Epigenetic Epidemiology

Karin B. Michels
Editor

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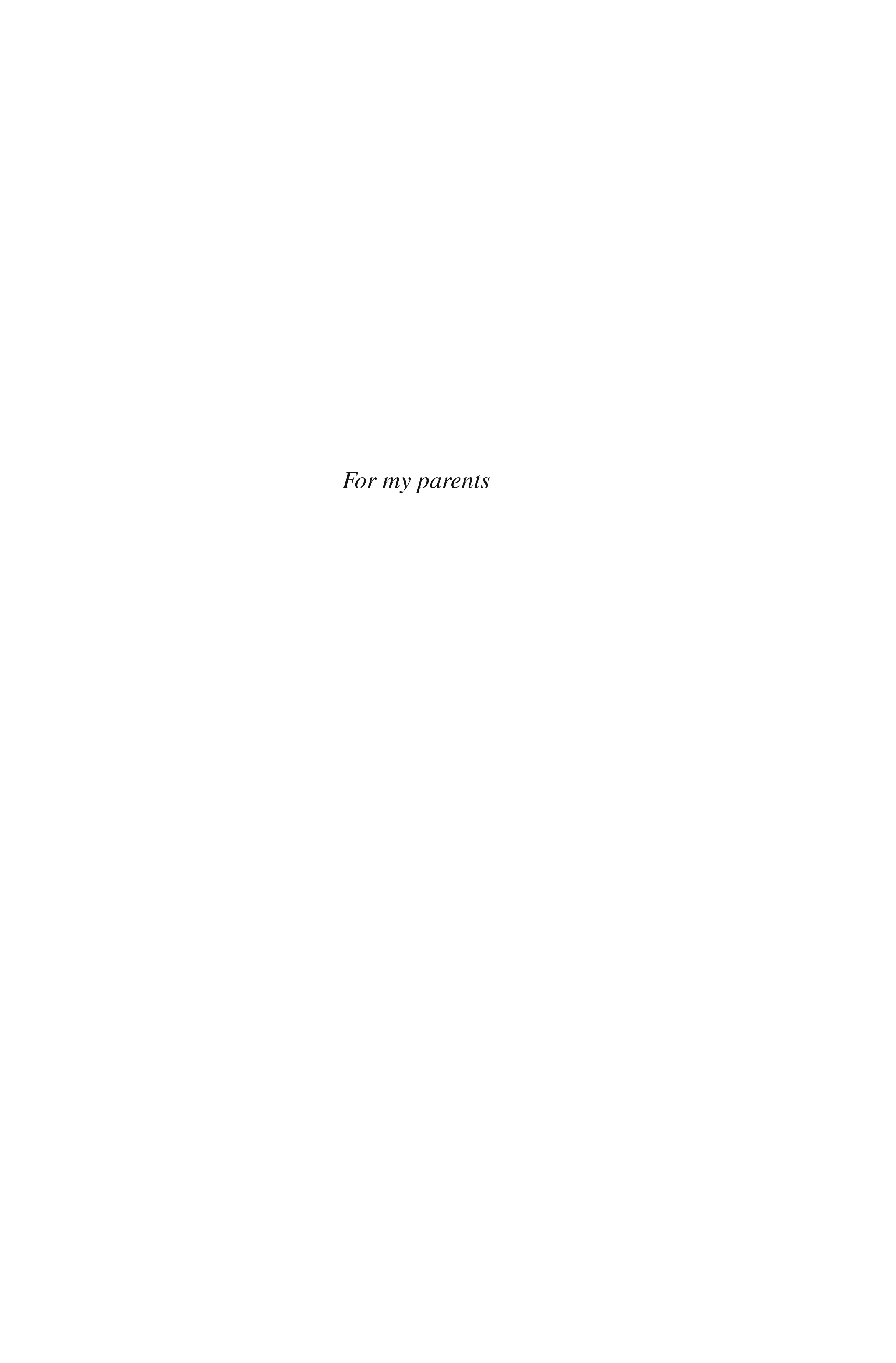

For my parents

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Chapter 1
Introduction

Karin B. Michels

This book joins two fields, epidemiology and epigenetics, to take advantage of their respective strengths in creating the science of epigenetic epidemiology. Epidemiology is the study of the frequency, distribution, and determinants of health and disease in humans. As a science fundamental to the study of public health, epidemiology is concerned with the prevention and effective control of disease. Epidemiology has early roots with the Greek physician Hippocrates, was essential in resolving classic infectious disease epidemics such as cholera, and takes center stage in unveiling the causes of the chronic disease epidemics of our times including cardiovascular disease, diabetes, and cancer [1].

Epigenetics is the science of non-genetic mitotically heritable variation in the gene expression potential [2]. Gene expression represents the cell-specific response to intracellular and extracellular signals; epigenetic mechanisms such as DNA methylation and histone modification govern the ability to respond appropriately to these signals. While the field of epigenetics has evolved over the past three decades, interest is now increasing exponentially. Most work has been conducted in plants and animal models. While intriguing and important observations have emerged, many fundamental questions in epigenetic mechanisms in humans remain unanswered, providing abundant opportunities for discovery in the context of epidemiologic studies.

These two "epi" sciences (epidemiology: upon the people; epigenetics: above genetics) meet at the intersection of interindividual epigenetic variation and the distribution of disease [3]. Epigenetic epidemiology is defined as the study of the associations between epigenetic variation and the risk of disease in humans [2]. Marrying a bench science and a population science creates both challenges and opportunities. The amalgamation of the two fields creates a new science that supports

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the study of the role of epigenetic modifications in human disease etiology, the appreciation of epigenetics as a possible mechanistic link between environmental exposures and disease outcomes, and the discovery of new disease biomarkers [3]. Since epigenetic states are amenable to changes induced by environmental stressors, identifying factors that create or correct disease-specific patterns provides new possibilities for prevention and treatment.

Epidemiology and epigenetics share the elements of time and variability. The risk of disease among individuals varies with age and is influenced by environmental factors. The epigenetic code – unlike the genetic code – is modifiable [4] and, while fairly robust [5] changes with age [6–8] and as a result of environmental influences [3, 9]. Epigenetic epidemiology relies on associations between those epigenetic marks with considerable interindividual variability and the incidence of disease.

This book is intended to be a resource for epidemiologists and epigeneticists alike. It provides insights into the mechanisms and methods in both fields to enable scientists to learn from each other, collaborate, and conduct superior studies. Epidemiologists wishing to incorporate an epigenetic component into their epidemiologic study will find useful tools here such as guidance on the appropriate epigenetic methods and specifics about strengths and weaknesses of various laboratory assays. Epigeneticists will find relevant information on how to embed their research ideas into a population-based study, how to choose their study population and design, what pitfalls to watch out for, and the appropriate statistical analyses of their research findings. The book also provides information on the formation of the epigenome during development, the role of genomic imprinting, the potential effect of assisted reproductive technology on the epigenome, the influence of age and environmental factors on the epigenetic profile, and the role of epigenetics as a mechanistic underpinning of the developmental origins of health and disease. The contributors to this book have collected and summarized the state of the art in epigenetic epidemiologic research on a number of important diseases including cancer, infectious diseases, inflammation and rheumatoid arthritis, asthma, autism and other neurodevelopmental disorders, psychiatric disorders, diabetes, obesity and metabolic disorders, and atherosclerosis.

I hope this book conveys a sense of the excitement pervading the nascer field of epigenetic epidemiology

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Chapter 2
The Human Epigenome

Peter A. Jones and Gangning Liang

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Abstract The output of the genome is controlled by the interaction of transcription factors with the epigenome. Epigenetic processes such as DNA methylation, histone modification, histone variants, non-coding RNAs and nucleosomal remodeling machines interact with each other to ensure stable states of gene expression. These processes can become dysregulated during aging, exposure to environmental stressors and the development of cancer and other diseases. DNA methylation patterns can be relatively easily read by high throughput techniques and provide information reflecting the influence of the environment and aging on the functionality of the epigenome. Analysis of DNA methylation patterns therefore provides an exciting new route to understanding how the environment interacts with the epigenome to cause disease. Despite the promise of DNA methylation patterns for epidemiologic studies, caution in interpreting data from surrogate tissues is necessary and cellular heterogeneity can also complicate interpretation of the data. In addition,

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DNA methylation within the body of genes can influence the response of the genome to the environment. Hypomethylation of repetitive elements can lead to genomic instability and ectopic gene expression. Methylation of coding regions can directly increase the rate of spontaneous hydrolytic mutations and increase the mutational frequency induced by carcinogens and radiation. Epigenetic processes can therefore contribute in multiple ways to the development of human diseases particularly cancer.

List of Abbreviations

ChIP	chromatin immunoprecipitation
DNMT1	DNA methyltransferase I
DNMT3A	DNA methyltransferase 3A
DNMT3B	DNA methyltransferase 3B
ncRNA	non-coding RNA
SAM	S-adenosine methionine
TDG	thymine DNA glycosylases
UDG	DNA glycosylase enzymes

2.1 Introduction

The genetic information encoded in the DNA of living organisms has to be read and interpreted in cells in such a way that its expression is highly controlled in response to developmental and environmental cues. Eukaryotic organisms, unlike prokaryotes, package their DNA into chromatin in which the fundamental building block is the nucleosome consisting of ~146 bp of DNA wrapped around an octamer of histones (Fig. 2.1). This packaging is essential to fit the DNA into the confines of the mammalian nucleus and also to provide functionality in different cell types. The combination of DNA and histones within the nucleosome is inherently refractory to transcription and nucleosomes have to be moved around or even evicted from particular places to allow gene expression to occur. This chromatin substrate, which is read by transcription factors in differentiated cell types, is what constitutes the epigenome. The accessibility or lack thereof of the genetic code is governed by chemical modifications which are applied to both the DNA and the protein components of chromatin and recent advances in high-throughput technologies now allow us to read these epigenetic modifications in their entireties in differentiated cell types.

