocortical carcinoma;* Ab, *benign adrenocortical tumor;* As, *astrocytoma;* C, *Cardiac atrial tumor;* g, *germinoma;* G, *gonadoblastoma;* GG, *ganglioneuroma;* H, *hepatoblastoma;* Hg, *Hemangiotelioma;* IC1-GOM, gain of methylation in imprinting center 1; IC2-LOM, *loss of methylation in imprinting center 2;* L, *leukemia;* M, *melanoma;* My, *Myopepithelial cell carcinoma;* N, *neuroblastoma;* NET, *neuroendocrine tumors;* Np, *nephroblastoma;* P, *Pancreatoblastoma;* Ph, *pheochromocytoma;* R, *rhabdomyosarcoma;* S, *sarcoma;* T, *thyroid carcinoma;* UPD(11)pat, *Paternal uniparental disomy of chromosome 11;* W, *Wilms Tumor; (−): not applied.*

BWS HB neoplasms differ from those non-syndromic ones with earlier median age 6 versus 16 months and *CTNNB1* exon 3 mutation commonly among sporadic HB suggesting different oncogenic drivers [180,181]. In fact, recurrent copy number alterations were found in BWS HB across chromosomes 1p36.3, 2q35, 10q11.2, 12q21.31-q23.1, 15, 16, and 17. Transcriptomic profiles associated to BWS HB, showed an enrichment in the chromatin organization and the histone methylation genes, as well as in the genomic imprinting factors that drive oncogenesis, with a subset of 139 genes differentially expressed between BWS tissues and controls (mainly the upregulation of *HDAC1, IL-2, MAPK8, MYC,* and *TGFBR* and the downregulation of *HRAS, IL-6, SHC,* and *WNT1).* Major eight differentially expressed genes were highlighted when comparing tumor versus non-tumoral BWS samples (*ARID4A, ATRX, F-Box, FBXW7, GFI1B, HDAC3, KMT2E,* and *RTF*). Furthermore, a miRNA signature was specific to BWS HB (89 miRNA) with upregulated miR-30b-3p responsible for the genes' downregulation such as *FOXO3* and *JAK2* and downregulated miR-3622a-5p that increased the expression of CDKN2A, HDAC1, and MAPK8. These genes activation and repression create in liver the suitable transcriptional environment for overgrowth and cancer development and open insights to their consideration as diagnostic or therapeutic targets [182].

The BWS tumors' methyloma exhibited a global hypomethylation and a specific CpG islands hypermethylation that need to be confirmed with larger series of BWS HB tumors.

28.5.4 Silver–Russell syndrome

Silver–Russell syndrome (SRS; OMIM #180860), first described in 1953, has an incidence ranging from 1:30,000 to 1:100,000 [84,85]. Netchine–Harbison clinical score was established in 2015 with at least 4/6 criteria to consider SRS: prenatal and postnatal growth retardation, feeding difficulties, neonatal relative macrocephaly, body asymmetry, and protruding forehead [194]. The clinical score system leads the decision to perform genetic analysis that is positive in about 60%–70% of the affected persons. A negative genetic analysis should not exclude SRS.

Several epimutations and mutations of the imprinting region at the chromosome 11p15 leads to a growth restriction phenotype and are associated with SRS: paternal IC1 LOM, maternal duplication of both IC1/IC2 domains, maternal duplication of IC2 domain, maternal *CDKN1C* gain-of-function mutation and paternal *IGF2* loss-of-function mutation. Less than 10% of cases are due to a maternal UPD of chromosome 7 (Fig. 28.3.II) [195–198].

Some clinical signs have different frequencies depending on the underlying molecular mechanisms. Some candidate SRS genes on chromosome 7 were suggested such as *GRB10* (7p12.1) and *MEST* (7q32) [199–201]. Patients with IC1 LOM have more frequent dysmorphic or malformative features, than those with maternal UPD(7). The prominent heels is almost constant sign in patients with maternal UPD(7) [202]. Patients with maternal UPD(7) have more frequently impaired neurocognitive development, although there is great variability in expression, ranging from children with normal development to some with major learning difficulties, language delay with referral for speech therapy, and autism spectrum disorders [194,202–205].

SRS patients have digestive problems or malnutrition in over 70% cases, with the possibility to use an appetite stimulant to improve weight gain in a child capable of eating or switch to enteral feeding by gastrostomy tube in case of extreme feeding difficulties or gastroesophageal reflux [205a]. SRS is associated with an adult height reduction. Patients do benefit from GH treatment but do not reach the final adult height. Additional benefits of GH treatment are the body composition improvement, the motor development, and the increased appetite [206–209].

Adrenarche can be early and aggressive in SRS, particularly in those with paternal IC1 LOM, participating in the height decline. Promising results were observed with the combination of GH treatment and gonadotropin releasing hormone analog treatment from the initiation of puberty to at least 2 years [203,210]. Currently, treatment approaches in SRS aim to manage only the symptoms and do not provide a satisfactory rescue of all clinical signs. Future corrections with epi-editors, as done for the above syndromes might activate IGF2 expression.

28.6 Conclusions

The imprinting disorders belong to the group of rare diseases due to their low prevalence. These diseases involve a limited number of patients that present symptoms of a great severity and require a multidisciplinary approach, leading not to set them at the first-line priority in terms of researcher programs. All this makes the pharmaceutical companies not interested in developing drugs for a small cohort of patients. We point to the necessity to set international databases and research collaborations to support patients and families with these rare disorders. Great efforts were made by the European Network for Human Congenital Imprinting Disorders that counts more than 20 European countries in order to standardize the clinical and the molecular diagnostics, promote basic science research, and develop new therapeutic strategies, as shown by the new progress with epigenome editors.

Key points

- Genomic imprinting disorders are orphan diseases that arise from genetic or epigenetic abnormalities within monoallelic expressed genes from a parental-specific allele.
- Promising epigenetic drugs targeting writers and erasers could change the epigenetic marks but they lack locus specificity.
- New tools for gene therapy such as CRISPR/dCas9 have experienced renewed interest and opened the way to test them in the field of imprinting disorders. The delivery systems are yet to be adapted.

List of abbreviations

3C	Chromosome Conformation Capture
5caC	5-Carboxyl-cytosine
5 fC	5-Formyl-cytosine
5hmC	5-Hydroxymethylcytosine
5hmU	5-Hydroxymethyluracil
5 mC	Methylated cytosine
AAV	Adeno-associated virus
AID	Activation-induced deaminase
APOBEC	ApoliPOprotéins B mRNA Editing enzyme, Catalytic polypeptide-like
AS	Angelman syndrome
ASOs	Antisense oligonucleotides
BWS	Beckwith–Wiedemann syndrome
CRISPR-Cas9	Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein nine

CRISPRa	CRISPR-mediated activation process
CRISPRi	CRISPR interference
crRNA	CRISPR RNA
dCas9	DeadCas9
DMRs	Differentially methylated regions
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltransferases
DSB	Double-strand break
GH	Growth hormone therapy
gRNA	Guide RNA
HAT	Histone acetyltransferase
HB	Hepatoblastoma
HDAC	Histone deacetylase
HDM	Histone demethylase
HMT	Histone methyltransferase
IC	Imprinting control centers
IC1-GOM	Gain of methylation in the imprinting center 1
IC1-LOM	Goss of methylation in the imprinting center 1
IC2-LOM	Loss of methylation in the imprinting center 2
iPSC	Induced Pluripotent Stem Cell
IUGR	intrauterine growth retardation
KRAB	Kruppel-associated box-A-domain zinc finger protein
LncRNAs	Long noncoding RNAs
MBD	Methyl-CpG-binding domain protein
MeCP2	Methyl CpG binding protein 2
NGS	Next-Generation Sequencing
PTMs	Posttranslational modifications
PWS	Prader−Willi syndrome
SAM	Synergistic activation mediator
SnoRNA	Small nucleolar RNA
SRS	Silver−Russell syndrome
SSB	Single-strand breaks
TAD	Topologically associating domains
TALEN	Transcription activator-like effector nuclease
TET	Ten-eleven translocation family
tracrRNA	Trans-activating crRNAc
UPD	Uniparental disomy
UPD(11)pat	Paternal uniparental disomy of chromosome 11
ZFN	Zinc Finger Nuclease

Authors contributions

Hela Sassi: wrote the original draft, participated in the review and the revision of the manuscript.
Léa Guerrini Rousseau: participated in the revision of the manuscript.
Jacques Grill: participated in the revision of the manuscript.
Etienne Rouleau: participated in the revision of the manuscript.

Conflict of interest

Hela Sassi: recipient for a grant within the framework of DUERTECC (European University Diploma of Translational and Clinical Research in Cancerology) as a researcher physician.

Léa Guerrini Rousseau: none.

Jacques Grill: none.

Etienne Rouleau: none.

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CHAPTER

Stem cell epigenetics in development and disease

29

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29.1 Introduction

At its root, cellular identity is governed by the exquisite organization of chromatin in 3D space. As would be covered in other chapters, genome organization broadly follows a hierarchy of scale. Chromosome territories and nuclear localization examine the position of chromosomes or genomic loci in nuclear space. Segregation of the genome into eu or heterochromatin, A/B compartments, and topologically associated domains (TADs) interrogate the organization of large-scale chromatin structures. Finer modalities of genome organization include chromatin interactions between regulatory elements (such as promoters, enhancers, and insulators), dynamic interplay between chromatin remodellers and various chromatin binding proteins such as transcription factors, DNA modifications (DNA methylation and histone modifications), and biophysical parameters such as supercoiling and nucleosome positioning. Cell-type specification can be conceptually illustrated by the Waddington landscape, where cells traverse through "chreodes" or developmental valleys to become increasingly differentiated. The ridges between valleys represent epigenetic barriers separating lineages, while the imagery of a hill conjures up the difficulty of reverting to a more potent state. In modern parlance, epigenetic changes are progressively acquired during embryonic and fetal development in the form of chromatin modifications and organization. In this chapter on the epigenetics of stem cells in development and disease, we provide an overview of the epigenetic dynamics in pluripotent stem cells, germ cells, and adult stem cells, and how they change in the context of human diseases. The restoration of "wild-type" epigenetic marks in relevant stem cell populations may contribute toward the reversion of undesirable disease states.

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Epigenetics in Human Disease. https://doi.org/10.1016/B978-0-443-21863-7.00014-7
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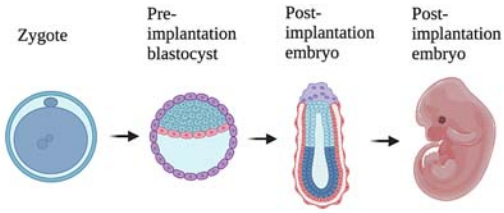
29.2 Chromatin dynamics during embryogenesis

During development, the embryo undergoes a series of conserved and extensive epigenetic alterations that represent changes in cellular identity (Fig. 29.1). Upon fertilization, a wave of epigenetic resetting occurs, which restores totipotency from differentiated gametes as part of the maternal-to-zygotic transition (MZT) [1,2]. To refresh epigenetic memory and maintain sex-matched, allele-specific gene expression, both maternal and paternal genomes undergo extensive epigenetic reprogramming, with changes in DNA methylation and histone modifications. At the two-cell stage in humans, a wave of DNA demethylation occurs [3,4]—the paternal genome is actively demethylated through the methylcytosine dioxygenase TET proteins (TET1, TET2, and TET3) [5], while the maternal genome primarily undergoes a replication-dependent dilution of DNA methylation [6]. Global H3K4me3 levels remain relatively constant through embryonic development [7], even as broad H3K4me3 domains enriched in gene bodies in gametes relocalize to transcription start sites (TSS) upon ZGA [8−10]. Mixed lineage leukemia proteins 3 and 4 (MLL3 and MLL4)-deposited H3K4me1 decorate enhancers [11] and, in conjunction with chromatin modifiers p300 and BRG1, may overlap with H3K27ac or H3K27me3 to indicate "active" or "poised" state [12]. Concomitant with the downregulation of histone deacetylase 1 (HDAC1) and upregulation of coactivators p300 and CREB-binding protein (CBP) [13], H3K27ac level increases during blastulation [14]. Repressive H3K27me3 marks become more abundant through embryonic development [15], accumulates at the



Epigenetic mark	Function
H3K4me1	Active/Poised
H3K4me3	Active
H3K27ac	Active
H3K9me2	Repressed
H3K27me3	Repressed/Poised
DNA methylation	Repressed



FIGURE 29.1

Epigenetic dynamics of embryogenesis. Global abundance of histone modifications and DNA methylation are correlated with shading (right).

promoters and enhancers of developmental genes [16,17], and is functionally involved in X inactivation in female cells post-implantation [18]. In addition, H3K27me3 may play a role in mediating extra long-range interactions between regulatory elements, where these interactions are progressively established during development through polycomb repressive complexes 1 and 2 (PRC1 and PRC2) [19–21]. H3K9me2, which is laid down by a G9a-containing protein complex, is enriched over gene bodies and decommissioned enhancers [22]. Although repressive H3K9me2 marks remain largely invariant, a subset of sites (~ 5%), such as the enhancers of *Esrrb* and *Prdm1* [23], experience increased deposition post-implantation and during lineage commitment [24].

At the nuclear periphery, lamina-associated domains (LADs) are established de novo at the two-cell stage with a genome coverage of ~42% and progressively extend in size to ~61% at the eight-cell stage [25]. Interestingly, maternal LADs appear to be more stochastic between cells and are more euchromatic [25]. During cell-fate transitions, genes integral to stemness are "locked" away at the nuclear periphery and tissue-specific genes are released to the nuclear core [26–28]. In all, global trends support a discriminating increase in chromatin modifications that progressively confer more epigenetic constraints and lineage-specific nuances. Indeed, electron spectroscopic imaging revealed that chromatin in early embryos is largely diffuse and becomes quickly compacted into discrete domains during development [29]. This likely corresponds with the observation of noisier DNase I hypersensitivity (DHS) signals and a more promiscuous transcriptome in early embryogenesis [30].

Higher-order chromatin architecture undergoes extensive remodeling during embryogenesis, providing the context for, or reflecting changes in transcriptional programs. Interphase chromatin can typically be partitioned into multi-Mb-sized A or B compartments, representing active and repressed chromatin respectively [31,32]. A-type compartments are associated with active histone marks (H3K4me1, H3K4me3 H3K27ac, H3K36me3, H3K79me2), enriched for actively transcribed genes, and open chromatin [33]. B-type compartments contain peri-centromeric and telomeric heterochromatin, are enriched for repressive epigenetic marks (H3K9me2, H3K9me3, H3K27me3), and overlap with LADs [33]. Intriguingly, in oocytes, the maternal genome lacks compartmentalization [34], potentially reflecting the transcriptionally inert phase post-fertilization [35]. In contrast, the paternal genome retains A/B compartmentalization, which could be a remnant of sperm protamine-mediated compaction or differences in establishment timing [34]. Through embryogenesis and lineage commitment, A/B compartments exhibit remarkable plasticity, with about 36% of compartments switched between human embryonic stem cells (hESCs) and several hESC-derived lineages [36].

TADs are Mb-sized domains whose boundaries constrain physical chromatin interactions within their space. Similar to observations between different somatic cell types [37], TADs are predominantly invariant between hESCs and hESC-derived lineages, with subtle changes within individual TADs or sub-TADs [24]. Intra-TAD interactions increase or decrease in frequency according to changes in lineage-specific gene activity and in conjunction with dynamic alterations in DHS, active histone marks, CCCTC-binding factor (CTCF), and cohesin binding [24]. TAD maintenance and reorganization during differentiation can be mediated by certain key transcription factors. For instance, together with Mediator subunit Med1 [38] and through its intrinsically disordered domains [39], Oct4 anchors phase-separated condensates at ESC-specific super-enhancers governing *Esrrb*, *Trim28, Mir290*, and *Nanog* [40]. The degradation of these OCT4 condensates disrupts TAD organization and affects cell fate determination in vitro [41].

29.3 Epigenetics of embryo-derived pluripotent stem cells

Pluripotent stem cells possess the ability to differentiate into all three primary germ layers: the ectoderm, mesoderm, and endoderm. While there are many cell types that are considered pluripotent during embryogenesis, each has degrees of pluripotency that have important functional consequences. Pluripotency presents as an interconvertible spectrum between a naïve (or ground) state and a primed state. Broadly, these states correspond with the preimplantation blastocyst and post-implantation embryos, respectively. Epigenetic hallmarks of mouse preimplantation epiblast cells include the presence of two active X chromosomes in female embryos, DNA hypomethylation [42,43] and an insensitivity of DNA methyltransferase (DNMT1, DNMT3A, DNMT3B) knockouts to steady-state self-renewal [44,45]. In general, stem cells derived from epiblasts and cultured in vitro are predisposed to take on the epigenetic profiles of their origins, with ESCs being associated with preimplantation blastocysts [46]. Epiblast-derived stem cells (EpiSCs) and epiblast-derived stem cell–like cells (EpiSCLCs) correspond to temporal stages of post-implantation epiblast cells. Accordingly, they lack markers of naïve pluripotency and cannot withstand a loss of DNA methylation [47]. Functionally, these cells are diametrically opposed to ESCs, in that they readily differentiate when subjected to MEK signaling inhibition [48] and cannot form chimeras when microinjected into preimplantation blastocysts [49,50], although they can still differentiate into three germ layers in vitro [50].

Specific culture conditions largely influence where these stem cells sit in the gradation of pluripotency. Historically, mouse ESCs (mESCs) were derived from blastocyst explants [51,52] and cultured in media containing serum and leukemia inhibitory factor (LIF) [53,54]. LIF, through the JAK-STAT3 (Janus kinase-signal transducer and activator of transcription 3) signaling pathway, allowed mESCs to proliferate without the use of feeder cells [53,54], but the presence of serum engendered a heterogenous population of cells with varying levels of epigenetic modifications and transcriptional differences in contrast to inner cell mass (ICM) cells [7]. The introduction of small molecule inhibitors to MEK and glycogen synthase kinase 3 (GSK3) signaling (also known as 2i) ameliorated the lack of uniformity and stabilized the cells as "ground-state" ESCs [7,55], which bore a close epigenetic resemblance to preimplantation blastocysts. Moreover, the loss of DNMT1 and other epigenetic modulators, such as the polycomb embryonic ectoderm development (EED) protein, DICER, and methyl-CpG binding domain protein 3 (MBD3), can result in "hyper-naïve" mESCs that are resistant to differentiation [47,56]. In contrast, these epigenetic regulators are indispensable to primed pluripotent cells [57]. Primed EpiSCs are traditionally maintained in FGF2/Activin A-containing media [58] or, alternatively, with GSK3 and tankyrase inhibitors (GSK3i/IWR1) [59], which abrogate Wnt/β-catenin signaling. Different culture conditions guide cells toward identities from various regions of the embryo, likely reflecting micro-environmental nuances within post-implantation embryos. Canonical FGF2/activin A-cultured EpiSCs are transcriptionally and functionally similar to the anterior primitive streak [60], while GSK3i/IWR1-cultured cells resemble posterior-proximal epiblasts [61]. Even within the FGF2/activin A condition, subsets of cells exist that map to different temporal stages of post-implantation embryonic development [62]. These observations imply that shades of pluripotency manifest as a confluence of multiple epigenetic and molecular signals that can be influenced extraneously.

Mouse ESCs and EpiSCs can switch between naïve and primed pluripotent states depending on the supplementation provided. Overexpression of naïve factors, such as KLF4, NANOG, and MYC,

or exposure to 2i/LIF conditions can revert EpiSCs or explanted post-implantation epiblasts back to naive PSCs, along with the key characteristics of naïvety—X reactivation, global DNA hypomethylation, and activation of E-cadherin [63,64]. Other naïve pluripotency factors, such as ESRRβ and PRDM14, can act as adjuvants for this process as well [65]. When PSCs are conditioned from serum/LIF to 2i/LIF, pioneer factors OCT4, SOX2, and NANOG initiate epigenetic remodeling by accessing nucleosomal DNA, rewiring promoter−enhancer interactions genome-wide, and inhibiting DNA methylation at their binding sites [66−69]. In parallel, MEK inhibition through 2i leads to a decrease in polycomb repressive complex 2 (PRC2) activity and reduced levels of H3K27me3 deposition on regulatory elements [70]. Conversely, moving toward a more primed pluripotent state from 2i/LIF to serum/LIF, global DNA methylation increases, but regulatory elements denoted by DNase I hypersensitivity remain refractory to methylation until stronger signaling pathways are activated [71]. Under primed conditions with FGF2/activin A signaling, naïve ESCs or ICM cells are converted to a more primed pluripotent state [63,71,72] that is indiscernible from EpiSCs from E6.5 mouse embryos [73].

Human ESCs and mouse ESCs share similarities but differ in important ways. Unlike their mouse counterparts, transforming growth factor β1(TGFβ1)/FGF2/activin signaling underpins the maintenance of hESCs derived from ICMs and induced pluripotent stem cells (iPSCs) [74,75], reflecting primed pluripotency. In turn, activin/nodal signaling is responsible for the deposition of H3K4me3 by the DPY30-COMPASS histone methyltransferase complex onto the promoters of pluripotency and cell fate determining genes such as *OCT4, NANOG*, *PRDM14, LEFTY1,* and *NODAL* [74,76]. Other evidence that traditional hESCs are akin to mEpiSCs include a lack of DNA hypomethylation, presence of H3K27me3 on developmental genes, signs of X-inactivation, sensitivity to MEK inhibition, and intolerance of DNMT knockouts [44,77−79]. That said, hESCs express certain naïve pluripotency factors such as *KLF17*, *DPPA3*, *NANOG*, and *PRD*M14 [78], of which *NANOG* and *PRDM14* are necessary for maintaining pluripotency in vitro [80], suggesting an intermediary configuration between naïve and primed pluripotency. Emulating studies in rodents, progress has been made to define conditions for shifting hPSCs back toward a naïve epigenetic landscape. A concoction containing 2i/LIF, FGF2 and TGFβ 1/activin A, and inhibitors against Jun N-terminal kinase (JNK), atypical protein kinase C (aPKC), RHO-associated protein kinase 1 (ROCK), and p38 resulted in canonical characteristics or naïve pluripotency: H3K27me3 demethylation, increase in global DNA hypomethylation through repressed DNMT3B [81], repression of cell fate markers (CD24, ZIC2) [82], and an enhancer switching toward dormant distal enhancers [83]. In all, pluripotency manifests as an interchangeable continuum that can be influenced by external factors. Even though they can self-renew and may appear morphologically similar, epigenetic landscapes confer specific cellular identities that affect function and lineage commitment.

29.4 Epigenetic changes during reprogramming to generate induced pluripotent stem cells

In a seminal work by the Yamanaka laboratory, mature somatic cells were reprogrammed to become iPSCs by the overexpression of key pluripotency drivers (*Oct3/4*, *Klf4*, *Sox2*, and *cMyc*) [84−89], in opposition to the Waddington landscape. Selection with Nanog instead of Fbx15 resulted in germline-competent iPSCs with functional pluripotency [85]. The characterization of bona fide iPSCs entails

comparability to ESCs in terms of morphology (cell—rounded with large nucleolus and scant cytoplasm; colony—flat, tightly packed with distinct boundaries [90]), expression of pluripotency markers and telomerase reverse transcriptase (*TERT*), and differentiation potential (ability to form embryoid bodies, teratomas in immunodeficient mice, and viable chimera). Fully reprogrammed iPSCs would be practical equivalents to ESCs, possessing the capacity of producing viable progenies by tetraploid complementation [91], albeit at low efficiencies.

By and large, reprogramming follows a stepwise process whereby cells begin to divide faster and lose their differentiated features as they switch off somatic gene expression [92,93]. Metabolism pivots from oxidative phosphorylation to glycolysis [94], and ESC markers like SSEA1 and alkaline phosphatase are upregulated [95,96]. Until this stage, the ectopic expression of reprogramming factors is still required to prevent the reversion back toward an earlier state [95,96]. Later, pluripotency-associated genes (for example, *Nanog* and *Esrrb*) are activated, while developmental genes are repressed, with some acquiring bivalent chromatin states of H3K4me3 and H3K27me3 [92,97]. To be fully reprogrammed, somatic cells must overcome epigenetic barriers to reflect the chromatin landscape in ESCs. For instance, condensed chromatin in the promoter and enhancer regions of pluripotency genes limits the ability of Yamanaka factors to bind for transcription activation and expression [98]. In addition, in "pre-iPSCs," which have ESC-like morphology but lack pluripotency-related gene expression, *Nanog* and *Oct4* retain DNA methylation unlike their hypomethylated state in ESCs [92]. Heterochromatin-related H3K9me3 and H3K27me3 must be remodeled to attain the open, hyperdynamism of ESC chromatin with loosely associated histones, chromatin remodelers, and heterchromatin protein 1 (HP1) [99]. The H3K27me3-specific demethylase KDM6A is an essential player for reprogramming, and its loss impedes the activation of pluripotency mediating genes such as *Sall1*, *Sall4*, and *Utf1* [100]. Conversely, when overexpressed, KDM6A can enhance the efficiency of reprogramming when using Yamanaka factors [101] as well as somatic cell nuclear transfer [102]. Epigenetic modifiers such as trichostatin A (class I and II HDAC inhibitor) and 5-azacytidine (DNMT inhibitor) can also enhance reprogramming efficiency through nucleosome remodeling, elevated global H3K27ac expression, and DNA demethylation [103−108].

Broadly, iPSCs share epigenetic similarities with ESCs—H3K27me3 and H3K4me3 signals are almost identical [86,109], and chromatin accessibility patterns are highly concordant [110]. Nonetheless, there are still salient differences, either as a consequence of epigenetic vestiges from the tissue of origin or from de novo errors. Epigenetic memory manifests in the form of DNA methylation [106,111,112], chromatin architecture [112], miRNAs [113], and histone marks [114], which increase their ability and tendency to differentiate along lineages related to the tissue of origin [106,111−114]. Comparative genomic analyses revealed tissue-specific DNA methylation signatures in fully reprogrammed iPSCs that corresponded to their origins [106]. The less discriminate alteration of DNA methylation during reprogramming also results in aberrant imprinting that may affect downstream differentiation [96,115,116]. Interestingly, $\sim$ 50% of differentially methylated regions between iPSCs and ESCs are not known to relate to reprogramming or pluripotency but are de novo errors that are not present in somatic cells [114]. Moreover, reprogrammed cells can retain chromatin loops from their tissue of origin. In iPSCs derived from neural progenitor cells (NPCs), genes such as *Sox2* and *Klf4* preserve physical interactions with decommissioned, NPC-specific enhancers in addition to interactions with their typical pluripotent enhancers [117].

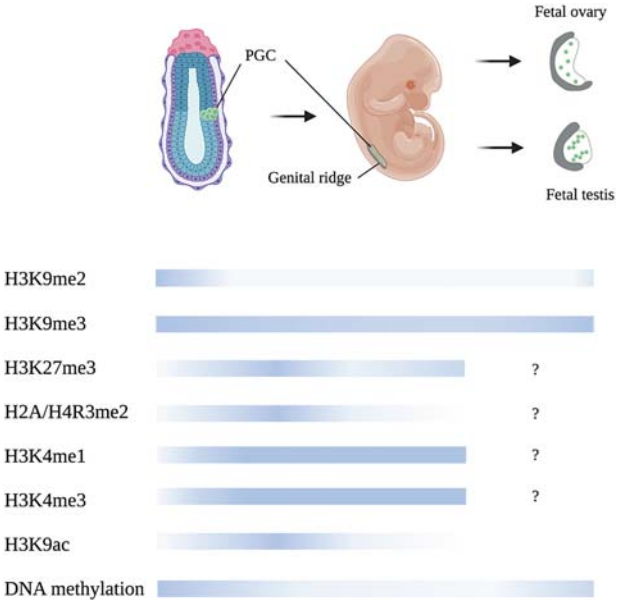


FIGURE 29.2

Epigenetic dynamics of germ cell development. Global abundance of histone modifications and DNA methylation are correlated with shading (right). Question marks indicate a contemporary lack of information.

29.4.1 Epigenetics in germ cell development

Germ cells are responsible for the generation of totipotency and the transmission of genetic and epigenetic information to subsequent generations. As such, germ cell development is a conserved process with key hallmarks that are defined by extensive epigenetic changes (Fig. 29.2). Primordial germ cells (PGCs) are specified during gastrulation from the pluripotent epiblast through BMP signaling [118] in tandem with the maintenance of *OCT4* expression and reactivation of other pluripotency transcription factors [119–121]. During gestation, PGCs migrate from the yolk sac wall to the genital ridges, at which they will undergo colonization, followed by maturation and gametogenesis [119,120]. To eliminate epigenetic memory while maintaining the fidelity of sex-specific imprints from the parental generation to offspring, PGCs undergo broad epigenetic reprogramming, including changes in DNA methylation and histone modifications [122]. Sex-specific imprinted genes are reset [123], together with the reactivation of the X chromosome in female PGCs. Herein, we will discuss major epigenetic changes in pregonadal and gonadal PGCs, which relates to their migration to the genital ridges.

29.4.2 Epigenetics in pregonadal PGCs

Much of what we know about the cell states of PGCs come from mammalian models. Even then, to circumvent the lack of sufficient cell numbers, many chromatin immunoprecipitation (ChIP) experiments have been performed in mouse PGC-like cells (mPGCLCs) that were induced from mEpiLCs by treating with cytokines BMP4, stem cell factor (SCF), LIF, and epidermal growth factor (EGF). In humans, ethical issues surrounding post-implantation embryos and the lack of access entail the use of

human PGCLCs that are thought to approximate early hPGCs but are ultimately of an indeterminate developmental stage [124,125]. Briefly, hPGCLCs can be acquired from incipient mesoderm-like cells (iMeLCs), epiblast-like cells (EpiLCs), or mesendoderm precursors with the same cocktail of cytokines as in mPGCLCs [124,125].

Upon specification from PGC precursors, repressive H3K9me2 levels are reduced globally, although H3K9me3 remains decorated at chromocenters [126]. H3K27me3-mediated facultative heterochromatin increases gradually [127], alongside repressive H2A/H4R3me2, which increases as a corollary of nuclear PRMT5 activity and BLIMP1 binding [128]. *Prmt5* is necessary for germ cell development—*Prmt5*-null mPGCs undergo apoptosis, with complete germ cell loss by E15.5 [129]. In contrast, there is a general decline in DNA methylation in part due to the downregulation of *DNMT3A*, *DNMT3B*, and *DNMT1* recruitment factor *Uhrf1*, although many loci retain DNA methylation [130]. Deletion of *DNMT1* is sufficient for the precocious loss of DNA methylation at functionally relevant sites such as imprinted regions [131].

In hPGCLC cultures, there is no consensus on whether global DNA methylation profiles change upon induction. Evidence suggests that external signaling cues, which are contingent on culture and induction conditions, affect DNA methylation through the regulation of *DNMT3B* and *UHRF1* [132−134]. Immunofluorescence staining of hPGCLCs reflects lower H3K9me2 and higher H3K27me3 levels [132,134], analogous to mPGCs [134,135]. Human PGCLCs share certain epigenetic resemblance to naïve pluripotent cells, with the deletion of TFAP2C-bound enhancers impairing hPGCLC induction [136]. However, the absence of direct comparisons with early hPGCs in vivo limits the interpretation of these findings [124].

29.4.3 Epigenetics in gonadal primordial germ cells

The epigenetic dynamics that PGCs experience prior to colonization are a harbinger to more extensive changes subsequently. At this stage, PGCs are epigenetically distinct from their somatic neighbors, with global DNA methylation levels reaching their nadir in the developmental cycle [135,137]. The depletion of DNA methylation persists even in fetal germ cells (FGCs) that are more developmentally mature at around week 11−19 [137,138]. DNA re-methylation occurs during sex-specific differentiation of FGCs, but insights into the process remain elusive. Experimental evidence for re-methylation ranges from weeks 8 to 19 in male germ cells and around week 11 for female germ cells [137]. *UHRF1, DNMT3A,* and *DNMT3B* are repressed during sex determination, suggesting that the decrease in DNA methylation is likely due to replication-dependent turnover, consistent with findings in hPGCLCs. Although widespread, only 12% of demethylated promoters are upregulated, and the associated genes are ontologically enriched in germline-specific genes such as *DAZL* [138,139]. Nonetheless, depletion of DNA methylation is discriminate, with retention at certain neurological and metabolic-related loci and transposable elements, implicating the heredity of disorders such as obesity, schizophrenia, and multiple sclerosis [135].

Concomitant to changes in DNA methylation, gonadal PGCs have drastically distinct chromatin states and a remodeled chromatin architecture that reflects a less structured transcriptional environment. The nucleus increases in size, with lower levels of repressive H3K9me3 as compared with nearby somatic cells [122]. H3K9me2 remains basal while H3K27me3, which saw an earlier increase during migration, diminishes as well [135,141]. Activating H3K4me1, H3K4me3, and H3K9ac are enriched to levels comparable with somatic cells [122]. Post-reprogramming, the nucleus recompacts

and chromatin condenses [137,142], and H3K27me3 and H3K9me3 marks are reestablished, together with the return of heterochromatic puncta in immunofluorescence stains [122,126].

PRC1 and PRC2 complexes are intimately involved in germ cell development. A deletion of PRC2 subunit *Eed* resulted in the loss of H3K27me3, leading to developmental defects in PGCs due to a precocious activation of sex-specific differentiation genes [143]. Canonical PRC1 complexes regulate lineage specification and development by organizing genes into three-dimensional networks [21] and through higher-order chromatin compaction [144]. On the other hand, non-canonical PRC1 complexes regulate genes by driving the ubiquitination of H2A (H2AK119ub) [145]. Accordingly, *Rnf2* knockouts did not affect H3K27me3, but drove early expression of meiosis-initiating *Stra8* upon retinoic acid (RA) induction [145,146]. Deleting both *Rnf2* and *Ring1* brought about fewer PGCs but is insufficient to functionally alter meiosis timing [145]. Sex-specific differences in relation to the regulation of H3K27me3 are prevalent. Kdm6a, an X-linked H3K27 demethylase, is redundant for maintaining male fertility but causes embryonic lethality in *Kdm6a*-null females [147]. The loss of PRC2-mediated H3K27me3 also affects female PGCs more than in males [145].

Sex-specific differences in development go beyond PGCs and can in fact affect the entire embryo. One reason for this difference relates to the presence of an additional X chromosome in females compared with males, which has implications for gene expression. One such example is the *DKC1* gene, an X-linked gene that encodes the dyskerin protein, a major component of the telomerase enzyme complex. Dyskerin is expressed at higher levels in female cells than in male cells before X chromosome inactivation occurs [148,149]. This is hypothesized to have lasting effects for health and aging, with lower telomerase activity in the developing male embryo leading to shorter telomere lengths in male cells at birth compared with female cells [148]. In fact, some genes on the X chromosome escape inactivation during development [150], with potential health implications. For example, *KDM6A* and *KDM5C*, which encode histone demethylases that act on H3K27 and H3K4, respectively [151], escape X inactivation during development [152−154]. KDM5C is expressed at higher levels in females than in males [155], and KDM5C dosage underlies sex differences in adiposity [155]. Thus, while conserved epigenetic dynamics underlie morphological changes during different stages of germ cell development, there may be sex-specific differences in terms of specific epigenetic regulators involved in maintaining or laying down the epigenetic marks. Accordingly, epigenetic dysregulation stemming from the mutation of these epigenetic modulators can lead to sex-differentiated diseases.

29.5 Germ cell disorders

29.5.1 Germ cell tumor

Germ cell tumors (GCTs) are rare tumors that initiate in the gonads. They are derived from stem cells in the early embryo or germlines [156]. GCTs can be divided into two categories: germ cell neoplasia in situ (GCNIS)−derived and non−GCNIS-derived tumors [157]. Testicular tumors are the most common tumors in teenagers and young men, most of which are testicular germ cell tumors (TGCTs) [158]. TGCTs can be further divided into seminomas and non-seminomas: non-seminomas include teratomas, yolk sac tumors, and choriocarcinomas [159]. Abnormal DNA methylation patterns are a possible cause of TGCTs. During development, hPGCs undergo waves of DNA demethylation and re-methylation, and abnormalities in these processes may lead to the occurrence of tumors. DNA

methylation levels in seminoma are very low, with almost no CpG island methylation, whereas DNA methylation levels in non-seminoma are higher in agreement with those in other solid tumors [159]. Accordingly, DNMT3A isoform 2 (DNMT3A2), a de novo DNA methyltransferase, is overexpressed in non-seminoma TGCT samples [160]. Researchers have also identified some differentially methylated genes between TGCTs and normal samples, including *APAF1* and *RASSF1A* [161]. These differentially methylated genes are also considered possible causes for TGCTs. Another study found DNMT3B expression as a negative marker for the prognosis of stage I testicular seminoma [162]. The methylation status of TGCTs may also contribute to tumor formation. TGCTs have low levels of repressive H3K9me2 and H3K27me3 but high levels of active H3K9 acetylation, H3K4 methylation, and histone variant H2A.Z [163]. Such an active state may also increase the chance of getting tumors to some extent. In addition, some signaling pathways such as PIWI/piRNA may also play a role in the formation of TGCTs [159].

29.5.2 Male infertility

Infertility affects around 8%−12% of couples worldwide, with males accounting for about 50% [164]. Male infertility is a complex disease in which both genetic factors and epigenetic factors play a role [165]. Abnormal sperm count, motility, and morphology can cause male infertility. Genome-wide DNA methylation differences that lead to abnormal gene expression have been found between normal and clinically abnormal sperms [165]—14.4% of participants have abnormal paternally imprinted methylation patterns, while 20.6% of participants have abnormal maternally imprinted methylation patterns [166]. In addition, differential DNA methylation patterns are also present in patients with X chromosome aneuploidy. For example, Klinefelter syndrome (KS), characterized by an extra X chromosome in males (47, XXY), affects around 1:500−1:1000 males and causes infertility [167]. KS is generally associated with DNA hypermethylation and, at specific loci, DNA hypomethylation. Candidate genes were obtained by combining analyses of both methylome and transcriptome, with HEN methyltransferase 1 (*HENMT1*) and eukaryotic translation initiation factor 2 subunit 3 (*EIF2S3*) emerging as genes putatively responsible for hypogonadism and azoospermia [168]. At the same time, aberrant histone modification is another important cause of male infertility. Genome-wide analysis on sperms from fertile and infertile men revealed a decrease in H3K4 methylation or H3K27 methylation in a few imprinted genes [169]. Another study examined histone marks on low activity and high activity sperms and found a higher level of H3K79me2, H3K4me, H3K4me3, H3K9me2, H3K36me3 in low activity sperms compared with high activity sperms [170]. Abnormal histone methylation patterns might cause the abnormal activation or repression of some key genes, leading to the decrease in sperm function. The transformation of histone to protamine is an important process in spermatogenesis, and high levels of H4 acetylation are crucial to complete this transformation [171]. Accordingly, abnormal sperms have lowered H4 acetylation and abnormalities in H4K20 and H3K9 methylation [172].

29.5.3 Female infertility

The epigenetic basis surrounding female infertility is relatively less well studied, in part due to the difficulties in obtaining female reproductive cells. Moreover, epigenetic changes are likely to differ in each underlying case of infertility, such as in polycystic ovary syndrome (PCOS)-, endometriosis-,

age-, and nutritional status—related infertility [173]. These epigenetic changes may also not be limited to that in germ cells. For example, in PCOS, the most common cause of female infertility [174], DNA methylation changes have been found in the adipose tissues and skeletal muscles of patients, which may affect metabolism, and in turn fertility [174]. Differentially methylated regions have been found in ovarian tissues from women with PCOS compared with healthy individuals, although their role in disease etiology and fertility remains unclear. In a small study examining genome-wide DNA methylation profiles of women with PCOS against controls, researchers found significantly increased methylation levels in CpG island shores in women with PCOS [175]. Paradoxically, a separate study found over 7000 differentially methylated sites, with more hypomethylated sites within CpG islands in PCOS patients, with an enrichment for the involvement of p53 and NOD-like receptor signaling pathways [176]. Other studies examining DNA methylation in other ovarian tissues such as ovarian granulosa cells have also revealed differences in DNA methylation in the promoters of genes regulating hormonal signaling and androgen metabolism [177—179]. In particular, subsets of PCOS appear to be driven by separate regulatory pathways, with hyperandrogenism PCOS marked by dysregulation in HDAC-recruiter *NCOR1*, *HDAC3*, and peroxisome proliferator—activator reception gamma 1 (*PPARG1*) [163]. As in males, abnormal DNA methylation patterns are also linked to X chromosome number aneuploidy in females. Turner syndrome (TS), characterized by a depletion of X chromosome in females (45, X), leads to female infertility [180]. Global methylome and transcriptome profile of leukocytes from TS individuals revealed extensive DNA hypomethylation and a key role for *KDM6A* in disease etiology and infertility [181]. Further studies are required to determine causality as well as other epigenetic mechanisms that may lead to female infertility.

29.6 Epigenetics in adult stem cells and their relationship with disease

Adult stem cells are rare populations of multipotent or unipotent stem cells present within each tissue compartment that are capable of self-renewal and generation of lineage-restricted mature cell types during injury and regular cell turnover. Several populations of adult stem cells exist in the human, including hematopoietic stem cells, neural stem cells, and mesenchymal stem cells (MSCs), among others [182]. Unlike PSCs, adult stem cells have limited differentiation potential and a finite capacity for self-renewal. Adult stem cells are typically maintained in niches in a state of quiescence until stimulated to exit and proliferate [183]. An important consideration for adult stem cells is the balance between self-renewal and differentiation, to ensure the continuance of a stem cell population and to generate mature cells of specific lineages when necessary. As with PSCs, the differentiation of adult stem cells typically follows the silencing of genes regulating self-renewal and the activation of cell type—specific genes, with changes in DNA methylation and histone modifications driving changes in gene expression. As there are numerous sources of adult stem cells, here, we cover only the major adult stem cell sources.

29.6.1 Epigenetics in hematopoietic stem cells and dysregulation giving rise to hematopoietic malignancies

Hematopoietic stem cells (HSCs) have the cardinal characteristic of hematopoietic reconstitution upon transplantation in hemoablated subjects. They are thought to sit at the Waddingtonian vertex in blood development, with the ability to generate all mature blood cell types. DNA methylation plays a major

role in HSC self-renewal and differentiation, where whole-genome bisulfite sequencing demonstrated that differentiation of HSCs to all blood lineages involves widespread changes in DNA methylation [184,185]. Accordingly, the DNA methyltransferases DNMT1, DNMT3A, and DNMT3B have all been implicated in HSC differentiation. *DNMT1*-null HSC cells exhibited maturation defects when transplanted into lethally irradiated mice, with the most significant effects on myeloid lineages. Bisulfite sequencing and global gene expression analysis revealed that the loss of *DNMT1* led to changes in DNA methylation across erythroid-specific genes *Gata1* and *Car1*, with corresponding gene expression changes specific to short-term HSCs, which cannot self-renew [186]. Finally, HSCs lacking the de novo DNA methyltransferases DNMT3A and DNMT3B have an impaired capacity for long-term reconstitution in transplantation assays [187]. Interestingly, lethally irradiated *DNMT3A*-null mutant mice possess a defect in hematopoietic differentiation over serial transplantation but not in HSC self-renewal, suggesting distinct regulatory mechanisms governed by separate DNA methyltransferases [188].

Besides DNA methylation, histone modifications also play key roles in HSC self-renewal and differentiation. Bmi-1, a member of the PRC1 family, maintains the HSC niche by downregulating the expression of $p16^{Ink4a}$ and $p19^{Arf}$ [189] and regulating HSC senescence [190]. Monocytic leukemia zinc finger protein (MOZ) and HBO1/KAT7, which are histone acetyltransferases (HATs) for H3K9ac and H3K14ac, respectively, promote the expression of *Hox* genes, *Egr1*, *Erg*, *Gata2*, and *Pbx1*, among others in a transcriptional network necessary for self-renewal and quiescence in HSCs [190,191]. A knockout of *Jarid1b*/*KDM5b*, a H3K4me2/3 demethylase that is highly expressed in embryonic stem cell and an oncogene driving melanoma [192], breast cancer [193], and leukemia [194], impairs HSC self-renewal but not steady-state blood development [195]. Separately, Setd2, one of several histone methyltransferases for H3K36, is responsible for regulating key genes for quiescence and differentiation through the inhibition of the Nsd1/2/3 transcriptional complex, which in turn controls transcription elongation [196]. Finally, histone ubiquitination also plays a role in regulating HSC function [197]. Conditional deletion of the H2A deubiquitinase *Usp16* in the bone marrow led to a reduction in mature and progenitor populations within the hematopoietic hierarchy, revealing a role in HSC lineage commitment [197].

Given the importance of DNA methylation and histone modifications in regulating HSC self-renewal and differentiation, it is unsurprising that alterations in chromatin regulators can lead to hematological malignancies. Perturbation in the normal differentiation of HSCs and its progenitors can lead to three major types of malignancies: leukemias, lymphomas, and myelomas [198]. DNMT3A mutations are among the most commonly mutated genes in AML patients [199,200]. MLL, a H3K4 methyltransferase, is also a commonly affected gene in infant leukemia patients and secondary AML patients, with gene rearrangements occurring in over 50% of infant acute leukemia cases and AML cases [201]. A number of studies have shown that AMLs with underlying MLL rearrangements display aberrant hematopoietic progenitor self-renewal, with underlying changes in global H3K4 and H3K79 methylation [202,203]. Evidently, epigenetics play a major role in malignant transformation in diseases related to HSCs.

29.6.2 Epigenetics in neural stem cells and dysregulation giving rise to neurological disorders

Neural stem cells (NSCs) are multipotent stem cells capable of giving rise to neurons and glial cells in the adult brain [203]. As in HSCs, DNA methylation plays a major role in regulating NSC

differentiation. For example, the loss of DNMT3A in mESC-derived mNSCs led to an altered equilibrium between self-renewal and differentiation, leading to impaired neuronal differentiation during postnatal neurogenesis [204]. Mechanistically, DNMT3a facilitates DNA methylation at proximal promoter and nonpromoter regions, directly and indirectly regulating the expression of neurogenic genes [205]. A recent study utilizing bisulfite sequencing further revealed that DNMT3A and DNMT3B establish neuron-specific methylomes to facilitate transcription of neuronal genes during adult hippocampal NSC differentiation and that the loss of these proteins led to impaired functional maturation of newborn neurons [206]. Additionally, the DNA methylation "reader," methyl-CpG binding domain 1 (MBD1), was recently shown to be important in maintaining NSCs and their ability to differentiate to neuronal lineages [207], further fortifying the importance of DNA methylation in adult neurogenesis. In addition, Tet1 knockout mice had fewer neural progenitor cells (NPCs) in the subgranular zone of 4-month-old mice, due to impaired NPC proliferation, as well as altered expression and methylation involved in NPC proliferation, demonstrating a role for DNA methylation in maintaining the NPC pool of adult mice brains [208].

Histone modifications are also associated with neuronal and glial differentiation. Lysine-specific demethylase 1 (LSD1), which is important for maintaining hESC identity and in differentiation [209], regulates the proliferation of neural stem cells as well [199,210]. Loss of LSD1 leads to vastly reduced NSC proliferation, which affects the self-renewal of NSCs. H3K4me3, H3K27me3, and H3K36me3 signatures may also have a hand in directing NPCs into different subsets of neural stem/progenitor cells (NSPCs), further supporting the importance of histone methylation in NSC and NSPC maintenance and identity [211]. On the other hand, differentiation down the neural lineage is associated with maintenance of histone acetylation, while differentiation to astrocyte and oligodendrocytes is associated with histone deacetylation [212]. The use of valproic acid (VPA), an HDAC inhibitor, prevented H3 and H4 deacetylation of neural progenitors and induced neuronal differentiation of isolated rat neural progenitors in the hippocampus, while inhibiting astrocyte and oligodendrocyte differentiation [213,214]. In all, combinations of histone marks appear to be pivotal in determining the cell fate of NSCs.

Chromatin remodeling also plays an important role in NSCs [215,216]. The BAF chromatin remodeling complex functions in adult neurogenesis and deletion of the BAF170 subunit lead to altered self-renewal and differentiation of NSPCs in the mouse brain [215,216]. Brg1, a core subunit of the SWI/SNF-like BAF chromatin remodeling complex, was also found to be important in adult NSC maintenance, with the loss of Brg1 leading to reduced NSC and NSPC maintenance, and reduced NSC response to physiological stimulation [215].

Misregulation of epigenetics in NSCs has been linked to diseases such as neurodegenerative diseases and brain tumors. Rett syndrome, a neurodevelopmental disorder characterized by progressive decline in cognitive and physical abilities, also has a basis in epigenetics. It is caused by mutations in the methyl-CpG-binding protein MeCP2 [217]. While the mechanism of disease progression remains unclear, there is some evidence that MeCP2 mutations may impair its binding to 5hmC and affect chromatin structure and DNA methylation [218,219]. Perturbation in DNA methylation and hydroxymethylation of neural stem cells may also play a role in neurological diseases such as Alzheimer's disease. A study examining global DNA methylation and histone modifications found that brains of patients with AD had an epigenetic signature characterized by DNA hypermethylation compared with age-matched controls. CpG islands in the promoter region of *BDNF*, which encodes brain-derived neurotrophic factor (BDNF) important for regulating the survival and growth of neurons [220], were also differentially methylated compared with healthy controls [221]. Epigenetic mis-regulation

also plays a role in brain tumor formation. In a recent study comparing DNA methylation in glioma stem cells compared with NSCs, researchers found altered levels of 5mC globally, with heterogenous epigenomic marks on genes related to DNA damage response and cell proliferation [222].

29.6.3 Epigenetics in bone marrow MSCs and dysregulation giving rise to diseases

MSCs are adult stem cells that are present in various tissue compartments, including the bone marrow, adipose tissues, and umbilical cord [223]. In each of these tissue compartments, MSCs can self-renew and differentiate into various lineages present in the tissues to maintain tissue homeostasis. In general, MSCs can differentiate into osteoblasts, adipocytes, and chondrocytes, although the efficacy may vary depending on their origins [224,225]. Herein, we will focus on bone marrow MSCs (BMMSCs), which are well studied and characterized.

Like ESCs, DNA methylation plays a major role in the regulation of BMMSC self-renewal. However, there are slight differences in the regulation by these epigenetic marks. For example, in ESCs, lineage-specific promoters associated with terminal differentiation are often DNA methylated [226], but promoters of genes related to lineage-specification are often hypomethylated in BMMSCs [227]. These differences in methylation status can be calibrated by the expression levels of *Oct4* and *Nanog* [228], which are upregulated under certain in vitro conditions. Thereafter, *DNMT1* expression increases, indirectly repressing genes associated with differentiation, and in all, suppress spontaneous differentiation in BMMSCs [228]. Lineage specification can be influenced through dynamics in DNA methylation. The in vitro hypermethylation of the *Trip10* promoter pushed MSCs toward neurogenic and osteogenic lineages rather than self-renewal [229]. DNA hydroxymethylases Tet1 and Tet2 regulate methylation at the *P2rX7* promoter, such that Tet1 and Tet2 double knockouts led to hypermethylation and an accumulation of miR-297a-5p, miR-297b-5p, and miR-297c-5p, which ultimately inhibited the canonical osteogenic Runx2 signaling pathway [230]. The involvement of miRNAs in MSC differentiation is further supported by shRNA-mediated knockdowns of the miRNA processing enzymes Dicer and Drosha, which resulted in impaired osteogenesis and adipogenesis [231–234].

Histone modification dynamics are associated with MSC self-renewal and differentiation. Changes in H3K9ac, H4K5ac, H3K4me1, H3K4me3, and H3K36me3 marks accompanied adipocyte differentiation from BMMSCs at adipocyte-specific genes *Adipoq* and *Lipe*. Interestingly, H3K9ac peaks in BMMSC-derived osteoblasts largely overlapped ($\sim$ 90%) with those in BMMSCs as compared with BMMSC-derived adipocytes ($\sim$ 70%), suggesting that BMMSCs possess epigenetic signatures of their resident tissues and are predisposed to osteoblast lineages [235]. In osteogenic differentiating BMMSCs, the deposition of H3K4me3 corresponds to increased *RUNX2* transcription. In MSC-derived chondrogenic lineages, H3K4me3 and H3K9ac signals are prepatterned near the TSS of genes related to skeletal development, the extracellular matrix, and chondrogenic differentiation [236]. Besides specific histone modifications, there also exists tissue-specific mechanisms of histone modification. For example, the loss of histone demethylase *KDM5A* in BMMSCs promoted osteogenic differentiation [237]. On the other hand, the loss of the H3K4 methyltransferase *ASH1L* diverted cell fate determination toward adipogenesis via the ablation of H3K4me3 on osteogenic and chondrogenic genes (*Osx*, *Runx2*, and *Sox9*) but not on adipogenic genes (*Pparg*) [236].

A major disease arising from BMMSC dysregulation is osteoporosis. Osteoporosis is often associated with aging, with an accumulation of epigenetic insults often contributing to disease pathogenesis. In a mouse model of osteoporosis, a reduction in histone acetylation repressed Wnt signaling and led to defects in bone formation [238]. In humans, a study comparing the methylation profiles of MSCs isolated from patients with osteoporotic fractures showed differentially methylated loci linked to proliferation and osteogenic differentiation, with an unexpected increased activity at *RUNX2* and *OSX* [239]. However, when harvested and cultured in osteogenic media, the MSCs failed to differentiate, likely due to the persistence of *RUNX2* activation, which must be switched off for terminal differentiation, indicating that a dysregulation of epigenetic dynamics can potentially be pathogenic. Primary osteoporosis is also associated with mutations in pre-miR-2861, which reduced *Runx2* expression through the activation of *HDAC5* [240]. Epigenetic dysregulation of MSCs from other tissue compartments drives disease pathogenesis. For example, oculo-facio-cardio-dental (OFCD) syndrome, a rare disease characterized by eye, facial, heart, and dental abnormalities [241], is caused by mutations in *BCOR*. In MSCs isolated from the root apical papilla (dental MSC) of an OFCD patient, the *BCOR* mutation was found to increase H3K4me3 and H3K36me2, leading to aberrant reactivating transcription of target genes [242]. Evidently, epigenetic regulation in MSCs is crucial for their proper function and for health.

29.7 Conclusion

Epigenetics underpins important facets of stem cell identity and differentiation. It defines and contextualizes transcriptional programs that determine cellular identity and function. Importantly, cells that appear morphologically similar may possess substantially distinct chromatin architectures, with implications for the differentiation potential of stem cells. Moreover, as highlighted in this chapter, misregulation of epigenetic pathways in adult stem cells can lead to disease. As such, a better understanding of the epigenetic landscape and regulation of various stem cell populations has broad implications in human health.

Stem cells represent a potent modality for cell replacement therapies. The use of patient-specific iPSCs may avoid allogenic immune rejection by the recipient, although limitations persist. However, our use of iPSCs is hindered by obstacles such as immaturity and heterogeneity, in part due to inefficient reprogramming of the iPSCs and underlying residual epigenetic marks from the donor cell type. As such, iPSC-derived cell products are often contaminated with the presence of partially differentiated, self-renewing cells that may become tumorigenic. Our ability to use hPSCs for therapeutic purposes may thus be improved by a deeper understanding of epigenetic mechanisms regulating their identity and function, for better reprogramming and differentiation to desired cell types.

In adult stem cells, understanding the epigenetic mechanisms underlying disease pathogenesis will open new avenues for treatments based on epigenetic drugs, as evident in many clinical trials today. Overall, a better understanding of the changes in epigenetic landscape during development and lineage commitment, especially in the context of diseases when regulation goes awry, is pivotal for engineering cell-based products and developing therapeutic modalities that are fit for real-world use.

Acknowledgments

L.Y.L. is supported by the NMRC Open Fund-Young Individual Research Grant (OF-YIRG) OFYIRG21jun-0035. D.W.K. is supported by the NUS Research Scholarship. A.K.K.T. is supported by IMCB, A*STAR, FY2019 SingHealth Duke-NUS Surgery Academic Clinical Programme Research Support Programme Grant, Precision Medicine and Personalised Therapeutics Joint Research Grant 2019, the 2nd A*STAR-AMED Joint Grant Call 192B9002, NUHSRO/2021/035/NUSMed/04/NUS-IMCB Joint Lab/LOA, Paris-NUS 2021-06-R/UP-NUS (ANR-18-IDEX-0001), OFIRG21jun-0097, CSASI21jun-0006, MTCIRG21-0071, SDDC/FY2021/EX/93-A147, FY 2022 Interstellar Initiative Beyond grant and H22G0a0005. Figures were created with BioRender.com.

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CHAPTER

30 Noncoding RNA regulatory networks, epigenetics, and programming stem cell renewal and differentiation: implications for stem cell therapy

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30.1 Major types of stem cells

Stem cells are, by definition, capable of self-renewal and can be guided by specific signaling mechanisms to differentiate into selected cell lineages. The capacity for self-renewal, i.e., for parent stem cells to produce daughter cells that are functionally and phenotypically identical to the parent cell, means that at least theoretically, every tissue and organ with a resident population of stem cells has a nearly indefinite capacity for repair and regeneration. Moreover, the capacity of stem cells to replenish lost and damaged cells and to repair tissues makes these cells useful to harness for therapeutic purposes.

Broadly, major classes of endogenous stem cells include embryonic stem cells (ESCs), fetal stem cells, and adult (or tissue) stem cells. These cells range in their capacity for differentiation. "Totipotent" stem cells, which exist only in very early development, have the capacity to generate an entire organism. They are formed after fertilization, from early divisions of the zygote through the resulting morula, or artificially derived through transfer of somatic cell nuclei into an oocyte (Fig. 30.1). (For an interesting essay into the history of nuclear transfer and cloning, see Ref. [1]). The cells of the morula subsequently form a blastocyst, where they differentiate into two cell layers, an outer trophectoderm, which will contribute to the placenta, and an inner cell mass, which will become the embryo proper. "Pluripotent" stem cells, which are isolated from the inner cell mass, are capable of differentiating into the three germ layers: ectoderm, mesoderm, and endoderm.[1] Cells from these lineages can form an entire organism. Finally, within each fetal and adult tissue, resident stem cells continue to generate, with variable efficiency, replacement cells through the life of the tissue and organism. These cells normally exhibit restricted potential, ranging from multipotency, to bi- or even unipotency, to form tissue-specific cell types. For example, hematopoietic stem cells are multipotent because they can generate a wide variety of lymphoid, erythroid, and myeloid cell lineages. Mesenchymal stem cells are

[1]This is often referred to as "trilineage differentiation."

Epigenetics in Human Disease. https://doi.org/10.1016/B978-0-443-21863-7.00015-9
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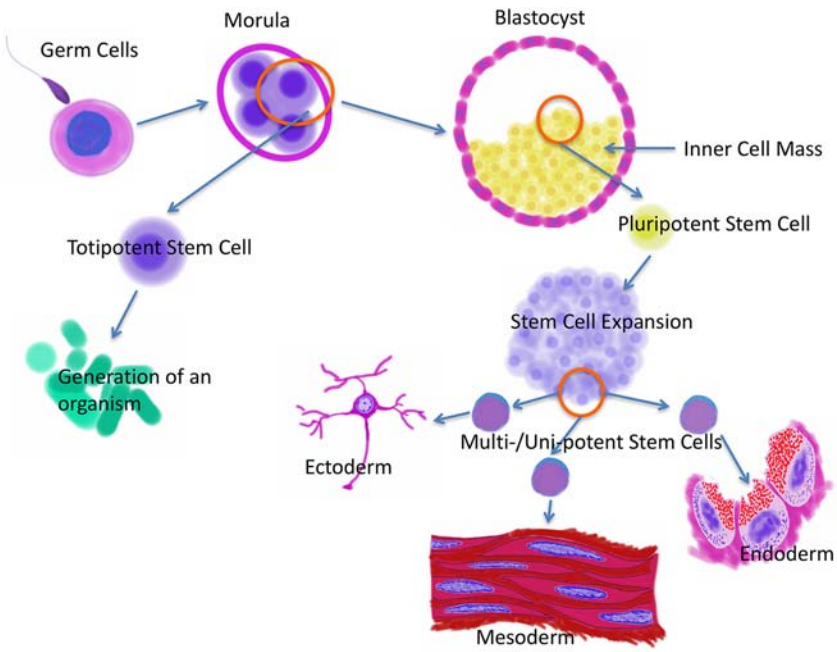

FIGURE 30.1

Schematic for the general maturation of stem cells and loss of differentiation potential.

also multipotent as they can generate osteoblasts, chondroblasts, adipocytes, and fibroblasts among a wide variety of cells. In contrast, neural stem cells exhibit more restricted capacity to differentiate into neurons and glia, and epidermal stem cells are unipotent as they only differentiate into keratinocytes. An extensive review of the types of stem cells and their properties is beyond the scope of this chapter. (The reader is referred to several curated public resources [e.g., National Institutes of Health, https://stemcells.nih.gov/info/basics]).

Conventional theories hold that stem cells exhibit a hierarchy of "stemness" with a specific stem cell at the top of the hierarchy, producing daughter progeny with limited self-renewal and differentiation capacity, as is the case with the hematopoietic system. However, studies have called into question conventional notions of a rigid stem cell hierarchy. For example, the G-protein-coupled receptor Lgr5 (Gpr49) marks a population of rapidly cycling cells within intestinal crypts that have the capacity to generate every other cell of the intestinal epithelium [2]. However, when crypt Lgr5+ cells were depleted, a rare population of slowly proliferating cells positive for Bmi1 (a member of the polycomb transcription regulatory complex), that reside above the Lgr5+ cells within the intestinal crypts, exhibit a parallel capacity to regenerate the intestinal epithelium including the Lgr5+ population [3]. Lineage tracing and transcriptional profiling suggests that these Bmi1+ cells are more restricted precursors derived from Lgr5+ cells, which under homeostatic conditions normally produce enteroendocrine and Paneth cells but are unable to generate enterocytes [4]. However, in the face of damage, as with Lgr5+ cell ablation, Bmi1+ cells can dedifferentiate into the Lgr5+ stem cells, which

can in turn produce the full complement of intestinal cells [5,6]. In subsequent sections, we will discuss research that shows that the process of stem cell maturation can be hijacked or even reversed by experimentally controlling genetic and epigenetic regulatory networks.

30.1.1 Stem cell renewal and the pluripotency transcription factor network: lessons from induced pluripotent stem cell biology

In the 1950s, John Gurdon successfully cloned a frog by transferring the nucleus of a somatic cell into an enucleated oocyte [7]. This seminal work demonstrated the possibility of reprogramming a cell's nucleus to a more nascent state of development. Subsequent work by Brigid Hogan in 1992 showed that unipotent primordial germ cells could be reprogrammed into pluripotent ESC-like cultures, termed "embryonic germ cells" [8]. Finally, in 2007, two research groups independently demonstrated that they could revert fully differentiated *human* cells to a pluripotent state by overexpressing genes known to be important for maintaining ESC pluripotency [9,10]. The research group led by Shinya Yamanaka used a combination of four transcription factors, Oct3/4 (POU5F1), Sox2, Klf4, and c-Myc (also known as Myc) to reprogram human dermal fibroblasts to express properties of ESCs (Fig. 30.2). The research group lead by James Thomson, in contrast, used an overlapping combination of transcription factors, Oct3/4, Sox2, Nanog, and Lin28, to accomplish the same purpose. Both research groups verified that the resultant induced pluripotent stem cells (iPSCs) had the capacity to differentiate into cells belonging to all three germ layers. The ability of these factors to confer pluripotency characteristics to a wide variety of differentiated somatic cell types derived from diverse tissue sources

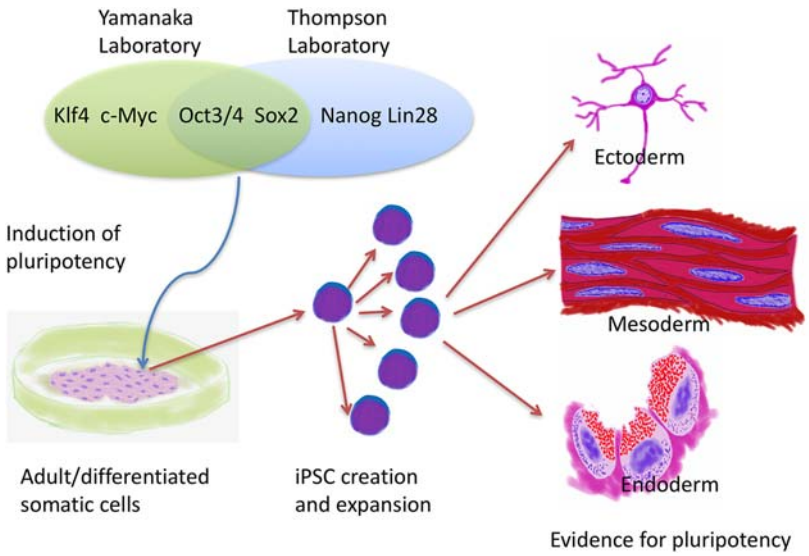

FIGURE 30.2

Schematic for the induction of pluripotency in somatic cells by the pluripotency network of transcription factors. *iPSC*, induced pluripotent stem cell.

of various species (reviewed in Refs. [11−13]) suggests that they are at the core of a highly conserved pluripotency network.

The extensive research that has followed reveals the stochastic nature of induced pluripotency. Many, if not all, cell types can be reprogrammed successfully to initiate iPSC formation; however, within each cell type only a small proportion of cells successfully complete reprogramming [14,15], indicating that cells must progress through several barriers to complete the reprogramming process. iPSC reprogramming begins with downregulation of differentiation marks, followed by increased expression of early pluripotency genes, such as *SSEA* family members [16,17], with concomitant cellular mesenchymal−epithelial transition (MET),[2] concluded by stable activation of the complete pluripotency networks. This final step involves expression of Oct4, Nanog, Sox2, and Klf4 from endogenous loci, which demonstrates that the transcription factors used for inducing pluripotency are crucial for maintaining the pluripotent state [15,16,18,19].

This pluripotency factor network has evolved functionally through vertebrate evolution, to target new downstream genes, and presumably new tissue- and species-specific stem cell renewal and differentiation programs. For example, the fibroblast growth factor 4 (*FGF4*) gene is present in vertebrates but only emerged in recent eutherian mammal evolution, as a transcriptional target of Oct3/4 and Sox2 [20,21]. FGF4 in turn mediates the transition between stem cell renewal and cell differentiation [22], implying that Oct3/4-induced, FGF4 autocrine/paracrine signaling may have evolved to limit the renewal capacity of the pluripotency transcription factor network, specifically in mammals. These types of data suggest evolutionary diversification in the control of stem cell renewal and maturation. Interestingly, the FGF family itself has expanded in numbers significantly during vertebrate and even recent mammalian development [23] and has come to play a critical role in mediating lineage specification of stem cells [24]. Although the core pluripotency network has remained relatively evolutionarily conserved (stem cells in amphibian [25] and mammalian species [9,10] make use of an overlapping complement of pluripotency factors), its interaction with lineage specification networks is evolutionarily diverse. As we will discuss below, some of this evolutionarily recent regulatory complexity is because of the emergence of novel epigenetic programs.

30.1.2 Regulatory networks and control of stem cell differentiation

Growth factors constitute an effective means for guiding multipotent stem cells down selected differentiation pathways because of their capacity to activate specific transcription factor networks (e.g., Fig. 30.3). In mouse ESCs, FGF4 signaling directs differentiation toward a neural (ectoderm) lineage by activating ERK1/2 (MAPK3/1) [26]. On the other hand, intracellular Sprouty1 (Spry1) overexpression can prevent neural lineage progression and instead promote mesoderm lineage differentiation [26]. Furthermore, within the mesoderm lineage, other growth factors, the bone morphogenetic proteins (BMPs) 4 and 7 for example, promote osteoblast differentiation by inducing the expression of the transcription factor Dlx5 [27,28]. Similarly, Wnt family members, Wnt1 and Wnt10b, promote expression of osteoblast transcription factors such as Runx2 and Dlx5, thus directing differentiation of mesenchymal stem cells toward an osteoblast lineage, while inhibiting adipocyte lineage

[2]The term "mesenchymal−epithelial transition" (MET) describes the process whereby previously invasive mesenchymal-like epithelial cells revert to a noninvasive epithelial-like state.

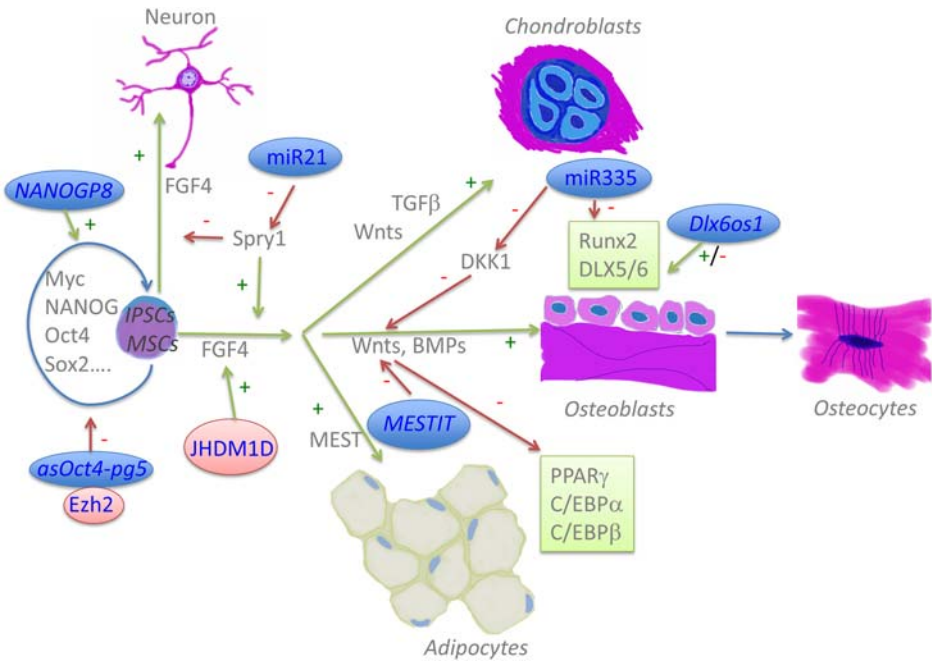

FIGURE 30.3

Schematic of the competing network of transcription factors and signaling molecules, overlaid with epigenetic/noncoding RNAs (ncRNAs) regulatory networks, that direct stem cells down specific mesoderm or ectoderm lineages. *Green boxes* indicate core transcription factors necessary for osteoblast and adipocyte lineage-specific differentiation. This schematic depicts examples of ncRNAs (*blue ovals*) and chromatin modification factors (*pink ovals*) that control the balance between signaling molecules and transcription factors, to direct stem cell differentiation.

differentiation, in part, by inhibiting adipocyte-associated transcription factors such as PPARγ, C/EBPα, and C/EBPβ [29].

Interestingly, in portions of the cranial ectomesenchyme, Dlx5/6, which can encourage osteoblast differentiation, can also induce expression of the transcription factor Hand2, which negatively regulates Dlx5/6-driven osteogenic differentiation. The expression of Hand2 results in the transformation of primitive stem cells into tissues of the tongue, whereas its absence results in a Dlx5/6-driven transformation to bone in a murine model [30]. Specific lineage commitment is, therefore, dependent on the contextual cues provided by competing signaling molecules. Moreover, a limited set of interacting secreted signaling factors and a related network of intracellular signaling cascades and transcription factors can clearly drive divergent differentiation from a common cohort of stem cells.

The core pluripotency network arguably emerged early in vertebrate evolution to support two main functions, to promote stem cell renewal while simultaneously suppressing differentiation, and appears to be largely similar from one stem cell compartment to the next. Differentiation, on the other hand, is likely to involve a diversity of maturation genes that adapt differentiating cells to specific tissues and

organs. It is therefore likely that there is a layer of cellular regulation that adapts the common pluripotency network to cell-, tissue-, and even organism-specific differentiation. Several pieces of evidence indirectly point to an intervening regulatory layer between stem cell renewal and differentiation. First, although stem cells reside in every tissue and organ, not all stem cells have an equivalent capacity to regenerate or repair tissues and organs. Even cells that share a common developmental lineage, i.e., mesenchymal stem cells, can exhibit varying differentiation potential related to their tissues of origin [31,32], suggesting the emergence of non−genetically programmed regulatory mechanisms. Second, the biology of stem cells is intimately associated with evolution and speciation. Similar types of stem cells in different organisms produce diverse body plans and exhibit divergent regenerative capacities. For example, in python, but not in humans, an episode of feeding produces a large and rapid increase in enterocytes in the small intestine [33], suggesting that stem cell renewal and maturation is remarkably attuned to the environment, but in a species-specific manner. Stem cells in amphibian [25] and mammalian species [9,10] make use of an overlapping complement of pluripotency factors, yet amphibian stem cells, but not mammalian, have the capacity to regenerate complex tissues such as limbs. Finally, not only do stem cells in older organisms exhibit diminished regenerative capacities [34], but stem cells can exhibit altered patterns of lineage commitment with age; e.g., aging mesenchymal stem cells favor adipocyte rather than osteoblast differentiation [35]. The question then is, "*why is there such diversity in stem cell differentiation potential from tissue type, speciation, and age?*" One answer to this question might lie in the existence of a new and relatively poorly understood network of regulatory mechanisms collectively termed, "epigenetics."

30.2 A brief overview of epigenetics

Epigenetics—meaning "over the genome"—was coined by the developmental biologist, Conrad Waddington, based on early observations that an environmental stimulus (heat) administered to a cohort of fruit flies, produced multigenerational alterations in wing structure without any apparent change in the fly genome [36]. Epigenetics refers to a dynamic regulatory network that is superimposed over, and therefore influences, the interpretation of genetic information contained within a cell. At the level of the organism, epigenetics serves to promote adaptation and is increasingly thought to be a major mechanism for speciation, and at the molecular level, a mechanism to control cellular differentiation and homeostasis. Epigenetic regulatory networks have been found to be critical facilitators of the successful transformation of stem cells into tissues and organs but may also serve the aberrant transformation of stem cells in cancer. A detailed and comprehensive overview of the field of epigenetics is well beyond the scope of this chapter. Excellent recent reviews have outlined the history and basic mechanisms underlying epigenetics [37,38] and detailed their relevance to tissue and organism development [39,40] and to cancer mechanisms [41]. A variety of cellular mechanisms that regulate nuclear chromatin structure and control gene transcription and translation are collectively classified as "epigenetic mechanisms."

30.2.1 DNA methylation, histone modifications, and gene imprinting

Epigenetic instructions can be encoded in direct chemical modifications to DNA or to the histones that provide structure to the DNA. Direct chemical modifications to DNA predominately occur through methylation of cytosine base residues. Historically, it was believed that DNA methyltransferases

(DNMTs) methylate cytosine residues exclusively at GC-rich gene promoters (termed "CpG [5′-cytosine-phosphate-guanine-3′] islands") to inactivate genes [42]. Recent studies have shown that a substantial amount of cytosine methylation occurs at non-CpG sites, that, along with conventional CpG methylation, can mediate gene repression by the recruitment of methyl-CpG–binding proteins and the segregation of chromosome regions into the nuclear heterochromatin [43]. Alternatively, DNA methylation within gene bodies has been positively correlated with transcript expression and is thought to be important for alternative splicing or regulating intragenic promoters, but the exact function and mechanisms of regulation are currently unknown and outside of the scope of this chapter (reviewed in Ref. [44]). Canonically, demethylation of DNA, either passively or actively by enzymes such as Tet1, is thought to increase gene transcription [45–47]. Methyl-CpG–binding proteins, such as MECP2, recruit histone-modifying complexes (see Section 30.3.1) to associated DNA, representing another level of epigenetic regulation [48].

Posttranslational histone modifications alter the compactness of nucleosomes to regulate gene expression. The methylation of histones, such as di- or trimethylation of histone H3 on lysine 4 (H3K4me2/me3) or monomethylation on lysine 27 (H3K27me1), as well as histone acetylation, result in increased activation, whereas di- and trimethylation on H3K9 and H3K27 are associated with transcriptional repression [49,50]. Histones can also be directly ejected from chromatin through ATP-dependent chromatin remodeling complexes, resulting in a loss of nucleosome structure [51]. All of these epigenetic modifications work in concert to regulate chromatin state and gene expression.

During vertebrate evolution, the emergence of mammals, particularly eutherian mammals, was accompanied by an important variation in the epigenetic program, the phenomenon of "*gene imprinting*," where one allele (either the paternal or the maternal allele) of a gene undergoes DNA methylation, and is silenced. The dominant model for transcription at these loci is that it proceeds from the remaining active allele. Frequently, the non-silenced allele exhibits posttranslational histone modifications such as trimethylation of lysine 4 (H3K4me3) that are known to facilitate transcription activation [52]. The human genome is predicted to contain at least 156 imprinted genes [53], and many of these do not overlap with the cohort of imprinted genes in the mouse [54], suggesting the likelihood of shifts in imprinting with mammalian speciation. The net effect is to decrease the gene dosage in tissues, and the emergence of this phenomenon with mammalian evolution is thought to be a mechanism for the control of fetal size. Paternal alleles are thought to promote, whereas maternal alleles are thought to constrain, fetal growth [55]. The implication of imprinting as an epigenetic phenomenon that regulates stem cells is enormous. Because of their capacity to control tissue growth [56], it is likely that imprinted genes play an important role in stem cell maturation [57]. The species variation in gene imprinting suggests that the epigenetic controls over stem cell renewal and maturation are likely to be species-specific. Moreover, gene imprinting may vary as a function of the state of cellular differentiation. For example, neurons of the adult mouse brain have been shown to exhibit monoallelic expression of the Angelman syndrome locus, *Ube3a*, whereas glial fibrillary acidic protein (GFAP)–positive cells of the ventricular zone have been found to exhibit biallelic expression from this locus [58]. As GFAP-positive cells adjacent to the lateral ventricles of the brain are presumptive neural stem cells [59], these data suggest that the gene dosage of *Ube3a* is decreased during the differentiation of neural stem cells by epigenetic mechanisms. Collectively, these data suggest that the epigenetic programming of stem cells may vary as a function of both species and tissue of origin, and that the replication of tissue- and species-specific epigenetic programs will be critical for the successful therapeutic manipulation of stem cells.