The roles of the different modifications during development and stability of differentiated states are now beginning to become apparent, as are the switching mechanisms which occur during the development of a human from a fertilized egg. Epigenetic information is heritable in somatic cells and can be copied after DNA synthesis and mitosis to ensure stability of cellular states. However, the epigenome

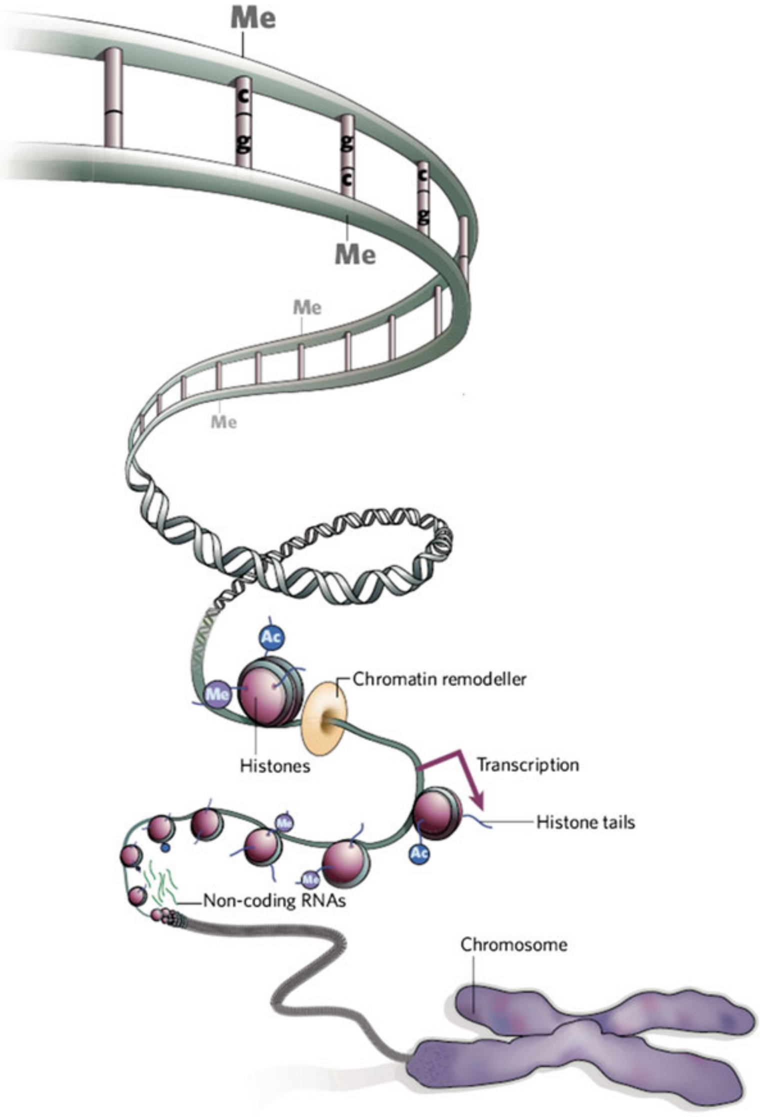

Fig. 2.1 Epigenetic processes. The DNA in living cells is complexed with proteins and RNA to fit into the structural confines of the mammalian nucleus. Most DNA is found in nucleosomes which contain about 146 base pairs of DNA wound around a histone octamer. The DNA can be modified by the application of methyl groups to cytosine residues in the simple palindromic sequence CpG. The tails of the histones and some internal amino acid residues are subject to posttranslational modifications which have significance in terms of dictating transcriptional competence. Nucleosome remodelers are necessary to expose regions of DNA so that it is accessible to the transcriptional and regulatory machinery. Non-coding RNAs also participate in the organization and functionality of chromatin. The various covalent marks communicate with each other and with remodeling machines to define the structure of different epigenomes and different cell types (Reprinted with permission from *Nature* [1])

is susceptible to alterations induced by the environment, nutrition, and other factors, so that potential changes in the packaging of genetic information can subsequently be copied in differentiated cells giving rise to both normal and abnormal cell states. Missteps in epigenetic processes can give rise to cancer and possibly to several other human diseases. We are just beginning to understand the multiple effects of the environment on epigenetic modifications and since these are potentially reversible, there is the possibility that several different diseases which have an epigenetic basis may be subject to pharmacological rectification [1].

2.2 Four Interacting Systems of Epigenetic Control

DNA in the nucleus of the cell is wrapped around an octamer containing 8 histone molecules in the fundamental structure of the nucleosome. The nucleosome contains about 146 bp of DNA and packages the DNA into the confines of the nucleus and also controls the output of the genome (Fig. 2.1). The nucleosome compactness is quite refractory to the initiation of transcription and nucleosomes need to be physically moved by nanomolecular machines to open up the DNA and allow transcription factors to initiate transcription.

The packaging and output is controlled by interaction between the various molecules which constitute chromatin and these systems interact with each other as depicted in Fig. 2.2. A variety of covalent marks and the presence of distinct histone variants, together with the involvement of non-coding RNA, are essential to the proper control of gene activity. DNA may become modified by the application of methyl groups to the 5 position of the cytosine ring and patterns of DNA methylation which are established during early development and differentiation can be copied giving rise to somatically heritable states of gene expression which can be passed from one daughter cell to the next.

The nucleosome contains two copies each of the basic histone proteins, H2A, H2B, H3 and H4, and the tails of these histones are subject to a large number of covalent modifications which convey information regarding the stability of the nucleosome and its accessibility to transcription (Fig. 2.2). Some of these covalent modifications, such as acetylation and methylation of particular lysine residues are associated with active gene transcription. On the other hand, modifications such as methylation of other lysine residues can result in transcriptional silencing. In addition, certain histone variants such as histone H3.3 or histone H2AZ are inserted into nucleosomes within specific places in the transcriptional unit and play important roles in gene activation or repression.

The covalent modification of DNA and of histones communicate with each other at a biochemical level in ways which are just now being unraveled. Together these processes can collaborate to ensure stable states of transcriptional competency and we should soon have a much better understanding of how alterations in the nucleosomal constituents collaborate to cause these relatively stable competency states.

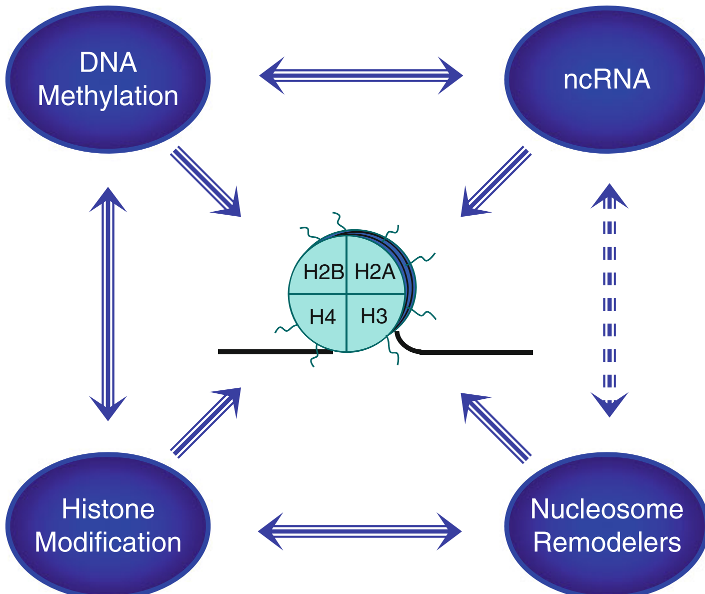

Fig. 2.2 Four interacting systems ensure epigenetic control. The four epigenetic processes outlined (among others) communicate to ensure somatically heritable states of gene expression in the context of the nucleosome. For example, DNA methylation and histone modification interact with each other and chromatin remodeling machines to position nucleosomes into active or repressive states. DNA methylation can also control the expression of non-coding RNAs which in turn can alter DNA methylation states in plants and possibly in human cells. These systems are mutually self reinforcing and therefore can both initiate silencing and maintain previously silenced states

Figure 2.2 also depicts the role of nucleosomal remodeling in epigenetic processes. As mentioned earlier, the presence of nucleosomes at transcriptional start sites is inherently refractory to transcriptional initiation and a whole series of multi protein complexes uses the energy of ATP to move nucleosomes around and expose different parts of the DNA thus allowing transcription to occur. Unfortunately, little is now known as to how the covalent marks on the DNA and histone proteins communicate with these remodeling machines.

Recent work has also suggested the potentially important role of non-coding RNA (ncRNA) species in the establishment and stability of epigenetic states. The role of RNA is quite well understood in organisms such as yeast and also in plants where it has been shown directly that ncRNAs can lead to DNA methylation and histone modifications which are important for keeping particular chromosomal regions silent by the formation of more densely packed configurations. Also certain microRNAs can downregulate chromatin modifiers such as DNA methyltransferases [2] or histone methyltransferases [3, 4]. The exact role of RNA in human epigenetic states is not clear at the present but it seems very likely that it will play a major role in their establishment and stability.

Recent developments in chromatin immunoprecipitation (ChIP) procedures and the coupling of these approaches with high throughput sequencing now makes it relatively simple to map the distribution of the different epigenetic marks on a genome-wide basis. It is likely that the availability of epigenomic maps will make it easier for us to understand how the marks communicate with each other and their potential roles in human development and disease.

Although it is clear that all of the processes outlined in Fig. 2.2 are contributing to epigenetic behavior and that all of them might be potentially altered by different environmental and nutritional influences. It is very likely that nutrition and the environment cause immediate and potentially reversible alterations to histone modifications which could be the subject of detailed epidemiologic studies. This review will, however, focus on the promise and potential of DNA methylation analysis for epidemiologic studies because the 5-methylcytosine mark is inherently more stable than the chromatin structure and the code of DNA methylation can be more easily and quantitatively read so that its role in disease states can be better understood.