30.2.2 Epigenetics of noncoding RNAs

In the abstract of a 2007 article [60], John Mattick proposed that, "*the epigenetic trajectories of differentiation and development are primarily programmed by feed-forward* [noncoding][3] *RNA regulatory networks and that most of the information required for multicellular development is embedded in these networks.*" Because "differentiation" and "development" programs implicitly involve the maturation of stem cells, the hypothesis advanced by Mattick implies that non-protein−coding RNAs (ncRNAs) are a significant source of epigenetic control over stem cell maturation programs.

Sequencing the human genome showed unexpectedly that the human genome contains a surprisingly small number of protein-coding genes, i.e., just 19,804 (The Ensembl database [http://www.ensembl.org/], GRCh38.p13 [Genome Reference Consortium Human Build 38] [61]). Moreover, the number of protein-coding genes in humans is less than those annotated in mouse (22,213), zebrafish (25,545), and the invertebrate, *Caenorhabditis elegans* (19,997). In humans, protein-coding genes are less than 8% of total gene transcripts, while in *C. elegans* they are 33% of gene transcripts. Clearly the protein-coding gene content of animal chromosomes does not change dramatically with vertebrate and mammalian evolution, and consequently, cannot readily explain the complexity of stem cell programs that generate the human body plan. However, recent evidence indicates that other products of genome transcription, particularly ncRNAs, do exhibit increased representation within genomes with increasing evolutionary complexity [60]. A 2007 report of the ENCODE (Encyclopedia of DNA Elements) project suggested that the human genome is "*pervasively transcribed, such that the majority of its bases are associated with at least one primary transcript*" [62]. This paper showed that the human genome is filled with large numbers of transcription start sites that code for ncRNAs and that regions of the genome that had hitherto been thought of as "silent," were in fact, transcriptionally active. It is likely that, in terms of sheer numbers (25,134 ncRNA-encoding genes and 15,240 pseudogenes, which are presumptive ncRNAs, have been annotated to date in GRCh38.p13), ncRNAs will dwarf the numbers of protein-coding genes. RNA molecules can participate in a variety of cell functions because they exhibit two important functions. Firstly, their primary nucleotide sequence complementarity can give RNA molecules the capacity to bind other RNA and DNA molecules with exquisite specificity. Secondly, RNAs can fold into a variety of functional shapes in the presence of helicase chaperones to participate in RNA−protein complexes [63]. Therefore, ncRNAs can effectively serve as adapter molecules to target regulatory protein complexes to DNA or other RNAs, with important consequences for stem cell renewal.

The family of ncRNAs ranges in size from small, microRNAs (miRNAs) and small-interfering RNAs (siRNAs, 17−24 nucleotides), and piwi-interacting RNAs (piRNAs, 25−30 nucleotides), to extremely long ncRNAs (lncRNAs), such as the 19.3-kb X chromosome inactivation factor, *XIST* [64]. Both lncRNAs (reviewed in Ref. [65]) and short ncRNAs (reviewed in Ref. [66]) have roles in chromatin reorganization, and consequently, epigenetic programming.

The function of miRNAs as an epigenetic regulator has largely been through their functions as inhibitor of protein translation, by binding the 3′-untranslated region of mRNAs, recruiting argonaute (AGO) proteins and the RNA-induced silencing complex (RISC), and initiating mRNA degradation [67]. However, recent deep-sequencing data suggest an additional layer of epigenetic regulation through nuclear actions, as substantial numbers of processed miRNAs traffic back into the nucleus

[3]Author inserted text in brackets.

([68], reviewed in Ref. [69]). A subset of miRNAs are regularly localized to the nucleus, containing nuclear localization elements [70,71], while nuclear localization/expression of other miRNAs can be increased in response to cellular stress [72–74] or decreased in response to cellular events, as has been seen over neuronal development [75]. Nuclear-localized miRNAs can bind specifically to target proteins, as aptamers, to interfere with protein function [73], destabilize RNA transcripts following transcription [76], including lncRNAs such as the primary transcripts for other miRNAs [77], and bind promoter sequences resulting in transcriptional gene activation [78–80] or transcriptional gene silencing [81]. miRNA-induced transcriptional gene activation and silencing occur largely through AGO-dependent recruitment of histone epigenetic modification machinery ([78,81] reviewed in Ref. [66]) and is part of complex milieu of mechanisms used by miRNAs to control stem cell pluripotency and differentiation (see Section 30.3.2).

lncRNAs are also multifunctional with respect to how they modify gene expression. This is due to their ability to both bind DNA and RNA with their primary sequence and through their two- and three-dimensional folding patterns that allow these molecules to also bind proteins. In the cytoplasm, lncRNAs regulate mRNA stability and turnover through direct binding to mRNAs transcripts, where they can block miRNA binding sites to stabilize mRNA expression, or by binding miRNAs and preventing the miRNAs from binding their target mRNAs (reviewed in Ref. [82]). lncRNAs bind RNA binding proteins (RBPs) to prevent RBP association with mRNAs or to recruit RBPs to mRNAs to facilitate stabilization, degradation, or translation of mRNAs (reviewed in Ref. [82]). RBPs are not the only proteins that can bind lncRNAs. For example, a number of nuclear receptors, including sex steroid receptors, are co-activated by stem loop structures of the lncRNA *SRA* binding as receptor ligands (reviewed in Ref. [83]). lncRNAs also bind to and coordinate the activity of epigenetic modification machinery at target gene loci. The lncRNA *HOTAIR* binds >800 genomic loci, a majority of which are associated with H3K27me3 [84]. *HOTAIR* acts as a scaffold to coordinate H3K4 demethylation and H3K27 methylation through the association of polycomb repressive complex 2 (mediates H3K27 methylation) with the 5′ end of *HOTAIR* and lysine demethylase 1 (mediates H3K4 demethylation) with the 3′ end of *HOTAIR* [85]. lncRNAs can also provide scaffolds for chromatin loops and bring distal regulatory elements near active transcription loci [86,87].

RNAs can also be epigenetically modified to impact function, with more than 100 types of modifications identified [88]. Pseudo-uridylation, i.e., the attachment of a uridine to the lncRNA structure through a carbon-carbon bond, of *SRA* is required for its activity as a co-activator of nuclear steroid receptors and can be targeted to reduce estrogen and androgen receptor signaling [89]. Methylation of adenosine (N6-methyl-adenosine) is the most common modification to lncRNAs (reviewed in Ref. [90]) and has been shown to be integral for the relationship of lncRNA *linc-1281* and stem cell differentiation (see Section 30.3.1.2).

30.3 Epigenetic programming of stem cells

30.3.1 Epigenetic programming, early development, and the core pluripotency transcription factor network

The ability to successfully reprogram fully differentiated somatic cells toward totipotent or pluripotent states using transfer of somatic cell nuclei into oocytes or by overexpressing core pluripotency

transcription factors (Section 30.1) indicates that differentiation and dedifferentiation impose reversible epigenetic changes rather than irreversible genetic changes on the genome. Characterization of epigenetic states during embryogenesis, as well as recent studies with cultured ESCs and iPSCs, has shed light onto the complex interplay between core pluripotency factors, epigenetics, and ncRNAs (Fig. 30.4).

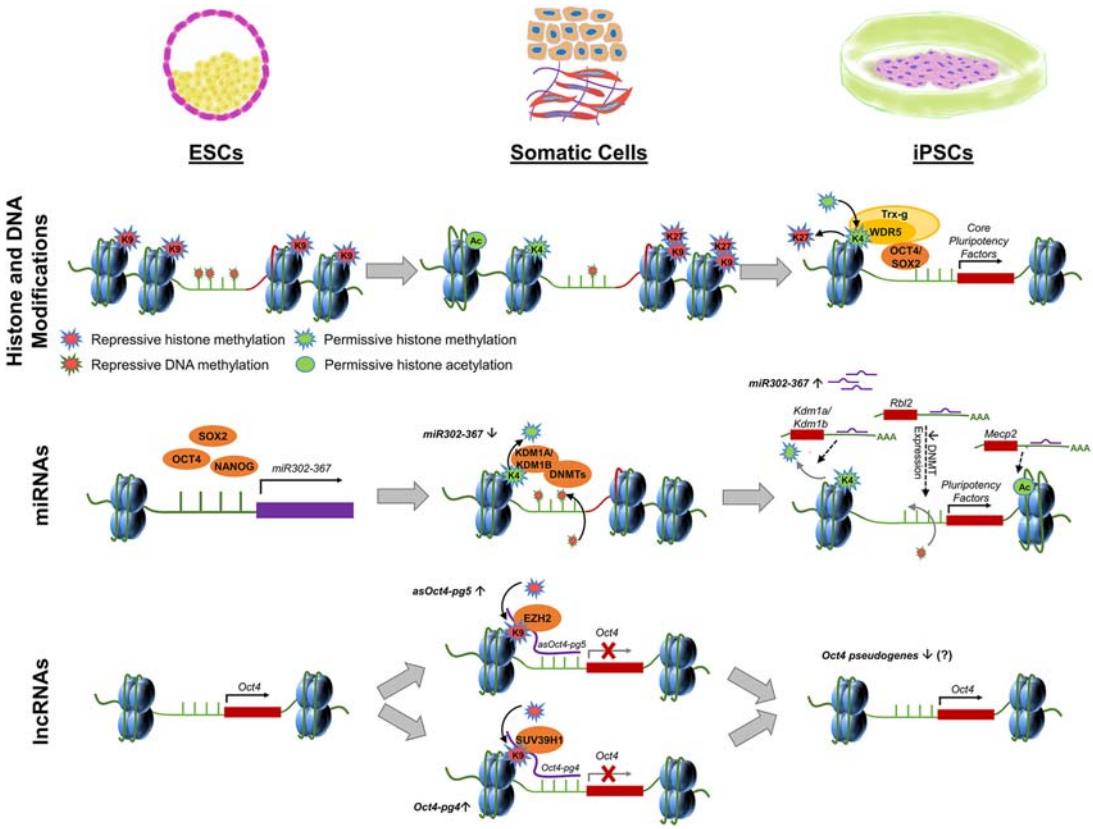

FIGURE 30.4

Interplay between epigenetic states and stem cell potential. In ESCs, histone methylation at H3K9 and DNA methylation represses expression of the maternal genome. In somatic cells, open (eu-) and closed (hetero-) chromatin compete to regulate gene expression in a complex histone code. During reprogramming, epigenetic modifiers are recruited by pluripotency factors to add permissive and remove nonpermissive histone modifications and pluripotency genes are hypomethylated to allow for expression. miRNAs and lncRNAs also regulate pluripotency gene, with increased expression of miR302-367 miRNAs facilitating expression of pluripotency factors during iPSC reprogramming while Oct4 lncRNAs inhibit expression of the pluripotency gene, *Oct4*. *DNMTs*, DNA methyltransferases; *ESCs*, embryonic stem cells; *iPSCs*, induced pluripotent stem cells; *lncRNAs*, long noncoding RNA; *miRNAs*, microRNAs. Purple denotes ncRNA, red denotes protein coding genes. For clarity, only one strand of DNA is shown.

30.3.1.1 Interaction of histone and DNA epigenetic modifications in pluripotency

Rapid and massive epigenetic changes occur during early development [91]. In preimplantation blastocysts, there is active global demethylation of the paternal genome by the Tet3 demethylase and passive demethylation of the maternal genome, which is thought to unveil the transcriptional potential of the entire genome allowing for totipotency and pluripotency [92–96]. Coincident with active demethylation of the zygotic paternal genome is the replacement of paternal protamines (which substitute for histones in spermatogenesis) with maternally inherited histones [91,97]. These newly incorporated histones initially undergo acetylation, favoring an open chromatin state, with histone methylation occurring later. The zygotic maternal genome, on the other hand, is resistant to histone modifications and nucleosome remodeling throughout early embryogenesis, explaining the relative permissiveness of the male genome to transcription from the late zygote stage [98]. The retention of posttranslational histone modifications in the maternal genome, particularly repressive H3K9me2 marks, is believed to protect it from active DNA demethylation by recruiting Dppa3, preventing Tet3 binding [99]. Additionally, reestablishment of DNA methylation in the pluripotent inner cell mass is thought to occur via H3K9me3-mediated recruitment of Dnmt3a, whereas permissive H3K4me3 marks inhibit Dnmt3-associated DNA methylation [100,101]. These mechanisms interact with other chromatin remodeling mechanisms, including the five known mammalian ATP-dependent chromatin remodeling complexes: BAF, ISWI, CHD, INO90, and SWR1. All of these complexes are involved in nucleosome remodeling and in the maintenance of pluripotency [51,102]. Actions of the BAF complex have been particularly studied within ESCs and iPSCs because of its vital role in early embryogenesis. The Brg1 ATPase serves as the core catalytic subunit of the 15 component BAF complex [103,104]. Deletion of Brg1 or the Baf155 or Baf47 subunits independently results in preimplantation embryonic failure because of inability of totipotent cells to form the trophectoderm or the pluripotent inner cell mass [105–107]. Likewise, in vitro studies show that loss of Brg1 and Baf155 impairs iPSC and ESC self-renewal and results in aberrant premature differentiation, whereas overexpression of these proteins improves iPSC reprogramming efficiency [103,108]. An additional chromatin architecture remodeling ATPase, SMCHD1, is expressed in the mouse maternal oocyte and its expression prevents precocious expression of the anterior-posterior patterning *Hox* genes following implantation, likely by chromosomal remodeling at repressive H3K27me3 loci in the *Hox* genes loci [109].

Coordination of epigenetic mechanisms to regulate pluripotency and differentiation can be further seen in the genetic loci of core pluripotency factors, or "Yamanaka factors" during iPSC induction. Work in iPSCs has shown that demethylation of promoters of core pluripotency factors, including *Nanog*, *Oct4*, and *Zfp42,* during late stages of reprogramming is crucial for successful iPSC induction [110], and that incomplete demethylation of these loci at later stages of pluripotency induction underlies incomplete human iPSC reprogramming [111]. Similarly, members of the core pluripotency network, *Sox2*, *Klf4*, and *c-Myc*, as well as developmentally regulated genes, gain transcriptionally permissive H3K4me2 at their promoters and enhancers [112,113]. Furthermore, the loci that gain H3K4me2 are enriched for targets of Oct4 and Sox2. This suggests that either histones at these loci first acquire H3K4me2, allowing for Oct4 and Sox2 binding, or that the initial binding of Oct4 and Sox2 to DNA recruits chromatin-modifying complexes. The latter option seems more likely given that Oct4, Sox2, and Klf4 bind preferentially to closed chromatin domains (indicated by resistance to DNAse I-induced degradation) immediately after induction of pluripotency and Oct4 and Sox2 proteins

associate with Wdr5, a core component of the H3K4me2/3 methylating trithorax-group protein complex (Trx-g), within human iPSCs and mouse ESCs (Fig. 30.4) [114,115]. Ultimately, these same loci that gain permissive H3K4 methylation also lose repressive H3K27me3 marks, indicating that these histone posttranslational modifications set the stage for transcription of these loci during later stages of reprogramming [113,116]. Analysis of the *Oct4* and *Nanog* loci reveals hypomethylated, nucleosome-depleted enhancer and promoter regions for both genes in human ESCs [117]. Oct4 and Sox2 bind these nucleosome-depleted regions (NDRs), promoting transcription. Interestingly, during ESC differentiation, NDRs are lost from the *Oct4* and *Nanog* promoter and enhancer regions. Loss of NDRs at these loci allows for immediate, but reversible, repression of Oct4 and Nanog expression, whereas the subsequent gradual gain of CpG methylation can result in long-term repression. The temporal concordance of NDR loss with decreased Oct4 expression during human ESC differentiation suggests that Oct4 acts to maintain the NDRs and therefore can regulate its own expression in a feed-forward mechanism. Indeed, knockdown of Oct4 recapitulates loss of NDRs seen during ESC differentiation suggesting that Oct4 may be required for the formation or maintenance of NDRs [117]. These findings raise the question of "*what specific chromatin remodeling complex(es) interact with Oct4 to form or maintain the NDRs?*"

Chromatin remodeling BAF complex has been implicated in Oct4 maintenance of NDRs. Knockdown of Brg1 and Baf155, or Baf170 in human ESCs, reduces Oct4 levels, suggesting that the BAF complex is the chromatin remodeler that interacts with Oct4 to maintain NDRs at the *Oct4* and *Nanog* loci [103,108]. In support of this view, immunoprecipitation assays reveal BAF complex components predominately associate with pluripotency factors, particularly Oct4, within murine ESCs, and that these components are enriched at genetic elements predicted to be bound by Oct4 [118]. No components of any other chromatin remodeling complex were enriched at these Oct4-associated genetic elements. Additionally, deletion of either Oct4 and Brg1 in murine pluripotent cell lines reduces chromatin accessibility at Oct4 target sites and reduces the binding of other pluripotency factors, including Nanog and Sox2 [119]. Overexpression of Baf155 and Brg1 in iPSCs accelerated demethylation and increased deposition of transcriptionally permissive H3K4me3 marks at promoters of pluripotency factor genes, including *Oct4* and *Tcf3* [51,118]. Taken together, these data suggest that Oct4 interacts with the BAF complex, guiding it to genetic loci where it creates NDRs, allowing other pluripotency factors to bind, and ultimately promote transcription.

The specific factors with which the BAF complex interacts to regulate DNA methylation and posttranslational histone modifications to maintain NDRs are not well understood. In mouse embryonic stem cells, Yin Yang 1 (YY1) interacts with both Oct4 and the BAF complex [120]. While promoters occupied by YY1 were largely associated with transcriptionally permissive H3K4me3 (~75%), co-occupation of promoters with Smarca4 further increased rates of H3K4me3 (~80%) with 95% of genes with co-occupied promoters containing H3K4me3. The transcriptional activation role is discrete from the known role of YY1 to mediate transcriptional silencing. For example, in differentiating murine myoblasts, pro-differentiation genes are silenced by the binding of YY1 which allows for the binding of Ezh2, a mediator of transcriptionally repressive H3K27me3, and Hdac1, a histone deacetylase [121]. Therefore, YY1 may have an Ezh2-independent function when interacting with Oct4 and the BAF complex to maintain stem cell pluripotency. Interestingly, the BAF complex is known to interact with a number of lncRNAs which may play a role in recruiting the necessary additional factors chromatin remodeling [122].

30.3.1.2 Role of ncRNAs in coordinating pluripotency-associated epigenetic mechanisms

The interactions of the BAF complex with the lncRNA *XIST* provides an example of lncRNA regulation of gene expression and additional epigenetic regulators. X chromosome inactivation is the process by which X chromosome expression is controlled and equalized across the sexes. In the process of X chromosome inactivation, one randomly selected X chromosome remains active while additional X chromosomes (e.g., one for both sex chromosome-normal XX females, and for males with Klinefelter syndrome, with a karyotype of 47,XXY [123]) are condensed into heterochromatin or a "Barr body" [124]. Condensation of X chromosomes relies on the lncRNA *Xist* to coordinate epigenetic modifications on the chromosome to mediate condensation as well as the expression of an antisense lncRNA transcript, *Tsix*, to protect the active X chromosome from condensation [125]. The BAF complex is integral for is X-chromosome inactivation by *Xist*. Recently, using a mouse ESC model in which each X chromosome is fluorescently tagged either red or green to track X chromosome inactivation, knockdown of BAF complex members *Smarca4* or *Smarcc1* was found to impair X chromosome inactivation [126]. The BAF complex was shown to be critical for nucleosome remodeling to open promoters and allow the establishment of DNA methylation to inactivate a X chromosome. Disruption to appropriate *Xist* expression and X chromosome inactivation may be a mechanism underlying the impact of developmental teratogens, such as prenatal alcohol exposure, on stem cells. We found, in the mouse developing cortex, *Xist* expression is decreased following prenatal alcohol exposure, with increased expression of a number of genes located in inactivated regions of the X chromosome in female cells [127]. This change in *Xist* expression was particularly impactful in the neural stem cell populations, as *Xist* was shown to be a hub gene, likely coordinating altered expression for genes across the transcriptome, including transcripts from autosomes. We have also found in cell culture models of neural stem cells, that developmental alcohol exposure induces the aberrant maturation of the BAF complex following ethanol exposure [128]. Given the known link between the BAF complex and successful X chromosome inactivation, these data collectively point to a mechanism whereby environmental perturbations may result in the long-term reprogramming of stem cell behavior specifically in females, resulting in sex-specific developmental outcomes.

A further analysis of Myc function is also instructive for assessing the contribution of lncRNAs to stem cell programming. Myc directly binds to, and strongly represses, the transcription of *Gata6*, a transcription factor that promotes endodermal differentiation of stem cells. However, while the expression of the *Gata6* mRNA transcript is repressed during stem cell renewal, not all transcription at the *Gata6* gene locus is suppressed. The mouse *Gata6* gene locus is also the source of a lncRNA [129], termed *Gata6bt* (for Gata6 bidirectional transcript), that is transcribed in the antisense orientation relative to Gata6. The expression of *Gata6bt* is inversely correlated with the mRNA transcript for Gata6, being highly expressed in stem cells and suppressed during differentiation. A non-homologous lncRNA for GATA6 identified in the human, *GATA6-AS1*, has high expression during endoderm differentiation from ESCs, positively regulates GATA6 expression, and loss of *GATA6-AS1* impairs endoderm differentiation [130]. *Gata6bt*, like Myc, may serve as a negative regulator of Gata6 transcription [129], to maintain stem cell pluripotency while *GATA6-AS1* may serve as a positive regulator of *GATA6* transcription [130]. Both GATA6 lncRNAs, like other large antisense ncRNAs (*HOTAIR* for example [85]), may regulate chromatin state by serving as scaffolds for the assembly of histone modification complexes.

A study by Guttman et al. showed that a large network of long intergenic ncRNAs (termed lincRNAs) maintain the pluripotency of mouse stem cells [131]. Guttman et al. identified at least 26 lincRNAs that positively regulated the activity of Oct4 and Nanog. Using Oct4 and Nanog promoters linked to a luciferase reporter, the authors demonstrated that suppression of any one of these lincRNAs resulted in significant suppression of promoter activity for these pluripotency factors. Interestingly, these data imply that each of the 26 lincRNAs exhibits independent, nonredundant control over the Oct4 and Nanog promoters and suggests that all these lincRNAs are required to maintain these components of the minimal pluripotency transcription factor network. The promoter of one of these lincRNAs, *linc-1552*, is bound by Oct4, Klf4, Nanog, and c-Myc and is itself crucial for promoting Oct4 and Nanog expression, suggesting a feed-forward regulatory interaction. The recruitment of RNA-binding proteins by lncRNAs can lead to epigenetic reprogramming that alters the fate of stem cells [132]. Interestingly, *linc-1552* also partners with the WDR5 protein, which we described previously to be a core component of the Trx-g complex [133]. This evidence indicates that *linc-1552*, in addition to other lncRNAs, binds and stabilizes WDR5, guiding the Trx-g complex to the loci of pluripotency genes in ESCs where it deposits H3K4me2/3, promoting transcription.

Some of these lncRNAs that regulate expression of pluripotency factors are transcribed from pseudogenes that arise from imperfect duplications of genes encoding pluripotency factors. *In silico* screens have found that mammalian genomes are specifically enriched in pseudogenes for Oct4. The human genome contains eight pseudogenes for Oct3/4, compared with a relative paucity of pseudogenes for other non-pluripotency-related transcription factors, such as the two confirmed pseudogenes for Nanog [134−136]. The Oct4 pseudogene family has been recently found to exert complex and mutually interdependent epigenetic regulation of the Oct4 promoter. *Oct4-pseudogene 5*, for example, generates an antisense transcript (*asOct4-pg5*), that has been shown in a human breast cancer cell line to serve as a negative regulator of Oct4, in part by recruiting the histone methyl transferase, Ezh2 to the Oct3/4 promoter and increasing H3K9me2 and H3K9me3 histone methylation marks [137]. *Oct4-pseudogene 4* (*Oct4-pg4*) was also shown to repress Oct4 expression [137]. *Oct4-pg4* binds to the Oct4 promoter to recruit the histone-lysine N-methyltransferase, SUV39H1, which catalyzes repressive H3K9 methylation to suppress Oct4 expression [135] (Figs. 30.3 and 30.4). Likewise, overexpression of *Oct4-pg4* causes premature differentiation in mouse ESCs [135]. Similarly, in mouse neural stem cells, *Oct4-pseudogene 9* (*Oct4-pg9*) functionally antagonizes *Oct4*, an outcome which was validated by single cell RNAseq in which only 2% of cells expressing either *Oct4-pseudogene 9* (*Oct4-pg9*) or *Oct4,* co-express the other transcript [138]. Taken together, these data lend further support to Mattick's hypothesis, which states that extensive ncRNA regulatory networks [60] are likely to control pluripotency and stem cell renewal.

miRNAs are another group of ncRNAs that are crucial for early development as evidenced by embryonic lethality in mice with knockout of miRNA processing enzymes, DGCR8 and Dicer [139,140]. Similarly, deletion or knockdown of DGCR8 or Dicer in ESCs impairs proliferation, expression of pluripotency markers, and differentiation capacity [141−143]. Profiling of miRNAs reveals the members of the miR17-92, miR302-367, and miR290-295 clusters to be highly expressed during early stages of induced stem cell programming and within ESCs, suggesting a relationship with the pluripotency network [143−145]. Indeed, Oct4, Sox2, and Nanog directly bind to promoters for both miR302-367 and miR290-295 clusters, promoting their expression in mouse and human ESCs (Fig. 30.4) [146,147]. In turn, increased expression of both miR290-295 and miR302-367 clusters enhances reprogramming efficiency, with expression of miR302-367 cluster miRNAs capable of

independently inducing pluripotency in mouse and human cells without co-transfection of core pluripotency factors [148–150]. Within these clusters, mir302, miR291, miR294, and miR295 share identical seed sequences, suggesting that they promote reprogramming through regulating overlapping targets. These miRNAs target a number of cell cycle regulators, including the G1/S regulator Cyclin D1, to promote transition and cell cycle progression (reviewed in Ref. [151]). These miRNAs also target the lysine demethylases KDM1A and KDM1B, which normally stabilize DNMTs, representing another avenue through which they promote demethylation at promoters of *OCT4*, *SOX2*, and other loci during reprogramming [152]. Aside from their role in DNA methylation, KDM1A and KDM1B also demethylate H3K4me1/2, which we previously stated are found at loci targeted by Oct4, Sox2, and other pluripotency factors. Thus, repression of KDM1A and KDM1B by these miRNAs stabilizes H3K4me1/2. In addition to H3K4me1/2, these miRNAs also stabilize histone acetylation through repression of MECP2, which normally binds methylated DNA to recruit histone deacetylases (Fig. 30.4) [152,153].

miRNAs are also important for cellular reprogramming and iPSC induction. As described earlier (Section 30.1.1), MET is an intermediate stage of reprogramming. The miR200 family promotes MET by repressing members of the epithelial–mesenchymal transition (EMT) pathway, which is the conversion of epithelial cells to a mesenchymal-like state [154–156]. Accordingly, miR200 family members are increased during later stages of reprogramming. This reactivation of miR200 expression during reprogramming is dependent on Tet-mediated demethylation of miR200 family promoters. miR200 is a critical downstream mediator of Tet's actions during reprogramming as the severely attenuated reprogramming efficiency in Tet knockout cells was rescued by overexpression of miR200 family members [157].

Taken together, miRNAs that are induced early in pluripotency create a euchromatic state characterized by transcriptionally permissive histone modifications and DNA hypomethylation (Fig. 30.4).

30.3.2 Epigenetic programming, noncoding RNAs, and early stem cell differentiation programs

In contrast to the previously mentioned miRNAs, let-7 miRNA family expression is low in murine pluripotent cells but increases during differentiation [158,159]. The relationship between let-7 and the pluripotency network is relatively straightforward. Lin28, a member of the pluripotency network, and one of the factors used for somatic cell reprogramming by James Thomson, has a double negative feedback relationship with let-7. Lin28 is decreased during differentiation by direct binding of let-7 to *Lin28* mRNA resulting in *Lin28* degradation [160]. Yet, during stem cell maintenance, Lin28 binds pri-let-7 and interferes with its maturation [143,159]. In turn, reduction of let-7 prevents the destabilization of two transcription factors, HMGA2 and ARID3B, thereby promoting the transcription of *Oct4* and *Sox2* [161]. c-Myc, a reprogramming factor, transactivates the *Lin28* promoter to further facilitate let-7 repression [145,162]. Inhibitors of let-7 can substitute for c-Myc to increase reprogramming efficiency, suggesting that much of c-Myc's role in reprogramming is through modulating miRNA expression [159].

Indeed, c-Myc also promotes cell proliferation, in part, by binding to the common promoter for miR17-5p and miR20a, in the miR17-92 miRNA cluster (Fig. 30.5) [163]. miR20a represses translation of retinoblastoma-like protein 2 (Rbl2) [164], and, as Rbl2 and other members of the retinoblastoma family increase DNA methylation by promoting expression of DNMTs [165–167], miR20a

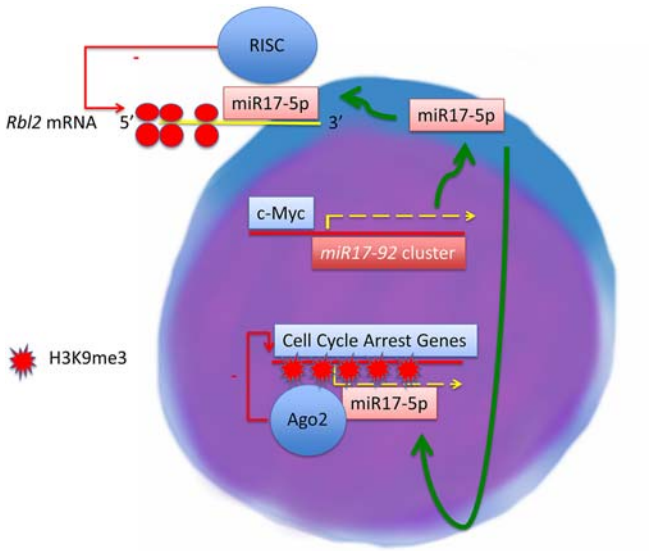

FIGURE 30.5

Example of interactions between a pluripotency factor, Myc, and the non-coding RNA/epigenetics network. Myc promotes the expression of the miR17-92 miRNA cluster, which is processed and transported to the cytoplasm, and directs translational repression of cell cycle arrest genes by the RNA-induced silencing complex (RISC). However, miRNAs are also localized to the nucleus, where they can bind to nascent mRNA transcripts and recruit Argonaut proteins (Ago2) and promote histone methylation marks (e.g., H3K9me3) that result in suppressed transcription of cell cycle arrest proteins. Collectively, these factors contribute to Myc-mediated epigenetic control over stem cell renewal and maintenance of pluripotency.

might interfere with DNA methylation. However, miR20a and miR17-5p (another member of the miR17-92 cluster), also translocate to the nucleus, and recruit AGO complexes to promoters of target genes (in this case, a cyclin-dependent kinase inhibitor CDKN2B/INK4B), resulting in increased H3K9me3, a repressive epigenetic mark, and promote the formation of heterochromatin [168]. These apparently contradictory data suggest that Myc-mediated epigenetic programming is complex. The association between Myc and the miR17-92 cluster also provides a mechanistic hypothesis that explains the observed diversification in DNA methylation patterns that reshape the nuclear heterochromatin landscape during cellular reprogramming whereby some genes gain repressive epigenetic marks and others lose them [169].

Additional contradictory regulation of stem cell proliferation and differentiation occurs with let-7 and its interactions with the lncRNAs like *linc-1281*. In mESCs, *linc-1281* gradually decreases during differentiation in a manner similar to *Oct4* and *Nanog*. Intriguingly, through m^6A methylation, *linc-1281* can promote differentiation of mESCs, likely by regulating timing of expression for the let-7/Lin28 axis. *linc-1281* m^6A allows for the binding and regulation of let-7 expression, as loss of m^6A or *linc-1281* expression can increase let-7 expression and decrease Lin28 expression. These let-7/Lin28 expression changes have been considered pro-differentiation, but when these occur with loss

of *linc-1281*, differentiation is decreased, suggesting a role for *linc-1281* m^6A as a timing regulator of differentiation through the let-7/Lin28 axis [170].

Given their integral roles in regulating pluripotency and exit from pluripotency, it follows that interactions between ncRNAs and other epigenetic mechanisms also regulate early stem cell maturation. The *Mest/Peg (Paternally Expressed Gene)-1* imprinted locus provides an example of how these interactions between epigenetic mechanisms gate stem cell differentiation. Analysis of the *Peg-1* locus (human chromosome 7:130,125,573−130,146,478) shows the presence of two CpG islands, coinciding with transcription factor−binding sites and constituting two presumptive promoters. Interestingly, these regions, particularly at the second CpG island, also coincide with a high density of activating acetylation (H3K27Ac) and methylation (H3K4me3 and H3K4me1) marks on histones, suggesting differential activation of maternal and paternal alleles. While the exon structure of MEST appears to be conserved through vertebrate evolution, two ncRNA transcripts that arise from the *Mest/Peg* locus, a miRNA (miR335) and a long antisense transcript overlapping the presumptive promoter region (*MESTIT1*), arose late in evolution, in eutherian mammals and primates, respectively. Like MEST, *MESTIT1* also exhibits monoallelic expression from the paternal allele and may recruit heterochromatin marks to the parental gene. We previously observed that the MEST intronic miRNA, miR335, is expressed at moderate levels in mouse neural stem cells and that miR335 knockdown results in increased neural stem cell proliferation [171]. The MEST/miR335 locus also regulates differentiation of mesenchymal stem cells, by interacting with core differentiation signaling programs. We previously discussed evidence, for example, that Wnt signaling directs mesenchymal stem cells toward osteoblast-specific differentiation and inhibits adipocyte differentiation. MEST blocks Wnt signaling by inhibiting posttranslational processing of the Wnt coreceptor LRP6, and knockdown of MEST prevents adipogenesis and results in reduced expression of the adipogenic factors C/EBPα and PPARγ in a human cell line (Fig. 30.3) [172]. The MEST intronic miRNA on the other hand, is highly expressed in human bone marrow−derived mesenchymal stem cells compared with differentiated cells belonging to the mesoderm lineage, and its expression decreases during osteoblast and adipocyte differentiation [173]. miR335 promotes Wnt signaling by suppressing the expression of the Wnt antagonist DKK1 [174]. However, miR335 also acts as a direct negative regulator of Runx2, a factor required for osteogenic differentiation [173]. Collectively, these data suggest that osteoblast differentiation from mesenchymal stem cells is facilitated by careful temporal choreography of gene expression from the imprinted *MEST/miR335* locus.

Numerous other ncRNAs have been found to regulate neural development. We previously presented evidence for FGF4 as an example of an early differentiation gene that directed stem cells toward a neural ectoderm lineage in preference to mesoderm or endoderm lineages. Spry1, the negative regulator of FGF4 signaling, is a target of the miRNA miR21 [175], which is highly expressed in neural lineage-committed stem cells [171]. Therefore, in the presence of miR21, FGF4-programmed stem cells will preferentially commit to neural lineages (Fig. 30.3). Later during neural differentiation, another miRNA, miR153, function as a gate keeper for neural stem cells by inhibiting neurogenesis. Overexpression of miR153 has been shown to inhibit neurogenesis during mitogen withdrawal−induced differentiation of ex vivo mouse neural stem cell cultures [176−178]. Additionally, when miR153 expression is increased during early murine cortical development in vivo, developing neurons show decreased doublecortin, a marker of neuronal maturation [179−181]. These effects are thought to be due, in part, to miR153 targeting NFIA and NFIB [178], two members of the nuclear factor 1

transcription factor family, which are known to promote neurogenesis and cortical development [182,183].

Upregulation of two miRNAs, miR124 and miR9, has also been implicated in promoting neurogenesis. Both the miRNAs increase in expression during early development, and while the expression of miR124 continues to increase until adulthood, the expression of miR9 peaks during a second-trimester equivalent period, during which high rates of neurogenesis occur [184–187]. miR9 and miR124 are expressed in neurogenic regions during mouse development with miR9 predominantly localized to the ventricular zone and miR124 in the developing telencephalon [188–190]. The role of these miRNAs in neurogenesis is highly conserved as loss of miR9 expression in early zebra fish development results in microcephaly and dysregulation of head structure, and these impacts on neurodevelopment are consistent with decreased production of early neurons and reduced cortical thickness seen in a mouse model [191,192]. miR9, along with its passenger strand–derived miRNA, miR9*, and miR124 regulate neurogenesis and cortical growth, in part, by governing maturation of the BAF complex. Neural precursor cells possess a unique BAF complex, termed npBAF, which contains the Baf53a and Baf45a subunits [193]. Both subunits are targeted and repressed by miR9/9* and miR124, facilitating the switch in subunits to create the neuronal BAF complex, nBAF [194]. The npBAF complex is necessary for cortical growth, as it promotes proliferation and expansion of neural precursors, whereas the nBAF complex is required for murine postmitotic neural development and dendritic morphogenesis [193,195]. The BAF complex itself binds to the *miR9* locus on mouse chromosome 13, promoting miR9 expression. Therefore, there is a feed-forward relationship whereby the BAF complex drives miR9/9* expression that mediates the maturation of npBAF to nBAF through suppression of Baf53a and Baf45a [128].

30.3.3 ncRNAs as biomarkers of stem cell function

ncRNAs are not just present intracellularly, as both lncRNAs and miRNAs can be packaged, e.g., in extracellular vesicles, and released into circulation (reviewed in Refs. [196,197]). The expression of ncRNAs in the blood is thought to be reflective of cellular biology and, therefore, can be biomarkers of aberrant cell function, including stem cell behavior. A key example of the potential for ncRNAs in circulation to be biomarkers of stem cell biology comes from cancer studies. Cancer can be thought of as a "gain-of-function" mutation in somatic cells where they inappropriately have stem-cell-like behaviors [198]. Part of the "gain-of-function" can include the expression of stem cell-associated lncRNAs (reviewed in Ref. [199]) and miRNAs (reviewed in Ref. [200]). Both lncRNAs and miRNAs have been evaluated at biomarkers of cancer. For example, as we previously mentioned (see Section 30.3.2), decreased miR335 is associated with increased neural stem cell proliferation [171]. For patients with non-Hodgkin lymphoma, miR335 and a miR335 lncRNA sponge, *NEAT1* are expressed in blood plasma. For individuals with high grade, or fast-growing, lymphoma, miR335 expression was decreased and *NEAT1* was increased, and low levels of miR335 co-incident with high levels of *NEAT1* were indicative decreased overall survival [201].

We have shown that prenatal drug exposures can impact human plasma miRNAs and alterations to the plasma miRNA milieu can indicate previous exposures and outcomes, including decreased growth, and potentially mediate the negative sequelae following these exposures. miRNAs present in maternal plasma can indicate which neonates will show impaired growth and neurodevelopment 6 months after birth in response to heavy alcohol exposure in utero [202]. Preclinically, these infant-outcome-

predictive miRNAs directly impaired placental growth and development, inhibiting EMT, a crucial pathway for appropriate placental development [203]. Plasma miRNAs present in infancy can also predict growth and neurodevelopmental deficits following prenatal alcohol exposure (PAE) [204] and withdrawal following prenatal opioid exposures [205]. Intriguingly, a group of infant miRNAs partially mediates the relationship between PAE and growth deficits [204]. These data support a compelling, but yet untested, hypothesis that PAE-altered miRNAs may directly impact stem cell biology as a mechanism of impeding infant growth, particularly as stem cells throughout the body are teratogenic targets of PAE [206]. Moreover, the miRNAs mentioned previously that are part of ethanol-induced reprogramming of neural stem cells [128,171,191] may be altered, in part, through extracellular miRNA transmission, as we have seen for miR140 [207].

Collectively these data show that the RNA networks needed to maintain pluripotency in stem cells can be leveraged to serve as biomarkers of stem cell function, including the aberrant stem cell function seen in cancer. ncRNAs also have the potential to be therapeutic targets, including to impact neural stem cell biology, as has been shown with administration of miR124 to promote neurogenesis from transplanted neural stem cells following stroke (see Case Study: miRNAs in Stem Cell Therapy for Ischemic Stroke).

30.4 Consideration of sex as a key biological variable in stem cell therapies targeting noncoding RNA networks

As research continues to assess the utility of stem cell therapy to combat human disease progression and symptomology, it is important to acknowledge a few key biological factors that may influence the efficacy of stem cell treatments and the role of ncRNAs in the behavior of these therapeutic stem cells. One such factor is the biological sex of cells, which in published experiments is overwhelmingly under-reported [208] and when reported, largely biases toward including male (XY) cells exclusively. Similarly, databases of commercially available cell-lines often fail to identify the sex of the donor, from both human and animal-derived cells [209].

ESCs are an attractive option for developing regenerative therapeutic agents. In humans, male and female sex chromosome-defined pluripotent stems cells demonstrate distinct epigenetic landscapes, with more than 200 differentially expressed autosomal genes between male and female stem cells [210]. These differences contribute to sex-specific differentiation in response to estrone, a steroid precursor to estrogen, as well as differences in stem cell proliferation, possibly attributable to the Y chromosome-exclusive gene *SRY* in males. Furthermore, sex hormones, can drive changes in ESC growth and differentiation. Prior research has demonstrated that androgens, steroid hormones that regulate the development of male characteristics, promote human ESC differentiation, and suppress ESC self-renewal, without affecting ESC proliferation [211]. Meanwhile, estrogens, which contribute to the development and regulation of female reproduction and sex characteristics, increase the expression of proto-oncogenes and mouse ESC proliferation [212]. In smooth muscle progenitor cells derived from human iPSCs, male and female cells demonstrate similar differentiation efficiency, while female cells demonstrate higher proliferation rates both natively and follow estrogen stimulation [213]. Human iPSCs in particular have been recognized for their utility in investigating sex difference in the development of neurodegenerative diseases [214,215]. However, stem cells derived from rodents have also uncovered sex-specific regeneration capabilities [216]. In addition to ESCs and iPSCs,

biological sex may further influence the growth and proliferation of mesenchymal, hematopoietic, muscle-derived, and neural stem cells (for a comprehensive review, see Ref. [217]).

Researchers are beginning to identify ncRNAs that exhibit genetic sex-dependent variations in expression [127,204,218,219]. Differences in expression have been associated with disease outcomes, including pregnancy complications [219], infant growth and vulnerability to prenatal teratogen exposure [127,204], and depression [218]. Moreover, in murine embryonic stem cells, the majority of sex-specific ncRNAs are encoded on autosomes [220], indicating that sex-specific expression of ncRNAs is widespread throughout the genome. The scope of sex-differences in ncRNA expression in iPSCs is unknown, but at least one aforementioned lncRNA, *Xist*, has been implicated in female-biased phenomena in iPSCs. The presence of more than one X chromosome in somatic cells initiates X chromosome dosage compensation, the random silencing of X chromosomes to equalize the expressed X-linked genes to those found in male (XY) somatic cells. Importantly, human iPSCs retain their inactive X chromosomes from parent tissue, unlike murine iPSCs. Moreover, reprogramming and repeated cell culturing can lead to abnormal X chromosome expression in human iPSCs [217]. For instance, reactivation of a previously inactive X chromosome can result in unpredictable transcription and epigenetic instability following differentiation [221]. In high-passage female human iPSCs, this reactivation has been associated with the loss of *XIST* and irreversible expression of genes on the previously inactive X chromosome [222]. Reductions of *XIST* are further correlated with increased expression of X-linked oncogenes, faster cellular growth rates and poorer differentiation, highlighting an important sexual distinction between male and female-derived iPSCs [223]. Importantly, while *XIST* is well-understood to epigenetically inactivate one of the X chromosomes, it has also recently been found to be a multi-functional lncRNA that can also bind to and sequester miRNAs, to regulate, for example, self-renewal properties of cancer stem cells [224]. Clearly its role as a miRNA sponge is limited to cells with more than one X chromosome, emphasizing the role of sex differences in the ncRNA control of stem cell renewal and maturation.

Although the number of cell therapies targeting sex-specific ncRNA networks for treatment are currently limited, given the known sex-specific RNA profiles in mammalian species, and the explicit role of ncRNAs in stem cell differentiation and pluripotency, it stands to reason that sex-biased RNA networks would contribute to the efficacy of stem cell treatments. This argument is best supported by clinical trials using stem cell intervention in cancer treatments. In cancerous tissue, sex-specific lncRNAs are associated with changes in cancerous cell growth, cell cycle progression, and proliferation, while modulating gene expression through interactions with select miRNAs [225]. lncRNAs also have distinct interactions with sex hormones. Estrogen has been shown to regulate several noncoding RNAs, including *ERINA*, *H19*, *TROJAN*, and *MALAT1*, while androgen-regulated lncRNAs include *ARLNC1*, *LINC00304*, *SARCC*, and *PART1*. Notably, *HOTAIR* interacts with both androgen and estrogen receptors, and may play a distinct role in sexually dimorphic stem cell activity. Differences in the availability of RNA-binding proteins between men and women [226,227] may further impact lncRNA function and, therefore, treatment efficacy following stem cell delivery.

Separately, the biological sex of the patient who is a recipient of stem cell therapies may also play a role in treatment efficacy. In a rodent model of muscular dystrophy, XX muscle-derived stem cell transplantation produced better outcomes in both male and female subjects [216]; however, female recipients experienced more regeneration overall than males, independent of donor cell sex. This would suggest that matching of donor and patient sex may not always be optimal. This notion has been

further supported by a clinical study investigating the relationship between donor and recipient sex and recovery outcomes following allogeneic hematopoietic cell transplantation [228]. This meta-analysis collated data from 450+ transplantation centers worldwide and included almost 12,000 adult patients living with different forms of cancer prior to transplantation. Overall, female recipients experienced greater cancer survival rates and reduced disease-progression than males, regardless of donor sex. Male recipients, however, experienced increased (12%) instances of cancer relapse when receiving cells from male donors compared to female donors.

Although far more research is required to determine how donor-recipient sex matching influences treatment outcomes, future clinical trials incorporating stem cell therapies will need to sufficiently power their studies to account for biological sex. In 2015, an initial report on the efficacy of intracoronary cell therapy in patients who had experienced an acute myocardial infarction determined that cell treatment did not improve patient health outcomes [229]. However, this initial report had not assessed whether treatment outcomes varied by patient sex, which was addressed in a follow-up publication several years later [230]. In fact, women who received cell transplantation were significantly less likely to experience stroke, heart attacks and death, than women who did not receive treatment. However, as only ~20% of patients from the original study were female, a lack of treatment effects in males had previously "washed out" the improvements produced in females. Therefore, it is important for researchers to not only report donor and recipient sex, but to recruit sufficient sample sizes to consider sex in statistical assessments, even when prior evidence for differences between men and women is yet unreported.

Taken together, ongoing research in the development of stem cell therapies would be enhanced by reporting and controlling for biological sex. In addition to elucidating the efficacy of donor-recipient sex matching (or alternatively, optimized mis-matching) for greater treatment success, these data may be particularly helpful in expanding upon and refining (a) treatments for diseases which disproportionally affect one sex, and (b) treatments known to produce distinct improvement outcomes between male and female patients [231]. Furthermore, the contribution of ncRNAs to these sex differences, particularly sex differences in autosomal ncRNAs, is drastically understudied. Future research investigating the sex differences of ncRNAs in stem cells could identify targets to enhance the efficacy of stem cell treatments.

30.5 Final comments

In this chapter, we introduced cellular pathways governing the normal maturation of ESCs and somatic stem cells into different developmental trajectories. We have described how the epigenetic and ncRNA environment interact with these pathways to either maintain the stem cell state or drive differentiation. Manipulation of the epigenetic and ncRNA environment improved reprogramming efficiencies of somatic cells or, in some contexts, was capable of independently inducing dedifferentiation and reprogramming. We also discussed the potential for ncRNAs released from the cell and present in the circulation to be biomarkers for aberrant stem cell behavior. Finally, we discussed the importance of considering key biological variables, in particular genetic sex, when leveraging ncRNAs and stem cell biology as therapeutics. Thus, wider adoption of stem cell—based therapies will be contingent on a clearer understanding of the stem cell epigenome during self-renewal and through successive stages of maturation and how the epigenome is modified by key biological variables including genetic sex.

Chapter key points

- Non-protein—coding RNAs are important regulators of pluripotency and differentiation during normal stem cell development and can drive aspects of the induction of pluripotent stem cells.
- Core pluripotency factors and epigenetics, including non-coding RNAs, are a complex, and understudied, network of factors that are critical regulators of stem cell activity.
- Noncoding RNAs have potential as biomarkers of aberrant stem cell function and are promising targets for intervention to improve stem cell therapies.
- Far more research is needed to understand how patient and donor characteristics, like chromosomal sex, influence the efficacy of disease treatments incorporating stem cell transplantation.

miRNAs in stem cell therapy for ischemic stroke

Yang [232].

Objective

To create a new and more efficacious delivery system for miRNA therapy for stem cell therapy following ischemic stroke, a novel delivery system, utilizing nontoxic and nonimmunogenic metal-organic frameworks, was evaluated for the ability to deliver miR124 without degradation and promote neural-directed differentiation of NSCs in a mouse model of middle cerebral artery occlusion ischemic stroke.

Rationale

NSC therapy is well recognized for its potential to treat patients with ischemic stroke; however, previous issues had been identified with NSC transplantation including limited neuronal differentiation. This inability to produce sufficient numbers of neurons has limited the advancement of NSC therapy into clinical trials. Here, the authors leveraged the known role for miR124 to promote neurogenesis to improve NSC differentiation. Efficacy of miRNA delivery can be limited by degradation and a lack of uptake into target cells, but these limitations can be overcome using an appropriate nanoparticle delivery system to protect the miRNAs and increase internalization into target cells.

Innovation

This study was innovative for two reasons: first, for using miRNA therapeutics to overcome limitations to the success of stem cell transplantation to treat stroke outcomes; and second, for using advanced nanostructures called metal-organic frameworks (MOFs) — porous crystalline structures with large internal surface areas and versatile surface bonding sites — to deliver miR-124 to a living animal and assess stem cell—induced recovery from ischemic stroke.

Audience

This research was performed by scientists from Shandong University in Jinan, China. The corresponding article for this research was published in a scientific, peer-reviewed journal from the American Chemical Society in September 2022. This article would be informative reading for other research scientists, as well as drug developers and clinicians interested in current advancements for the treatment of ischemic stroke.

The process, workflow, and actions taken

Generation and transplantation of Ca-MOF@miR-124 nanoparticles for the treatment of middle cerebral artery occlusion in mice, a model of ischemic stroke.

Step #1: Loading of miR-124 on Ca-MOF nanoparticles		
Objective To load miR-124 into Ca-MOF nanoparticles for transplantation and subsequent uptake into host cells.	**Methods and equipment** miR-124 was loaded onto Ca-MOF to form Ca-MOF@miR-124 nanoparticles by mixing appropriate amounts of miR-124 and Ca-MOF nanoparticles in deionized water. Agarose gel electrophoresis was performed to investigate the miR-124 loading capacity of Ca-MOF.	**Results** miR-124 was successfully loaded on Ca-MOF nanoparticles in a concentration-dependent manner.
Step #2: Lysosomal tracer labeling		
Objective To confirm that Ca-MOF@miR-124 nanoparticles can be successfully endocytosed in NSCs, with miR-124 released in lysosomes and delivered to the cytoplasm.	**Methods and equipment** NSC lysosomes and miR-124 were prelabeled with lysosome tracker (red) and 6-carboxyfluorescein (green), respectively. NSCs, with lysosomes prelabeled (red), were then cultured with Ca-MOF@miR-124 nanoparticles (miR-124 prelabeled, green), and the distribution of miR-124 in cells was visualized after 2 and 36 h with a confocal microscope.	**Results** At 2 h, green fluorescence overlapped with red fluorescence, indicating Ca-MOF@miR-124 nanoparticles were taken up by NSCs and internalized by lysosomes. At 36 h, the green fluorescence area had dispersed and desegregated from the red fluorescence area, interpreted to mean that miR-124 had escaped from lysosomes and was released into the cytoplasm.
Step #3: NSC differentiation following treatment with Ca-MOF@miR-124 nanoparticles		
Objective To assess the impact of Ca-MOF@miR-124 nanoparticles on neuronal differentiation of NSCs.	**Methods and equipment** Untreated NSCs, or NSCs treated with Ca-MOF nanoparticles, miR-124, or Ca-MOF@miR-124 nanoparticles, were cultured for 7 days. RNA was isolated from the	**Results** Nestin expression, a marker for NSC identity, was decreased and neuronal lineage markers were increased following Ca-MOF@miR-124 nanoparticle treatment

Continued

	NSCs and qPCR was used to compare the expression of neural differentiation and maturation stage–specific genes.	compared to all other groups alone, indicating neuronal-directed differentiation.
Step #4: Ischemic stroke modeling and stem cell administration		
Objective To test the efficacy of stem cell transplantation, with and without Ca-MOF@miR-124, as therapy for ischemic stroke in a mouse model middle cerebral artery occlusion (MCAO).	**Methods and equipment** Male C57Bl/6J mice underwent surgery to transiently occlude the middle cerebral artery. Subjects were randomly assigned to receive stereotaxic injections into the center of the cerebral infarct. These injections contained NSCs together with miR-124 alone or Ca-MOF@miR-124 nanoparticles. A week later, subjects were evaluated for neurological deficits before sacrificing for tissue collection. The size of the cerebral infarct was determined. Using immunohistochemistry, the amount of apoptosis and altered neuronal morphology/pathology were visualized within the infarct region.	**Results** In subjects that received the middle cerebral artery occlusion surgery, the cerebral infarct size was the smallest among subjects who received the synthesized Ca-MOF@miR-124 nanoparticles and NSCs. The NSC/Ca-MOF@miR-124 group also had the lowest neurological deficit scores and lowest amount of apoptotic cells compared to all other MCAO ± treatment groups. Together, these findings indicate that the Ca-MOF@miR-124 nanoparticles improved outcomes following stroke, including more than NSC transplantation alone.

Results

In this experiment, miR-124 was conjugated to the surface of Ca-MOF by hydrogen bonds to form Ca-MOF@miR-124 nanoparticles. This conjugation prevented the degradation of miR-124 by nucleases. Ca-MOF@miR-124 nanoparticles were successfully internalized into NSCs and aggregated in lysosomes. Furthermore, the hydrogen bonds were rapidly broken at an acidic pH in the lysosome, facilitating the release of miR-124 and its release into the cytosol. The improvements in the delivery and internalization of miR-124 achieved by Ca-MOF@miR-124 nanoparticles allowed miR-124 to promote the differentiation of NSCs into neurons with electrophysiological function within 5 days in vitro. More importantly, Ca-MOF@miR-124 nanoparticles enhanced the therapeutic effects of NSC therapy on ischemic stroke outcomes.

Learning and knowledge outcomes

These findings highlight the therapeutic potential of modulating miRNA expression to impact stem cell behavior and increase efficacy of stem cell therapies. The delivery system described here could enhance the utility of NSC therapy following stroke and could also be used in other neurodegenerative diseases.

Article link: https://pubs.acs.org/doi/full/10.1021/acsnano.2c04886.

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CHAPTER

Aging and disease: the epigenetic bridge

31

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31.1 Introduction

Conrad Hal Waddington, in 1942, was the first to define Epigenetics as "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being." At that time, the biochemical nature of genes was unknown as well as their role as repositories and transmitters of the genetic information. Waddington imagined the epigenetics as a conceptual model to explain his theory sustaining that different interaction between the genes and their surroundings (or, we could say their "environment") could result in different phenotypes, starting from the same genetic material. He used the metaphor of the "epigenetic landscape" to explain the biological development. Waddington stated that cell fates were established during the development similarly to a stone (a marble) that rolls down from high places to the point of lowest local elevation; the increasing irreversibility associated with cell type differentiation was imagined as due to ridges, rising along the slope where the stone is rolling down, directing the marble into different valleys [1].

More recently, Holliday defined epigenetics in a more formal way as "the study of the mechanisms of temporal and spatial control of gene activity during the development of complex organisms" [2]. According to this definition, the term "epigenetic" could be used to describe anything other than DNA sequence itself that is able to influence differentiation, physiology, and fate of a cell or of a whole organism. Actually, the present use of the term is more narrow, referring to the combined epigenetic modifications of a given domain of DNA sequence, characterized by heritability (over rounds of cell division and sometimes transgenerationally), that do not involve changes to the underlying DNA sequence. On this basis, epigenetics was recently defined as "the study of any potentially stable and, ideally, heritable change in gene expression or cellular phenotype that occurs without changes in Watson-Crick base pairing of DNA" [3]. Epigenetic modifications include (among other) methylation of cytosine residues of DNA and posttranslational modifications of the histone proteins. Specific combinations of epigenetic modifications determine the conformation of the chromatin fiber, thereby having the possibility to regulate the transcriptional potential of the associated genes.

Despite the advances in our knowledge about cell differentiation and epigenetic phenomena, and with the unavoidable adjustments and corrections, Waddington's model still represents a nice visualization of the epigenetics. As a matter of fact, it appears really useful to suggest that aging processes are particularly prone to epigenetic mechanisms. The notion Waddington could not know, indeed, was

Epigenetics in Human Disease. https://doi.org/10.1016/B978-0-443-21863-7.00016-0
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that once differentiation has been completed (i.e., the "lowest local elevation" has been reached) cell fate could be not definitive; on the contrary, the phenotype of differentiated cells still evolves either with the normal aging processes or as a consequence of external (pathogenic) stimuli. To resume and apply Waddington's model to aging, we can imagine that erosive processes can change the shape of the slope and of the surrounding of the stone, causing the reprise of its rolling down through new ridges and valleys. According to this view, the terminally differentiated cell is subjected to "environmental" stimuli (originated either from the organism itself or from the external environment) able to induce changes in gene expression through epigenetic mechanisms. The higher the mountain, the longer the slope; consequently, the stone encounters many more possibilities to be subjected to changes of directions and shape. This view recalls the idea that a longer life (of a cell or organism) is associated to a more frequent probability that epigenetic changes arise, possibly causing aging-associated dysregulation. On the basis of this metaphoric view, aging (and aging-associated diseases) represents the inevitable companion of a long life. Fortunately, epigenetic modifications are, by definition, reversible; consequently, they are potential targets for pharmacological interventions aimed at reestablishing the correct epigenome. Interestingly, recent evidences indicate that the epigenome can be also modulated by nonpharmacological intervention like diet, physical exercise, lifestyle, and behavioral stresses. This research field is defined as "geroscience" and moves on the observation that aging is the major risk factor for several complex diseases; on the attempt to identify the mechanistic links between aging and age-related diseases, geroscience naturally looks at the substantial remodeling the epigenome observed during aging [4].

In the present chapter, evidences related to the connection between epigenetics and aging are presented and discussed in the light of the most recent advances in this field of the bio-medical research. Particular attention is devoted to the aging brain, which appears to be the organ most interested by normal and pathological aging processes, due to the relevance of neurodegeneration among the age-associated diseases and to the recent scientific evidences indicating substantial involvement of epigenetic phenomena in brain aging.

31.2 Genes and aging

A common saying among geriatricians sounds like "Life is a chronic disease with unpropitious outcome." Probably, daily observing the aged and diseased patients negatively influences humor and mood of clinicians and researchers working on aging, but the sentence is undeniably correct. Indeed, humans cannot escape (as far as we know) aging and, in case, aging-related diseases [5]. Aging, although not considered a disease, is itself associated with progressive, cognitive, physical, and physiological impairment; moreover, many diseases are age-related. The main risk factor for Late Onset Alzheimer's Disease (LOAD), for instance, is the aging, since the incidence of the Disease is constantly increasing with age [6].

As a matter of fact, evolution of the human species has allowed the possibility to constantly improve life quality, particularly in terms of better hygiene, nutrition, protection, and medical care. These improvements contributed not just to the increasing of the average life expectancy but also, in many cases, to reach and spend the oldest age in better physical and cognitive conditions than in the past. Despite this progress in life expectancy, it is interesting to note that almost no progresses were observed for the oldest age that it is possible to reach (the "maximum lifespan potential"); moreover, in

association with the increased life expectancy, many (and sometimes new) diseases show an increased morbidity dependent on aging [7].

The existence of a genetic determinant of life duration is supported by the apparent impossibility of going beyond a certain maximum lifespan potential and also by the observations indicating that this potential seems to be determined and characteristic for each species. This information induced the theory that even if we could cure or prevent the diseases most responsible for human death, we will be able to just further extend life expectancy, but won't be able to significantly overcome the maximum lifespan potential determined by the advent of fatal age-associated physiological impairment [8]. This hypothesis depicts the age-related diseases, mainly the neurodegenerative diseases, as the price to pay to survive cancer; in other words, the organism has two possible fates during the aging: facing cells prone to errors that would shift toward a relatively longer (cancerous) life, or coping with the progressive cell impairment and death due to physiological cellular degeneration.

Molecular and cellular degeneration, resulting in the aging of the whole organism, are often evident and clearly observable although the initial and causal molecular mechanisms are difficult to be studied because of their extreme complexity, interconnections, presence of side-effects and concurrent external and environmental factors [9]. The study of the picture representing the age-associated diseases is complicated by the possible early start of the pathological mechanisms, possibly initiating in early age, and also by the above-cited difference in the regulation of aging mechanisms in different organisms, which makes difficult to use surrogated animal models to study human aging.

In this context, it is not easy to hypothesize a unifying and comprehensive theory of aging. Initially, it was postulated that biological aging was dependent on changes residing in the information-containing molecules, leading to formulate the "error theory," the "redundant message theory," the "codon restriction theory," and the "transcriptional event theory" [10]. In a second moment, attention of the researchers has been shifted on the hypothesis that possible "gerontogenes," mainly involved in DNA repair, could be responsible for cellular senescence [10]. To summarize, it is possible to differentiate two major groups of theories: stochastic and genetic; however, it becomes evident that these groups could be, in some case, not mutually exclusive.

A list of the principal theories explaining causes and possible mechanisms of aging is here reported [9,11]:

1. Evolutionary: evolution presses the organisms to reach the reproductive age, procreate, and care for the offspring. According to this point of view, the physiology of an organism after the end of the reproductive period could be the manifestation of the epigenetic events occurring on the basis of the genetic development during the previous stage of the life. The conclusion is that cellular senescence could be the price to pay in order to avoid other damages, like the tumorigenesis, potentially caused by the prolonged expression of the genes involved in the reaching of the reproductive fitness [12].
2. Stochastic: the stochastic theories are generally based on the idea that aging is caused by casual damages to cell molecules, particularly to DNA.
   - 2.1. Mutation and repair: DNA is subjected to continuous damages that induce mutations. The effect is cumulative and becomes increasingly evident because of the loss of the DNA repair ability [13].
   - 2.2. Error-catastrophe: casual errors in the synthesized proteins are normally by-passed by the normal molecular turn-over, but errors in the molecules involved in protein or DNA

synthesis or in postsynthetic modification could represent the start of a process of error amplification, resulting in the accumulation of wrong molecules able to interfere with normal cell physiology [14].

2.3. Protein modification: the worsening of the enzymatic activities in aging could be a consequence of the altered postsynthetic modifications, altered turnover, and proteins cross-linking [14].

2.4. Oxidative stress: it is one of the most investigated areas of cellular senescence; the involvement of free radicals and the alteration of the oxidative status in aging have been characterized in several models and organisms and in different pathologies associated with the older age, like Alzheimer's disease and Parkinson's disease. The balance between pro- and antioxidants in the cell is finely and complexly regulated and the impairment of this regulation is critical to mitochondrial, cellular, and tissue physiology during aging [15].

3. Genetic: in the genetic (or developmental) theories, aging is considered as a programmed and genetically controlled process of maturation, successive to the development of organism or cell. These theories are supported by the elevate species-specificity of the maximum lifespan but are in contrast with the variable control and manifestation of aging in different individuals of a same species.

3.1. Longevity genes: there are several evidences about the existence of genetic elements able to regulate senescence, in particular responsible for the regulation of the maximum lifespan. Studies regarding the role of genes involved in the increment of lifespan were primarily performed on "simple" eukaryotes like yeast and *C. elegans*, but significant data also arise from studies on *D. melanogaster* and rodents [16,17].

3.2. Aging syndromes: existence of a number of human genetic diseases (Hutchinson's Syndrome, Werner's Syndrome, Down's Syndrome) displaying some characteristics distinctive of accelerated aging, lead to hypothesize that aging could be a kind of disease itself, regulated by specific genes. Recently, different transgenic mouse models showing aging phenotypes similar to those observed in humans were also settled [18].

3.3. Neuroendocrine theory: it is based on the importance of the hormones secreted in the brain (hypothalamic, pituitary, and adrenal hormones) in the regulation of organismic aging and on the decrement in brain neurons [19].

3.4. Immunologic theory: it is based on the decreased T-cells response and increased autoimmune reactions during aging [20]. As for the neuroendocrine theory, the weak point is that complex immune and neuronal systems are not present in simple eukaryotes although they show characteristics of aging comparable to higher organisms.

3.5. Cellular senescence: cellular cultures were used as a model for the comprehension of senescence processes due to their usefulness to study the basic molecular mechanisms, unlike the whole organisms. Data on the genetic effectors responsible for the regulation of cell senescence sustain the hypothesis that organismic aging reflects the senescence of single cell lines or tissues. Cellular senescence is often indicated as "replicative senescence," since the genes involved in this phenomenon are mainly genes related to the replication machinery and since the cellular senescence becomes evident through decline in growth rate and proliferative activity and alterations in the signal transduction and adaptive response pathways. All these alterations characterize a senescent cell growth status, which is quite different from the young cells [21]. The first event characterized as a potential

cellular clock was the mechanism of telomere shortening [22]. The function of telomeres (sequences of noncoding DNA at the end of chromosomes) is to avoid the degradation of coding regions and fusion with other DNA sequences; the reduction of the telomeric length with cell aging is the main evidence in cellular senescence. Other two genes of the replicative machinery, retinoblastoma and p53, are well known to be involved in cell senescence; their activity is generally increased in senescent cells [23]. The complexity of the mechanisms of cell cycle progression and the balance between positive and negative regulators is due to the importance of this regulation in order to avoid the onset of tumorigenic events in the cell. These data are consistent with the hypothesis that cellular senescence has evolved as a mechanism of tumor suppression [9].

3.6. Cell death: strictly linked to the mechanisms of cellular replication and senescence, the mechanism of apoptosis is considered as a cause of aging since it consist of a process of active, gene-dependent, and injury-independent cell death [24].

More recently, evidences that epigenetic mechanisms could have a role in cellular degeneration and aging are supported by technical advances allowing a detailed study of the epigenome and of the epigenetic mechanisms and by the discovery of a complex, non-Mendelian, nature of many age-associated disorders.

In yeast and mice, significant changes in gene expression during cellular degeneration are related to significant and net loss of heterochromatin, with consequent overexpression of heterochromatin-associated silenced genes [25]. Changes in chromatin structure are associated with epigenetic modifications that consist of DNA methylation, DNA hydroxymethylation, histone posttranscriptional modifications (methylation, acetylation, and phosphorylation) and ATP-mediated chromatin modifications. DNA is associated to euchromatin in actively transcripted loci in proliferating cells; these loci mainly contain genes involved in cell growth regulation and in basal cell metabolism [26]. For this reason, it has been proposed that loss of repressive chromatin domains (heterochromatin) may contribute to cellular degeneration and aging processes.

The observed heterochromatin loss is in agreement with, and apparently linked to, the generally observed loss of DNA methylation in the elderly [27,28]. Other experimental evidences also point out the spreading of DNA methylation and the presence of hypermethylated DNA in senescence and cancer [29,30]; on the one hand, these contrasting data stress the complexity of the epigenetic phenomena and contribute to confound the picture but, on the other, the contrast can be clarified when DNA methylation is analyzed at a sequence-specific level in regions and sequences undergoing changes of methylation during aging. Observed global changes in DNA methylation, at genomic level, could indeed be due to changes in methylation of bulk or nonregulatory DNA regions. The role of these changes is not yet ascertained, and we cannot conclude it is directly associated to changes in gene expression, unlike methylation changes in promoters and other regulatory DNA sequences. However, the evidence about the general DNA methylation decreasing in proliferating cell cultures and in normal organismic aging, but not in immortal cell lines, and the concurrent impairment of the DNA methyltransferase activity, stress the hypothesis that DNA methylation is susceptible of aging-linked variations and that these variations have a specific role in the aging processes [31]. One of the main questions to be ascertained in this sense involves the "cause or consequence" role of DNA methylation in aging.

DNA methylation is strictly related to histone modification and "packing"; in turn, histone modifications can influence gene transcription by modulating chromatin assembly. Histone acetylation is the most studied modification; it is regulated by the classical histone deacetylases (HDAC) family and by the SIR2 (silent information regulator) family. It is worthwhile to underline that the SIR2 family was also identified as involved in regulation of lifespan in other eukaryotes [32,33].

Other epigenetic modifications, such as histone methylation and ATP-mediated chromatin remodeling, are not yet well characterized for their role in cellular degeneration and aging, but, as a matter of fact, it was showed that euchromatinization of specific domains or, vice versa, heterochromatinization of specific growth genes, due to alterations in balance of chromatin modifying molecules, is modulated during aging [34].

A third class of epigenetic modifiers is commonly identified in the family of short noncoding RNA called microRNAs (miRNAs). These molecules can target one or more messenger RNAs, altering their stability and therefore modulating the gene expression. Whether miRNAs, commonly referred to in the biological literature as somehow being epigenetic, also meet the criteria of the epigenetic heritability is still very much an open question. MicroRNAs can be transmitted, but given the apparently stochastic mode of their transmission, it is not evident they can indeed give rise to stably inherited traits not encoded by the offspring DNA [35,36]. A recent review presented an intriguing hypothetical model, whereby miRNAs might be transmitted from the parental germlines to the developing embryo by circulating vesicles, and then stably acquired [37]. No doubts, instead, that specific miRNAs are involved in the aging and neurodegenerative processes targeting aging-associated genes [38,39].

Besides their role in normal cellular senescence, epigenetic mechanisms are also to be considered in the light of their association (DNA methylation in particular) with cancer onset and development, since it is well known that cancer onset increases with aging.

Methylation, aging, and cancer are representatively connected to the observed CpG island methylation increasing associated to the estrogen receptor (ER) gene, which resulted age-associated in the normal human colonic mucosa. ER gene hypermethylation was also found in all colonic tumors examined including small adenomas, suggesting that methylation-dependent inactivation of the ER gene in the aging colorectal mucosa is one early event in colorectal tumorigenesis. Other CpG islands in promoter regions of several genes exhibit age-related hypermethylation in colon mucosa [30,40]. Most of the CpG islands found hypermethylated in primary colon tumors resulted hypermethylated to a lesser extent in the aging colon, but a minor number of islands resulted hypermethylated only in subsets of colon cancers. These findings stress the hypothesis that two kinds of methylations exist: (1) one age-related methylation present in the normal mucosa as a function of the age and (2) a cancer-related methylation, not observed in normal colon. The mechanisms responsible for the concurrent induction of global DNA hypomethylation and sequence-specific increase in de novo methylation with aging are still an open field of research. It is well known that different DNA-methyltransferases (DNMTs) enzymes with peculiar functions exist. Maintenance methylation of hemimethylated DNA during cellular replication is guarantee by DNMT1, which is the most abundant. Other DNMTs (namely, DNMT3a and DNMT3b) have a high affinity for unmethylated DNA and were, for this reason, indicated as de novo methyltransferases, able to methylate DNA without preexisting hemimethylation. These methylation mechanisms are schematized in Fig. 31.1. Since the levels of DNMT1 decrease with aging, it can be hypothesized that the overall decrease in DNA methylation can be referred to maintenance methylation, whereas the sequence-specific increase in de novo methylation depends on the increased activity of the other DNMTs [41]. The observed general decreasing in total

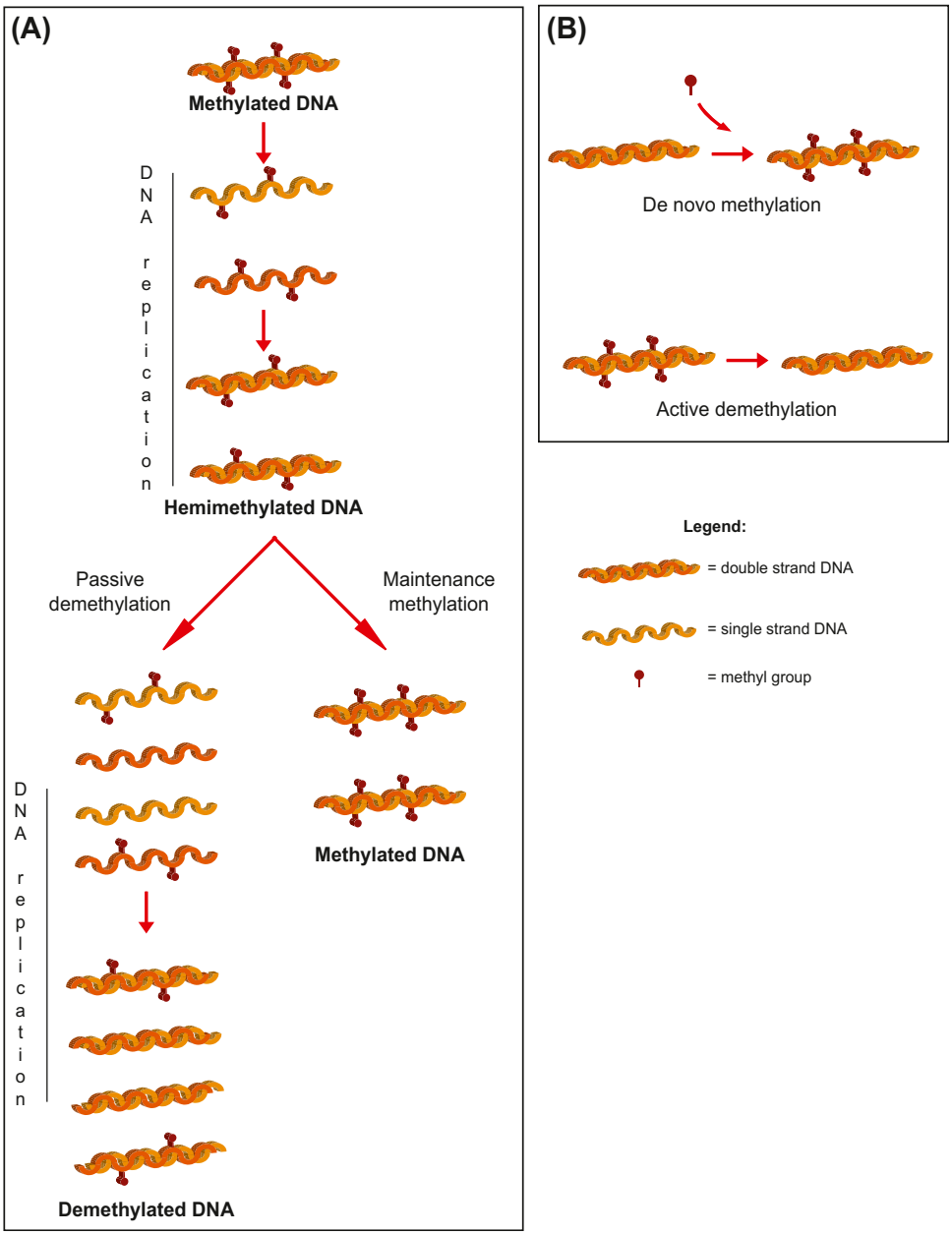

FIGURE 31.1

Schematic representation of the DNA methylation reactions: maintenance methylation, passive demethylation, de novo methylation, and active demethylation. (A) After rounds of cell divisions, methylated sequences result demethylated in neo-synthesized DNA molecules (passive methylation) unless the maintenance methylase activity guarantees the transmission of the methylation pattern retained by the parental DNA strand. (B) De novo methylase activity has the ability to methylate DNA sequences independently on the presence of methyl groups in one of the two strands, whereas active demethylase activity has the ability to remove methyl groups; both these activities can be exerted in absence of cellular replication.

genomic methylation with aging in different organisms and the apparently finite number of cell divisions characteristic of most somatic cells stress the view of overall DNA methylation (or even methyl-cytosine residues) loss as a cellular countdown mechanism to trigger normal cellular senescence or degeneration.

More recently, thanks to the power of the genome-wide studies comparing younger to older subjects, it was possible to confirm on a large-scale basis that methylation changes (both in the direction of hyper- and hypo-methylation) are associated with aging, both in humans [42–44] and in animal models [45]. Even after these recent results, the idea that the methylation status of a larger part of the examined genes and sequences seem unchanged during aging [41] is, so far, still preserved. This is not at all, of course, a "negative" or controversial result for the disciples of the epigenetic theory of aging, but it just points out the idea that the age-associated epigenetic drift targets specific genes involved in aging processes. The alterations in CpG island methylation are crucial to modulate the binding of transcription factors and methyl-DNA binding proteins. Aberrant methylation of CpG islands in the promoter region may contribute to the progressive inactivation of growth-inhibitory genes during aging, resulting in the clonal selection of cells with growth advantage toward cancer development.

DNA methylation also presents a link with the regulation of telomerase activity, another key factor in the regulation of senescence. Human telomerase consists of an RNA component (hTR) and a catalytic subunit with reverse transcriptase activity (hTERT). Most differentiated somatic cells lack detectable telomerase activity, in contrast to the high activities found in immortalized cancer cells, germ line cells, and stem cells and the high GC content of the hTERT promoter indicates that DNA methylation may be important in controlling hTERT expression. One emerging theory indicates that telomere control and DNA methylation may be strictly involved in the aging processes; in fact DNMT1 expression results decreased in aging human fibroblasts, with consequent decreasing of methylation activity in the cells, but hTERT was able to activate DNMT1 expression avoiding the loss of methylation [46].

All the above-reported data evidence that the methylation pattern established during the development is not stable or definitive in the adult life and, in particular, during aging. Therefore, the epigenetic approach seems promising for the understanding of the mechanisms that regulate aging and age-related diseases, whereas the consideration that the preservation of epigenetic patterns may help the maintaining of a healthy status could provide the basis for a therapeutic and preventive action.