2.3 The Basics of DNA Methylation

About 1% of the cytosine residues in human DNA become methylated after the DNA is synthesized by the application of a methyl group from S-adenosine methionine (SAM) to the 5 position of the cytosine ring (Fig. 2.3a). The modification occurs very shortly after DNA has been synthesized although there clearly is some methylation which occurs hours after the DNA has left the replication fork [5]. There are at least three enzymes which are responsible for setting up and maintaining DNA methylation patterns. DNA methyltransferase 3A (DNMT3A) and DNMT3B are thought to act early in development and can apply methyl groups to unmethylated and hemimethylated DNA (in which one strand has methylation and the other not). DNMT1 is thought to act primarily as a "maintenance enzyme" [6, 7] in that it has a preference for hemimethylated DNA and is the most active DNA methyltransferase in somatic cells [8]. All three of these enzymes have been shown, in gene knockout experiments in mice to be essential for mouse development demonstrating that DNA methylation is required for mammalian development.

These enzymes were thought to act largely in an autonomous manner with the DNMT3A and 3B "*de novo* methylases" required for early establishment of methylation patterns and DNMT1 then taking over and faithfully copy these patterns once they had been established. However, we have recently argued that this is unlikely to be the case and have proposed a cooperativity between the enzymes in order to appropriately maintain DNA methylation patterns in normal and transformed cells [9]. The methylation activities of these enzymes are also regulated by accessory proteins which alter the methylating capability of the enzymes. For example, the catalytically inactive form of DNMT3 (DNMT3L) is strongly expressed in embryonic stem cells and is highly stimulatory to the DNA methylating activity of both DNMT3A and 3B [8].

Fig. 2.3 Covalent modifications in DNA. (**a**) Almost all of the cytosine methylation in human DNA occurs in the simple palindrome CpG in which either both cytosines are methylated or neither are methylated as shown. The recent demonstration of 5-hydroxymethylcytosine in DNA suggests that a certain number of these sites might be further modified by the TET proteins as indicated above. Recently non-CpG methylation in the sequence CHG (where *H* represents any base other than *G*) have been observed in human embryonic stem cells. These very recent discoveries complicate the further dissection of the role of 5-methylcytosine in gene control and need to be considered in epidemiologic studies. (**b**) Cytosine residues in DNA are modified by the application of a methyl group from S-adenosyl methionine to the 5 position of the cytosine ring. Recently the TET proteins have been shown to be capable of further modification of the 5-methylcytosine to 5-hydroxymethylcytosine. This might represent a pathway to demethylation or alternatively have consequences for gene control

The existence of tissue-specific patterns of DNA methylation has been known for a long time and these patterns are known to be strongly associated with gene expression. Methylation of gene promoters is commonly linked to silencing whereas methylation in the gene body does not block transcription or silence alterative transcripts in a tissue specific manner [10]. The complexity of maintaining DNA methylation patterns is not completely understood, however, errors in the process can occur under normal conditions such as aging and in abnormal situations such as those which occur as a result of exposure to environmental insults. For this reason, the study of DNA methylation patterns in normal and diseased states has become of great importance. However, many of the observed alterations may have no direct role in the aging process or disease and it is still very difficult to distinguish between causative changes and alterations which have no functional consequence.

Almost all of the methylation of cytosine residues in somatic cell DNA occurs in the simple palindromic sequence, CpG (Fig. 2.3a). Most studies relating to DNA modification have focused on this covalent addition of the methyl group, however, it has recently become clear that other sequences and other modifications are also present in human DNA. For example, genome-wide studies in human embryonic stem cells have shown a high proportion of non-CpG methylation in particular regions of human DNA in this early developmental state [11]. The physiologic significance of this is not understood,

however, it may be due to the very high level of the DNMT3A and 3B enzymes in embryonic stem cells causing methylation at sites which subsequently lose their methylation at later stages of development possibly because the substrate generated after DNA synthesis would not be recognized and inherited by DNMT1.

Until very recently it was believed that 5-methylcytosine was the only modified base in human DNA but tremendous excitement has also been generated by the detection of 5-hydroxymethylcytosine in brain cell DNA and also in leukemia cells [12, 13]. The TET (Ten-Eleven Translocation) proteins are capable of oxidizing 5-methylcytosine to 5-hydroxymethylcytosine possibly on a pathway to active demethylation or to fulfill some currently unknown physiologic function (Fig. 2.3b). The hydroxymethylation state is not distinguished from 5-methylcytosine by standard bisulfite sequencing technology which is used to map DNA methylation in human cells. Thus, there is the potential for this modification to have been missed until now and it will likely attract a great deal of interest over the next few years.

A variety of methodologies have been used to determine DNA methylation levels and patterns, including restriction enzyme degradation, high pressure liquid chromatography and bisulfite sequencing among others. The fact that the mark can be read in DNA extracted from formalin-fixed material and seems to be stable in specimens which have been kept for a long time has encouraged the use of DNA methylation as a marker for environmental exposures with the goal of determining the influence of these exposures on epigenetic processes.

2.4 Shaping of the Genome by DNA Methylation

DNA cytosine methylation has had a profound effect on the structure of the genome because the application of the methyl group to the 5 position on the cytosine ring creates mutational hotspots in DNA [14]. The methylation of DNA in germ cells has resulted in the depletion of the methylation acceptor site CpG during the course of evolution [15]. Cytosine residues paired with guanines are known to undergo spontaneous hydrolytic deamination reactions of the order of 100 deaminations per genome per day (Fig. 2.4). The product of this deamination is a uracil residue which is not normally found in DNA and which can be rapidly and accurately repaired by ubiquitous and highly expressed uracil DNA glycosylase enzymes (UDG) which remove the uracil base and this results in the reinsertion of the cytosine residue so that no mutational events occur.

The presence of a methyl group on the 5 position of the DNA increases the rate of spontaneous deamination by about 2.5-fold [16], but more importantly, results in the generation of a thymine residue as a deamination product rather than a uracil. Thymine, being a normal constituent of DNA, is more difficult to repair. Although thymine DNA glycosylases (TDG) or MBD4 are capable of repairing most deamination sites in the correct direction there is an increased possibility of the C to T transition mutation occurring following DNA methylation and deamination. As mentioned above, this process has led to the depletion of CpG sites in the bulk of human DNA because most of the CpG sites in germ cells are methylated and

Fig. 2.4 5-Methylcytosine as a mutational hotspot. CpG sites in DNA are hotspots for C to T transition mutations in human DNA. Both cytosine and 5-methylcytosine can undergo spontaneous hydrolytic deamination to form uracil and thymine respectively. Uracil is not a DNA base and is rapidly and accurately repaired by uracil DNA glycosylase (UDG). Thymine, being a natural component of DNA, is more difficult to accurately repair by thymine DNA glycosylase (TDG) or methyl binding protein domain 4 (MBD4). This has led to the loss of CpG sites which previously were methylated in the germline during evolution and has led to the generation of CpG islands which somehow have escaped methylation in the germline. CpG methylation in the germline contributes to about 30% of all new disease causing familial mutations in humans [15]. In somatic cells, it can lead to mutations in tumor suppressor genes thus causing cancer

therefore subject to this increased mutability. Regions of DNA which are not methylated in germ cells have escaped this depletion of CpG sites and this has resulted in the presence of so-called "CpG islands" which are small regions of DNA about 1 kb in length which occur in the promoters of a substantial portion of human genes [17]. These CpG islands usually remain unmethylated in all normal tissues and this is associated with transcriptional competency. On the other hand, the abnormal methylation of these CpG sites can cause gene silencing resulting in cancer and other diseases.

Figure 2.5 outlines how some cytosine methylations can be involved in normal gene control and can directly interact with the environment. About 50% of human genes contain unmethylated CpG islands in their promoters and first exons, whereas repetitive elements including Alus and LINES tend to be methylated, as do the coding portions of genes within the exons as indicated. Abnormal methylation of the CpG islands, which can be the result of copying errors associated with cell division, aging, diet or exposure to carcinogens or other environmental stressors can result in the silencing of genes as indicated in Fig. 2.5a. This process has been well studied in cancer where between 1% and 10% of the CpG islands within genes have acquired abnormal methylation patterns during transformation [18]. The Fig. 2.5b also shows that demethylation of repetitive elements such as those within Alus and LINES can frequently occur [19] and this alteration is often related to disease outcomes in epidemiologic studies since

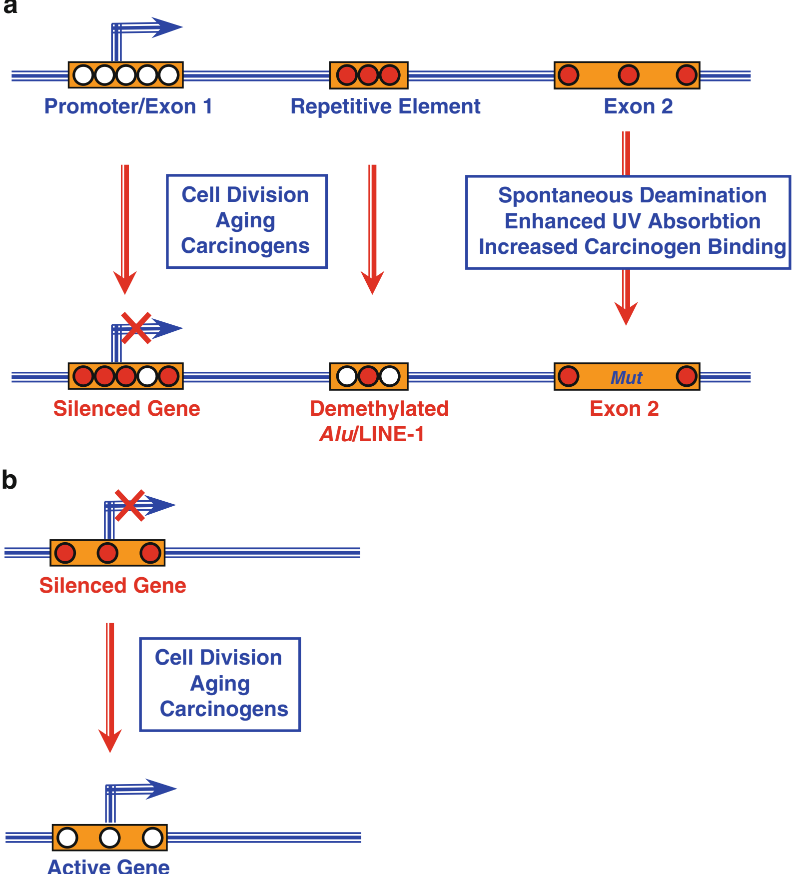

Fig. 2.5 The methylation status and effects of methylation on the carcinogenic process. (**a**) A promoter CpG island containing gene in which the gene is actively expressed due to a lack of methylation (shown as open circles) at the transcriptional start site. Oncogene and/or repetitive elements such as Alus or LINES located in the gene body are generally extensively methylated (closed circles) as are the CpG sites which are found within the coding regions of the gene. Promoter CpG islands can undergo inappropriate silencing and methylation of the CpG sites in response to aging, cell division, nutrition or exposure to environmental carcinogens. Demethylation of repetitive elements has often been observed during carcinogenesis and can be easily measured because of the high copy number of these elements in human DNA. The methylation of CpG sites within the exon can increase the rate of somatic mutations directly by increasing the frequency of C to T transition mutations. It also can alter the interaction of the DNA with the environment. For example, it can increase UV absorption and increase the binding of carcinogens to DNA. (**b**) Often overlooked, is the demethylation of non-CpG island promoters which can result in inappropriate gene inactivation as opposed to silencing during carcinogenesis

these elements are abundant in DNA and their methylation status can be measured relatively easily using quantitative techniques such as pyrosequencing [20].