31.3 The dynamic methylome

The idea that epigenetic changes are responsible for aging and age-related diseases directly implies that the epigenome is a dynamic entity, undergoing modulation after particular stimuli. DNA methylation was long time considered as the repository of the epigenetic "message" since the methylation pattern could be transmitted to the offspring and, once established at the end of the developmental processes, could be maintained during cell replication in a semi-conservative manner. This feature is permitted by the ability of the maintenance DNMT(s) to replicate in the newly synthesized DNA strand the methylation pattern of the parent strand. For the last 2 decades it was supposed that the only mechanism leading to DNA demethylation could be the passive, replication-dependent, loss of methyl moieties occurring when the maintenance DNMT activity was defective during cell replication. This hypothesis stressed the impossibility that DNA demethylation could occur

in adult organisms, at least in nonproliferating tissues, depicting a DNA methylation pattern that is, once established, relatively fixed and stable [47]. These assumptions were initially contrasted by the identification of putative DNA demethylase activity (Fig. 31.1) in different candidate proteins like 5-methylcytosine-DNA glycosylase, Gadd45a, MBD2, MBD2b, MBD3, MBD4 [48–54]. It was even evidenced that DNMTs could be associated with demethylase activity [51,55,56]. Among these putative demethylases, MBD2 is the best characterized thanks to the pioneering work by the group of M. Szyf: they hypothesized that MBD2 can directly bind to methylated CpGs removing only the methyl group without the cytosine excision postulated by others [52]. Data are still not definitive and the possibility that MBD2 could interact with other (effectors) proteins could not be excluded; if the real nature of the active DNA demethylase is not yet definitely revealed, the existence of such enzymatic activity cannot be ignored longer [57]. In our laboratory, we obtained earlier indirect indications that rapid demethylation, not compatible with the time necessary for cellular replication, occurred at a specific CpG site of myogenin gene promoter during myogenic differentiation in vitro [58]. More recently, we obtained evidences, in the same experimental system, that DNA demethylation pattern of myogenin gene promoter is regulated by a dynamic balance between DNA methylase and demethylase activities and that inhibition of DNMTs results in improved active demethylation, nonnecessarily dependent on DNA replication [59]. In a different experimental model, more strictly connected to age-related disease processes, we were able to demonstrate that DNA methylase and demethylase activities could be modulated in the adult brain. Using a transgenic mouse model of Alzheimer's disease we showed that the inhibition of the metabolic pathway that generates the methyl donor S-adenosylmethionine resulted in the impairment of the methylase activities and the improvement of the demethylase activity in mice brain—a tissue known for its scarce cellular proliferation. On the contrary, the supplementation of the methyl donor resulted in increased DNA methylation and decreased DNA demethylation activities. These modulations had a direct effect on the methylation pattern of Presenilin1 (PSEN1) gene, which is involved in amyloid processing in Alzheimer's disease [60,61]. We can therefore conclude that the methylation pattern of specific genes is not fixed in adult and nonproliferating tissues but undergoes dynamic regulation under appropriate stimuli.

31.3.1 Epigenetic dynamics in the aging brain

The brain is certainly the human organ most hit by aging: it shows sensitive impairment yet in normal aging, and appears more prone than other organs to the occurrence of aging-related diseases. Neurodegenerative disorders represent the main class of age-associated diseases and, among these, Alzheimer's disease represents the most prevalent form of neurodegenerative disease. Moreover, many of these disorders have been recently associated with epigenetic events. For these reasons, it seems of particular relevance the discussion of the epigenetic changes occurring in the brain and observed in adulthood and aging; an excellent and comprehensive review of these mechanisms was recently published by J. Rogers and colleagues [62].

As previously discussed, the knowledge that postmitotic cells could present functional alterations of the methylation patterns is a quite recent acquisition. In the last years, it was demonstrated that dynamic and rapid (active) DNA methylation and demethylation are present in postmitotic cells and, in particular, in neurons [51,56,63] stressing the involvement of epigenetic processes in neuronal plasticity. The paper by Murgatroyd and colleagues [56] has to be considered as a milestone in this area. In their work, they demonstrated that early life stress in mice was associated to behavioral changes in

adult life via a mechanism involving epigenetic modifications of hypothalamic neurons. Early exposure of mice to environmental stress during the first 10 days of life resulted associated to impaired avoidance learning, sustained hyperactivity of the hypothalamic-pituitary-adrenal axis, and corticosterone and pituitary adrenocorticotropin prohormone hypersecretion. These effects were linked to arginine-vasopressin (AVP) overexpression by parvocellular neurons of the hypothalamic paraventricular nucleus, and to hypomethylation of specific CpG sites within the AVP gene. The authors also showed the age-dependent hypomethylation of AVP gene at different CpG sites in control mice, although hypomethylation of CpGs within a CpG island included in the AVP enhancer region resulted responsible for AVP overexpression in mice that underwent the early-life stress. Hypomethylation of this CpG island was specific to the paraventricular nucleus, suggesting a possible mechanism for the observed epigenetic change that involves the binding of MeCP2 protein to the CpG sites in this island. The authors finally demonstrated that phosphorylation of MeCP2 by calmodulin-dependent protein kinase II decreased MeCP2 occupancy of CpGs in the CpG island, enhancing gene expression. Experimental models taking advantage of early-life stresses represent promising approaches to study the modification of adult stress response, cognition and behavior, induced by epigenetic modifications [64,65].

A different approach was used to study the age-dependent decrease of caspase-3 in rat brain, associated to alterations of the methylation pattern of specific CpG sites in the promoter of the gene [66]. The promoter sequence interested by methylation alteration lies in a region of the promoter necessary for its activity. This region is predicted to bind the transcription factors ETS-1 and -2, which are important, besides in controlling caspase-3 transcription, in regulating neuronal differentiation and death. Since these two factors seem not altered during aging, it is possible to appreciate the relevance of methylation status, mediating transcription factors binding and activity.

Dynamic changes of methylation patterns even showed cyclical regulation associated to cyclical activation/inactivation of transcription [51,63]. In this model, DNMT3a and 3b are responsible for deamination of methylated CpG sites, leading to a process of active demethylation mediated by an excision/repair mechanisms; DNA demethylation induces gene transcription, followed by MeCP2 and DNMTs new recruitment responsible for new DNA methylation and gene silencing.

While much is known about DNA methylation mechanisms and the possible role of DNA methylation, dysregulation has become an intense field of research in respect to aging and age-related neurodegeneration [67], little is known about the relevance to the aging processes of DNA hydroxymethylation, a relatively new epigenetic modification recently identified [68]. 5-Hydroxymethyl cytosine (5-hmC) is generated by the action of the ten-eleven translocation enzymes (TET), which are able to hydroxylate methylated cytosines [69,70]. 5-hmCs are then formed on after a previous cytosine methylation and are not efficiently recognized by methyl-binding proteins [71,72]. Unlike DNA methylation, hydroxymethylation is generally associated to euchromatinization and gene expression [73,74]. A role for this epigenetic modification in aging could be hypothesized on the basis of the work by Chouliaras and colleagues that recently showed increase of hydroxymethylation in aging mouse hippocampus associated with increases in Dnmt3a levels [75]; interestingly, this increase was prevented by caloric restriction (CR) [76].

As for DNA methylation and demethylation, a recent study suggested that also histone acetylation and deacetylation are subjected to dynamic and rapid changes that can influence gene transcription [77]. Histone modifications are involved in several neurobiological processes in the differentiated brain, operating dynamic regulatory mechanisms in postmitotic neurons: posttraumatic stress

disorders [78], addiction [79], choline acetyltransferase activity regulation [80], GDNF and BDNF transcription [81], and microglial apoptosis [82]. Moreover, histone modifications are known to be associated to different neurological and neurodegenerative disorders, including Parkinson's disease [81], motor neuron disease [83], multiple sclerosis [84], and X-linked mental retardation [85].

Another class of molecules with transcriptional regulatory functions, today included in the list of the epigenetic mechanisms, is represented by the microRNAs (miRNAs). The study of these regulatory molecules is facing an increasing boost in the very recent times since miRNAs were found associated to different physiological and pathological processes, also involving the brain, and appear modulated in postmitotic neurons [86]. For example, miRNA-329, miRNA-134, and miRNA-381, which are induced by neuronal activity, seem to be essential for the dendritic outgrowth of hippocampal neurons [87] and were also investigated in various neurological disorders [88].

31.3.2 The complexity of the age-associated epigenetic changes

Both normal and pathological aging processes in the brain are characterized by increased DNA damage, synaptic dysfunction, brain shrinkage, structural brain changes and cognitive decline [89–91]. As a matter of fact, specific brain regions as cortex and hippocampus appear more prone to be interested by these aging-related changes [92–94]. This region-specificity could be also observed when alterations of gene transcription are analyzed. These alterations can be summarized, on the one hand, in downregulation of genes devoted to maintain synaptic plasticity, neurotrophic support to neurons, neurotransmitters synthesis and DNA repair machinery and, on the other, in upregulation of immune-related genes [66,95,96]. A role for epigenetic mechanisms, which are responsible for regulating gene expression, is now clearly claimed in these aging-related changes [97–99].

As previously stated for Alzheimer's disease, aging is the most evident risk factor associated with aging-related diseases [100]. Unfortunately, the causal or consequential nature of this association is not at all yet clear, although a number of possible concurrent alterations were identified both in normal and pathological aging; these include oxidative stress, sexual hormones effects, calcium dyshomeostasis, neurotransmitters and glucocorticoids deregulation, neuroinflammation, diabetes-associated alterations, neurovascular deficits, toxic protein deposition, and transcriptional alterations of many genes [97,101–109]. Epigenetic modifications, being involved in aging-associated changes in different experimental models and organisms, could represent a link between normal and pathological aging [110].

Like in other organisms [111], progressive, age-related, and genome-wide DNA hypomethylation was observed in humans both in in vivo and in in vitro models [112,113] and was associated with the concurrent DNMT1 impairment [41,114]. Age-associated loss of DNA methylation was also associated with the parallel increase in S-adenosylhomocysteine [115] causing the inhibition of methyltransferase reactions (as discussed in depth in the prosecution of this chapter). Besides this general loss of methylation, specific hypomethylation of both coding and noncoding regions was observed during aging.

As for noncoding regions, since these are normally repressed by methylation, it seems possible that hypomethylation processes activating repetitive sequences, retrotransposons, and endogenous retroviruses occurring with aging could induce chromosome instability and retroviruses activation [116]. Moreover, a very recent paper evidenced that hypomethylation of noncoding sequences like LINE-1 is possibly associated to neurodegeneration and, in particular, to LOAD [117].

The effect of the age-related hypomethylation on coding regions and specific genes in humans was also particularly studied in LOAD, probably because it is the most frequent pathology in older people. The work by Tohgi and his group, for example, showed hypomethylation of Amyloid Precursor Protein (APP) gene promoter in the brain of older people and also complex variations in methylation patterns of Tau and RAGE (Receptor for Advanced Glycation End-products) gene promoters [118–120]. Other researchers evidenced the age-dependent hypomethylation of regulatory regions of the immune/inflammatory antigen CD11a [121].

Examples of gene-specific hypermethylation related to aging were also evidenced, together with the silencing of the associated genes. This was particularly observed for genes associated to CpG islands like tumor suppressor genes, ER genes, and insulin-like growth factor 2 [122,123].

The very complex draft of the age-dependent changes in DNA methylation patterns, representing both hypo- and hypermethylation events at specific DNA sequences, is further complicated by the presence of tissue-specific patterns. Once again it is the brain that offers a clear example of this complexity, since it was demonstrated that methylation patterns are region-specific [124].

Finally, a further degree of complexity is added by the possibility that many of the above-discussed changes in the epigenome of the aging brain could have an unsuspected early (even developmental) origin. The theory that early-life events could induce epigenetic modifications that are phenotypically silent until the middle or the old age, accumulating potential toxic features (amyloid deposition, for example) during many years of asymptomatic state, is well summarized by the LEARn (latent early-life associated regulation) model of age-related neurological disorders [125]. Experimental evidences of this model were given by the above-mentioned effect of early-life stress that results in epigenetic modifications and behavioral deficits in adult age [56,65] and by the finding that early exposure of monkeys to Pb decreased DNMT activity and had effects on amyloid-beta (Aβ) deposition in late life [93]. Also the work performed in our laboratory so far, seems supporting the LEARn theory since we observed that early interventions on methylation machinery in AD transgenic mice are able to modulate PSEN1 expression and Aβ deposition [60,61,126].

Besides the interest about specific loci in aging and the study of differentially methylated regions (DMRs) along the genome, the concept of the general epigenetic drift can be useful for better understanding the association between epigenetics and healthy versus diseased aging, as recently showed in a study that took advantage of the calculation of the "epigenetic age," its association with cognitive decline, and its use as a possible "predictor" of dementia [127]. Similarly, the concept of "epigenetic assimilation" has been formulated following studies on the epigenetic drift in human brain and could represent, in the future, a useful tool to better understand the molecular mechanisms of aging and the origins of diseases for which age is a risk factor [128].

31.4 Healthy and pathological aging

The variability of age-associated cognitive change is related to many factors influenced by demographic, social, educational, medical, nutritional, and biological stimuli. Biological factors include both genetic and epigenetic mechanisms. Epigenetic modifications, such as DNA methylation and histone acetylation regulate replication and transcription and are responsible for chromatin (re) modeling, chromosome stability, and DNA imprinting. Among the different epigenetic modifications, DNA methylation plays a pivotal role; methylation homeostasis is fundamental for normal brain

physiology, whereas the impairment of methylation reactions and particularly DNA methylation is associated with markers related to neurodegeneration. Moreover, epigenetic mechanisms are greatly involved in brain development during the early life and the adult neurogenesis and are also involved in the onset and development of AD and other neurodegenerative disorders [129]. The hypothesis that gene transcription in brain is epigenetically regulated by DNA methylation much more than tissue-specific expression in other organs is now largely accepted. This hypothesis is supported by the strong association between age-related low methylation status and cognitive deficits or neurological and neurodegenerative pathologies [5,27,28]. However, it is still not completely clear whether epigenetic changes actually represent a cause or a consequence of the disease. This gap is due to the high complexity of the epigenetic mechanisms and of their regulation during aging; moreover it is possible that epigenetic studies on pathological aging could be biased by the involvement of subjects in an advanced stage of disease or, in any case, subjects in which the epigenetic changes started even many years before the comparison of the first symptoms. An active role for epigenetics in normal and pathological aging must meet two conditions: specific epigenetic changes must occur during aging and they must be functionally associated with the aged and/or the diseased phenotype. Assuming that specific epigenetic modifications can have a direct functional outcome in aging or age-related diseases, it is also essential to establish whether they depend on genetic, environmental, or stochastic factors [67]. Few objections could be moved to the statement that the two cited conditions (the specificity of the epigenetic changes and the functional association to a phenotype) are demonstrated in the relationship between aging and cancer. As a matter of fact, epigenetic modifications play a major role in cancer, influencing tumor outcome by interfering with key senescence pathways [130]. As for other physiopathological mechanisms, a direct and causal role can be, so far, just supposed; the complexity of the immune system, for example, opens the possibility to predict that a higher-order, supragenetic regulation is indispensable for generation of its constituents and control of its functions during aging [131].

In human brain, a recent study attempted to quantify the extent and the identity of epigenetic changes in the aging process. DNA methylation at >27,000 CpG sites throughout the human genome, was examined in frontal cortex, temporal cortex, pons, and cerebellum from 387 subjects between 1 and 102 years of age. The authors were able to demonstrate the presence of CpG loci, mainly associated to CpG islands, which showed highly significant correlation between DNA methylation and age; they also confirmed the positive correlation between age and DNA methylation level. Moreover, the loci showing the most significant association with age were physically close to genes and involved in DNA binding and regulation of transcription, suggesting that specific age-dependent DNA methylation changes could be responsible for regulating gene expression in the human brain [132].

A very intriguing and useful model to study normal and pathological aging is represented by the monozygotic twins discordant for the occurrence of age-related diseases. Monozygotic twin siblings share the same genotype because they are derived from the same zygote. Despite the appearance, they frequently present phenotypic differences, such as their susceptibility to disease. Recent studies suggest that phenotypic discordance between monozygotic twins could be at least in part due to epigenetic differences and factors changing over their lifetime. The epigenetic drift occurring during the development is probably resulting by a combination of stochastic and environmental factors [133]. One example is represented by a study on twins discordant for Lewy body dementia that allowed postulating that epigenetic factors could play a role in Lewy body pathology [134]. In another elegant work taking advantage of a rare set of monozygotic twins discordant for AD, it was possible to observe

significantly reduced levels of DNA methylation in temporal neocortex neuronal nuclei of the AD twin. This result stressed the hypothesis that the effects of life events on AD risk could be mediated by epigenetic mechanisms, providing a more general potential explanation for AD discordance despite genetic similarities [135].

We can conclude that aging is a process characterized by genetic and epigenetic interactions, where epigenetics has an important function in determining phenotypic differences. Epigenetics also plays a key role in the development of diseases associated with aging and explains the relationship between individual genetic background, environment, aging, and disease [136].

31.4.1 Environment, epigenetics, and aging

The discussion about the studies on monozygotic twins also helps to introduce another fundamental concept regarding epigenetic changes occurring with aging: it is the idea that epigenetic mechanisms could be triggered by environmental factors or, in a slightly different point of view, that epigenetics exerts the role of mediator of environmental stimuli. The term "environmental" encompasses, in this case, many different processes and conditions occurring outside but entering in contact with the organism. These environmental factors include, among others: stresses (physical and behavioral), nutritional factors, pollutants and pesticides, chemicals, metals, physical exercise, and lifestyle. All these factors are able to cause, in the organism, biological effects that could determine (or contribute to) the onset and progression of the disease; many of these factors were found associated with the induction of epigenetic changes, as depicted in Fig. 31.2. Obviously, these factors become increasingly relevant with aging to the healthy or pathological status of an individual, due to the increased possibility to encounter different environmental "hits" or to cumulate the reiterated effects of one of these factors.

Since monozygotic twins are genetically identical, they are considered as ideal "experimental models" to study the role of environmental factors as determinants of complex diseases and phenotypes. As in the case of the above-mentioned study on twins discordant for AD, for example, the AD twin presented a history of contact with chemical species [135]. Another example of association between acute environmental stimuli and epigenetic-dependent disease phenotypes is given by the observed increase of CpG-island promoter hypermethylation in tumor suppressor genes in the oral mucosa of smokers (reviewed in 136). Environmental factors are increasingly claimed as responsible for neurodegeneration-related modifications; a link between environmental-induced epigenetic modification, oxidation, and repair of AD related genes, is discussed in depth in a comprehensive review [137].

One of the most intriguing interactions between environment and epigenome is represented by the discussed results evidencing that even an apparently "mild" environmental factor, as the behavioral stress (since it does not involve a physical contact of the individual with any chemical species), could result in a long lasting alteration of epigenetic markers, leading to functional alterations. This surprising effect was very well demonstrated by the above-discussed study showing that perinatal behavioral stress in rats was responsible for altered AVP or BDNF expression, mediated by DNA methylation [56,65]. Another "mild" environmental factor that seems involved in the development of healthy or pathological aging is represented by the physical exercise. Physical exercise improves the efficiency of the capillary system and increases the oxygen supply to the brain, thus enhancing metabolic activity and oxygen intake in neurons, and increasing neurotrophin levels and resistance to stress. Regular exercise and active lifestyle during adulthood have been associated with reduced risk

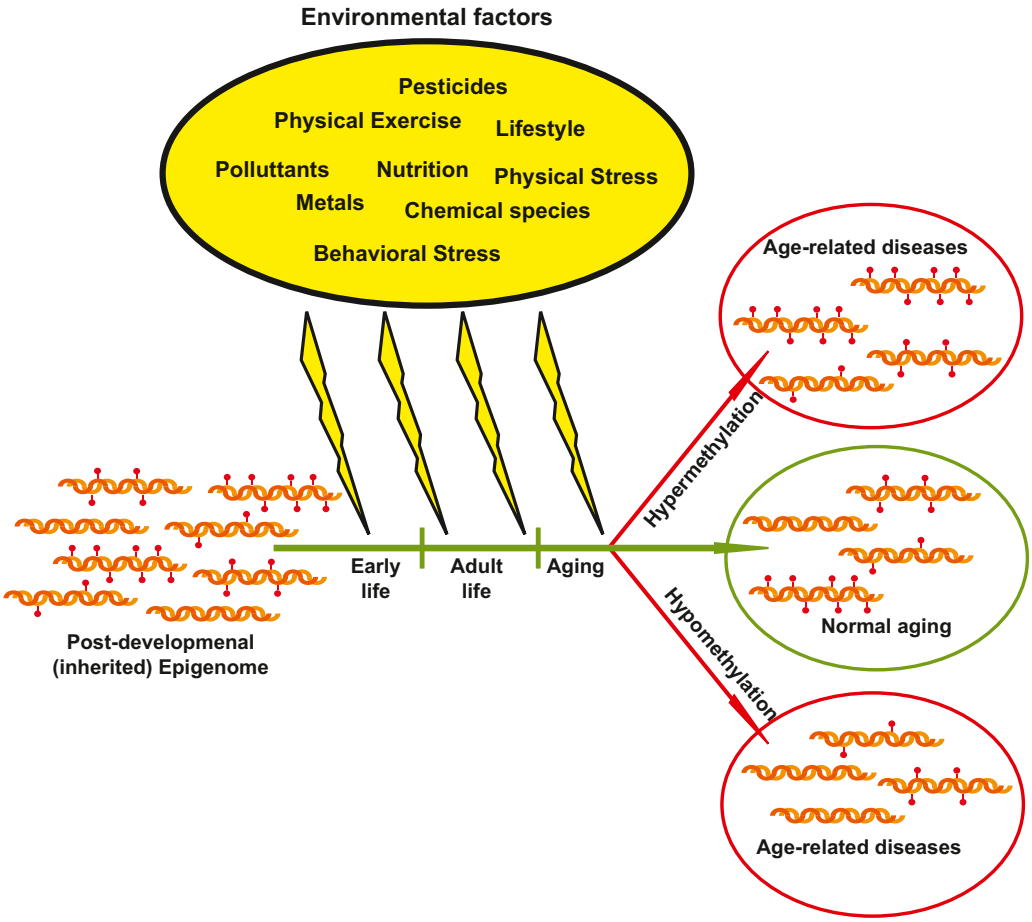

FIGURE 31.2

Many environmental factors can interfere with the organism, inducing epigenetic modifications. DNA methylation patterns established after developmental processes, for example, can be modified in sense of local and sequence-specific hyper- or (more frequently) hypomethylation. These alterations can be responsible for deviations from the normal aging processes, resulting in higher susceptibility to age-associated disease.

and protective effects for mild cognitive impairment and AD. Similarly, studies in animal models show that physical activity has positive physiological and cognitive effects that correlate with changes in transcriptional profiles possibly mediated by epigenetic modifications [138].

Nutrition and diet represent another environmental factor that can exert its influence on aging. Dietary exposures can have consequences even many years later and this observation raises questions about the mechanisms through which such exposures are "remembered" and how they can result in altered disease risk. There is growing evidence that epigenetic mechanisms may mediate the effects of

nutrients, micronutrients, and even nonnutrient dietary factors nutrition and may be causal for the development of complex diseases [139]. Alterations in DNA methylation during aging can depend on alterations in dietary status, and the great influence of nutritional components on health and lifespan is largely accepted. Among the various mechanisms by which nutritional elements could affect the progress of aging, two pathways involve DNA methylation: the first involves the supply of metabolites of the S-adenosylmethionine cycle (and will be extensively discussed in the next paragraph), whereas the second is referred to elements able to directly modify the DNMT activity (selenium, cadmium, and nickel). However, other nutritional factors seem able to determine epigenetic modifications without directly perturbing the core of the methylation reactions. One example is given by the link between under- and overnutrition during pregnancy and the consequent (later in life) development of diseases such as diabetes and obesity. Epigenetic modifications may be one mechanism by which exposure to an altered intrauterine milieu may influence the onset of these disturbances much later in life. As a matter of fact, it was demonstrated that epigenetic modifications affecting processes important to glucose regulation and insulin secretion are present in the pancreatic β-cells and muscle of the intrauterine growth-retarded offspring, characteristics essential to the pathophysiology of type-2 diabetes. Moreover, epigenetic regulation of gene expression contributes to both adipocyte determination and differentiation in in vitro models [140]. The epigenetic connection between nutrition and age-related diseases was well presented by Tollefsbol and colleagues in relation to cancer onset [41], whereas the work performed in my laboratory in relation to the connection between nutrition and epigenetics in LOAD and the role of nutrition in the modulation of methylation reaction were summarized in a recent commentary [141] and will be here exposed more in detail.

31.4.2 Nutrition, epigenetics, and aging

When we are clear in mind that mild environmental stimuli have the potential to modify the epigenome, it is easy to understand that nutrition represents probably the main among these stimuli, in terms of continuous exposition and of variety of bioactive molecules introduced. Today, diet is considered as a relevant source of epigenetic variation not only during embryonic and early life but also during aging [142]. The observation that dietary exposures can have consequences even many years after the "event" and this observation opens the question regarding the mechanisms through which such exposures are "remembered" and how they can result in risks for unhealthy aging or AD [143]. Epigenetics has the characteristics to give the answer, since the epigenetic modifications are able to mediate the effects of macronutrients, micronutrients, and even nonnutrient dietary factors and may be causal for the development of complex diseases [139]. Among the different epigenetic markers, DNA methylation is particularly correlated to nutrition. Different mechanisms allow nutritional elements (vitamin B deficiency, alcohol, pesticides, and metals) to affect the progress of aging and are associated with AD risk; among these, two pathways are related to DNA methylation: the first involves the supply of metabolites of the S-adenosylmethionine cycle (also known as "one-carbon metabolism") [141,144], whereas the second is referred to elements that can directly modify the DNMT activity (selenium, cadmium, and nickel). However, other nutritional factors can modulate the epigenetic modifications without directly perturbing the core of the one-carbon metabolism or the methylation reactions. The effects of under- and overnutrition during pregnancy and the consequent (later in life) development of diseases are increasingly studied for the relevance in the areas of obesity, diabetes, and metabolic syndrome [145]. It was demonstrated that epigenetic modifications affecting

processes important to glucose regulation and insulin secretion are present in the pancreatic β-cells and muscle of the intrauterine growth-retarded offspring, characteristics essential to the pathophysiology of type-2 diabetes. Moreover, epigenetic regulation of gene expression contributes to both adipocyte determination and differentiation in in vitro models [140]. The epigenetic connection between nutrition and age-related diseases is largely investigated in relation to cancer onset [41].

The influences of nutritional factors on the regulation of the epigenetic marks have been extensively investigated; however few studies so far considered the role of specific food components in regulating epigenetic patterns during neurodegeneration and AD. The "nutriepigenomic" appears to have a great potential in the field of neurodegenerative and age-related diseases and promises the opportunity to treat or, at least, to implement standard pharmacological interventions [146].

The most recent evidences describing the epigenetic changes induced by nutritional interventions in the adulthood mainly consider four main "epigenetic diets" that can be identified as: caloric restriction, supplementation with nutrients involved in one-carbon metabolism, supplementation with bioactive food components and ketogenic diets [147].

Finally the connection between nutrition, with specific attention to B vitamins and folate, and epigenetics in AD and the role of nutrition in the methylation reaction homeostasis represent an attractive area of investigation which is discussed in Section 31.5 of this chapter.

31.4.3 Epigenetics and age-associated diseases

Epigenetic modifications appear to be causative of, or at least involved in, an increasing number of human diseases. As previously discussed, a modern and developing concept points to the fetal or perinatal origin of adult diseases, and the adaptation response to environmental stimuli leads to increased susceptibility to age-associated diseases [148]. Although the mechanisms mediating and expressing this "memory" of the early life throughout the aging are not clearly unraveled, it is clear that an epigenetic basis exists. Apparently, the consequent increased susceptibility to the disease recapitulates as well the mechanisms typical of the decline observed in normal aging. The involvement of multiple organ systems in the pathological aging phenotype can be assimilated to the "frail syndrome." *Frailty* is defined as a nonspecific state of global vulnerability reflecting multisystem pathology due to decreased adaptation to stressors and reduced functional reserve that becomes not sufficient to maintain and repair the aging body [149,150]. Clinically, frailty is characterized by low physical activity, global weakness, low muscle strength, fatigability, general slowness, and loss of weight [151]. As a matter of fact, frailty is strictly related to epigenetic mechanisms. It was demonstrated that global DNA methylation levels were correlated to the frailty status and that the worsening of frailty was significantly associated with DNA demethylation [152]. Identification of the role of epigenetic drift in the onset of frail status also represents the opportunity to underline the connection between epigenetics and other age-associated diseases. Part of the frail phenotype is, in fact, connected to other disease typical of the old age and characterized by evident epigenetic bases. As a matter of fact, significant high levels of inflammatory markers and activation of the clotting cascade were found in frail patients; these markers are, as well known, also risk factors for cardiovascular diseases (CVD) [153]. An emerging theory identifies an epigenetic basis also for the chronic low-grade inflammation typical of aging, generated by the increasing in the production of proinflammatory cytokines and other markers that lead to the definition of "inflamm-aging" status. This status is, in turn, comparable to frailty due to the multiorgan (brain, liver) and different tissue (adipose, muscle) contribution [154].

Finally, this complex picture involving inflammation and multiorgan contribution to the aged phenotype, finds a further piece of the jigsaw in the epigenetic basis of another complex disease like diabetes. Diabetes also involves alterations of the inflammatory markers and undergoes environmental influences mediated mainly by the nutrient intake; thus aging, frailty, nutrition, early life-events, and epigenetics are, once again, connected [148,155–157]. A very important concept emerging from these studies is that malnutrition is often associated with aging but that this deficit should be seen in terms of quality and variety of foods rather than in terms of quantity [155].

Epigenetics, disease, and aging find connection also in another complex relationship represented by the telomere attrition and the onset of cancer. Telomere attrition and mutations accumulation due to the deficit of the DNA repair machinery occurring with age is one of the main causes of age-associated genome instability. Telomeres are composed by G-rich repetitive DNA sequences (TTAGGG); these sequences are bound to complexes and specialized proteins, localized at the end of linear chromosomes. Telomere length shortens with age as a consequence of decreased DNA methylation, until the minimal required length necessary to maintain telomere structure and function is reached [158–160]. The activation of the mechanism responsible for the maintenance of telomeric length (telomerase) is considered a hallmark of cancer while attrition is associated with the aging phenotype [161] depending on the response of the DNA damage checkpoint [162]; as previously discussed, the human telomerase reverse transcriptase gene (hTERT) is regulated by epigenetic mechanisms [163]. Recently, different models of transgenic mice deleted for the shelterin proteins (the major complex bound to telomeres) have been generated and could help the future study on the role of telomeric attrition and instability in aging and cancer [160]. Besides the epigenetic regulation of the telomeric length, cancer onset is clearly associated to epigenetics via the transcriptional repression of tumor-suppressor genes by CpG island promoter hyper-methylation (reviewed in Ref. [164]); in addition to classical tumor suppressor and DNA repair genes, cancer-associated hypermethylation also affects genes involved in premature aging and miRNAs with growth-inhibitory functions.

Epigenetic changes associated to aging and very often induced by environmental stimuli seem therefore responsible for the possible onset of different, although strictly interconnected, pathologies typical of the elderly. The reversal of this concept is maybe even more interesting: if external stimuli can cause epigenetic drift leading to diseased aging, then the opposite stimuli can be beneficial. Moreover, since epigenetic modifications are, intrinsically, reversible, epigenetic drugs can be used to treat major problems of health. The efficacy and safety of these drugs depend upon the efficiency of the pharmacogenetic and pharmacoepigenetic processes involved. Since the information available on the pharmacoepigenomics of most drugs is very limited, specific studies should be incorporated in drug development and personalized treatments [165].

31.4.4 The fleeting DNA hydroxymethylation in the aging-associated diseases

While DNA methylation is known for decades, a new “epigenetic” modification of the DNA such as 5-hydroxymethylation has been very recently described. Rapidly, also the enzymes responsible for the establishment and removal of this modification (e.g., TET1, TET2, TET3) have been characterized [166]. In a very short time, the presence of 5-hydroxymethylcytosine (5hmC) has been found associated to many physiological and pathological processes, including the aging process and the onset and development of age-associated diseases. Similarly to the other epigenetic modifications, it was demonstrated that 5-hmC levels could be modulated by different environmental factors including

vitamin C, phenobarbital, hydroquinone, and environmental allergens [167]. At the same time, altered DNA hydroxymethylation profiles were observed in different diseases including melanoma [168] and prostate cancer [169,170], developmental diseases, and late-onset diseases such as Huntington's disease, AD, and neuropsychiatric disorders [171].

Through the presence of their functional groups, 5mC and 5hmC seem to exert opposite effects on gene expression; whereas DNA methylation is generally associated with gene silencing, 5hmC seems mainly associated with increased gene expression. As already discussed, DNA methylation plays a fundamental neurodevelopmental and neuroprotective role, and the environmental induced alteration of methylation pattern may cause neurodegenerative processes. Similarly, the levels of 5hmC in brain increase progressively with aging, but hydroxymethylation pattern seems to be changed or compromised in case of neurodegenerative diseases [172] such as AD [173]. Actually, data so far seem still not sufficient to draw a clear role for 5hmC in AD or even to infer which direction (hyper- or hypo-hydroxymethylation) is associated to the disease. As a matter of fact, studies in AD models and in tissues from AD patients reported controversial results. In mouse models of AD both increased [174] and decreased [175] 5hmC levels were found, or even no changes associated with mice aging [176]. Studies on AD mouse models could be potentially limited since they represent the "genetic" form of the disease, rather than the sporadic and age-dependent form. However, studies in AD patients showed similarly contrasting result: although differentially hydroxymethylated loci were found in AD brains [177,178], both hyper- [179] and hypo-hydroxymethylation [180] were reported.

These inconsistent data could possibly be explained by a different point of view on the role of DNA hydroxymethylation: 5hmC originates from hydroxylation of 5-mC but the reaction doesn't end at this stage. On the contrary, further oxidation of the modified base results in final net DNA demethylation; additionally, TET proteins could exert a role in gene regulation independently on their enzymatic function [181]. 5hmC was initially considered a stable intermediate between methylation and demethylation and this idea has raised questions about the functions of 5-hmC in gene regulation in cells, tissues, and organs in response to environmental exposure [167]. After the great enthusiasm of the first hour for the discovery of a new epigenetic mark that carried with it new perspectives in the comprehension of the gene regulation processes and also in the clinical treatment, more cautious positions seem now opportune [182]. As a matter of fact, the rapid turnover of 5-hmC and the speed of the steps leading from methylated to demethylated cytosine, passing through hydroxymethylation, may induce to consider the hydroxymethylcytosine as a "not-so-stable" intermediate of 5-mC transformation. According to this point of view, is emerging the idea that 5-hmC half life is too short to impact gene regulation. In addition, robust evidence for 5-hmC transmission through cell divisions, a fundamental characteristic of the epigenetic modifications, are still lacking. In conclusion, more recent theories indicate that 5-hmC could unlikely be an epigenetic mark "per se" but rather the long time chased mechanisms responsible for "active" DNA demethylation, i.e., DNA demethylation in absence of cellular replication [183−185]. Since the two hypotheses are still not completely demonstrated or accepted, it will be wise to maintain a cautious approach in defining the functional role of 5hmC.

31.4.5 miRNAs in neurodegeneration

miRNAs are RNA molecules composed of about 22 nucleotides on average, synthesized from non-coding regions of the genome or from the DNA strand complementary to these coding for specific messenger RNA (mRNAs). miRNAs are present in plants, animals, and viruses and exert the role of

regulatory factors of gene expression by binding to messenger RNAs. This is usually a role of negative regulators of mRNA expression since they bind complementary sequences in the 3′-UTR of target mRNAs, causing repression by reducing the accessibility to the sequence for the translation machinery, or even by mediating the degradation of the target mRNAs [186]. Therefore, miRNAs are included in the list of the epigenetic factors that can modulate gene expression, although the lack of evidence assessing the transgenerational heritability of miRNAs still promote debates regarding the opportunity to include these molecules among the epigenetic modifiers. miRNAs are known to be transmitted to the progeny through the female germline (i.e., the oocyte); however, the casual way of transmission of these molecules through the meiotic oocyte division and to the embryo could mine the confidence toward including the miRNAs among the epigenetic modifications [187,188].

In AD, the dysfunction of a wide range of microRNAs promotes the generation and insufficient clearance of amyloid-β (Aβ) and increases tau plaques which are the pathophysiological markers of AD. Disturbance of these microRNAs is associated with mitochondrial dysfunction, oxidative damage, inflammation, apolipoprotein E4 (APOE4) pathogenic process, synaptic loss, and cognitive deficits induced by AD [189]. Differential gene expression analyses confirmed the involvement of several miRNAS, specifically miR-129-5p, miR-132-5p, miR-138-5p, and miR-195-5p in AD pathogenesis and highlight several novel potentially relevant target mRNAs [190]. In particular, it has been disposed that miRNAs are involved in neurodegenerative diseases by four main mechanisms including the imbalance between proinflammatory and neuroprotective cells in microglia and astrocytes, NLRP3 inflammasome, oxidative stress, mitochondrial dysfunction, and inflammatory mediators [38].

For example, studies on miR-124, one of the most abundant microRNAs in the central nervous system, have shown that its dysregulation is related to the occurrence and development of pathology within the central nervous system [191]. Another well-studied miRNA known for its role in neurodegeneration is the miR-155. miR-155 plays main roles in several physiological and pathological mechanisms, such as Down syndrome (DS), immunity and inflammation, and in AD. For this reason it is studied as potential therapeutic target. The miR-155 is one of the overexpressed miRNAs in patients that contribute directly and indirectly to the onset or progression of the neurodegeneration. Since the miR-155 can simultaneously reduce the translation of several genes at post-transcriptional levels, targeting the miR-155 might set the stage for the treatment of different pathogenic mechanisms in which it is involved. In AD, in particular, it seems to act through the regulation of BACE1, responsible for the amyloidogenic pathway [192]. Many other miRNAs (miR-22-3p, miR-29 family, miR-106b, miR-143-3p, miR-212-3p, miR-384, miR-485) have been found to be associated with neurodegeneration, either with "protective" or "pathological" roles, by targeting genes directly involved in amyloidogenesis and tau phosphorylation or indirectly involved in the neuroinflammatory and oxidative processes associated to the disease [39,189,193].

Many other classes of noncoding RNAs (ncRNA) are increasingly capturing the attention of the researchers for their role in the modulation of gene expression and protein synthesis and functions. Among these, the long noncoding RNAs (lncRNAs) are a largely studied class with several evidences linking their expression to aging and neurodegenerative disease. However, the mechanisms of action of the lncRNAs are various, ranging from the transcriptional to the translational and posttranslational levels, making it even more difficult than for the miRNAs their clear inclusion in the family of the epigenetic modifications. While further consensus is needed to classify lncRNAs as epigenetic modifiers, a comprehensive overview of their role in aging and neurodegeneration is reviewed in Ref. [194].

31.5 One-carbon metabolism

Studies on epigenetics exponentially increased, evident by the raise, from a scarce dozen in 1980 to over 5000 papers published in 2010, of PubMed citations involving epigenetics. Despite this great increase, one could suspect that a real knowledge of the biochemical basis regulating epigenetic mechanisms (DNA methylation, for instance) is often missing in these works; as a matter of fact, we can see that PubMed citations in which "epigenetics" and "homocysteine" are both present are only 96 in the period 1980–2010. This observation can be considered as a clue that the biochemical pathway underneath the transfer of methyl groups (the most relevant epigenetic modification) is quite unknown, even though this pathway (known as "One-carbon metabolism," being characterized by the transfer of monocarbonic units like the methyl group –CH3) has a number of biochemical reactions comparable to ATP pathway.

One-carbon metabolism, also known as homocysteine (HCY) cycle, is a complex biochemical pathway regulated by the presence of folate, vitamin B12, and vitamin B6 (among other metabolites) and leading to the production of methyl donor molecule S-adenosylmethionine (SAM). A schematic and partial (focused on SAM and B vitamins) representation of this metabolic cycle is reported in Fig. 31.3. SAM can donate the methyl group to different substrates (lipids, proteins, and DNA) being

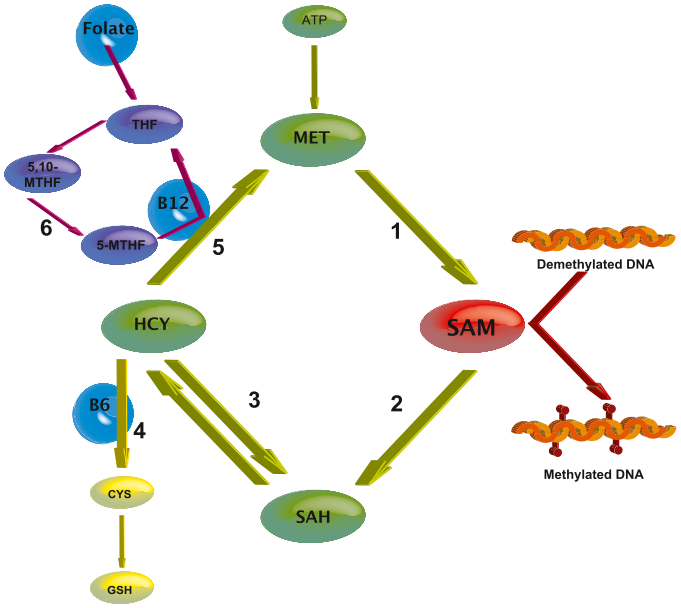

FIGURE 31.3

Schematic representation of the most relevant reactions comprises in the "one-carbon metabolism," emphasizing vitamin cofactors cycle (remethylation) and SAM-dependent DNA methylation (transmethylation). Abbreviations: *Met*, methionine; *SAM*, S-adenosylmethionine; *SAH*, S-adenosylhomocysteine; *HCY*, homocysteine; *CYS*, cystathionine, *GSH*, glutathione; *THF*, tetrahydrofolate; *MTHF*, methyltetrahydrofolate; *B12*, vitamin B12; *B6*, vitamin B6; 1, methionine adenosyltransferase (MAT); 2, methyltransferase(s); 3, SAH hydrolase; 4, cystathionine-β-synthase (CBS); 5, methionine synthase; 6, methylenetetrahydrofolate reductase (MTHFR).

converted into S-adenosylhomocysteine (SAH), a strong competitive inhibitor of methyltransferases. SAH is then hydrolyzed to adenosine and HCY, a thiol containing amino acid produced during the methionine metabolism via the adenosylated compound SAM, once formed is either converted to cysteine by transsulfuration (further leading to glutathione synthesis), remethylated to form methionine or exported to blood [195−198]. In the remethylation pathway HCY is remethylated by the vitamin B12-dependent enzyme methionine synthase (MS) using 5-methyltetrahydrofolate as cosubstrate. Alternatively, mainly in liver, betaine can donate a methyl group in a vitamin B12-independent reaction, catalyzed by betaine-homocysteine methyltransferase (BHMT). In the transsulfuration pathway, HCY can condense with serine to form cystathionine in a reaction catalyzed by the cystathionine beta synthase (CBS), a vitamin B6-dependent enzyme, and the cystathionine is hydrolyzed to cysteine (Cys); it is not sure if this pathway is normally active in the brain, due to the lack of the enzyme γ-cystathionase, but a recent study demonstrated the presence of a functional (maybe alternative) transsulfuration pathway also in this tissue [199]. Cys is used for protein synthesis, metabolized to sulfate or used for glutathione (GSH) synthesis. The tripeptide GSH is the most abundant intracellular nonprotein thiol, and it is a versatile reductant, serving multiple biological functions, acting, among others, as a quencher of free radicals and a cosubstrate in the enzymatic reduction of peroxides [200].

HCY accumulation causes the accumulation of SAH because of the reversibility of the reaction converting SAH to HCY and adenosine (Ado); the equilibrium dynamics favors SAH synthesis. The reaction proceeds in the hydrolytic direction only if HCY and Ado are efficiently removed. At least in brain and other tissues, SAH is a DNA methyltransferase inhibitor, which reinforces DNA hypomethylation [201]. Therefore, an alteration of the metabolism through either remethylation or transsulfuration pathways can lead to hyperhomocysteinemia, decreasing of SAM/SAH ratio (often indicated as the "methylation potential," MP) and alteration of GSH levels, suggesting that hypomethylation is a mechanism through which HCY is involved in vascular disease and AD, together with the oxidative damage [5,202−204].

31.5.1 One-carbon metabolism in aging and neurodegeneration

Methylation of CpG dinucleotides plays an important role in regulation of gene expression in the brain. It was discovered that brain specific promoter-related sequences are surprisingly enriched in CpG sites. This leads to the conclusion that it is likely that brain-specific transcription is regulated by methylation at an epigenetic level much more frequently than tissue-specific expression in other organs. Low methylation status is strongly associated with neurological and cognitive deficits. Many epidemiological studies have shown that factors connected to low methylation status such as elevated total homocysteine, low folate or low vitamin B12 levels are associated with increased risk of cognitive decline, dementia, and brain atrophy. In recent years, hyperhomocysteinemia has begun to be widely considered a risk factor in AD, and this may be ascribed to alteration of the S-adenosylmethionine/homocysteine (SAM/HCY) metabolism [205]. Indeed, a frequently observed condition in AD-affected people is the increase of plasmatic HCY, sometimes along with the decrease of vitamin B12, B6, and folate uptake [206]. In AD, the loss of precise control through gene methylation may alter a delicate equilibrium among the three enzymes (α-, β-, and γ-secretases) known to be involved in the production of either amyloid-beta (Aβ) or other nondangerous catabolites [207].

It is well established that α-secretase cleavage of APP does not produce the amyloidogenic peptides and that, on the contrary, they are produced by the activity of β-secretases that generate an N-terminal soluble fragment and a C-terminal fragment that is sequentially cleaved by γ-secretase to produce Aβ peptides [208]. The alteration of SAM/SAH ratio is tightly related to the altered expression of two genes involved in APP metabolism, finally producing the accumulation of Aβ peptide in the senile plaque. In the past 50 years, SAM has been shown to be perhaps the most frequently used substrate, after ATP, and therefore occupies a central position in human health, disease, and aging. SAM is known to be the primary methyl-donor present in eukaryotes, and it is involved in methylation of target molecules like DNA, RNA, proteins, lipids, and polyamines synthesis [209]. SAM appears to be altered in some neurological disorders, including LOAD [27]. About 95% of SAM is engaged in methylation reactions. These metabolic alterations may be responsible for the generalized reduction of DNA methylation observed in aging, which can lead to overactivation of methylation-controlled genes.

One-carbon metabolism imbalance could be also induced by folate deficiency, frequently observed in individuals over 65 years of age and primarily caused by malabsorption or nutritional deficiency. Folate deficiency is associated with mild cognitive impairment, dementia, AD, and depressive syndromes both in healthy aging and neuropsychiatric diseases. No clear evidence exist that folate supplementation can improve cognition or contrast cognitive impairment. On the one hand, these population-based studies stress the role of SAM as a better epigenetically based intervention in aging and in aging-associated diseases, on the other hand they point out the need for additional and targeted studies [210].

DNA methylation represents an important mechanism for epigenetic control of gene expression and the maintenance of genome integrity. Methyl deficiency results in global hypomethylation of the genome. Hence, methyl deficiency leads to disturbances in gene expression. Interestingly, the aging process leads to similar changes in the methylation pattern.

31.6 Epigenetics and neurodegeneration: the Alzheimer's disease paradigm

The sporadic form of Alzheimer's disease (LOAD) is considered multifactorial. Genetic, nutritional, metabolic, environmental, and social factors are associated with onset and progression of the disease [81,126,211] but, despite the extent of researches in this area, most causes of LOAD remain obscure. The complex, interconnected, non-Mendelian etiology of LOAD suggests that epigenetic components could be involved and epigenetic modifications could represent candidate effectors mechanisms mediating the above-mentioned risk factors in AD onset and progression. Though it is already well known that epigenetic changes could act in several physiological and pathological processes, few papers pointed the attention on epigenetic regulation of aging and neurodegeneration. Nevertheless, it is evident that whereas genetic factors are clearly associated with early onset form of AD, epigenetic factors could be more easily linked to LOAD, since the epigenome is prone to changes during development and also aging [212–214]. As a matter of fact, the epigenetic mechanisms can be considered as a link between environmental stimuli and their effect on the genome and on the pathologies. For these reasons, LOAD research is facing an increasing interest in the study of epigenetic mechanisms possibly involved in the pathology [62,141,215]. Due to the large development

of wide-scale analysis techniques and their application to the study of epigenome, it has been possible to demonstrate that DNA methylation in individuals changes over time [216]. Moreover, DNA methylation pattern in human brain is different in different brain areas [126] and can dynamically change during the lifespan [217]. Finally, it has been demonstrated the existence of epigenetic changes in LOAD patients that can easily contribute to the onset and development of disease [218] evidencing in particular that, among others, PSEN1 gene was differentially methylated and that there was a correlation between gene demethylation and LOAD. In agreement with this result, we also demonstrated that PSEN1 promoter demethylation is responsible for the overexpression of the gene [61,126].

As previously described, methylation metabolism is dependent on the "one-carbon" metabolism (Fig. 31.3). High HCY and low B vitamin levels are positively associated with LOAD [219–221], even if the cause-effect relationship is still not completely demonstrated. In this scenario, epigenome modulation could represent the molecular link between hyperhomocysteinemia and LOAD course and onset [222–224]. Moreover, one-carbon metabolism alteration and consequent methylation reactions unbalance (i.e., loss of methyl groups) during aging represents one of the mechanisms by which environmental and dietary factors can promote LOAD [81,192,225–227]. Finally, the finding that one-carbon metabolism alterations can affect also tau processing [228] stresses the hypothesis that this metabolism, and methylation reactions in particular, could retain a central role in LOAD, connecting both amyloidogenic and neurofibrillary pathways.

Two genes directly involved in AD showed changes in methylation pattern. It was demonstrated that APP gene promoter was differentially modulated [229] and it was found hypomethylated in postmortem brains of AD patients [117,230]. Besides APP, also PSEN1 has been found hypomethylated both in AD patients [218] and in our experimental models characterized by unbalanced methylation pathway [59,60,126].

31.6.1 Aged AD mice and epigenetics

These studies, spacing from specific gene to large-scale studies, both in LOAD patients and in experimental models, are very well summarized in two comprehensive reviews [62,231]. Studies on LOAD subjects evidenced that DNA methylation, the main epigenetic modification, is associated to Alzheimer mechanisms. Studies on experimental models, although not supported by a final proof in AD patients, had the fundamental value of investigating the molecular mechanisms linking DNA methylation to the onset and the progression of the AD-like features observed in these models. Several groups investigated the role of epigenetics in AD in different areas of cell biology as Aβ processing and scavenging, tau phosphorylation, inflammation, apoptosis, cell cycle dysregulation, ApoE expression (summarized in 62).

In our laboratory, during the past 10 years, we addressed our studies to the area of DNA methylation in human neuroblastoma cells [232,233] and then in a murine model of Alzheimer's disease [126], developing an experimental model of nutritionally induced hyperhomocysteinemia. Taking advantage of the massive and rapid amyloid deposition of the TgCRND8 mice, carrying a double mutated human APP gene, we were able to demonstrate that hyperhomocysteinemia induced by B vitamins deficiency (Folate, B12, and B6) caused an alteration in SAM and SAH levels, inhibiting SAM-dependent methyltransferases. This alteration was responsible for disequilibrium in the DNA methylation machinery consisting in the decrease of the methyltransferase and increase of the demethylase activities [60]. The DNA methylation impairment was correlated to the site-specific

demethylation of PSEN1 promoter, with consequent gene overexpression [61]. Other studies found similar results in a different cellular model [234] also suggesting the role of DNA hypomethylation in the increase of PSEN1 expression and amyloid production.

More recently, the ability of SAM to contrast hypomethylation, diminish the amyloid pathology, and restore cognitive capabilities was also demonstrated in a different strain of APP mice, confirming the result obtained in our laboratory, but also pointing out that SAM could have slightly different effects at low and high doses [235]. Further possible application of SAM treatment to neurodegenerative diseases in humans should therefore take into account these data.

Obviously, all the murine AD models generated so far are rather models of the familial, genetic disease, but are necessary to investigate the effect of the treatment on the AD-like symptoms (i.e., amyloid and tau accumulation and cognitive impairment). However, at least in the case of the TgCRN8 mice used in our lab, we superimposed the B vitamin deficiency mimicking one of the risk factors of the sporadic AD.

If the involvement of DNA methylation in LOAD is now largely accepted, its causal or consequential role still remains to be ascertained [129,236]. One hypothesis is that alteration of methylation reactions could be dependent on other major pathways involved in LOAD onset, representing just a marker of the disease. Experimental evidences in this sense are given by the finding that exogenous Aβ 1−40 seems to induce the hypermethylation of Neprilysin gene [237]. To prove that PSEN1 hypomethylation is a cause and not a consequence of the AD-like phenotype in our mouse model, we analyzed the methylation pattern in old mice (presenting increased Aβ pathology) versus young mice and in transgenic versus wild-type mice (which are not affected at all by Aβ pathology); our results (submitted at the moment this chapter was written) clearly indicate that PSEN1 methylation is not dependent on Aβ levels, since similar methylation patterns were found in young and old mice, both transgenic and wild-type. This result further stresses the idea that epigenetics plays a pivotal and fundamental role in the onset and progression of age-related diseases like LOAD.

31.6.2 Rediscovered non-CpG methylation

Almost all epigenetics textbooks and the vast majority of scientific articles report, so far, that DNA methylation almost exclusively occurs cytosines associated with CpG dinucleotides in mammals [238−241]. As a matter of fact, methylation of cytosines moieties associated with CpA, CpC, and CpT dinucleotides, i.e., the so-called "non-CpG methylation," has been poorly documented in mammals and has been described as an epigenetic mark of limited extent and characteristic of specific cell types, such as stem cells, or specific developmental stages. DNA methylation levels and associated patterns of distribution across the genome are extremely variable depending on cell type, differentiation stage, tissue, and age. In embryonic stem cells, high levels of non-CpG methylation have been observed with respect to other cells. In mouse, non-CpG methylation in embryonic stem cells (ESCs) has been calculated to be among 15% and 20% of total 5-mCs [242] and associated with the activity of de novo DNMTs (3a, 3b, and L). These data were further confirmed also in human and in larger genome fractions, taking advantage of genome-wide mapping of cytosine methylation. In fact, in human ESCs and induced pluripotent SCs (IPSCs), non-CpG methylation levels ranging from 12% to 25% were observed together with the functional association of non-CpG methylation in gene bodies with gene expression [243−245].

More recently, growing evidence indicate that unexpected high levels of non-CpG methylation (25%−35% of total DNA methylation) are present in adult brain, both in mice and humans [246−250]. Most importantly, the observed non-CpG methylation appears to be established during neuronal development and maturation depending to de novo methylase activities and that it is functionally correlated with regulation of gene expression. One interesting observation even points out that non-CpG methylation established and accumulated in human neurons, during the developmental processes of the brain, is conserved throughout the adult age and reach, at specific stages, the 53% of total 5-mC, representing the main form of neuronal DNA methylation [251]. At present, we can affirm that non-CpG methylation plays a functional role also in adult mammalian tissues [252,253] including human brain [239]. Despite the growing number of evidence pointing out relevant and functional non-CpG methylation, the topic is still debated because of the lack of historically consolidated data. In this framework, our laboratory recently proposed a possible explanation for the tardive recognition of non-CpG methylation extent and provided further evidence that non-CpG methylation is associated with AD in humans. In fact, we recently reported that non-CpG methylation has been so far underestimated due to a technical bias consequent to the large use of software-designed primers to be used in the PCR step following the bisulfite modification of genomic DNA [59,254]. These primers, generally known as "methprimers" are indeed designed by several softwares assuming that non-CpG cytosines are always (or at least in the large majority of the cases) demethylated; the obtained primer sequences include therefore mutated bases in correspondence of the non-CpG moieties, according to the transformation induced by the bisulfite treatment. It is clear that in case of discrete presence of non-CpG methylation, the methylated non-CpG sequences cannot be recognized by these primers, leading to the selective amplification of DNA sequences with low non-CpG methylation. The approach used in our lab, on the contrary, takes advantage of PCR primers that are designed considering the uncertainty of non-CpG methylation status: on the practical point of view, these primers are designed to match sequences with few cytosines; moreover, when cytosines are present, the corresponding positions on the primers are synthesized as "degenerated" bases, in order to accommodate both methylated and nonmethylated statuses [254]. This approach allowed us to demonstrate that non-CpG methylation is present at specific loci, as the two proinflammatory cytokines IL-1b and IL-6, in the brain and is differentially modulated in the brain of AD patients [255,256]. Interestingly, the non-CpG methylation patterns seems particularly prone to be altered by modulation of the one-carbon metabolism through "environmental" interventions (like dietary supplements) than CpG methylation [257].

Finally, a recent work by our lab highlighted that PSEN1 is differentially methylated, with at CpG and non-CpG moieties in the human brain during neurodevelopment, aging, and neurodegeneration, stressing once again the role of the epigenetics in the onset of the pathology [258].

31.6.3 The DNA methylation-miRNAs interplay

A class of short noncoding RNAs, also called microRNAs (miRNAs), can bind complementary sequences on cellular mRNAs, usually causing the mRNA degradation and translation inhibition. Because of this effect, miRNAs are listed among the epigenetic factors as modulator of gene expression. How miRNAs expression is, in turn, regulated is still the object of active investigation, but DNA methylation seems to play a central role. The "one-carbon" metabolism is responsible, as previously discussed, for the metabolic regulation of transmethylation reactions and, therefore, for DNA methylation. It is then reasonable that DNA methylation modulates miRNAs expression alike it does

with mRNAs expression. Moreover, recent evidence indicates that, vice versa, miRNAs are associated with DNA methylation modulation, in a mutual cross-talk [36]. The concept above described is schematized in Fig. 31.4.

Studies on miRNAs and their functional roles have exponentially increased in the past decade, leading to the description of hundreds of different miRNAs in human and allowing the association of most of them to specific transcripts [259]. It is worthy of note that several miRNAs have been associated with specific pathological states [260], promoting the study of these molecules as new biomarkers to monitor and, hopefully, modify the course of the disease. Moreover, despite the interest and the efforts spent studying miRNAs and their functional role, a final consensus seems also lacking regarding how the expression of these molecules is regulated [261]. It is acknowledged that miRNAs synthesis may be regulated by DNA methylation, but studies also point out that the reciprocal relationship occurs, when miRNAs modulate target gene expression through DNA methylation-associated mechanisms. Recent evidence add further information to the previously known complex network in which miRNAs and DNA methylation are mutually linked adding a new layer of complexity to the posttranscriptional regulation of gene expression [262].

DNA methylation can inhibit the mRNAs transcription of coding genes by methylating cytosines in the regulatory regions. The repression appears more stable when a CpG island is present (i.e., a region rich in CpGs moieties), or more prone to dynamic changes when the gene promoter is not associated with CpG islands. miRNAs synthesis, just like for coding genes, can be repressed by methylation of the cytosines in the promoter sequence of genomic regions encoding for miRNAs [262]. Recent reports have disclosed the existence of the interplay between DNA methylation and miRNAs expression. These studies are particularly abundant in cancer models, in which it was possible to prove the

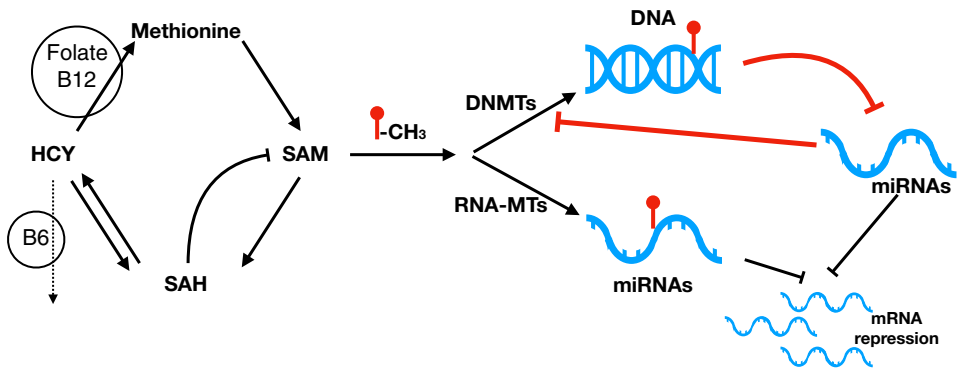

FIGURE 31.4

The mutual relationship between one-carbon metabolism and miRNAs. The one-carbon metabolism (*left*) is responsible for the homeostasis of the transmethylation reactions (-CH3). miRNAs normally repress mRNA expression by binding to target messenger; DNA methylation of miRNAs genes can suppress miRNAs production and, in turn, miRNAs can repress DNMTs causing genome hypomethylation (*right, up*). RNA-methyltransferases (RNA-MT) can add methyl groups directly to miRNA molecules, modulating their activity (*right, down*).

presence of feedback modulation between the expression of specific miRNAs and the methylation-dependent regulation in the pathology. Several miRNAs (miR-200, miR-29 family, miR-375, miR-34, and others) appear to be regulated by DNA methylation in different types of cancer and metabolic diseases. Conversely, other miRNAs (miR-301, miR-449, miR-148, miR-101, miR-214, miR-128, and others) retain the potential to modulate the DNA methylation pattern mainly targeting the regulation of the DNMTs expression [263–266].

Although miRNAs modulation leading to altered biological functions is mainly studied in carcinogenesis, recent findings put their biological functions and regulation in the way to other pathological conditions. For example, in the most common brain cancer in adults, glioblastoma multiforme (GBM), miR-211 acts as a tumor suppressor when downregulated by EZH2-induced H3K27 trimethylation and promoter methylation [267]. The methylation-dependent modulation of this miRNA is also involved in other pathologies of the central nervous system, as observed in epileptic seizures, associated to acute traumatic stress and with miR-211 upregulation [268]. Neurodegenerative diseases and brain disorders represent an expanding area of interest to study the correlation between miRNAs regulation and DNA methylation. A further example is given by the miR-608, which has a role as suppressor of AChE, CDC42, and IL-6; the downregulation of these molecules cause acute anxiety. miR-608 target CDC42 interacts with collybistin in GABAergic neurons and is actively involved in the formation of the anxiolytic GABAA receptor synapse [269]. Noteworthy, it has been demonstrated that miR-608 is epigenetically regulated in PC3 cells by the methylation of a CpG-island close to the TSS of the gene that silences the miRNA gene via transcriptive inhibition [270].

Another interesting aspect of the connection between miRNAs and DNA methylation is related to the role that miRNAs may have in the response to treatments. For example, the DNA methylation-mediated silencing of the miR-211 is responsible for the reduced sensitivity of melanoma cells to cisplatin [271]. This observation suggests that the epigenetic modulation of specific miRNAs could be used as a way to reinforce pharmacological treatments.

Certain miRNAs have the potential to target directly DNA methyltransferases causing their repression at the transcriptional or posttranscriptional level. It is expected that this repression affects the whole genome methylation since the DNMTs are ubiquitous.

Since the mutual interplay between DNA methylation and miRNAs has been ascertained, we should start to consider these two epigenetic mechanisms not just as independent events but as a piece of complex and multifaceted machinery retaining the ability to modulate gene expression in different ways. This should be particularly relevant when considering the epigenetic events involved in disease onset and progression; degenerative and neurodegenerative diseases, in particular, show epigenetic basis or contribution. Being the epigenetic modifications, including methylation and miRNAs, potentially reversible, it is advisable to look at the "big picture" (i.e., the possible presence of the two different mechanisms, methylation and miRNAs) when approaching the study of a specific disease and the efficacy of potential epigenetic therapeutical interventions.

The study of the role that the one-carbon metabolism may have in regulating both miRNAs and DNA methylation appears of extreme relevance to understanding the basic metabolism underlying these epigenetic changes. Preliminary (unpublished) results in our laboratory have shown that the alteration of one-carbon metabolism obtained by vitamin B deficiency, responsible for gene-specific hypomethylation in Alzheimer's disease models [60,272], alters the expression profile of cancer-associated miRNAs in neuroblastoma cells.

31.7 Conclusion

The great technological advance in biomedical research makes possible the constant increase of our ability to study the extremely complex phenomena encompassing the different, but highly interconnected, epigenetic modifications. This ability opened the window, in the recent years, on a previously hidden scenario where epigenetics retains a causal role in mediating the effects that environmental stimuli exert in the organism. The increasing knowledge about the regulation of the epigenetic mechanisms and the interest on the epigenetics of aging is witnessed by the large number of interesting and comprehensive reviews published in the very recent years [273–281]. This growing area of the science is particularly relevant to the study of aging associated processes, because the aging organism is increasingly exposed to continuous and different external stimuli. Now that the window is open on the mechanisms possibly responsible for the shifting from healthy to pathological aging, new questions raise together with the first results. How many, and how long, stimuli are necessary to induce changes in the normal aging processes? In which manner the organism translates these stimuli in processes that alter epigenetic modifications? Why environmental-induced epigenetic changes have different tissue, cell population, genomic sequence, or even DNA site specificity? Alternatively, is this different susceptibility an endogenous characteristic of these tissues, cells, or sequences? Are these "toxic" epigenetic modifications really reversible? And, in this case, are these modifications "druggable," in order to hypothesize epigenetic intervention and therapies? Deciphering the epigenome and its relevance to the aging processes is probably one of the most promising challenges for the researchers in the coming years and, due to its extreme complexity, it surely requires the interaction of different skills and knowledge in an interdisciplinary effort [282].

List of abbreviations

AD	Alzheimer's disease
Ado	adenosine
APP	amyloid precursor protein
AVP	arginine-vasopressin
Aβ	beta amyloid protein
CBS	cystathionine beta synthase
CpG	cytosine-guanine dinucleotide
CR	caloric restriction
Cys	cysteine
DMR	differentially methylated region
DNMT	DNA methyltransferase
ESCs	embryonic stem cells
GSH	glutathione
HCY	homocysteine
HDAC	histone deacetylase
hmC	hydroxymethylcytosine
hTERT	human telomerase reverse transcriptase gene
LEARn	latent early-life associated regulation
lncRNA	long noncoding RNA
LOAD	Late Onset Alzheimer's disease

MBD	methyl binding domain
mC	methylcytosine
miRNA	micro-RNA
MP	methylation potential
non-CpG	CA, CT, CC dinucleotides
PCR	polymerase chain reaction
PSEN1	Presenilin1
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
TET	ten eleven translocase
UTR	untranslated region

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CHAPTER

Epigenetic programming of human disease and aging

32

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32.1 Introduction

Aging has been defined as a biological process that leads to decline functioning of the organism at different levels in a time-dependent way. Such decline makes the individual more vulnerable to the development of neurodegenerative, musculoskeletal, cardiovascular, and metabolic diseases. In this context, research has focused on unveiling the factors involved in both the aging process and the age-related diseases development. Despite the current controversy regarding defining whether aging is a disease or not, there are many authors that aim to understand the molecular and cellular features that contribute to the aging process. The most outstanding effort is the contribution of Lopez-Otín and cols [1], which report nine hallmarks of aging, such as altered intracellular communication, genomic instability, telomere attrition, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and epigenetic alterations. Interestingly, there is a question about the impact of early stages of life in aging, suggesting that we are pre-conditioning the way that we age. Hence, in this chapter, we attempt to summarize the most relevant studies regarding the role of epigenetic programming of human aging and disease. First, we sketch a brief resume about the Developmental Origins of Health and Diseases (DOHaD) hypothesis, and then we complement this hypothesis with the recent epigenetic findings.

It has been stated that throughout life organisms experience epigenetic alterations in DNA methylation (DNAm) patterns, post-translational modifications of histones, chromatin remodeling and alterations in noncoding RNA expression, altogether these changes result in alterations in gene expression, reactivation of transposable elements, and genomic instability [2]; such changes represent the reversible heritable mechanisms that are responsible for the link between the genotype and the phenotype. Moreover, the occurrence of the epigenetic marks is driven by both external and internal stimuli while others are spontaneous. Nevertheless, these seem to influence the aging process triggering different phenotypes of the same process in each individual, even in the same species. For instance, it has been reported that exercise in humans alters the expression of skeletal muscle genes, these changes are mediated by epigenetic regulation [3], meaning that to different environmental stimuli, such as life style, diet, or drug consumption induces epigenetics alterations that impacts on physical appearance, behavior or lifespan. The same occurs during disease where the DNAm and

Epigenetics in Human Disease. https://doi.org/10.1016/B978-0-443-21863-7.00017-2
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changes in histone modification take place often, for instance in cancer it has been reported that promoter regions in tumor suppressor genes are hypermethylated leading to inactivation of the expression and function of these sets of genes [4]. In the same context many other human diseases such as autoimmune, neurodegenerative, cardiovascular, and metabolic showed epigenetic changes that are currently under investigation. Interestingly some of these diseases consider aging as a risk factor for their development.

There are a wide range of epigenetic modifications such as methylation, and chromatin modifications via acetylation or non-coding RNA (small interfering RNAs or microRNAs) participation which impact on gene expression. However, since DNAm is the best-known epigenetic process because it is relatively easy to study with the current technology and is the most replicated around the world, in this chapter we will focus on it.

32.1.1 How early life experiences may impact the way that we age?

Nowadays it is well accepted that an adverse intrauterine environment leads to the development of obesity and metabolic syndrome, and both increase the risk for chronic diseases such as hypertension, diabetes, or cancer, among others. This observation has been explained in the light of the Barker's hypothesis also known as the "thrifty phenotype hypothesis" suggesting that a fetus which is exposed to adverse intrauterine environments such as famines, violence, or hypercaloric diets reprogram both the endocrine and the metabolic status, providing energy for the organs essential at this life stage. However, further in life these individuals experience insulin resistance, dyslipidemia, and hypertension. This theory evolved into the Developmental Origins of Health and Diseases (DOHaD) derived from epidemiological studies of infant and adult mortality, which establishes that pathologies expressed in late life stages are due to variation in nutrition in early life [5–7]. As examples of the DOHaD hypothesis are the historical famines that evidence the transgenerational effects of undernutrition and stress. For instance, the Dutch cohort exposed to famine at the end of the World War II (1944–45) shows that individuals conceived before famine increase the risk for insulin resistance and impaired glucose tolerance in adulthood, while individual that were conceived during the famine show increased risk for dislipidemias and heart disease in adulthood [8]. As well, other studies report a strong association between early-life nutrition or maternal diet exposure with the risk of development of metabolic diseases such as Type 2 Diabetes Mellitus [9], neurodegenerative [10] and cardiovascular diseases [11] while other habits such as smoking and alcohol consumption during pregnancy are associated with cognitive impairment in the offspring [12] (Fig. 32.1).

Most of the studies mentioned above are epidemiological studies derived for observations performed across several populations at different time periods, however, current the epigenetic studies strengthen the DOHaD hypothesis and attract the attention of the health professional to know the potential risk factors and behaviors that may represent potential therapeutic targets to improve the population health.

32.1.2 How are epigenetics related to diseases and aging?

Epigenetic modifications are changes in the gene expression without alterations of the genome sequence, these modifications occur along organism lifespan in response to the environment and its behaviors (pollution, sociodemographic variables and lifestyle). Interestingly, current evidence

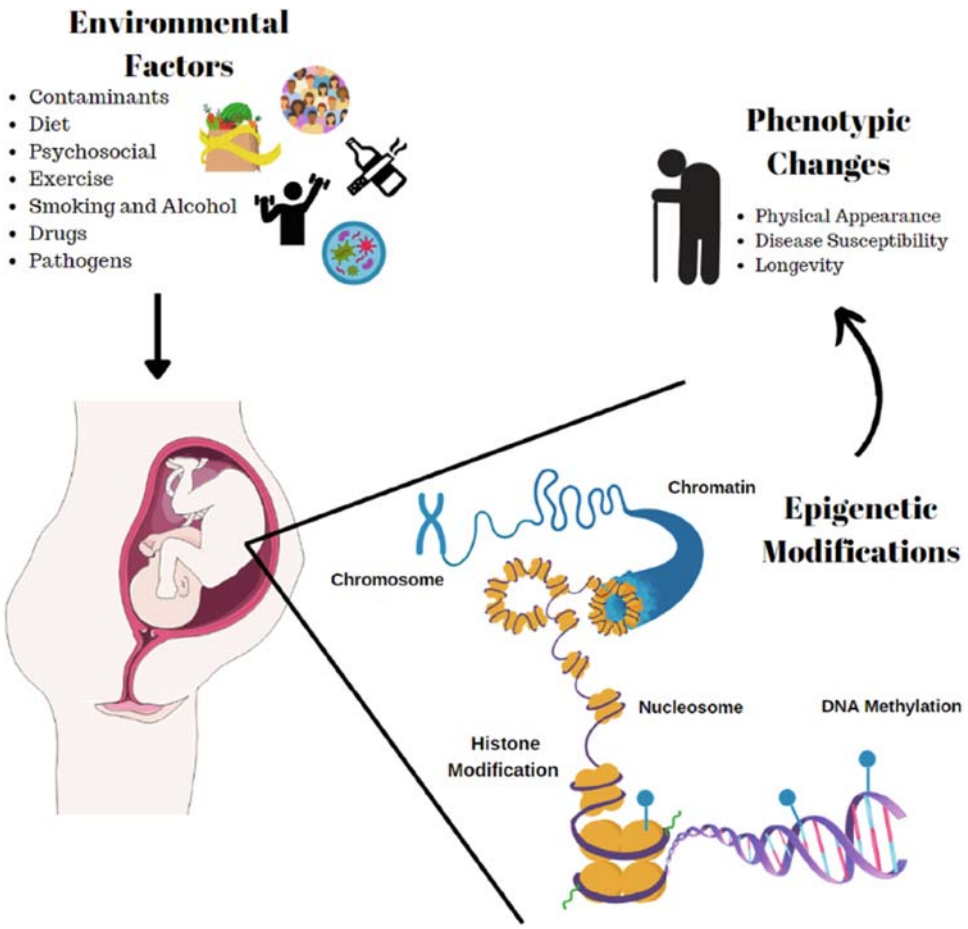

FIGURE 32.1

Epigenetics modulates the way that we age. This picture summarizes the environmental factors and the habits that affect the gene expression via the main epigenetic mechanisms.

demonstrates that these modifications condition the organism to the development of chronic diseases or the way that we age. Despite, there are four principal epigenetic process such as ATP-dependent chromatin-remodeling complexes, DNA and histone modifications, and non-coding RNAs, here we mainly focus on describing the DNA methylation (DNAm) since this is the most studied (due to the facility and reproducibility). DNAm occurs on the cytosines of the CG-dinucleotide rich genomic regions, also known as CpG islands, that are found in the promoters of the human genome and are associated to the transcriptional repression [13]. In this context, current evidence has demonstrated that epigenetic mechanisms are implicated in several aged related diseases, however, due to the vast amount of information on this topic, here we focus on the most studied and common during aging.

Moreover, in recent years, DNAm patterns have become an excellent tool to estimate biological age and predict the risk of mortality via the epigenetics clocks. The first generation of these clocks were performed in saliva samples analyzing methylation patterns associated with advancing age, and using the lasso penalized regression identify the main predictors of age [14]. The following generation of epigenetic clocks have become more popular nowadays, the most common was developed by Horvath, which is a multi-tissue epigenetic clock that reflects the impact of health and disease, it means that disease state accelerate the epigenetic clock, since this clock is near to zero in embryonic or induced pluripotent stem cells but is accelerated in obesity and other age-related diseases. The principle of this clock regards the correlation of a larger number of variables including sex, cognition, lower grip strength, lung function, and chronic diseases, with a set of CpGs that predict age across several types of cells and tissues [15,16]. Currently, it has been reported more than a 100 of epigenetic clocks however there are only few that are reliable [17,18]. For instance, it has been reported that acceleration in the Horvath's epigenetic clock is associated to individuals with Parkinson's disease, as well, samples derived from breast, kidney, lung, skin, prostate, ovarian and thyroid cancer show higher epigenetic age in contrast to non-tumoral samples. Osteoarthritis, an age-related disease characterized by cartilage degeneration, also shows a significant association with an increased epigenetic age. Additionally, regarding on the DOHaD hypothesis and the potential inheritance of the epigenetic marks in further generations it has been reported that offspring from women exposed to starvation, malnutrition, or undernourishment display aberrant DNAm patterns that increase the likelihood to develop cardio-metabolic, respiratory and cognitive-related diseases [19]. In line with the last observations, it has also documented that individuals exposed during childhood to adverse events such as abuse, violence, or psychological stress exhibit poor mental and physical health, and additionally they exhibit an increased acceleration of the epigenetic clock in females, mainly [20]. However, a healthy lifestyle has potential benefits for the epigenetic clocks, since exercise and balanced nutrition decrease the DNAm and concomitantly diminish the acceleration of the clock [21].

As we review in this section the role of epigenetics, particularly the DNAm, are crucial for understanding the way that we will age, hence in the following chapter we attempt to summarize the most outstanding studies about the role of epigenetics in programming aging and disease, focusing on the most common age-related diseases such as cancer, neurodegenerative, psychiatric, metabolic, and cardiovascular diseases.

32.2 Aging epigenetics and cancer

Cancer has been recognized as a multifactorial disease that results from the accumulation of both genetic and epigenetic alterations that has age as an important risk factor for its development, in part since genetic mutations and environmental damage accumulate as we get older [22]. Particularly, changes in cellular environment, inflammation, impaired immune response and accumulation of DNA damage have been associated to trigger the carcinogenesis [23]. Moreover, the loss of DNAm in both regulatory and repetitive elements, along with other epigenetic modifications, have been associated with genomic instability that promotes tumor formation; as well, it has been reported that hypermethylation on the Polycomb Group Target Proteins (PGTPs), involved in stem cell differentiation, occur in an age-dependent manner [24]. Interestingly, pre-malignant dysplastic tissues show that methylation sites in PGTPs CpGs are more methylated in comparison to non-tumoral samples leading to suggest a direct contribution of age in carcinogenesis by silencing genes that are suppressed in stem cells [25].