Figure 2.5a also shows that the gene body methylation which occurs in exons can have profound effects on carcinogenesis. For example, the spontaneous deamination

of these methylated sites can give rise to mutations in tumor suppressor genes [21]. The presence of 5-methylcytosine in the coding regions of genes increases the rate at which mutations are introduced by ultraviolet light during the development of skin cancers [22]. This is because 5-methylcytosine absorbs UV light at a wavelength more prevalent in sunlight than cytosine, thus increasing the chances of mutations. Pfeifer et al. [22] have also shown that methylated CpG dinucleotides are the preferred targets for G to T transversions which are the most common mutations induced in mammalian cells by benzo(a)pyrene derived from tobacco smoke. Analysis of the mutational spectrum in human cancers can therefore provide evidence of value to the epidemiologist because the pattern of mutations can provide evidence of the most likely environmental cause [23]. For example, the high prevalence of C to T transition mutations at CpG dinucleotides in the p53 gene in colorectal cancer argues that these are most likely induced by endogenous processes potentially involving increased cell division [24] rather than being caused directly by exposure to carcinogens in the gut.

Figure 2.5b also shows that many tissue-specific genes which do not have CpG islands in their promoters can be ectopically activated as a function of aging and cell division. The potential role of non-CpG island methylation in gene control has been largely neglected in the field even though there is strong evidence that methylation of such regions can preclude gene expression [25]. Several studies have recently pointed to widespread hypomethylation of such regions in tumors and apparently normal cells adjacent to the tumor [19]. Since chemical carcinogens can inhibit DNA methylation, these processes can potentially result in the ectopic activation of genes which could play a significant role in the tumorigenic process.

2.5 Effects of the Environment on DNA Methylation

Soon after the discovery of the presence of 5-methylcytosine in DNA, work began to determine whether the levels of the modified base were altered in cancer and to determine whether chemical carcinogens could directly influence the methylation process. Lapeyre and Becker [26] showed that primary hepatocarcinomas and transplantable mouse liver tumors contained decreased levels of 5-methylcytosine relative to normal liver. Subsequently human leukemias and other uncultured tumors were found to have altered levels of DNA methylation [27]. Many such studies showed alterations of the overall levels of DNA methylation in cancer cells, however, Feinberg and Vogelstein [28] were the first to show that the methylation of specific sites within individual gene bodies were decreased in uncultured tumors. These early studies summarized by Riggs and Jones [27] established clearly that DNA methylation was fundamentally altered in cell lines and cancers.

Given the emerging interest in the potential role of 5-methylcytosine in controlling gene expression [6, 7] there was increasing research activity in determining the potential role of DNA methylation in cancer. The thrust of this work was on the potential for carcinogens to heritably alter the regulation of genes rather than on their abilities to cause mutations as discussed earlier. Early pioneers such as

Drahovsky and Morris [29] began work to determine whether chemical carcinogens could influence DNA methylation reactions in the test tube. These studies, also summarized in Riggs and Jones [27], pointed strongly to the possibility that chemicals in the environment including benzo(a)pyrene might be able to influence the DNA methylation machinery and that this could participate in the oncogenic process. Evidence that this was indeed the case came from studies of Wilson and Jones [30] using cultured cells and Wilson et al. [31] using freshly explanted normal human bronchial epithelial cells. These studies provide a logical hypothesis that analysis of human DNA extracted from cancers might provide evidence for the nature of the chemical insult which caused the methylation change.

2.6 The Role of Aging

A potential effect of aging on DNA methylation was suggested by Wilson and Jones [30] who showed that the lifespan of cells in culture was linked to the rate of overall loss of 5-methylcytosine levels. Subsequent experiments by Mays-Hoopes et al. [32] showed that alterations in DNA cytosine methylation also occurred in the intercisternal A particles (IAP genes) within mice. These studies which demonstrated hypermethylation as a function of age again emphasized that DNA methylation levels, while somatically heritable, were not completely invariant and could be used as markers for aging and exposure to chemicals.

The pioneering work of Ahuja and Issa and colleagues [33] in showing that hypermethylation of CpG islands in the colonic epithelium of people without cancer could be directly linked to the age, was also of great value in showing that the epigenome reacts to the increased cell division which accompanies aging. More recent work in mice has shown widespread and tissue specific DNA methylation changes showing that epigenetic regulation is a common feature of aging in mammals [34]. Since aging is a major risk factor for cancer, these alterations might provide a biochemical basis for the subsequent development of tumors.

Observations that DNA methylation patterns can be profoundly altered in aging in people without cancer show the plasticity of the epigenome. They also underline the importance of using age-matched controls in epidemiologic studies to investigate their alterations in this process and the relevance to development of cancer and other diseases. As mentioned previously, many DNA methylation changes may have no known significance in terms of genome function making it important for the important causative alterations to be determined in the future.

2.7 The Use of Surrogate Tissues

Epigenetic landscapes are tissue-specific and contribute to the phenotype of the cell. Unlike genetic studies, in which all differentiated cells in a subject have essentially the same markers such as SNPs etc., one cannot assume that surrogate tissues will

necessarily have the same value in assessing the effect of the environment on a given marker such as DNA methylation. It is therefore not always feasible to take an easily available tissue such as peripheral blood and use this to examine DNA methylation changes which might be occurring in a different target tissue. Epigenomic epidemiologic studies are therefore more difficult to perform and evaluate than genetic epidemiologic studies. Another complicating factor, which limits the use of blood cells, is that they are a heterogeneous mixture whose composition can change dramatically in response to other cues such as infections etc. Because each specialized type of cell in the peripheral circulation would be expected to have a different epigenomic profile, a measured change might reflect a change in cellular composition rather than a change in the pattern in a given cell type. Since epidemiologic studies often demand a large number of subjects and sometimes repeat sampling, other cells to consider are buccal cells, urine sediments, sputum and epidermal cells which can be relatively easily obtained.

Despite these reservations, useful information can be obtained from peripheral blood DNA methylation studies which might have value in determining the influence of nutrition or age for example on particular epigenomic marker. For example, the DNA methylation patterns of imprinted genes which are possibly methylated to similar extents in different tissues might be suitable as a surrogate although this remains to be shown. Another would be the methylation status of repetitive DNAs such as Alus and LINEs which do not show a great deal of inter tissue variations and which have been successfully used to measure changes in response to benzene exposure [35]. It is also important to consider the potential biological significance of relatively small changes which might be uncovered by these studies if there is an attempt being made to link the changes to a particular disease state. For example, it is not known whether small changes in the methylation status of a given promoter necessarily translates an alteration in gene expression.

2.8 Appropriate Controls

As mentioned earlier, epigenetic analyses differ from genetic analysis because the epigenome is cell type specific and is altered by environmental factors. This makes the appropriate selection of normal controls of great importance. For example, comparison of the DNA methylation patterns in a set of tumors should be compared to age-matched controls because the epigenome is known to change with aging. Another complicating issue is the fact that epigenetic changes can often be observed in the cells surrounding the tumor. For example, we found that the entire urothelium of the bladders of patients with bladder cancer is altered with respect to DNA methylation patterns [19]. Thus, the surrounding normal tissue may already harbor DNA methylation changes which are either selected for in the tumor or more probably allow the tumor to grow by altering the integrity of the epithelium. Therefore, comparisons of normal appearing surrounding tissue to similar tissues from age-matched controls who do not have the particular disease of interest, is often necessary to fully appreciate the changes which occur during the process of transformation.

Despite this complication, the existence of DNA methylation changes in normal tissues surrounding a tumor may be of great value in understanding the mechanism of carcinogenesis. It is still not known whether these changes precede the formation of the tumor or are a response of the epithelium to the presence of a tumor in a bladder. It might be possible in the future to conduct these analyses on high risk populations without cancer and predict cancer susceptibility. However, this will be limited to easily biopsied tissue and may not be applicable to all cancers, like brain cancer.

2.9 Summary

Interacting epigenetic processes ensure the somatic heritability of differentiated cell states and are set up early in development. These processes reinforce each other and can be influenced by environmental factors to alter gene expression in heritable ways which can cause disease. DNA methylation is a particularly attractive epigenetic process for epidemiologic studies since DNA methylation patterns can be quantitatively measured, are known to influence gene expression when located in controlling regions of genes and are subject to alterations associated with aging and exposure to environmental toxins. High-throughput approaches allow for the concomitant analysis of thousands of DNA methylation sites in large numbers of samples thus opening the door to future studies to link the influence of the environment to the epigenome. Caution however needs to be used when interpreting DNA methylation data, particularly because many changes may have little functional significance and there is a need to examine the cell type of origin in order to gain useful information.