32.3 Epigenetics and neurodegenerative diseases

Another of the most common age-related diseases are the neurodegenerative diseases (ND) which represent a relevant health problem. ND is characterized by progressive brain cell dysfunction, including neurons, synapses, and glial cells [26], due to proteostasis impairment, gene expression, transcriptional regulation, and mitochondrial dysfunction [27]. However, it has been recognized that epigenetic mechanisms, particularly DNAm, are associated to trigger ND [28]. Also, clinical and sociodemographic variables such as body mass index, cholesterol ratios smoking, and socioeconomic status are associated to accelerate the epigenetic age measured by the most common epigenetic clocks. In fact, the Hannum, Horvath, PhenoAge, and with the amyloid beta load [29]. Similarly, another study suggests that aberrant patterns of DNAm are related to an increased acceleration of the epigenetic clock in Parkinson's disease [30].

32.3.1 Alzheimer's disease (AD)

AD, considered a precursor for dementia, is in the Top 10 worldwide diseases with the highest mortality affecting around 13.8 million people and is expected to rise over 115 million cases by 2050 specifically at the worldwide old population [31] AD is characterized by cognitive motor and behavioral alterations that decrease greatly life expectancy. At molecular and cellular levels, AD involves the deposition of amyloid plaques and intracellular neurofibrillary tangles of tau protein which unleash a progressive neuronal cell death [32]. Nowadays, AD seems heterogeneous in the factors that cause the disease. However, it has been associated with lifestyle, environmental factors like diet, physical activity and genetic factors that increase the early-onset AD (EOAD) and late-onset AD (LOAD) [33]. EOAD occurs in individuals younger than age 65 but it may manifest at 40 or 50 however those cases are relatively rare (less than 10% of AD cases) and is due to genetic mutations in Amyloid Precursor Protein (APP) [34]. While LOAD patients have low CpG density regions in different tissues, as a result many genes might be suppressed by DNAm [35].

Multiple studies have shown that genomic DNA is commonly hypermethylated and there are much less CpG density regions in LOAD patients increasing the risk to develop AD. Even several Genome-Wide Studies showed some genes such as presenilins, apolipoproteins, and methionine are responsible for directly causing the disease [35]. Interestingly, it has been reported that DNAm decreases in specific brain areas during AD, for instance, the HTERT gene involved in telomere length shortening and cancer promotion appears hypermethylated.

From the epigenetic perspective, it has been reported that DNA methylation alterations appear impaired in AD. Using machine learning methods to identify genetic risk factors that can elucidate the AD pathogenesis, a few studies summarize a number of gene-specific differences involved in different brain regions as can be seen in Table 32.1 [36].

32.3.2 Parkinson disease (PD)

PD is the second most common neurodegenerative disorder worldwide, around 0.3% of the general population present it but is mainly common in people older than 60 years old (80% approx.) representing 1.0% and 3.0% in people older than 80 years [49]. Aging has been recognized as the principal risk factor for PD, however, environmental factors (pesticides) and male gender also are relevant [50].

Table 32.1 Main DNAm marks related to Alzheimer's disease.

Disease	Gen	Associated pathway	Cell type affected	Observations	References
Alzheimer (early-onset)	Amyloid precursor protein (APP)	Amyloidogenic pathway Cell-signaling Aβ production	Neurons Glial cells	A mutation in chromosome 21 increase APP methylation resulting in an aberrant expression for this gene developing excessive accumulation of Aβ.	[37]
	Presenilin 1 (PSEN1) and Preselin 2 (PSEN2)	β-secretase (BACE1) production ϒ-secretase complex production	Neurons Glial cells	A mutation in chromosome 14 and chromosome 1 allows an hypomethylation for PSEN1 suppressing the complex formation for ϒ-Secretase complex. without this complex, the Aβ is downregulated and unleash insoluble peptides like Aβ42.	[38]
Alzheimer (old-onset)	Neprilysin (NEP)	Clearing Aβ deposition	Neurons	This gene encodes an enzyme that can degrade the Aβ peptide. However, if this gene is highly methylated it could cause negative effects like Aβ bioaccumulation in aged healthy brains.	[39]
	Microtubule-associated protein tau (MAPT)	Tau-protein production and microtubules stabilization for cells	Neurons	Located in chromosome 17, it has two haplotypes whose main function is to create tau-protein, however, an hypomethylated form causes an overexpression and subsequently a Tau protein bioaccumulation. Haplotype H1c has been associated with neurodegenerative	[40]

				diseases that include Parkinson's disease.	
	Apolipoprotein $E_{\varepsilon 4}$ ($APOE_{\varepsilon 4}$)	Stable lipoprotein structure Ligand for low-density lipoprotein Cholesterol and lipoprotein regulation for brain cells	Microglia Astrocytes	A polymorphism in alleles ε3 and ε4 due a hypermethylation on a.a position 112 and 158 resulted in elevated gene expression and impaired cellular pathways increasing Aβ proteins in the brain	[41]
	Glycogen synthase kinase 3β (GSK3β)	Creation of neurofibrillary tangles	Neurons	An hypermethylation for GSK3β is related to overexpression of tau protein and consequently increases Aβ protein bioaccumulation in the brain.	[42]
	Triggering receptor expressed on myeloid cells (TREM-2)	Phagocytosis control Modulates inflammatory signaling Modulate myeloid cells	Neurons Microglia	TREM2 deficiency or suppression by hypermethylation is related with augmented Aβ for patients, on the other hand an hypomethylation increases microglial pro-inflammatory cytokines levels generating neuronal loss.	[43]
	Complement receptor 1 (CR1)	Complement pathway Receptor from inflammatory protein Negative regulator in adaptive immunity	Microglia	CR1 is activated when Aβ levels increase and the innate immune system responds as a complement cascade. If there exists a hypermethylation for this gen, most of the tissues will be affected by immune-complex deposition and excessive complement activation.	[44,45]
	Clusterin (CLU)	Mediator of Aβ toxicity in brain and	Neurons Astrocytes	The CLU gene is expressed in all mammalian tissues and	[46]

Continued

Table 32.1 Main DNAm marks related to Alzheimer's disease.—cont'd

Disease	Gen	Associated pathway	Cell type affected	Observations	References
		blood−brain barrier Apoptosis complement	Microglia Blood−brain barrier	it is considered to have high levels of expression in pathological conditions like chronic inflammation of the brain. In AD patients, CLU gene is hypermethylated suppressing its interaction with soluble Aβ and increasing affected cortical areas with amyloid plaques.	
	Phosphatidylinositol binding clathrin assembly protein (PICALM)	Intracellular trafficking for neurons and postsynaptic structures	Neurons	The PICALM gene is highly involved in trans-vascular Aβ clearance. Hypermethylation suppresses its expression and its levels are reduced. PICALM is also associated in other neurodegenerative diseases like dementia and Parkinson's.	[47]
	Methylenetetra hydrofolate reductase (MTHFR)	Homocysteine regulation Methylenetetra hydrofolate reductase creation	Neurons	A polymorphism in nucleotides 667 (C to T substitution) and 1298 (A to C substitution) in chromosome 1 by methylation. An hypermethylation suppresses MTHFR enzyme production and cuts remethylation and homocysteine pathways, decreasing production of methionine, co-substrates for B6 and B12 vitamins.	[48]

Parkinsonism is the term used to refer to the whole clinical status and syndromes that include motor symptoms like bradykinesia, postural instability, rigidity, and resting tremors, such symptoms arise from the loss of dopaminergic neurons due to the deposition and accumulation of both α-synuclein and β-amyloid proteins [51,52]. PD is more common in older people; however, some mutations have been described rarely in younger people worldwide. To categorize the Age-of-Onset (AO) of PD, it has been split into Young Onset PD (YOPD), which has motor symptoms between 21 and 40 years, while Late Onset PD (LOPD) starts >65 years [53,54].

The DNA methylation patterns in PD demonstrate that DNAm-age acceleration is related to PD age at onset [30,55]. In Table 32.2 we summarize the genes that are methylated in PD, and the effects caused by the DNAm.

Table 32.2 Main DNAm marks related to Parkinson's disease.

Disease	Gen	Associated pathway	Cell type affected	Observations	References
Young Onset PD (YOPD)	PINK-1 (PARK-6)	PTEN protein production Regulation and control for mitochondrias (autophagy pathway)	Dopaminergic neurons	Located in chromosome 1, it can be found outside and inside of the mitochondrial membrane. An hypermethylated phase of this gen suppress the PTEN expression and increase autophagy of healthy mitochondrias. It shares a pathway with PRKN gen, which involves several proteins to promote a strong signal for the mitochondrial phagophore. An altered PINK/PRKN pathway results in damaged mitochondrias and progressive degeneration for neurons.	[56]

Continued

Table 32.2 Main DNAm marks related to Parkinson's disease.—cont'd

Disease	Gen	Associated pathway	Cell type affected	Observations	References
	PRKN (PARK2)	Parkin protein production (E3 ubiquitin-ligases) Regulation and control for ubiquitin-binding proteins Maintenance of vesicle in mitochondria	Dopaminergic neurons	Located in chromosome 6, it has multiple mutations and heterozygosity in some tissues with low cancer probability. PARK2 interacts with PINK-1 and DJ-1 to regulate mitochondrial dynamics, if there exists an overexpression caused by hypermethylation it may result in apoptosis in dopaminergic neurons. An hypomethylation status of this gen has been reported in leukemia patients.	[57,58]
	DJ-1 (PARK7)	DJ-1 antioxidant protein production Chaperone activity Regulation and control for mitochondrias (autophagy pathway) Protection against reactive oxidative species (ROS) Transcriptional regulation	Dopaminergic neurons	Located in chromosome 1, it has three mutations that include this gene overexpressed by PD. An overexpression of DJ-1 by both mutation or hypermethylation in the SN suppressed the protection of dopaminergic neurons. In addition to PD, DJ-1 has been	[59,60]

Table 32.2 Main DNAm marks related to Parkinson's disease.—cont'd

Disease	Gen	Associated pathway	Cell type affected	Observations	References
				used to act as an antioxidant in AD for APP protein in mice and human models.	
	SNCA (PARK1/ PARK4)	α-synuclein production and regulation	Dopaminergic neurons	Located in chromosome 4, it has 2 principal loci (PARK1 and PARK4) responsible for α-synuclein in presynaptic terminals from neurons. In hypomethylated form allows a bioaccumulation and formation of LB. This is one of the main diagnostic marks for PD patients (around 74%–78% specification).	[61,62]
	ATP13A2 (PARK9)	Regulation of cellular proteostasis ATPase type 5 codification (ATP13A2 protein)	Dopaminergic neurons	Located in chromosome 1, it has relevance in mutations to develop Kufor-Rakeb syndrome (KRS), a form of autosomal PD. An hypermethylation in this gene increases intracellular accumulation of ubiquitin proteins, specifically in the endo-/lysosomal network such as α-synuclein	[63,64]

Continued

Table 32.2 Main DNAm marks related to Parkinson's disease.—cont'd

Disease	Gen	Associated pathway	Cell type affected	Observations	References
				causing proteostasis and mitochondrial dysfunction.	
	SNCA (PARK1/ PARK4)	α-synuclein production and regulation	Dopaminergic neurons	Located in chromosome 4, it has 2 principal loci (PARK1 and PARK4) responsible for α-synuclein in presynaptic terminals from neurons. In hypomethylated form allows a bioaccumulation and formation of LB. This is one of the main diagnostic marks for PD patients (around 74%—78% specification).	[61,62]
Late Onset PD (LOPD)	VPS-35 (PARK-17)	Retromer compound for vesicular structures and golgi apparatus Maintenance of mitochondrial homeostasis and dynamics	Dopaminergic neurons	Located in chromosome 16, this gene is a critical component for proteins and compound recycling; it has an interaction with Drp1 from mitochondria. An hypermethylation in VSP-35 avoids the removal of Drp1 and creates an unbalanced mitochondrial vesicles regulation	[65,66]

Table 32.2 Main DNAm marks related to Parkinson's disease.—cont'd

Disease	Gen	Associated pathway	Cell type affected	Observations	References
				resulting in mitochondrial dysfunction and neural loss.	
	LRRK2 (PARK-8)	Dandarin protein production Vesicular trafficking participation (endosomal, lysosomal and autophagosomal pathways) Interaction with kinase activity and cell's structural framework	Dopaminergic neurons	Located in chromosome 12, it has 3 mutations whose G2019S and R1441G mutation are responsible for more than 40% of familial PD worldwide. In patients with PD it is commonly found LRRK2 protein levels elevated in the prefrontal cortex and it is caused by overexpression in this gen either by mutations or hypomethylation in CpG regions. LRRK2 hypomethylated gen is related with toxic species and α-synuclein bioaccumulation, because of this LRRK2 and SNCA has a "protective relation."	[67,68]
	PPARGC1A	PGC-1α production Regulator of cellular energy metabolism, microglial plasticity, and	Dopaminergic neurons Microglia	Located in chromosome 4, this gen covers PGC-1α levels to control cellular metabolism producing PGC-1α which is a	[69]

Continued

Table 32.2 Main DNAm marks related to Parkinson's disease.—cont'd

Disease	Gen	Associated pathway	Cell type affected	Observations	References
		mitochondrial function		transcriptional coactivator for nuclear receptors. Reduced expressions of PGC-1α by gene mutations (like PINK1 and PRKN) and PPARGC1A hypermethylation play an important relationship with PD.	
	ATXN2	Ataxin-2 production Involved in cell division, apoptosis and cell signaling	Dopaminergic neurons	Located in chromosome 12, heterozygous and error sequence in the first exon CAG has been reported as pathogenic expansion to develop ND that include AD and PD. It has been reported as a multi-disease gene involved in RNA metabolism, endocytosis and grow factor receptor tyrosine kinase trafficking. A mutation or suppression by hypermethylation represents a risk factor for patients to develop ND like atypical parkinsonism symptoms.	[51,70]

As depicted above the two most common age-related ND have specific DNAm patterns, that trigger the development of neuropathic changes causing a dysfunctional process for neuronal cells [71], such discoveries have been useful for identifying several proteins as biomarkers to make an accurate diagnostic for ND and other diseases and to develop therapies using site-specific DNAm editing approaches for targeted therapeutic design [72,73].

32.4 Psychiatric diseases

Nowadays, we know that the growing extension of life expectancy is accompanied by an increasing rate of degenerative diseases associated with lifestyle and aging, such as psychiatric diseases, among which predominate mainly in the elderly: bipolar disorder, schizophrenia, and major depressive disorder. Unlike some diseases, these are of complex etiology, however, recent findings show that the onset and development of these diseases cannot be described by a single gene, so it is believed that their clinical presentation is the result of the regulatory interaction of a large number of genes, where within the participation, regulation, and activation of these genes, environmental factors play a crucial role in contributing to their pathogenesis, because the individuals are in the womb of their mother, they are exposed to various factors such as diet, exercise, pollutants, etc. Factors that can generate epigenetic modifications, thus influencing gene expression and thus causing neuronal and behavioral changes that have been associated with mental disorders [74,75].

In this section, we discuss briefly these epigenetic modifications found within the psychiatric diseases commonly presented in the older adult and the relevance this has had for the better understanding of these diseases.

32.4.1 Major depressive disorder (MDD)

MDD is a disorder of the mind and body that affects about 6% of the adult population worldwide each year, and represents the leading cause of disability worldwide. Unfortunately, the prevalence of this disorder in the population increases yearly and the World Health Organization (WHO) predicts that it will generate the largest global burden by 2030 [76,77].

Particularly individuals from 60 years and more are the most affected due to the multiple factors such as health issues and access to healthcare systems, socioeconomic status, violence, abuse, widowhood, and social isolation. These stressful experiences induce inflammatory responses that concomitantly increase ROS and suppression of neurogenesis, which lead to depression [78]. However, when this disorder is present, it has also been shown to increase the risk of obesity, frailty, diabetes, cognitive impairment, and mortality since of all medical disorders, calculated by years of disability, MDD is the first significant contributor to chronic disease burden [76,78]. That is why there is currently a need to look at the broader and more complex perspective of the disorder and refine future directions to explore better treatments and diagnosis for MMD because in the case of treatment, current drugs that mainly modify monoamine homeostasis are insufficient to alleviate the complexity of symptoms (appetite and sleep disturbances, recurrent suicidal thoughts, distress, among others) [76], in addition to the fact that approximately 50% of patients do not achieve remission with first-line treatment; on the other hand, the diagnosis of MMD is characterized according to the Diagnostic and Statistical Manual of Mental Disorders, which is characterized by 2 or more weeks of depressed mood

and/or loss of interest and pleasure, along with symptoms such as change in sleep, weight, and energy [79]. However, this is not so specific and could be confused with other disorders. It is for all of the above that epigenetic studies focused on MMD in recent years have become relevant. It is important to mention that there are a vast number of studies that encompass the role of microRNAs as potential biomarkers of MMD [77], however, here we only focus on DNAm.

In recent years, genome-wide interaction studies have estimated that the genetic contribution to MDD is about 35%, suggesting that the likelihood of developing MDD is closely related to the environmental conditions to which the individual is exposed during childhood and adulthood such as stress, sedentary lifestyle, and unhealthy nutrition, an example of this is a meta-analysis of 26 studies where they found that childhood trauma, such as emotional abuse, was strongly associated with depression in adulthood [77]. Also other studies have shown that alternative and complementary medicine therapies, such as yoga, help to reduce depressive symptoms as a positive regulation in the levels of CX3XL1, GPR50, and RB genes has been seen; CX3XL1 is a cytokine found in the brain, especially in neurons with receptors in the microglia, in the case of the RB gene (a tumor suppressor gene), where an increase in the levels of this gene was seen, it took importance since depression has been associated with increased risk of cancer [76,77].

Further studies of various genes have found that patients with MDD have DNA hypermethylation at loci encoding brain-derived neurotrophic factor (BDNF) and SLC6A4 (the serotonin transporter gene). In other studies it has been analyzed that individuals with MDD exhibit altered DNAm pattern in genes related to the transport, metabolism, and biosynthesis of serotonin (NEGRI 1, 5-HT, DRD2, CELF 4, mTORbNRC31, SLCA4, FKBP5, SKA2, OXTR, LINGO3, POU3F1, ITGB1, MAO-A, 5-HTR, SLC6A), as well, it has been also reported that BDNF gene involved in serotonergic expression show decrease levels of DNAm [80,81]. Regarding the DOHaD hypothesis, it has been also demonstrated that MDD patients show higher epigenetic aging if they were subjected to childhood trauma [82].

Although there is still a long way to go to better understand how this disorder takes place as well as the factors that influence it, should be noted that as technology has increased and the number of individuals in the cohort has increased, epigenetic studies related to depression have been enriched, as well as studies, environmental and social agents that could trigger or be related to depression.

32.4.2 Bipolar disorder

Bipolar disorder (BD) is a severe neuropsychiatric disorder characterized by recurrent episodes of depression and mania that affect thought, perception, emotion, and social behavior, alternating with intervals of wellbeing which has been associated with accelerated aging, and recent research has begun to explore the possible anti-aging effects of bipolar disorder treatments [83,84].

Within this disorder, lithium has played an important role since it is the first-line treatment and it is the most economical mood stabilizer by 10 times, however its efficacy is not the same among all patients, it is estimated that 30% of patients are excellent responders. This is why over time the use of lithium is decreasing, due to the above is that in recent years, studies in epigenetics to better understand this disorder as well as the search for better treatments, even to predict the responsiveness to lithium. Among the GWAS analyses, one comparing responders to healthy controls found several SNPs in the AMA-type glutamate ionotropic receptor ionotropic subunit 2 (GRIA 2) gene that encodes for GluR2 of the AMPA ionotropic receptor and that GRIA2 expression has been shown to be down-regulated by

acute lithium treatment, where chronic lithium use has been shown to downregulate this gene in vitro and reduce synaptic expression of GluR2 in the hippocampus, this glutamate is of importance because it has been widely implicated in the pathophysiology of mood disorders and magnetic resonance spectroscopy imaging has demonstrated increased hippocampal glutamate concentrations in euthymic patients receiving prolonged treatment with lithium [85]. Hypomethylation at the BDNF in peripheral blood mononuclear cells (PBMC) has been seen in patients treated with lithium [86]. One study proposed GDF-15 as a biomarker for this disorder where they showed that GDF-15 levels were increased in the plasma of TB patients and these levels were closely associated with disease duration, however more studies are needed in the future for GDF-15 to be considered as a biomarker [87].

32.4.3 Schizophrenia

According to the WHO, Schizophrenia (SZ) is characterized by significant alterations in the perception of reality and changes in behavior [88]. Aging in patients with schizophrenia is associated with reduced longevity, but the possible biological factors associated with aging in this population have not yet been comprehensively investigated [87,89]. This disease has also been associated with certain factors such as diet, exercise, socioeconomic status, and smoking [90].

Several biomarkers of aging in schizophrenia have been proposed with conflicting results, leading to the idea that schizophrenia may be associated with segmental aging, i.e., some but not all features of aging may be accelerated. Epigenetic clocks based on DNA methylation 8, 9 are validated predictors of chronological age in all tissues and throughout life. Increased or decreased epigenetic age relative to chronological age is termed acceleration or deceleration. Age acceleration predicts increased risk of age-related disease 10 and all-cause mortality 11. Although aberrant DNA methylation has been consistently reported in schizophrenia 12, no significant accelerated aging has been observed. However, numerous epigenetic clocks have been developed in recent years that appear to capture different aspects of aging in addition to classical chronological age prediction, such as age-related morbidity and mortality 20 or telomere length 21. A recent large-scale DNA methylation study that simultaneously tested 14 epigenetic clocks covering different aspects of aging found that non-classical predictors of epigenetic aging (i.e., mortality telomere length clocks) were altered in cases of schizophrenia. In another study they suggest that epigenetic age acceleration may be involved in the clinical course of schizophrenia and may be a useful marker of relapse when measured in stages of remission [91].

More studies are still needed to better understand this disease, however, with the studies that have been done in the last few years, regarding mDNA in patients with SZ, hypermethylation has been seen in genes such as COMT and HTR2, in contrast to hypomethylation of the COMT gene [92–94].

32.5 Cardiovascular diseases

Cardiovascular diseases (CVDs), including heart failure, hypertension, atherosclerosis, and cardiomyopathy, are the leading cause of mortality worldwide. CVD is a system with broad interdependence with blood circulation and involves affections in heart, blood vessels, renal and nervous system [95]. Diverse risk factors such smoking, obesity, hypertension, and high cholesterol are the most common precursors for CVD which increase risk of diseases such as cardiomyopathies, coronary artery disease, and arrhythmias [96]. However, studies in 2014 showed that other factors such as aging, race, environmental physical, and social disorders have been related with CVD [97]. Aging has been involved in

several structural and function tissues, specifically in heart and vasculature show homeostatic imbalance, vascular stiffening, and ventricular wall thickness altering mechanical properties for tissues and high risk for calcium or collagen bioaccumulation and inflammation in extracellular matrix [98].

The most common disease for CVD is arteriosclerosis and atherosclerosis which is the cumulative result of endovascular inflammation, lipid oxidation, and plaque formation in artery walls. Aging and atherosclerosis have similar chemical pathways in vascular alterations such as aortic stiffening, due this it can be considered as an early-age—related manifestation and may emerge in childhood and increase risk at maturity [99].

Different studies have been discovering crucial mechanisms behind the deterioration of CVD structure and function, from these studies epigenetic alterations have a major impact to describe the relation between intrinsic genetic and extrinsic environmental influences [99].

As epigenetic changes are subject to environmental factors, DNAm has been one of the main studies to find a modification in genes and their relation with several diseases which include CVD [100]. New analysis technologies like genome-wide association studies (GWAS) and epigenome-wide association studies (EWAS) have been designed to detect associations between genetic variants or traits with biological mechanisms related with diseases using biological molecules and fine-mapping for genes [101].

Since, several analyses have shown that DNA methylation in genes plays important roles using the abnormal methylation status and their mechanism to develop CVD, in this section we describe the most common DNA methylation changes associated with CVD as response to the programming induced during the development of childhood and adulthood that impacts on the aging process.

32.5.1 Coronary heart disease (CAD)

Also known as Coronary Artery disease, CAD is the most common CVD and the major cause of death worldwide and during the last decades people older than 35 years have a high probability to develop this disease [102]. As with most complex diseases, CAD is modulated by genetic and lifestyle factors. The main factor involved to develop this disease is cholesterol which buildup plaques in heart's arteries allowing an alteration in blood circulation to cardiac muscle and surrounding tissues, however other factors like hypertension, obesity, and genetics may be a main risk to have CAD [103,104].

A recent study from Palou-Márquez et al. [105] showed a MOFA method in Framingham Offspring Study related to heart and CVD in people >65 years describe four factors associated with CVD risk driven by DNAm which include cardioprotective effects, angiogenesis and inflammation biomarkers. Similarly, in Table 32.3 we summarize the most methylated sites in CAD.

32.5.2 Heart failure (HF)

Is a clinical syndrome caused by both structural and functional cardiac systems resulting in reduced cardiac output to pump and fill with blood [110]. HF has been caused by hypertension, cardiomyopathy, arrhythmias, and valvular disease, and is considered as a global pandemic disease affecting around 26 million people worldwide who are >65 years [111]. Currently, several studies report the participation of epigenetic modification in the development and progression of HF. The most common altered methylation patterns correspond to cardiac development genes such as TBX5, HAND1, and NKX2.5 [112].

Table 32.3 Main DNAmethylation marks described in CAD.

Classification	Gen	Associated pathway	Cell type affected	Observations	References
Premature CAD (<65 years)	MTHFR (C677T)	Dyslipidemia	Cardiomyocytes	MTHFR is involved in folate metabolism and homocysteine production. Multiple investigations demonstrate a high impact of homocysteine polymorphisms (C677T and A1298C) levels in blood levels which cause damage in arterial endothelium. An hypomethylation in this gene causes a high production for homocysteine and reduces plasma folic acid concentrations allowing the development of CAD.	[106]
	MTTP	Dyslipidemia	Cardiomyocytes	MTTP is associated with a large subunit of microsomal triglyceride transfer protein, a crucial molecule for lipoprotein assembly, in cholesterol and fatty acid metabolism. An hypomethylation may cause an overexpression of cholesterol production and create lipid bioaccumulation in the vascular wall causing myocardial infarction.	[107]
Elder-onset CAD (>65 years)	SORT1	Cholesterol homeostasis	Cardiomyocytes	SORT1 encodes a multiligand sorting receptor which interacts with apolipoproteins and very low density	[108]

Continued

Table 32.3 Main DNAmethylation marks described in CAD.—cont'd

Classification	Gen	Associated pathway	Cell type affected	Observations	References
				lipids (VLDL) which allows their degradation. An hypermethylation of this gene cause a sortilin's suppression increasing the risk of hypercholesterolemia and higher CVD risk.	
	ESR1 and ESR2	Atheroprotective effects and homeostasis	Cardiomyocytes	They are the principal genes which encode the estrogen receptors Erα and Erβ, respectively. An hypermethylation in coronary atherosclerosis tissues may cause a mitochondrial down regulation causing an abnormal heart function.	[109]

32.6 Diabetes mellitus type 2 and obesity

Currently the prevalence of obesity is increasing at a considerable rate, which has caused that this event can no longer be explained by genetic factors alone, so it has been considered that a driver of this, are environmental and social factors as these days, has been seen in populations an increase in the consumption of fast-food high in calories and low physical activity. As part of epigenetics, DNAm has been seen as a link between obesity and DM2 with clinical conditions; for example, in people with obesity it has been seen that they have a higher methylation than lean people. In a study where DNA methylation was analyzed in CD4+ T cells, eight CpG sites were found which were associated with BMI and five with waist circumference, of which CPT1A, CD38, and PHGDH stand out as they were associated with both phenotypes; especially DNAm of intron 1 of CPT1A has been highlighted as it encodes the enzyme carnitine palmitoyltransferase 1A, which is involved in carnitine-dependent transport across the mitochondrial membrane when the oxidation of long-chain fatty acids is initiated and is very important in several metabolic processes [113].

In another study by Wahl et al. [114], where they analyzed more than 10,000 blood samples and managed to identify 187 CpG sites associated with BMI, where they used Mendelian randomization and thus were able to demonstrate that alterations in DNAm, are most often the result of obesity, but sometimes also the cause, in this study they managed to predict in some of the patients, the risk to present DM2 through DNAm sites related to obesity [114].

Adding to the list of CpG sites associated with BMI and waist circumference, in 2018, Dhana et al. found associations with both phenotypes in MS12 which encodes the RNA-binding protein 2 gene and has a role in post-transcriptional gene regulation which has been linked to eating behavior. In this study, they also suggest a direct link between DNAm, gene expression and obesity status for several genes in which SOCS3 was highlighted, with which obesity was associated with decreased methylation and increased gene expression, this has shown that increased expression of this gene during obesity induces some resistance to both insulin and leptin, with implications for blood glucose control and energy homeostasis [113,115].

During aging a more sedentary lifestyle contributes to insulin resistance and obesity, both closely related to DM2 development. Hence, depicting the age-related molecular mechanisms that predispose to obesity and DM2 will help to the development of new therapies and prevention strategies. In this context, it is relevant to develop tools for the identification of subjects at high risk of developing obesity and DM2. Currently, epigenetic biomarkers have already been identified, for example methylation of the ABCG1 locus was associated with an increased risk of DM2 and PHOSPHO1 with a lower risk. Other studies have shown that methylation in KLF14, FHL2, ZNF518B, and FAM123C are associated with future insulin secretion and the possibility of developing DM2, besides these sites seem to increase its methylation in an age-dependent manner [113].

32.7 Hypertension

Hypertension is a common and complex disorder that particularly affects the elderly and can result in heart failure, kidney failure, or stroke; it currently affects approximately $\leq$1 billion adults worldwide; this is of concern as the prevalence of this condition is increasing and is estimated to affect one-third of the population by 2025 [116,117,118].

Several studies have shown that there is an interaction between aging, genetics, and environmental factors and epigenetics contribute to the susceptibility to hypertension. For example, blood pressure variation and the development of hypertension has been associated with changes in DNAm in CpG sites related to blood pressure regulation. As well, alteration of blood pressure in adulthood has been related to prenatal exposure to air pollutants that induce a wide variety of alterations in DNAm of the DNMT1, DNMT3B, TET2, and thymine DNA glycosylase, TLR4I [119]. Additionally, it has been reported that epigenetic age acceleration is more significantly associated in women with hypertension as compared with their male counterparts, suggesting a close relationship between blood pressure and epigenetic aging as well as the role of steroid hormone decrease in women's health [120].

32.8 Epigenetics and frailty

Despite the controversy around frailty definition, here we will define this syndrome as a state of increased vulnerability to stressors as a result of decreased physiologic reserves, which leads to adverse health outcomes during aging. Taking advantage of the lack of consensus about frailty definition it has been suggested the potential use of an epigenetic clock based on DNAm to define objectively frailty. Interestingly, in several cohorts there is a significant association between the acceleration of the epigenetic age in frail individuals in comparison to non-frail individuals, and this is also useful to identify pre-frail individuals from the frail ones [121], suggesting that DNAm patterns

may contribute to the early, accurate and reliable detection of changes and deficits at both molecular and cellular labels, to prevent or delay the development of frailty.

32.9 Conclusions

As seen above, aging is one of the most important risk factors for the development of human diseases such as cancer, cardiovascular, psychiatric, and neurodegenerative disorders. Besides their genetic background, current research reveals that epigenetics plays an important role in understanding their pathogenesis. Here we identify that there is a huge amount of evidence that points to the crucial role of DNAm in programming aging and disease, suggesting that this epigenetic mechanism may be a potential target to improve the aging process and concomitantly prevent the development of chronic diseases.

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PART

Future research

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CHAPTER

Epigenetics, epidemiology, and public health

33

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33.1 Introduction

The term "epigenetics" was first proposed by C. H. Waddington in 1942 as a field of research aiming to "discover the processes involved in the mechanism by which the genes of the genotype bring about phenotypic effects" [1]. Epigenetic variation, which modifies gene expression without changing the underlying DNA sequence, is thought to play a critical role in the etiology of complex traits and diseases [2]. Many epigenetic studies are laboratory-based research investigating the molecular mechanisms underlying epigenetic variation. Epidemiological studies are required for the translation of epigenetic findings to disease prevention and the implementation of public health policies.

Epidemiology is the study of the distribution, causes, prevention, control, and treatment strategies of health-related states and diseases in the population. Epidemiology is the core of public health, and it has made great contributions to understanding disease etiology and implementing disease prevention strategies. For example, epidemiological studies have shown that smoking increases the risk of lung cancer, hypertension increases the risk of cardiovascular diseases, and water fluoridation reduces the risk of tooth decay. In recent years, traditional epidemiology has absorbed knowledge from other fields and used new technologies to better understand diseases and support public health action. Many new fields of epidemiology have emerged, such as molecular epidemiology and genetic epidemiology.

Epigenetic epidemiology, an interdisciplinary field combining epigenetic and epidemiological knowledge, tools, and techniques, is emerging as a critical element of research into diseases and public health [3–5]. Microarray technology has made genome-wide DNA methylation data available (at hundreds of thousands of methylation sites), and epigenome-wide association studies (EWAS) have emerged. Epigenetic epidemiology can be defined as "the integration of epigenetic analyses into population-based epidemiological research with the goal of identifying both the causes (that is, environmental, genetic or stochastic) and phenotypic consequences (that is, health and disease) of epigenomic variation" [3].

Epigenetics in Human Disease. https://doi.org/10.1016/B978-0-443-21863-7.00018-4
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In this chapter, we aimed to summarize the promising roles of epigenetics in epidemiology and public health, and current challenges in the practice of epigenetic epidemiological research. Given that DNA methylation is the most studied type of epigenetic modification, this chapter mainly discusses DNA methylation, but the issues discussed in this chapter, especially those related to the implementation of epigenetic epidemiological studies, are also applicable to other types of modifications, such as microRNA and histone modification.

33.2 Epigenetics in disease aetiology

33.2.1 Epigenetic variation as a disease cause

Imprinting disorder is the most relevant example of epigenetic variation causing diseases. Imprinting is a biological phenomenon in which certain genes are expressed in a parent-of-origin specific manner due to epigenetic modification. Several diseases are imprinting disorders, such as Angelman syndrome, Prader–Willi syndrome, and Beckwith–Wiedemann syndrome. Angelman syndrome and Prader–Willi syndrome are due to the loss of function of paternally- or maternally only expressed genes in the imprinted region 15q11-13 [6]. Usually, the paternal allele of the ubiquitin protein ligase E3A (*UBE3A*) gene is repressed due to imprinting and only the maternal allele is expressed. Impaired expression of the maternal allele, in most cases due to deletion, causes Angelman syndrome [7]. Prader–Willi syndrome results from the loss of function at the paternally inherited gene, the maternal copy of which is normally silenced by DNA methylation; for more details see Ref. [8]. Beckwith–Wiedemann syndrome is a pediatric overgrowth disorder, resulting from the abnormal DNA methylation of growth–related imprinted genes at the 11p15.5 region. This region contains two independent imprinting centers. In imprinting center 1, the insulin-like growth factor 2 (*IGF2*) gene is normally paternally expressed. The gain of DNA methylation at the maternal allele results in biallelic expression of *IGF2*. In imprinting center 2, the growth-restricting gene cyclin-dependent kinase inhibitor 1C (*CDKN1C*) is normally maternally expressed and the hypomethylated *KCNQ1* overlapping transcript 1 (*KCNQ1OT1*) gene is paternally expressed. Loss of DNA methylation at the maternal allele of *KCNQ1OT1* results in reduced expression of *CDKN1C* through regulation in *cis*; for more details see Ref. [9].

33.2.2 Epigenetic variation as a mediator of the effect of exposure

Epigenetic variation potentially plays a key role in mediating the effects of environmental exposures on diseases and other traits. There is some evidence that DNA methylation statistically mediates the effects of exposures on outcomes. Fasanelli et al. identified and replicated an association between hypomethylation in pre-diagnostic blood at two smoking-related cytosine-guanine dinucleotides (CpG) and increased lung cancer risk. Their analyses suggested that DNA methylation at these CpGs mediates >30% of the total effect of smoking on lung cancer risk [10]. Caution is nevertheless needed when interpreting these findings, as they could to a certain extent be attributed to residual confounding by smoking intensity [11]. Kupers et al. concluded that differential DNA methylation at the growth factor independent 1 transcriptional repressor (GFI1) gene mediates 12%–19% of the total effect of maternal smoking on offspring birth weight [12]. The findings of Meng and colleagues suggest that DNA methylation at CpG *cg21325723* in the MHC region mediates the interaction between single-

nucleotide polymorphism (SNP) *rs6933349* and smoking on the risk of anti-citrullinated peptide antibody-positive rheumatoid arthritis [13]. There is also evidence that DNA methylation mediates the effects of air pollutants. DNA methylation at inflammatory genes has been suggested to mediate the associations of air pollutants with coagulation [14], and with cardiovascular-related biomarkers [15].

The Developmental Origins of Health and Disease (DOHaD) hypothesis proposes that early-life exposures have influences on health in later-life. In this framework, epigenetic variation is thought to be one of the underlying biological mechanisms [16,17]. T cell DNA methylation at genes involved in insulin regulation and NF-κB signaling pathways has been suggested to mediate the effect of prenatal maternal stress on 13.5-year-old children's central adiposity and IFN-γ cytokine secretion, respectively [18,19]. There is also some evidence that the association between maternal adiposity and offspring adiposity may be tracked in offspring blood DNA methylation at birth [20]. However, the possibility that these associations are confounded, or mediated by other factors, cannot be ruled out.

There are several challenges in studying epigenetic variation as a mediator of environmental exposures. The first challenge is the causation between exposure, epigenetic variation, and outcome. In terms of epigenetic changes mediating early-life exposures' influences on later-life health, the investigation of causation requires robust associations to be established between early-life exposures and epigenetic changes, and between exposures-associated epigenetic changes and later-life health outcomes, and that persistent epigenetic changes are functionally relevant to health outcomes [21]. Methodologies such as the life-course approach [22], Mendelian randomization (MR) [23], and inference on causation from examination of familial confounding (ICE FALCON) [24] could be useful to address issues of causation; see the section "Causal inference" below for further details. The second challenge is establishing biological relevance. Several studies have investigated the mediating role of blood epigenetic variation on non-blood–related outcomes, such as the study on lung cancer mentioned above. However, results from these studies are merely statistical associations, and whether blood epigenetic variation is on the biological pathway between exposure and outcome is unclear. Using disease-relevant tissue is optimal.

Mediation by epigenetic variation is a potential biological mechanism underlying the association of genetic variants with complex diseases and traits, which is not well understood for many genetic variants identified by genome-wide association studies (GWAS). One example is DNA methylation at the major histocompatibility complex (MHC) region. The MHC region is one of the most gene-dense regions of the human genome and contains many genes involved in the immune system. Emerging evidence suggests that the associations of genetic variants in this region with some autoimmune diseases may be mediated by DNA methylation. For example, DNA methylation at two clusters within the MHC region has been suggested to mediate the genetic effects of this region on the risk of anti-citrullinated peptide antibody-positive rheumatoid arthritis [25]. Integration of genetic and epigenetic data could result in additional evidence and a deeper understanding of the biological mechanism underlying associations between genetic variants and diseases.