Although much neglected in the field, the methylation of cytosine residues within the coding regions of genes can directly contribute to carcinogenesis by increasing the frequency of both spontaneous and induced mutations. Analysis of the mutational spectrum in different disease states can give an indication of likely exogenous or endogenous causes. The large number of new epidemiologic studies including epigenetic analyses, such as those discussed in this book suggests that we are entering an age of epigenetic epidemiology and that much will be learned about the interaction of the epigenome and the environment and its relation to disease.

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Chapter 3
Considerations in the Design, Conduct, and Interpretation of Studies in Epigenetic Epidemiology

Karin B. Michels

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Abstract Studies in epigenetic epidemiology may identify epigenetic aberrations associated with disease, link environmental and lifestyle factors to the epigenetic profile, or unveil epigenetic mechanisms underlying statistical associations between risk factors and disease outcomes. Epidemiologic studies provide the framework for

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identifying epigenetic biomarkers for disease risk or early detection of disease. Appropriate design considerations for studies in epigenetic epidemiology are imperative for their success. The tissue specificity of epigenetic marks represents a challenge in epigenetic epidemiology, and identification of disease markers in easily accessible surrogate tissues are essential for large-scale population-based studies. Nested case-control studies using biospecimens collected prior to onset of disease provide appropriate data to identify epigenetic changes preceding disease. Selecting a representative study population with sufficiently large sample size and appropriate comparison group is crucial for the validity and reproducibility of the results. Challenges in epigenetic epidemiology studies include confounding and effect modification, and identifying epigenetic marks with sufficient systematic interindividual variation.

3.1 The Objectives of Studies in Epigenetic Epidemiology

Epidemiology is primarily concerned with the frequency, distribution, and determinants of health and disease in humans [1]. Epidemiologic studies connect risk factors with disease outcomes based on distributions, often producing observations that inform basic research to identify mechanisms. A familiar example is the link between cigarette smoking and lung cancer [2], which guided basic research studies to classify nicotine as a carcinogen. Integrating epigenetics into an epidemiologic study recognizes the mechanistic link between a risk factor and disease risk [3] (Fig. 3.1). Epidemiologic studies support an association between folate deficiency and neural tube defects (NTD) [4]. In a case-control study including 48 induced abortions with NTD and 49 elective induced abortions without NTDs, the risk of NTDs increased with decreasing levels of LINE-1 methylation in brain tissue [5]. Maternal serum folate levels were lower in NTD cases than in controls and positively correlated with DNA methylation in fetal brain tissue, with a stronger correlation among cases [6].

The implications of epigenetic modifications in the risk of human disease can also be explored using the framework of an epidemiologic study (Fig. 3.1). Loss of imprinting (LOI) of insulin-like growth factor (*IGF*) 2, defined as biallelic expression of the normally monoallellically expressed gene in a parent-of-origin fashion,

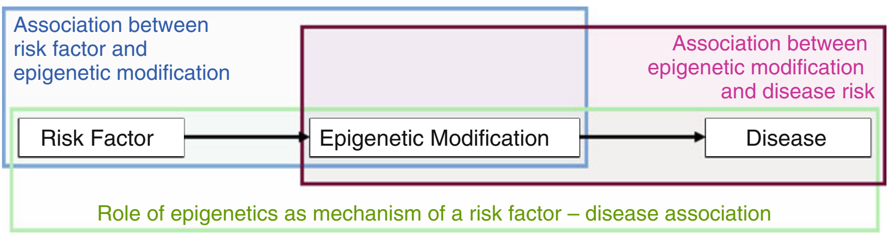

Fig. 3.1 Objectives of studies in epigenetic epidemiology

is an important etiologic factor in Beckwith-Wiedeman Syndrome [7] and Wilms' tumor [8] and was discovered by comparing the frequency of LOI of *IGF2* in children with and without these syndromes. We are only beginning to understand the role of epigenetic modifications in disease etiology; epigenetic epidemiology will provide the relevant methodologic underpinning to explore important correlations in human populations.

Whether aberrant DNA methylation, chromatin marks, or non-coding RNAs may be candidates for biomarkers of disease risk or early detection of disease can also be explored in epidemiologic studies. To date, few epigenetic biomarkers with acceptable sensitivity and specificity for disease have been identified [9–11]. Tissue-specificity of epigenetic patterns, requirements for sophisticated lab methods (e.g., capturing cancer DNA from serum) and equipment, and heterogeneity in sample collection and laboratory routines are just some of the obstacles that have hindered progress in identifying such biomarkers and moving them towards clinical application [12].

Observational studies also allow investigation of the influence of environmental and lifestyle factors on the epigenome (Fig. 3.1). Several lifestyle factors including alcohol consumption and smoking affect DNA methylation, as discussed in more detail in Chap. 12. Insights into environmental influences on the epigenome may provide targets for disease prevention.

Understanding the role of epigenetic changes in the disease process will stimulate the development of targeted interventions to prevent and treat disease. Indeed, the first epigenetic drugs have already been used to target hematologic cancers. DNA methyltransferase inhibitors (which induce hypomethylation) have been approved in the U.S. and elsewhere on the basis of randomized clinical trials to treat patients with myelodysplastic syndrome [13], and histone deacetylase inhibitors are available for the treatment of cutaneous T-cell lymphoma [14]. Moreover, DNA methylation may also predict response to therapy and foster individualized medicine. Promoter hypermethylation of the DNA repair protein MGMT is associated with a poor prognosis in various cancers because of the accumulation of mutations. However, these patients may respond well to alkylating drugs [15].

3.2 The Tissue Question

Due to its significant role in cell differentiation, one of the most profound characteristics of the epigenomic signature is its tissue specificity. Thus, the choice of the appropriate tissue is central to the success of an epigenetic epidemiology study. Changes in DNA methylation or chromatin structure are often restricted to the target organ affected by disease. Thus, when studying the contribution of epigenetics to cancer, it is important to microdissect tumor tissue to ensure a uniform cancer cell population. Moreover, tissue specificity can make epidemiologic studies of aberrant epigenetic marks very difficult. Not only is it challenging to collect samples of difficult to obtain target tissue from a sufficient number of patients with illnesses,

e.g., brain tissue from patients with Alzheimer's disease or pancreatic tissue from patients with pancreatic cancer, but it is nearly impossible to collect control tissues from healthy individuals to study the "normal" tissue-specific epigenome.

These barriers (as well as the problem of interindividual confounding discussed below) have seduced cancer researchers into using tumor-adjacent tissue that has been histologically rated tumor-free (from the same patient) for comparison. Of course, using control tissue samples from the same person avoids confounding by age or other interindividual differences. However, morphologically normal tissues adjacent to tumors harbor a number of genetic abnormalities [16–18], and epigenetic changes identified in cancer tissue have also been found in adjacent cancer-free tissue as far as 4 cm away from primary tumors [19–21]. This field effect renders the use of adjacent tissues in the quest for epimutations obsolete, and potential DNA methylation markers of tumorigenesis may be missed due to the use of inappropriate control tissue [22].

However, some tissue types are more accessible than others. For example, tissue harvested from invasive tumors in the intestinal wall of the colon displays characteristic epimutations when compared to colon mucosa from cancer-free patients [23]. Human skin biopsies show distinct methylation patterns according to sun exposure [24].

When obtaining tissues from a person with a particular disease, it is important to keep in mind that epigenetic changes may have either preceded or may be a result of the disease. Thus, target tissues should be obtained prior to disease onset to preclude reverse causation. This approach is unfortunately prohibitive for most tissues, given the number of individuals from whom target tissues would have to be harvested while they are phenotypically healthy. Prospective sampling may be possible only for specimens that can be reasonably easily collected such as blood, saliva, buccal cells, skin cells, urine, and feces.

Aberrant DNA methylation or histone modifications in such tissues may serve as surrogate markers of disease risk for many illnesses, however, the epigenetic pattern will not reflect the pattern in the target tissue. Such surrogate markers have been identified for some solid tumors [19, 25], and other chronic diseases including heart disease, asthma, and depression [26–28]. The use of blood to identify changes in DNA methylation associated with disease is hindered by the cell type-specificity of DNA methylation; any differences may be due to shifts in the distributions of the different cell types resulting from infection, inflammation, or disease. Alternatively, in patients with cancer it is possible to capture tumor DNA in the serum, however, identifying sufficient quantities of tumor DNA in the serum may prove impractical due to technical and quantitative challenges. Conversely, bladder cancer DNA has been retrieved from urine sediments [29].

3.3 Selecting the Epigenetic Mark to Study

The research question of interest generally determines the epigenetic mark to study. Generally, DNA methylation is a suitable marker for epidemiologic studies, because methyl groups are covalently bound to CpG dinucleotides and remain intact during routine DNA extraction, and cytosine methylation is fairly stable over the long term

if samples are properly processed and stored [30]. Very few epidemiologic studies have incorporated histone modifications, since the chromatin structure requires an additional immunoprecipitation step in sample preparation, which rules out the use of most existing biorepositories.

In the absence of reference epigenomes, the intraindividual variation of an epigenetic characteristic over time or the stochastic interindividual variation are not well understood. Epigenetic traits with large interindividual variation are the best candidates for epidemiologic studies, assuming that the majority of variation is systematic (and separates individuals with different characteristics such as differences in lifestyle or environmental factors or individuals with and without disease) rather than stochastic. Furthermore, for an epidemiologic study to detect interindividual variation in an epigenetic mark and link it to disease occurrence, interindividual variation has to exceed intraindividual variation. Some DNA methylation and genomic imprinting marks exhibit considerable interindividual variation and reasonable intraindividual stability over time [31], which makes them preferred markers for epidemiologic studies. However, a more detailed understanding of the DNA methylome will be necessary to identify prime candidates.

In epidemiologic studies the influence of environmental, nutritional, and lifestyle factors on the epigenome may be of interest. The focus will be on *de novo* methylation of unmethylated CpG islands in promoter regions. The stability of the methyl-cytosine bond makes active demethylation difficult to induce. While there is evidence of active demethylation in somatic cells [32], the mechanisms facilitating this process remain to be elucidated. Histone modifications are considered to be more volatile than CpG dinucleotide methylation and environmental factors may affect transcription also via this pathway. This makes chromatin an attractive target for studies in epigenetic epidemiology. The world of histone modifications, however, is complex and includes methylation, acetylation, phosphorylation, and ubiquitination making the choice of an appropriate candidate to study difficult.

Transcription is likely governed by the interplay of DNA methylation, histone modifications, and miRNAs [33]. Moreover, gene expression is not governed by epigenetic features alone, and changes in DNA methylation or chromatic structure may not affect transcription levels. Unmethylated gene promoters (or enhancers) do not necessarily facilitate expression. The functional relevance of methylation of individual CpGs within or outside of a CpG island is not always evident and may have pleiotrophic effects. Similarly, whether a difference in methylation of a few percent has functional consequences is likely gene-specific.

The candidate-gene approach targets DNA methylation or histone modification at certain candidate genes suspected or known to play a role in a specific disease process. To identify epigenetic changes associated with a disease or resulting from a particular exposure, e.g., folic acid supplementation or exposure to a chemical like bisphenol A, a genome-wide approach such as DNA methylation microarrays or ChIP-on-chip may be preferable to the candidate-gene approach in order to allow new discoveries (discussed in more detail in Chap. 4). However, genome-wide approaches are generally less sensitive than the assays used for candidate-gene approaches and rely heavily on sophisticated bioinformatics methods (see Chap. 5 for further details), which complicates interpretation of results. Various genome-wide

approaches are currently employed, and microarray-based methods, in particular the Illumina Infinium arrays for DNA methylation are widely used [34]. With the rapid advance in sequencing technologies, seq-based methods for both bisulfite converted DNA and chromatin immunoprecipitation are gaining popularity. Integrating genome-wide epigenetic scans into epidemiologic studies has become more feasible. For epigenome-wide association studies (EWAS), samples from prospective cohorts are required to ensure the temporal sequence of epimutations and disease incidence.

3.4 Study Designs in Epigenetic Epidemiology

A number of study designs can be employed in epigenetic epidemiology; the choice of the appropriate design depends on the research question (Table 3.1).

3.4.1 Cross-Sectional Studies

In a cross-sectional study, all factors of interest are assessed at one time point. For example, the proportion of individuals with a methylated CpG in a specific gene promoter in a population defined by a special characteristic, such as female smokers age 20–25 years, or the prevalence of LOI of a particular gene, say *IGF2*, in newborns, can be studied most easily with a cross-sectional design. Similarly, comparing the degree of global DNA methylation in two groups, e.g., among Caucasians age 60–65 years, males vs. females, can be accomplished effectively with a cross-sectional design.

Table 3.1 Study designs used in epigenetic epidemiology and their applications

Study design	Application
Cross-sectional study	Prevalence of an epigenetic mark in a well-defined population
Retrospective case-control study	Permanent epigenetic marks among individuals with and without disease
Cohort study	Epigenetic mechanisms underlying a risk factor-disease association
	Basis for nested case-control study
Nested (prospective) case-control study	Epigenetic marks predisposing to disease
	Biomarkers for early disease detection
	Biomarkers for disease risk
Intervention study	Effect of intervention on epigenetic pattern
Crossover study	Effect of epigenetic therapies on disease
Randomized controlled trial	
Family-based study	Transgenerational inheritance of epigenetic traits
Birth cohort	Influence of preconceptional and prenatal factors on establishment of the epigenome

3.4.2 Retrospective Case-Control Studies

In a case-control study, individuals with a disease and appropriately selected individuals free of the disease are sampled from the same source population. To study epigenetic variation, relevant biospecimens would be obtained and DNA methylation or histone modification assessed among cases and controls. In the context of epigenetics, this type of design bears similarities to a cross-sectional study, except for the particular control selection, which must be independent of the epigenetic state. The purpose of the controls is to estimate the epigenetic state of cases had they not contracted the disease under study; if the controls are not properly chosen, selection bias results. In neither the cross-sectional study nor the retrospective case-control study can it be determined whether the particular epigenetic signature assessed at the time of case and control selection preceded (and possibly caused) the disease among cases or whether it may be a consequence of (and possibly have been caused by) the disease. Therefore, epigenetic differences in retrospective case-control studies have to be interpreted with appropriate caution. Biomarkers for disease risk or early disease detection cannot be identified on the basis of retrospective case-control studies alone.

3.4.3 Cohort Studies

In a cohort, healthy individuals are recruited to participate in a longitudinal study over a certain period of follow-up time (weeks, months, years). At baseline, easy-to-obtain biospecimens are collected from all participants and stored. During follow-up, additional biospecimens may be collected. Such a biorepository provides the opportunity to study changes in DNA methylation or other epigenetic marks over time. During follow-up, disease outcomes are recorded. Cohort studies are usually large and may include many thousands of participants. Because it is not cost-effective to analyze the samples from all participants obtained at baseline and during follow-up, a nested case-control study is usually embedded in the cohort to study epigenetic marks predisposing to disease. In addition, cohort studies provide the opportunity to study epigenetic mechanisms underlying a risk factor-disease association. The major limitation of prospective studies is that for most target organs biospecimens cannot be obtained in healthy individuals.

3.4.4 Nested Case-Control Studies

A nested or prospective case-control study is embedded in a cohort study. All individuals who develop the disease of interest at any time during follow-up are selected, and appropriate controls (often 2 controls per case) are selected from those who remained free of the disease throughout follow-up. Since biospecimens from both cases and controls were obtained prior to the associating disease (and have been stored since

then), this study design permits associating the prediagnostic epigenetic status with disease outcome, i.e., it is clear that the epigenetic state reflected in the biospecimens preceded the diagnosis of disease and is not influenced by (phenotypically manifest) disease. Though the nested case-control study is a cost-effective study design, of course, prospective cohorts are generally expensive to maintain.

3.4.5 Intervention Studies

The effect of interventions such as folate and other supplements that affect the one-carbon metabolism on the epigenome or the effect of demethylating agents on disease progression can be studied in intervention studies. In an intervention study, the effect of supplements on the epigenetic pattern can be explored by comparing the profile before and after supplement use in the same person. In a randomized controlled trial, a demethylating agent is randomly assigned to treat half of the patients in the study, while the other half receives a different dose, a different drug, or even a placebo. Clinical outcomes are recorded in both groups.

3.4.6 Family-Based Studies

Transgenerational inheritance of epigenetic traits have to be studied using triads of mother, father, and child. For an epigenetic mark to be inherited transgenerationally, the putatively inherited epigenetic change must be present in both the contributed gametes and the offspring soma. Studying an additional generation would permit examining the persistent inheritance of the epigenetic mark. To date, transgenerational inheritance of epigenetic marks in humans has not been established.

3.4.7 Birth Cohorts

In a birth cohort, preconceptional and prenatal exposures can be assessed and related to DNA methylation, imprinting profiles, and chromatin states of the offspring at birth in tissues that can be easily obtained such as cord blood, cord, placenta, and saliva. Additional follow-up of the birth cohort permits tracking of developmental stages, anthropometric variables, disease outcomes, and changes in epigenetic marks over time.

3.4.8 Summary

Thus, if the goal is to identify epigenetic changes that may predispose to disease, a nested case-control study would be the most appropriate design. Similarly, epigenetic

biomarkers of susceptibility would be best studied using a nested case-control design. A retrospective case-control study may provide some useful information about an epigenetic marker of disease, but epimutations may actually be a consequence of the disease. The effect of a lifestyle factor such as nutrition or alcohol consumption on the epigenetic profile can be studied in either a cohort study or an intervention study. Epigenetics as a causal link connecting a risk factor and a disease requires samples from a cohort study, collected after the risk factor is manifest and before disease occurrence.

3.5 Other Methodologic Considerations in Epigenetic Epidemiology

Besides identifying the appropriate tissue, settling on the epigenetic marks to study, and choosing a suitable study design, other considerations are essential to ensure the success of the study (Table 3.2). Among them are the study population and its characteristics.

3.5.1 Choice of the Study Population

As only a sample of the population of interest can be studied, the characteristics of this sample determine the extent to which the results can be generalized to the population of interest. A convenience sample for a birth cohort may focus on women with uncomplicated deliveries because of the logistical ease of obtaining the biospecimens. However, the epigenetic profile among the newborns may differ from those of infants born prematurely or those whose mothers suffered from preeclampsia. Similarly, if individuals with certain characteristics are over- or underrepresented in the sample drawn, the result, e.g. the prevalence of hypermethylation of a particular tumor suppressor gene promoter, may not be representative of the prevalence in the underlying population of interest from which the sample was drawn.

3.5.2 Sample Size

One of the most important – and common – limitations of basic science research is small sample size. A sufficiently large sample is a fundamental requirement of a

Table 3.2 Some important considerations in planning and interpreting a study in epigenetic epidemiology

- Tissue-specificity: target tissue or surrogate tissue
- Restriction to well-defined representative population
- Sufficient sample size
- Effect modification
- Adjustment for confounding

high-quality study in epigenetic epidemiology: it increases the likelihood of a valid study result and is necessary to achieve adequate precision of the findings. Moderate to modest differences in epigenetic patterns between two groups may be important but cannot be detected in a small sample. Some factors may exert only modest changes on DNA methylation, e.g., diet or dietary supplements. A sufficiently large sample is necessary to detect such differences. Power calculations conducted during the planning phase of a study determine the number of participants necessary to detect a certain difference that may be of interest. Even if the expected difference is large, the number of individuals studied has to be sufficient to ensure precision of the results reflected in a narrow confidence interval around the measure of interest.

3.5.3 Effect Modification

Epigenetic marks are known not only to be tissue-specific but also to vary by race and ethnicity, sex, and age [35, 36]. If the prevalence of a particular epigenetic characteristic is of interest, e.g., the prevalence of LOI of *IGF2* among healthy individuals, such frequency is best described in the context of a well-defined substratum of the population, e.g., Caucasian females age 30–40 years. Inferences about the prevalence in other population subgroups, e.g., Hispanic males age 60–70 years, cannot be made unless the prevalence is assessed directly.