33.3 Epigenetic variation as a biomarker of exposure

Epigenetic marks are sensitive to the environment. DNA methylation has been most studied and found to be associated with lifestyle factors such as smoking [26–31], body mass index (BMI) [32–34], and alcohol consumption [35,36]. Epigenetic variation could serve as a biomarker to enhance exposure assessment.

In the case of smoking, CpG-specific DNA methylation in blood can potentially be used to improve smoking exposure assessment, including smoking initiation [26–29], cumulative smoking [26,37], and the time since quitting [26,38]. DNA methylation scores combining DNA methylation levels at multiple CpGs have been built to improve the assessment of lifetime exposure to smoking [29,39]. Importantly, the fact that the association of smoking with DNA methylation at certain loci are consistent across different ethnic populations [26–28] suggests that these smoking-sensitive loci are independent of the underlying genetic sequence, which increases their potential as smoking biomarkers. The value of blood DNA methylation as a smoking biomarker was further supported by the findings that DNA methylation at smoking-CpGs is related to serum cotinine concentration, and DNA methylation markers outperformed cotinine concentration in classifying smoking status [39]. Despite the promising perspective, caution is needed. The transient or persistent nature of smoking-induced DNA methylation changes needs to be distinguished. There is evidence that smoking-induced changes at some CpGs tend to reverse after the cessation of smoking [38], which may alter their utility as long-term biomarkers of smoking exposure.

Epigenetic variation can also be used to assess prenatal exposures; for example, DNA methylation of offspring could be used as a biomarker for maternal smoking [40], and the DNA methylation–based marker has been suggested to be associated with adulthood cancer risks [41]. Similarly, persistent epigenetic signatures are needed to be used as biomarkers of prenatal exposures, which have been shown to be the case for certain CpG sites [42].

Epigenetic variation can potentially be used to assess the timing of exposure as well. For example, one study observed that newborn cord blood DNA methylation was associated with maternal smoking during the time from conception to gestational week 18 only, but not with maternal smoking before conception or ceased by week 18 [43].

Epigenetic variation, especially DNA methylation, has also been suggested to be associated with outdoor environmental exposures, such as air pollution [44], ambient temperature [45,46], and surrounding greenness [47,48]. Specifically, the finding that a higher surrounding greenness is associated with a decelerated DNA methylation-based biological aging implies the potential public health value in changing the outdoor environment for healthy aging [48].

Another increasing application is to use epigenetic variation, especially DNA methylation, to predict lifestyle exposures and other health-associated factors. A review found that most of the relevant studies published before April 2020 focused on the prediction of age, biological aging, smoking, alcohol consumption, BMI, and diet [49]. Some of these predictors, such as those for aging and smoking [37,50–55], have been found to add to the prediction of health outcomes, i.e., beyond chronological age or smoking information collected via questionnaires [53,54,56]. These findings support the potential for using DNA methylation–based predictors of exposure to improve disease risk assessment. The review by Yousefi et al. provides more details about methodological aspects related to the development of epigenetic predictors [49].

The role of epigenetic variation in improving exposure assessment is promising. Epidemiological studies usually collect self-reported information for exposures of interest, which are vulnerable to recall bias and misclassification. Applying a well-validated epigenetic biomarker can potentially reduce such bias. This is at least supported by DNA methylation–based predictors of smoking, which appear to capture lifelong smoking exposure with less recall bias [41,53,54]. Many cohorts have archived biological samples, which can potentially be used to assess exposures prior to the sample collection time. This could be valuable if exposure information at that time was not well documented.

A well-developed blood epigenetic biomarker of prenatal exposure is of potential wide utility, given that blood samples of newborns are commonly collected through neonatal heel pricks.

33.4 Epigenetic variation as a biomarker of disease

Epigenetic variation may reflect disease development and progression in several aspects, such as being related to sub-clinical changes. Several studies have attempted to use epigenetic biomarkers in disease screening, diagnosis, and prognosis assessment. Such potential has been reviewed for cancer [57]. There are also some examples of the potential role of DNA methylation as a disease biomarker.

DNA methylation at the *POU* class 4 homeobox 3 (*POU4F3*) gene has been found to have a good performance in the triage of high-risk human papillomavirus-positive women [58]. Paired box 1 (*PAX1*) gene DNA methylation has also been suggested to be a biomarker of more advanced cervical abnormalities [59]. These results suggest that DNA methylation at *POU4F3* and *PAX1* have the potential to be useful targets for cervical cancer screening.

Regarding disease diagnosis, hypermethylation of the short stature homeobox 2 (*SHOX2*) gene in bronchial aspirates and plasma has been found to have high sensitivity and specificity for the classification of lung cancer patients [60,61]. This suggests that *SHOX2* DNA methylation may potentially improve early lung cancer diagnosis.

Regarding disease prognosis, long interspersed nuclear element-1 (*LINE-1*) hypomethylation in tumor has been found to be associated with higher mortality of colorectal cancer, independently of microsatellite instability [62]. This result suggests that tumor *LINE-1* methylation may be used as a biomarker of colorectal cancer prognosis.

The practical application of epigenetics to disease screening, diagnosis, and prognosis assessment still has a long way to go and more research is needed in several areas. Validation is essential; consistent results across multiple studies are currently lacking. As reviewed by Tang et al. inconsistent results across studies were observed in the context of blood-based DNA methylation biomarkers of breast cancer [63]. The broad accessibility of tissues also needs to be considered, as well as the usefulness of easily accessible tissues like peripheral blood. Another aspect that has been given little attention is whether combing epigenetic biomarkers with traditional markers leads to a substantially improved performance. Cost-effectiveness also needs to be evaluated because epigenetic measures are currently relatively expensive. One study published in 2013 concluded that a methylated septin 9 DNA plasma assay is less cost-effective than established colorectal cancer screening strategies [64].

33.4.1 Epigenetics in disease risk prediction and intervention

Epigenetics can play a role toward precision medicine. Individuals with different risk profiles can potentially be identified based on epigenetic variation and be further targeted for different risk-reducing intervention strategies.

There is an increasing body of evidence that DNA methylation could be useful in predicting risks of death and cancers. Epigenetic predictors of age based on DNA methylation at 71 [65] and 353 [66] CpGs have been proposed to reflect human biological aging, and found to be associated with increased risks of cancer and death [67,68]. DNA methylation−based biological aging predictors are rapidly increasing with new predictors such as PhenoAge [69], GrimAge [70], DunedinPoAm [71], and

DunedinPACE [72] recently developed; see Dugue et al. [73], Horvath et al. [74] and Rutledge et al. [75] for more details on this topic. A DNA methylation score based on measures at 10 CpGs has been reported to have a dose-response relationship with all-cause mortality [55]. DNA methylation at smoking-related CpGs has also been shown to predict the risk of lung cancer [76]. One study found that a DNA methylation score based on measures at two smoking-related CpGs was associated with all-cause and cardiovascular-disease-specific mortalities [37].

Some studies suggest that epigenetic variation has a comparable or better performance than conventional risk factors for disease risk prediction. One study found that DNA methylation at smoking-related CpGs outperformed smoking or DNA methylation at lung cancer-related CpGs in lung cancer risk prediction [10,76], and adding a smoking DNA methylation score to conventional risk factors in risk prediction models led to improvement in fatal cardiovascular disease risk stratification [37].

A review found that most of the studies using DNA methylation to predict disease published before April 2020 are for cancer, with tumor tissue as the major DNA source and cancer progression as the major outcome of interest [49]. It is important to distinguish whether the predictors are for disease detection or for disease risk assessment—the former is evaluating the likelihood of whether a disease is present at the time of assessment, while the latter is evaluating the future probability of developing the disease after the time of assessment. This distinction has generally not been made at least for breast cancer research, with predictors for detection being interpreted as predictors for risk assessment [77]. Most of the predictors reviewed by Yousefi et al. [49] are for disease detection. Typically, predictors used for disease detection have a better prediction performance than predictors used for risk assessment. For example, a DNA methylation–based predictor for breast cancer detection was found to have an Area Under the Receiver Operating Characteristic Curve (AUC) over 0.8 [78], while the AUC for a predictor for breast cancer risk assessment was around 0.65 [79].

Integrating epigenomics with other omics data such as genomics may also be of interest. A study [80] found that DNA methylation reflected a similar proportion of variation in BMI to that explained by a panel of SNPs. Furthermore, the DNA methylation profile and the SNPs "predicted" BMI in an essentially additive manner, which suggests that combining genetic and epigenetic profiles can reflect different aspects of obesity and improve the prediction. This was, however, not the case in the context of height, because the study found that DNA methylation did not account for additional variation in height after controlling for the SNPs of height. This illustrates the challenges and needs for further research in integrating multiple omics data to improve risk prediction. Nevertheless, combining omics data in disease risk prediction is a worthwhile pursuit.

It is important to note that a prospective association between epigenetic variation and disease is essential for the development of epigenetic tools for disease risk prediction. It requires these tools to be developed and validated in large prospective cohorts. However, neither causation nor functional knowledge is necessary for risk prediction. Even if the association between epigenetic variation and disease risk is due to confounders, or without plausible biological explanation, its usefulness as a risk assessment tool cannot necessarily be dismissed.

High-risk individuals identified by epigenetic tools are a potential target population for intervention. Epigenetic-based intervention can potentially be useful if the disease is a consequence of epigenetic variation. However, if the epigenetic variation does not cause the disease; non-epigenetic–based interventions can still be used on the high-risk individuals identified by epigenetic tools.

33.5 Epigenetics in disease treatment

Epigenetic variation can be a therapeutic target given its role in disease development and progression by modifying gene expression, e.g., by silencing genes involved in diseases. Epigenetic therapy has already played an important role in cancer treatment. DNA methyltransferase (DNMT) and histone deacetylase (HDAC) catalyze DNA methylation and histone modification, respectively. Targeting these enzymes, DNMT inhibitors and HDAC inhibitors are being used for cancer treatment. Several inhibitors have been approved by the US Food and Drug Administration or are being tested in clinical trials; for more details see Jones et al. [81]. These inhibitors are also used for the therapy of other diseases, such as neurological and immune disorders; for more details see Falkenberg et al. [82].

It is important to note that the causal relationship between epigenetic variation and disease outcome needs to be understood before the knowledge of epigenetic associations can be applied to disease treatment. Otherwise, any epigenetic-based therapeutic will not be efficacious [83]. Randomized controlled trials are the best epidemiological design for addressing this problem; more about such design is discussed in the following section.

33.6 Considerations regarding the implementation of epigenetic epidemiological studies

Though sharing similarities, EWAS and GWAS have distinct features. Germline genetic variation is static, identical across tissues, exactly prior to disease onset and cannot be impacted by environmental factors; therefore, in GWAS biases are better recognized and controlled. However, epigenetic variation is dynamic, tissue-specific, and environmentally sensitive. Its association with disease is subject to various biases, which requires careful consideration of the study design. Additionally, GWAS has a widely accepted strategy for study design, quality control, data preprocessing, and data analysis. This is, however, not the case for EWAS at present. EWAS studies are therefore more comparable to traditional epidemiological studies than GWAS. The implementation of epigenetic epidemiological studies needs to consider many more aspects. These considerations include the research question, study design, sample size, biological samples, and epigenetic measurement tool.

33.6.1 Research question

A research question can typically be classified into two types: one is to generate a hypothesis and the other is to test a hypothesis. These two types of research questions determine much of the downstream research. The study design, sample size, biological sample, and epigenetic measurement tool all depend on the research question. For example, an investigation of the efficacy of epigenetic enzyme inhibitors requires a randomized controlled trial design. A validation of a previous finding requires an adequate sample size not to miss important effect sizes. The development of disease biomarkers in easily accessible tissue might require peripheral blood as the biological sample. Scanning disease-related DNA methylation changes requires a measurement tool with high-throughput technology. Without a clear and pre-defined research question, studies are likely to be unsuccessful.

33.6.2 Study design

Study design is critical in traditional epidemiology research. Different designs have different applications with respective advantages and disadvantages (Table 33.1). This also applies to the field of epigenetic epidemiology.

33.6.2.1 Cross-sectional design

Cross-sectional designs are mainly used to investigate the distribution of epigenetic variation across populations with different characteristics. A substantial number of epigenetic studies are cross-sectional. In the cross-sectional design, subjects' characteristics and epigenetic measures are collected at the same time point or within a short time interval.

Results from the cross-sectional design are merely associations and they do not imply causation. However, these caveats do not necessarily dismiss the utility of cross-sectional studies. For example, several CpGs have been found to be cross-sectionally associated with smoking status, i.e., being differently methylated between smokers and non-smokers. If such associations are true (e.g., not being due to confounders), DNA methylation at these CpGs could still be used as biomarkers of smoking even if the causation of the association is unknown.

33.6.2.2 Case–control design

In a case–control design, cases are individuals with a specific characteristic, typically a disease, and controls are individuals without that characteristic. Epigenetic variation between cases and controls is compared to investigate the association between epigenetic variation and case status.

An important difference between EWAS and GWAS in a case–control design is the selection of controls. In traditional epidemiology, controls need to be selected from the source population from which cases are selected, to limit selection bias. However, GWAS sometimes does not have overly rigorous control selection but uses convenience controls. Bias arising from using convenience controls can be reduced by the low measurement error in genotyping, the absence of recall bias when studying inherited variants, the large sample size, the stringent criterion for statistical significance, the rigorous replication of findings and population stratification adjustment [84]. Given that EWAS is more like traditional epidemiological studies in the potential for bias, control selection is more important.

Caution is needed when interpreting results from case–control studies. Associations from a retrospective case–control design are cross-sectional. Whether the epigenetic variation precedes the disease or is secondary to the disease may be difficult to determine. Interpretation of results also depends on whether cases are prevalent or incident. If most of the cases are prevalent, the epigenetic variation cannot be interpreted as being related to disease onset, because it might be related to disease prognosis only. Additionally, the inference about risk made from the case–control design is for the population from which the controls are selected.

33.6.2.3 Prospective cohort design

Prospective cohort designs are longitudinal. In the context of epigenetics, biological samples and exposures of individuals are collected at baseline, typically in healthy individuals thought to be a representative of the general population. These individuals are followed for a period during which health outcomes are recorded. In this design, epigenetic variation precedes the health outcome; therefore, it overcomes some weaknesses of cross-sectional and case–control designs in making inference about causation. If biological samples and exposures are also collected during the follow-up,

Table 33.1 Pros and cons of epidemiological study designs in epigenetic epidemiology.

Design	Pros	Cons
Cross-sectional design	• Easy to conduct • Low cost • Enables investigation on the associations of epigenetic variation with participants' multiple characteristics	• Easy to be impacted by bias • Provides the weakest evidence of causation
Case–control design	• Easy to conduct • Low cost	• Easy to be impacted by bias • Provides weaker evidence of causation than prospective cohort design • Can only investigate the association with one disease/trait
Prospective cohort design	• Less impacted by bias than case–control design • Provides stronger evidence of causation than case–control design • Enables investigation on the associations of epigenetic variation with participants' multiple outcomes	• Costly • Bias may arise due to drop out during follow-up
Nested case–cohort design	• Cheaper than prospective cohort design • Less impacted by bias than case–control design • Provides stronger evidence of causation	• Can only investigate the association with one disease/trait

Continued

Table 33.1 Pros and cons of epidemiological study designs in epigenetic epidemiology.—cont'd

Design	Pros	Cons
	than retrospective case–control design	
Family-based design	• Enables investigation on the familial aggregation of epigenetic patterns and its causes • Enables investigation on epigenetic association controlling for familial confounding • Enables causal inference	• Families may be difficult to recruit • May be impacted by ascertainment bias
Experimental design	• Provides the strongest evidence of causation	• Very costly; however, may be the most cost-effective given the quality of evidence provided • May raise ethical issue • High standards for the design

the mediating role of epigenetic variation underlying the exposure-outcome association or of exposure underlying the epigenetic-outcome association can be investigated.

33.6.2.4 Nested case–control design

Cohort studies are typically very costly. A case–control study nested within a cohort is a cheaper solution that is widely used [85,86]. In the nested case–control design, at the time point when a case occurs during the follow-up, a control is selected from the individuals who remain free of disease. Only epigenetic variations from biological samples of the cases and controls, not from all individuals at baseline, are measured, which can reduce costs substantially. The nested case–control yields relative risk estimates comparable to those obtained from a cohort when incidence density sampling is used [87].

33.6.2.5 Family-based design

Family-based designs can be used to investigate familial aggregation of epigenetic patterns and potential causes, of which a special case is the twin design [88]. Some consider that it is difficult to

determine whether familial aggregation in epigenetic variation is caused by transgenerational epigenetic inheritance or shared genes and/or environment [16]; however, this mostly applies to family-based studies including only parents and offspring. This problem can be circumvented by an extended twin family design, specifically by a twin and sibling design. Another advantage of a twin and sibling design is that this design can minimize age and birth cohort effects occurring when studying familial aggregation, because siblings are of similar ages to, and of the same temporal generation as, the twins.

A limitation of some family-based studies for investigating familial aggregation in DNA methylation is that they have focused on limited age ranges, so these studies cannot provide an overview of DNA methylation familial aggregation over the whole human lifespan. By pooling data from multiple twin and family studies from which the age range of participants covers the whole lifespan, and adopting flexible pedigree analytical methods to model the changes in familial aggregation as a function of cohabitation and age, the LIfe Twin Epigenetic (LITE) study has provided evidence that environmental factors shared by relatives when they are cohabitating play an important role in causing the variation in genome-wide average DNA methylation and epigenetic aging across the lifespan [89–91], and early life affects late-life health through determining DNA methylation across the lifespan [92,93]. These insights could not be gained by studying twins and families of limited age ranges only.

Family-based designs can also be useful in studying epigenetic variation as the cause or outcome of a disease. For common diseases like breast cancer, under a multiplicative risk model, the underlying risk distribution is highly skewed—most individuals in the population are well below the average risk. Given that epidemiological studies make inference about the risk for the population from which controls are selected, epigenetic risk factors found by case–control studies with typical population-based controls are relevant to individuals, the vast majority of whom is at lower than average risk [94]. It is not known if these epigenetic risk factors apply to individuals at increased, if not high, risk. Cases and controls enriched for familial risk are essential in finding evidence relevant to individuals across the full continuum of risk. Family-based registries such as the Breast Cancer Family Registry [94] are precious resources for identifying such cases and controls. An example of family-based designs for the association between blood DNA methylation and breast cancer can be found in Ref. [95].

Family-based designs can also be used to control for potential familial confounding in epigenetic associations by comparing cases with their unaffected family members. The most typical comparison is within sibling pairs, and a special example is disease-discordant twin pairs. Such designs control for the confounding effects of shared genes and/or environment, and are more likely to measure causal associations. One study has investigated the association between within-sibship differences in BMI and in blood DNA methylation [96]. By studying the within-sibship difference, the confounding effects of shared genes and/or environment are canceled out.

Another application of family-based designs is to infer the causation between epigenetic variation and exposures or diseases; see the section "Causal inference" below for further details. A more detailed introduction about the value of family-based designs in epigenetic research can be found in Ref. [97].

33.6.2.6 Experimental design

In the context of epigenetics, experimental designs can be used to verify if epigenetic variation can be intervened on or be a successful therapy target. These designs include randomized community trials and randomized controlled trials. Eligible individuals are randomly assigned to several groups

receiving different interventions or treatments. Outcomes of interest across groups are compared. Epigenetic variation can be the outcome of interest or the treatment target. Many potential confounders are equally distributed across groups through randomization, thereby limiting biases inherent to other designs. Experimental designs are costly, which may limit their applications. However, given that the experimental design provides high quality of evidence, it may be the most cost-effective design. In addition, experimental designs might not be possible due to ethical issues, in which case observational studies might provide the best evidence possible.

33.6.3 Sample size

In epigenetic epidemiological studies, sample size contributes to the power to detect associations. In the context of DNA methylation, some studies have investigated this issue through simulation [98–100] by assuming study design, DNA methylation distribution and statistical methods; more details could be found in the publications cited.

The determination of adequate sample size in epigenetic epidemiology depends on several factors. It depends on the research question. If the question is for generating a hypothesis, the sample size may not need to be very large. However, if the question is for testing a hypothesis, the sample size needs to be adequate not to miss important effect sizes. It also depends on the statistical method. The influence of statistical methods has been reported by the simulation studies above. It also depends on the multiple testing adjustment procedure (see the Section 33.7.1 for further details); a more conservative procedure requires a larger sample size. Additionally, the repeatability of the measurement tool has also been shown to impact on the power; low repeatability can bias results toward the null [101].

The determination of adequate sample size in epigenetic epidemiology has two main challenges. First, in sample size estimation, the effect size to be not missed or to be detected is not straightforward to determine. Given the features of epigenetic variation, the effect size is potentially specific to tissue and population. There may be no prior knowledge of the effect size for the same tissue in a population with comparable characteristics. A pilot study may be helpful; however, a pilot study with a small sample size could be potentially misleading. Second, the theoretical sample size may be unrealistic to achieve in practice. The appropriate tissue may be not available for all subjects. The cost for a large sample size may be too high to be affordable. In practice, a trade-off between theoretical statistical power and practical research conditions is needed.

For identifying signals with meaningful effect sizes, volcano plots can be used. In a volcano plot, the x axis is the observed effect size, such as fold change or regression coefficient, and the y axis is the statistical *P*-value. Signals on the upper corners of the plot tend to be those with more meaningful effect sizes.

A single study might not be able to provide a sufficient sample size. One solution is pooling the data of multiple studies via a meta-analysis to increase the sample size. Meta-analysis could potentially minimizes the bias from studies with smaller sample sizes and provides more reliable findings. Exemplary studies are those from the Pregnancy and Childhood Epigenetics Consortium [102].

33.6.4 Biological sample

Biological sample is critical in epigenetic epidemiology. Many aspects related to biological sample, including tissue type, cell heterogeneity, collection, and storage, can impact the validity of the research and the interpretation of results.

Epigenetic variation can be tissue-specific, i.e., different tissues can have different epigenetic patterns. This implies that disease-related epigenetic changes might occur in relevant tissues only. Results from studying surrogate tissues are conceptually open to questions for many diseases, like the mediation results of blood methylation between smoking and lung cancer that are mentioned above. Results from surrogate tissues should therefore be interpreted with caution, and it is optimal to use disease-relevant tissues if possible.

However, for many diseases, the disease-relevant tissue is not easily available due to difficulties in the collection, cost, and ethics. Blood, saliva, and buccal swabs are easily accessible and commonly used surrogate tissues in epigenetic epidemiological studies. Given that these tissues can be easily accessed for the population, any disease association detected in these tissues is potentially important for public health translation. Using surrogate tissues requires cross-tissue research on epigenetic variation. In the context of DNA methylation, DNA methylation in blood and other tissues at some CpGs has been found to be weakly correlated [103]. On the other hand, some disease-associated CpGs appear to show consistent cross-tissue associations with diseases. For example, DNA methylation at the hypoxia-inducible factor 3-alpha (*HIF3A*) gene in blood and adipose tissue were both found to be associated with BMI, although with different magnitudes [104]. The cross-tissue correlation at this locus was found to be $\sim$0.4 [103]. However, it does not necessarily imply that this case is applicable to other CpGs, tissues or diseases; for example, in the same study, DNA methylation at *HIF3A* in skin was not associated with BMI [104]. A method has been developed to predict DNA methylation levels across human tissues, which can potentially be applied to recalibrate the DNA methylation level of a surrogate tissue to the DNA methylation level of the target tissue [105].

It is important to note that the choice of tissue depends on the research question. If the research question is to investigate biological causes of disease, it is more appropriate that the target tissue is disease-relevant tissue. However, if the research question is to derive a non-invasive biomarker of disease risk, then blood or saliva might be suitable even if less biologically relevant.

Another tissue-related issue is cell heterogeneity. Epigenetic variation is cell-specific. For example, different blood cell types have been found to have distinct DNA methylation [106]. Epigenetic variation in a tissue is typically a mixture of the variation in several types of cells. If the disease-associated epigenetic change is cell-specific, then the change may not be detected on the tissue level. In this case, cell sorting is needed to obtain purified cells. On the other hand, if the proportions of cells are associated with exposure/outcome of interest in a tissue, then cell composition could confound the epigenetic association. It has been shown that cell composition in the whole blood can change as a result of aging [107]. In the context of DNA methylation, methods have been developed for the deconvolution of blood cell proportions, such as the methods developed by Houseman et al. [108,109] and Salas et al. [110]—the latter could provide 56 immune profiles. The estimated cell composition could be adjusted in downstream statistical analysis. There are also other statistical methods that could control for the confounding of cell composition; see McGregor et al. [111] for more details. Most epigenetic epidemiological studies adjust for cell composition in their statistical models to avoid cell composition confounding the associations between DNA methylation and diseases, but the adjustment may not be warranted in all situations. For example, loss of precision due to unnecessary adjustment may be induced when the sample size is small, or investigators may be interested in overall methylation changes in whole blood.

The collection and storage of biological samples may also influence epigenetic variation. In epigenetic epidemiological studies, one consideration for collection and storage is preserving the

biological epigenetic variation and not introducing any artificial variation. To investigate disease etiology, the timing of the collection is important: samples taken prior to disease onset are needed. There is little evidence about the influence of the sample collection method on epigenetic variation. Regarding storage, DNA methylation is considered stable in frozen samples; however, this might not be the case for histone modification [4]. Differences induced by different collection or storage conditions between cases and controls could lead to false positive results. Many cohorts have archived biological samples at several time points. Differences in sample collection or storage across different time points might induce changes that could lead to erroneous conclusions when assessing longitudinal changes.

33.6.5 Measurement tool

There are several tools for quantifying epigenetic variation, especially for quantifying DNA methylation. These include high-performance liquid chromatography (HPLC) and repetitive element bisulfite sequencing for global DNA methylation quantification, bisulfite sequencing, methylation-specific polymerase chain reaction (MS-PCR or MSP), and bisulfite pyrosequencing for locus-specific DNA methylation quantification, and reduced representation bisulfite sequencing (RRBS), bisulfite-free sequencing techniques like TET-assisted pyridine borane sequencing (TPAS) and DNA methylation microarray (such as HumanMethylation450 BeadChip and Infinium MethylationEPIC array) for genome-wide DNA methylation quantification. Each method comes with a balance between cost, genome coverage, and measurement precision.

A critical issue related to the choice of measurement tool is the repeatability. For example, CpG-specific DNA methylation measured by the HumanMethylation450 BeadChip array has been found to have a wide-ranging repeatability [112,113]. One study found that, for middle-aged women, blood DNA methylation variation at many CpGs measured by the HumanMethylation450 BeadChip array is best explained by measurement error alone [114]. Low repeatability impacts the precision of the measurement, and measurement error can bias the association toward the null [101], which should be considered when interpreting results.

33.7 Challenges

Despite the promising prospect of applying epigenetic epidemiology to disease prevention and public health strategies, some challenges need to be addressed in this field. These challenges come from several aspects, such as our limited understanding of epigenetics, imperfect technologies, and high expectation of translation from epigenetic epidemiological findings to real-world public health strategies [115]. Here we summarize challenges in data analysis, interpretation of results, causal inference, and communication to the public.

33.7.1 Data analysis

There has been a substantial development of methods for microarray (such as the HumanMethylation450 BeadChip array and Infinium MethylationEPIC array) DNA methylation data analysis in recent years, on which we will focus in this section.

Data pre-processing and normalization are critical steps before statistical analysis. Many methods have been developed for background/color correction, within- and between-array normalization and probe type bias correction; see the review [116] for more details. Several studies have compared these normalization methods [116–119] and did not reach unanimous conclusions, at least in part due to that these studies used different evaluation criteria. Although no method is widely recognized as being preferable, strong associations between DNA methylation and disease/trait are typically robust to the normalization method used [118]. We have some suggestions for this issue. First, sensitivity analysis using different methods to investigate if the results depend on the primary normalization method may be desirable. Second, when meta-analyzing individual-level data from several studies, it may be preferable that each study adopts pre-processing pipeline that allows addressing its own study-specific technical variation. This strategy might avoid any systematic bias introduced by the same pre-processing pipeline.

Batch effects are one of the main sources of variation in high-throughput data. Batch effects do not only relate to the physical batch in the experiment but can also be related to various aspects of the experiment including processing time, lab conditions, technicians, reagents, etc. [120]. Batch effects can be minimized using a careful study design including samples being processed under standardized conditions and being equally distributed across chips, in particular regarding the outcome, main exposures and confounders [121]. There have been statistical methods developed to address the batch effects issue, such as *ComBat* [122] and surrogate variable analysis (SVA) [123]. Many studies also model batch effects in their statistical models (e.g., as random effects in mixed models). See the review [120] for more details about batch effects.

A commonly used analytic approach in EWAS is the locus-by-locus analysis, like what typically used for GWAS data, with the primary goal of detecting marginal associations. Some bioinformatic methods have been developed for this approach and are accompanied by statistical packages mostly implemented in the software R, such as *minfi*, *ChAMP*, and *wateRmelon* [124]. Some of these bioinformatics methods, however, are most appropriately used in an "experiment" design in which DNA methylation between groups are compared, like those investigating differential methylated regions between tumor and normal tissues. Most epigenetic epidemiological studies are much more complex so more sophisticated methods like regression modeling are commonly used. Multiple testing is a major problem for the locus-by-locus analysis. This is also addressable by commonly used adjustment methods. These include, for example, controlling the family-wise error rate (FWER) or type I error, such as the Bonferroni procedure, or controlling the false discovery rate (FDR), such as the procedure proposed by Benjamini and Hochberg [125]. The choice of adjustment method depends on the primary objective of the study. If the study aims at generating an hypothesis, a less stringent method that controls FDR could be used, while if the study aims at hypothesis testing, a conservative approach that controls type I error is usually preferred [126].

In EWAS, the explanation for and the control of genomic inflation is not necessarily the same as in GWAS. Genomic inflation in GWAS is assessed using the genomic inflation factor, the value of which is one in the absence of genomic inflation, under the assumption that only a small fraction of SNPs is truly associated with the trait so that the test statistics for the majority of SNPs should have a distribution similar to the null hypothesis that there is no association. A large genomic inflation factor in GWAS typically means there exists bias, such as population stratification [127]. However, it is not clear whether this holds for EWAS. The genomic inflation factor was found to be larger than 1.1 in

many EWASs. This may be due to the effects of exposures or diseases on DNA methylome are more likely to be global, so that small effects may exist for thousands or even millions of sites. Such phenomenon is similar to the polygenic inheritance and similarly substantial genomic inflation is expected in GWAS if the polygenic inheritance exists [128]. Nevertheless, different DNA methylation patterns have been observed across populations. Researchers have proposed to address such differences by adjusting for the principal components of genome-wide SNP or DNA methylation data, as is commonly done in GWAS analyses [129]. One caution is that the top principal components of genome-wide DNA methylation data may capture the global effects of exposures or diseases which may result in biasing the association estimate toward the null. There are also other statistical methods for addressing this issue [130].

There are other analytical approaches than the locus-by-locus one. DNA methylation sites can also be analyzed simultaneously using a regularized regression method. An example is the development of the methylation-based age predictor [66]. DNA methylation has spatial correlations, i.e., the methylation statuses of neighboring sites tend to be correlated [131]. Such correlations imply that DNA methylation changes might be better detectable at the scale of genomic regions. Several region-based methods such as bump hunting [132], DMRcate [133], and A-clustering [134] have been developed to investigate differentially methylated regions. The basic idea of both bump hunting and DMRcate is to apply a smoothing technique to the individual association statistics of methylation sites of a specific region to obtain a summary statistic from which the statistical inference is made. The basic idea of A-clustering is to identify clusters of correlated methylated sites first, and then use generalized estimating equations to estimate the association for the cluster, controlling for the correlation between sites; more details about these methods can be found in the publications cited previously. Gene Set Enrichment Analysis (GSEA) can be used to examine if a set of genes is over-represented for cases versus controls [135]. These methods take advantage of the fact that methylation patterns are strongly correlated in clusters (defined by a certain number of base pairs), and assume that a single CpG site is unlikely to result in gene expression changes. Another dimensionality reduction method has been shown to be useful: a summary DNA methylation measure (commonly the average or median DNA methylation level) over a cluster of sites is calculated and used in analyses. The cluster could be the whole genome, a specific region, or defined by clustering methods [85,86,136].

33.7.2 Interpretation of results

The interpretation of results from epigenetic epidemiological studies needs to consider many factors including study design, potential biases, and biological mechanisms.

As discussed above, different study designs have their own advantages and disadvantages, which can influence the interpretations of results. For example, associations between epigenetic variation and diseases found from retrospective case-control studies cannot be interpreted as demonstrating that epigenetic variation causes the disease, nor *vice versa*.

Results should be interpreted with caution given the presence of potential bias including measurement tools, data pre-processing approaches, and unknown factors specific to the study. A good study design is important for avoiding or minimizing these biases. Sensitivity analyses should also be applied to examine the existence and magnitude of bias. Validation is another important step for assessing the robustness of findings to other measurement tools. It potentially requires pyrosequencing to confirm the validity of loci identified by DNA methylation microarray. Replication in independent

studies is important as well. Findings that cannot be replicated are more likely due to bias existing in the original study.

The biological interpretation of results relies heavily on understanding the mechanism, and many discovered disease/trait-associated epigenetic changes are yet to be understood biologically. Collaborative works between epidemiologists, biologists, geneticists, and laboratory-based scientists are needed. The biological plausibility of epidemiological observations needs to be investigated by scientists from relevant fields. On the other hand, the epidemiological and public health relevance of findings from laboratory-based research needs to be investigated by epidemiologists.

33.7.3 Causal inference

Results from epigenetic epidemiological studies are more vulnerable to confounding and collider biases than GWAS, which brings the causation underlying the observed associations into question. A directed acyclic graph including epigenetic variation, exposure or outcome, and potential confounders and colliders in the study design phase can help researchers to better understand the possible relationships among these factors and try to avoid many biases [137]. Appropriate study designs such as prospective and experimental designs can improve causal inference, as well as appropriate applications of statistical methods such as those mentioned below.

Causal mediation analysis is a conventional method for investigating causation and mediation in epidemiology, and this method could also be applied to epigenetic epidemiological studies; see the section "Epigenetic variation as a mediator of the effect of exposure" above for exemplary studies.

MR can be used to test the causation between an exposure and an outcome by examining the association between exposure-associated genetic variants as instrumental variables and the outcome. This method has been widely applied using SNPs identified by GWAS. In the context of epigenetic epidemiology, a successful application of this method concluded that blood DNA methylation at the majority of 187 BMI-related CpGs is consequential to BMI [32]. The mediating role of epigenetic variation can be potentially investigated using a two-step MR design [23]. A study using such design found some evidence that cord blood DNA methylation mediates the association of maternal vitamin B12 level with children's cognition [138]. It is important to note that the validity of MR depends on certain assumptions, some of which should be given attention [139]. Additionally, it requires large-scale studies with genetic and epigenetic data available on the same sample. Even in a two-sample MR, i.e., the association of epigenetic variation with genetic variants and the association of epigenetic variation with disease/trait are assessed in two separate samples, the association of epigenetic variation with genetic variants still potentially need to be assessed using a large sample, like what was done in the study by Wahl et al. [32]. An international effort has been undertaken to identify the genetic variants associated with DNA methylation [140].

Another useful statistical method to assess causation is a regression model for family-based studies [141], and a special case is for twin studies [142]. This method examines whether factors shared by family members confound the observed cross-sectional associations. By performing between- and within-sibship analyses, a study suggested that the association between DNA methylation at the suppressor of cytokine signaling 3 (*SOCS3*) gene and BMI may be due to the confounding effects of factors shared within families [96].

A family-based statistical method, ICE FALCON [24], can also be adopted. ICE FALCON uses the data of pairs of relatives. Through examining cross-trait cross-pair associations and the changes in

regression coefficients before and after conditioning on the relative's exposure, ICE FALCON can make inference about the existence and direction of causation in the presence of familial confounding. ICE FALCON shares similarities with MR, as it essentially uses the relative's exposure as an instrumental variable, but it has many advantages over MR and is more powerful than MR; see Refs. [24,143] for further details about the comparison between ICE FALCON and MR. ICE FALCON has been applied to epigenetic epidemiological research and provided evidence that BMI and smoking have a causal effect on blood DNA methylation [31,34].

33.7.4 Communication to the public

Appropriate communication of research results to the public is essential for translating epigenetic epidemiological findings to public health strategies. The public is typically a non-expert audience, so researchers need to explain the results in lay language. Using some hooks might be helpful for attracting the attention of the public and facilitating communication, like the term "epigenetic clock." However, the communication should also avoid any over-interpretation of research findings, especially in the early stage of the field. The translation of epigenetic epidemiological findings to public health promotion is potentially limited by many unexpected factors. Additionally, and more importantly, the findings should be communicated with caution to avoid any ethical issues.

33.8 Summary

In this chapter, we summarize the promising role of epigenetics in understanding disease etiology, assessing relevant exposures, and using them for disease prediction, and therapy. We summarize the considerations regarding the implementation of epigenetic epidemiological studies including research question, study design, sample size, biological sample, and measurement tool. We also summarize several challenges in data analysis, interpretation of results, causal inference, and communication to the public. Combining epigenetics with epidemiology, with a careful study design and an appropriate interpretation of results, could result in an enhanced understanding of disease and a better strategy to improve public health.

List of abbreviations

AUC	Area Under the Receiver Operating Characteristic Curve
BMI	Body mass index
CDKN1C	Growth restricting gene cyclin-dependent kinase inhibitor 1C
CpG	Cytosine-guanine dinucleotide
DNMT	DNA methyltransferase
DOHaD	Developmental Origins of Health and Disease
EWAS	Epigenome-wide association study
FDR	False discovery rate
FWER	Family-wise error rate
GFI1	Growth factor independent 1 transcriptional repressor
GSEA	Gene Set Enrichment Analysis
GWAS	Genome-wide association study

HDAC	Histone deacetylase
HIF3A	Hypoxia-inducible factor 3-alpha
HPLC	High-performance liquid chromatography
ICE FALCON	Inference on causation from examination of familial confounding
IGF2	Insulin-like growth factor 2
KCNQ1OT1	KCNQ1 overlapping transcript 1
LINE-1	Long interspersed nuclear element-1
MHC	Major histocompatibility complex
MR	Mendelian randomization
MS-PCR	Methylation-specific polymerase chain reaction
OPERA	Odds per adjusted standard deviation
PAX1	Paired box 1
POU4F3	POU class 4 homeobox 3
RRBS	Reduced representation bisulfite sequencing
SHOX2	Short stature homeobox 2
SNP S	Single-nucleotide polymorphism
SOCS3	Suppressor of cytokine signaling 3
SVA	Surrogate variable analysis
UBE3A	Ubiquitin protein ligase E3A

Acknowledgments

SL is a National Health and Medicine Research Council Emerging Leadership Fellow (GNT2017373), and the chapter was written when he was supported by the Victorian Cancer Agency Early Career Research Fellowship ECRF19020. JLH is a Senior Principal Research Fellow of the National Health and Medical Research Council.

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Epigenetics in Human Disease

Third Edition

Edited by
Trygve Tollefsbol

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Volume ISBN
ISBN 978-0-443-21863-7
9 780443 218637

Set ISBN
ISBN 978-0-443-21811-8
9 780443 218118

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ACADEMIC PRESS
An imprint of Elsevier
elsevier.com/books-and-journals