When the goal is to study the relevance of an environmental factor for the epigenetic profile (e.g., the impact of smoking on DNA methylation) or the association between an epigenetic trait and a disease endpoint (e.g., a certain histone modification and the incidence of asthma), it is important to consider whether these associations may differ by sex, age, race or ethnicity, or other factors. Is there reason to suspect smoking may induce different methylation changes in Hispanics than in African-Americans? If the answer is yes or if there is sufficient uncertainty, the heterogeneity of this association ("effect modification") can be studied across ethnicity using a statistical test for interaction. If this test yields insignificant differences, the association between smoking and DNA methylation does not differ substantially between the different ethnic subgroups considered and can be assessed in a population with mixed ethnicity. Beware, however, that the strata of different ethnicities is not too small when applying the test for interaction. Similarly, whether smoking affects DNA methylation differently in men and women can be tested. If significant effect modification by sex is identified, the association between smoking and DNA methylation should be reported separately for males and females. Each of the population subsets created by stratification needs to be sufficiently large to generate valid and reliable stratum-specific estimates.

3.5.4 Confounding

A distinctly different consideration from the above described effect modification is whether a factor may influence the strength or even direction of an association.

When evaluating whether LINE-1 hypomethylation in mammary tissue is more common among women with or without breast cancer, the distribution of age may distort the findings. Women with breast cancer may be older than women free of breast cancer, since risk increases with age. However, global methylation of repetitive elements decreases with age. Unequal distribution of age may create a spurious or exaggerated association between LINE-1 hypomethylation and breast cancer that vanishes or at least diminishes after appropriate statistical adjustment for age differences. Confounding can be detected when proper statistical adjustment changes the association of interest, i.e., the effect estimates with and without statistical adjustment for the confounder differ. A factor is a confounder if it is associated with both factors of interest, the exposure or risk factor and the disease of interest but is not in the casual pathway between risk factor and disease outcome; in the above example age is associated with both LINE-1 methylation and breast cancer. Confounding is one of the most important threats to the validity of an epidemiologic study.

3.5.5 Misclassification

As the field of epigenetics matures, so do its methods. Genome-wide scans now allow us to assess the methylation status of an increasing number of individual CpGs within and outside of CpG islands. The assessment of loss of imprinting with allele-specific expression assays using pyrosequencing has become more quantitative; previous methods relied on gel electrophoresis and radioactive labeling. Each of these improvements in technology decreases random misclassification of the epigenetic state and improves precision. Misclassification may be further reduced by the next generation of genome-wide CpG dinucleotide methylation assessment methods. Correspondingly, increasingly sophisticated bioinformatics tools allow us to distinguish between true signals and noise.

3.6 Evaluating and Interpreting Results of Epigenetic Epidemiology Studies

The establishment of a benchmark of a "normal" or "disease-free" state is fundamental in genetics and epigenetics. Any departure from this state may be linked to diseases or other phenotypes of interest. If an epigenetic change is already manifest prior to diagnosis of a disease, it may be a candidate for an early detection biomarker [3]. If it is present prior to disease development, it may be useful as a biomarker of disease risk. To date, human reference epigenomes have not been established, although the first genome-wide, single-base-resolution of methylated cytosines was recently presented for human embryonic stem cells and fetal fibroblasts [37]. In the absence of reference epigenomes, results of epigenetic epidemiology studies are often based on comparisons of epigenetic marks between two or more groups.

In epidemiology, comparing two groups (i.e., individuals with and without a certain characteristic of interest) is a substitute for the ideal (but impossible) set of information: data on the same individual measured at the same time twice, once with the characteristic of interest and once without. For example, two sets of data on the same woman, once with regular consumption of one glass of wine per day and once without alcohol consumption, would allow inference of whether her subsequent breast cancer had also arisen if no alcohol was consumed, since all other factors are identical and no confounding was possible. Such counterfactual information would permit causal inference about the role of alcohol consumption in breast cancer etiology. Comparing the fate of two individuals, one with the characteristic and one without, even in the context of a randomized controlled trial, cannot exclude differences between these individuals besides the trait of interest.

Similarly, in epigenetic epidemiology information on the same person is not available twice: with and without a characteristic to see its bias-free effect on the epigenetic mark of interest, or with and without the epigenetic trait to evaluate its effect on disease risk. In order to reduce the risk of confounding, an intraindividual comparison can be conducted to explore the effect of an intervention, such as supplementation with methyl group donors but the different time points of assesment may still introduce bias.

While DNA methylation changes with age (discussed in more detail in Chap. 11), age-related changes occur slowly, and the degree of change differs for individual genes and repetitive elements. Hence, intraindividual variation in cytosine methylation is limited. Moreover, genome-wide methylation studies have generally revealed statistically significant interindividual differences in only a fraction of genes studied, suggesting that DNA methylation is reasonably tightly controlled [38, 39]. More profound differences are generally restricted to the comparison of cancerous and normal tissues [25, 40].

In the literature, most methylation differences between two groups of individuals with and without a certain characteristic are small and amount to less than 5%. This raises the question of the functional implication of differences in LINE-1 methylation of, let us say, 80% vs. 82% or in the DMR of an imprinted gene of 42% vs. 46%. While it is advisable to examine the expression profile of the gene studied, methylation and expression status of a gene have recently been found to be less closely correlated than previously assumed [41–43]. Nevertheless, downstream effects of methylation are possible but may be difficult to determine. Moreover, differences in methylation are often reported for individual CpGs, and the functional relevance of such differences may be difficult to assess. Neighboring CpGs seem to influence each other's DNA methylation states [44] and at least in cancer tissues, entire CpG islands rather than individual CpGs are aberrantly methylated [45].

The lack of precise definitions and standardization of epigenetic phenomena further complicates the interpretation of results of studies in epigenetic epidemiology. While hyper- and hypomethlation are terms commonly used in evaluating the results of global or locus-specific methylation experiments in populations, cut-off values applied in the literature vary considerably: above and below the median [46, 47], above and below the 25th and 75th percentile [5], statistically significantly higher or

lower than the controls [48], and percent methylated reference with a differently chosen cut-off for each gene studied [49]. Such lack of standardization makes results from different studies difficult to compare. Similarly, there is no distinct definition of loss of imprinting, even in individuals. Often the normally silenced allele is expressed but at a considerably lower level than the normally expressed allele. Yet there is no set threshold level that defines loss of imprinting, leaving it to the individual investigator to make the call. A more standardized approach may aid comparability across studies.

There is increasing evidence that DNA methylation is correlated with DNA sequence and single nucleotide polymorphisms, suggesting that DNA composition predisposes CpG islands to DNA methylation [39, 50]. Furthermore, DNA methylation, the chromatin state, and non-coding RNAs interrelate. For a comprehensive understanding of the structural and functional implications of epimutations, it may be necessary to examine genetics, methylation status, histone modification, non-coding RNAs, and expression in the loci studied.

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Chapter 4
Laboratory Methods in Epigenetic Epidemiology

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Abstract Epidemiology seeks to identify risk factors for disease and to quantify the association between environmental and lifestyle factors and disease frequency in human populations. Epigenetics may provide mechanistic clues on a cellular and molecular level behind epidemiologic links. Combining these two fields along with collecting relevant biospecimens will aid in understanding the benefits and the limitations of genome-wide and gene-specific approaches in decoding the epigenome and its functional relevance. This chapter addresses the importance of the experimental design, optimal preparation of biological samples in population-based studies, provide an overview of the available techniques to analyze **DNA methylation**, **gene expression** and **histone modification**, and highlight possible pitfalls of these approaches.

4.1 Introduction

Most scientific questions rely on good laboratory practices, and epigenetics is no different. At the start, scientists who want to perform epigenetic experiments must consider the choice of biological samples and study population, the study design, and the appropriate data analysis. This chapter provides a brief introduction to the most commonly used methods for epigenetic research.

4.2 Choice of the Study Design

4.2.1 Biospecimens and Materials

Once a hypothesis is established, it is important to first decide which samples are most suited to address the research aim. An individual has one genome but many different epigenomes, and an observation in one specific tissue type may not be the same in other tissues.

As described in Chap. 2, epigenetic alterations can affect different elements of the cells (DNA methylation, transcription levels and histone modifications) that all require specific preparation. The type and preparation of biospecimen determines which level of the epigenome can be assessed (Fig. 4.1).

- *Body fluids, fresh tissues or relevant cell lines*: if the samples are freshly collected, nucleic acid isolation (DNA and RNA), and/or histone modification analyses (if the number of cells in the sample is large enough) can be performed. Once frozen, cells burst and can no longer be sorted or isolated. In the context of epigenetic epidemiology, cell lines can be useful for development and optimization of experimental protocols, and to test the effect of certain drugs on loci of interest.
- *Formalin fixed Paraffin embedded tissues* (FFPE): up to now FFPE tissues have been used for DNA but not for RNA analysis, as the latter is often highly degraded. Although DNA is often fragmented, the ability to study large genomic

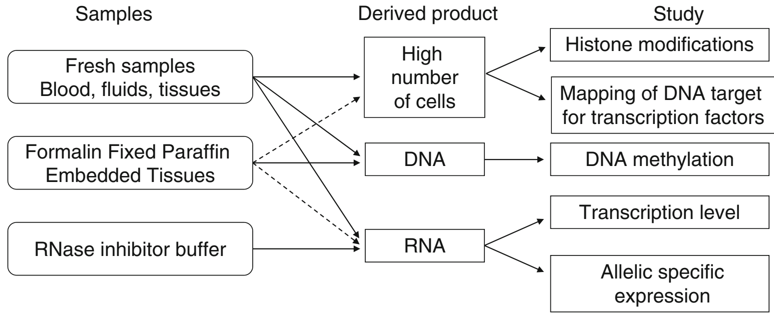

Fig. 4.1 Epigenetic studies possible according to sample availability

fragments (more than 500 base pair [bp]) may be impossible. New RNA kits have been recently developed enabling a better recovery of RNA from FFPE tissue, but the yield and quality do not allow all types of experiments (*e.g*. microarrays). Also FFPE are usually not suited for chromosomal immuno-precipitation (ChIP) analyses as the chromatin structure can be disrupted by fragmentation and the absence of good extraction methods [1].

- *Genomic DNA*: DNA can be isolated to assess cytosine methylation and hydroxy-cytosine-methylation analyses, immunoprecipitation targeting DNA (methyl cytosine) or DNA bound with supplemented transcription factors. However, it cannot be utilized for histone modification analyses since nucleosomes are disrupted during the extraction,
- *RNA*: RNA can be isolated for gene expression analyses and optimal conditions must be ensured to obtain high quality RNA. RNA can be extracted from tissues and bodily fluids. It is possible to isolate RNA from fresh frozen tissue and from frozen blood and buccal cells; however, addition of a buffer that inhibits RNAse at the time of collection (*e.g.* RNA later) is essential to aid in preventing RNA degradation. Unfortunately some experiments will become impossible to perform due to the initial cells lyses (*e.g.* micro-dissection, cell sorting, and phase separation for blood, and possibly expression microarrays).

4.2.2 *Experimental Backgrounds for Epidemiologic Epigenetic Research Studies*

Epigenetic studies require various expertise which may lead to the requirement for a multi-disciplinary team or collaborations. For each step of the process key aspects must be considered: (Fig. 4.2):

- *Sample collection:* Collection of samples may need to be performed by a pathologist or someone skilled in histology in order to select the desired cell-type since each differentiated tissue has a specific epigenome. Micro-dissection of tissue may be necessary to ensure a homogenous cell type.

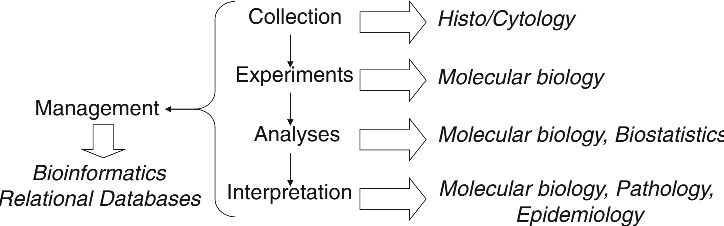

Fig. 4.2 Schematic summary of multiple-disciplines essential for good practice of epigenetic epidemiology studies

- *Experiments:* While commercially available kits have made lab experiments more robust, epigenetic experiments require a strong background in molecular biology to allow interpretation of the results at the cellular level and to avoid flaws in study design, conduct, and interpretation.
- *Analyses:* Statisticians and bioinformatics specialists can play a key role in experimental analyses especially since most of the new and high-throughput techniques require complex analyses (see Chap. 5). Moreover, it is important to involve statisticians at the study design level to ensure that the hypothesis can be properly addressed.
- *Interpretation:* Results should optimally be reviewed and interpreted by a multidisciplinary team including molecular biologists, pathologists, epidemiologist, statisticians, and other.
- *Sample and data management:* Epidemiologic studies typically involve a large number of samples, leading to a vast amount of samples and data to manage (for example, a study examining the methylation of 7 CpG sites in a gene, performed in 200 samples with replicates will produce 2,800 data points). Sample labeling and tracking could be made more efficient with the use of bar-coded tubes which are read by a scanner generating a unique sample barcode number. Spreadsheets are not an efficient solution for data management of this magnitude therefore; knowledge in bioinformatics or database management systems (SQL, Oracle, etc.) will be beneficial. Acquisition of a LIMS (Laboratory Information Management Systems) can become essential to deal with a growing through-put of the laboratory analyses.

4.3 Epigenetic Methods Targeting Genomic DNA: DNA Methylation and Hydroxymethylation

4.3.1 DNA Methylation

Discovered in the early twentieth century, 5-Methyl Cytosine (5mC; referred to as the fifth base in DNA) has been increasingly studied since the 1980s. Two different approaches are available: loci-specific and global estimation (Fig. 4.3).

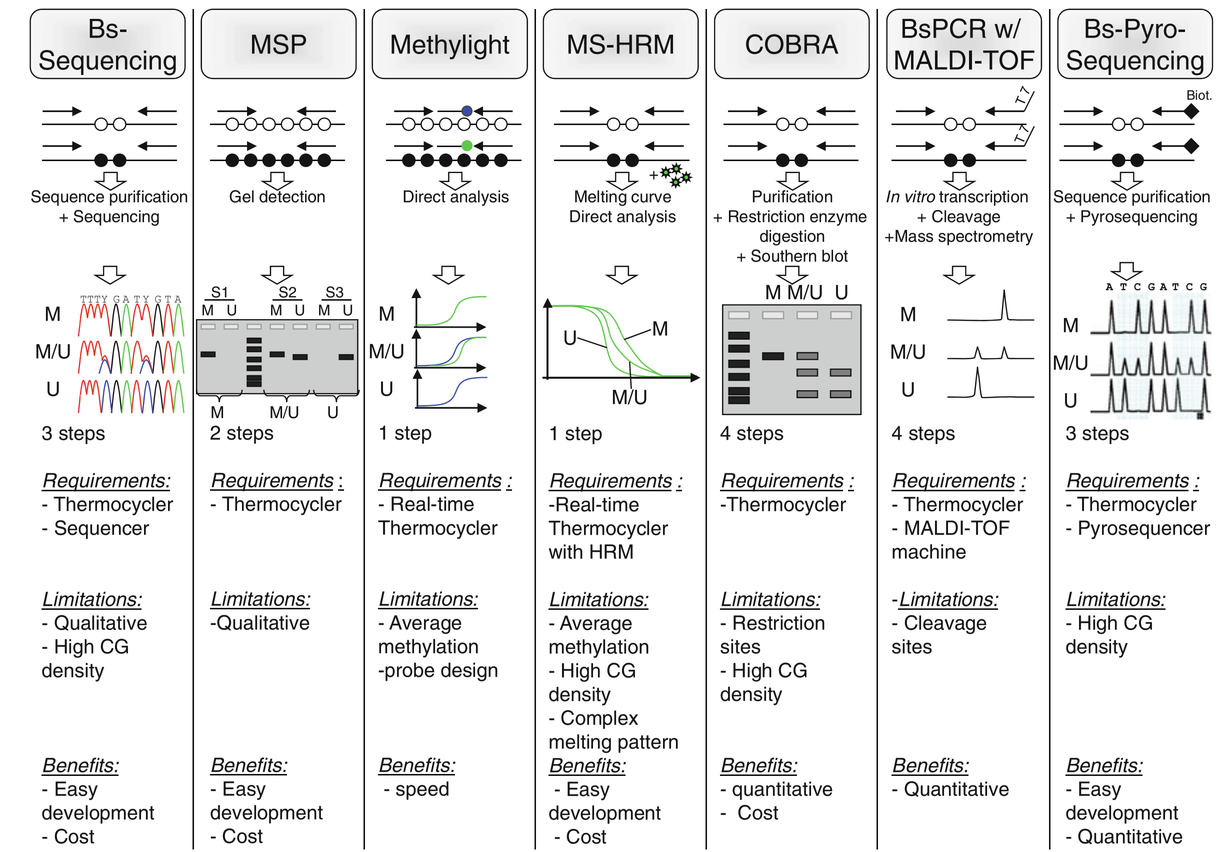

Fig. 4.3 DNA methylation methods. M: methylated; M/U: partially methylated; U: unmethylated; S1, S2, S3: sample 1, 2, 3

4.3.1.1 Loci Specific Analyses

Bisulfite treatment is a key requirement for many of the current techniques assessing DNA methylation. Various bisulfite treatment kits are now commercially available, enabling high recovery rate with a short incubation time, lower starting amounts of DNA and high conversion efficiency. Bisulfite treated DNA (BsDNA) results in single-stranded DNA of low complexity, since conversion of all unmethylated cytosines into uracil will drastically decrease the percentage of GC in the sequence. Different techniques can be used to evaluate the difference between the unmethylated and methylated DNA (see below). Single-stranded DNA, is very fragile and must be handled and stored properly (*e.g.* –20° C for short-term use, –80° C for long term storage and repeated freeze-thawing should be avoided).

- **Sensitive methods**
  Bisulfite sequencing (Bs-Sequencing) was one of the first techniques developed to assess DNA methylation [2]. Amplification is first performed on BsDNA followed by sequencing. Setting aside its cost, this technique has the benefit of providing information at each CpG sites in the sequence of interest, and can be useful as an exploratory step. However, the methylation levels cannot be quantified limiting the ability to draw associations with any transcriptional consequences.

  MSP (Methyl specific PCR): developed by Herman and colleagues in 1996 [3], this technique consists of an amplification specific to the methylated or unmethylated sequence. The targeted amplification is performed using primers with CG sites (or UG after treatment for the unmethylated DNA) in the 3′ sequence stabilizing the annealing specificity. This technique is sensitive, and requires the amplification of unmethylated sequence in order to confirm that the absence of an amplicon for the methylated sequence is really due to the lack of methylation and not a problem with the amplification protocol. MS-PCR can be performed using fluorescent primers and detection on capillary electrophoresis, as well as classical gel electrophoresis analyses.
- **Semi-quantitative methods**
  Methylight is a real-time MSP [4]. Primers and probes specific to the methylated or unmethylated sequences are used and the methylation ratio is calculated by the fluorescence ratio of the probes. The quantification of one amplicon is relative to the other making this technique semi-quantitative as it is not compared to a standard. Efficiencies of the two amplifications (methylated and unmethylated) have to be equivalent. This technique is dependent on the quality and the careful positioning of the probes. Moreover, the methylation ratio resulting from the experiment is an average of the CpG sites on the probe and the primers. It is important to note that a mutation at one of the sites will completely alter the percentage of methylation and should be avoided by carefully confirming the sequence of interest.

  MS-HRM (Methylation Specific High Resolution Melting curve analysis) is a recently developed method using new fluorescent dye chemistry [5]. Contrary to

Methylight, a PCR is performed using primers (independent of methylation) in the presence of a high saturating intercalating agent. After amplification, a melting curve is performed and is compared to a methylation scale obtaining a relative quantification of the methylation. As in Methylight, the percentage of methylation is only an average of all the CpG sites present within the amplicon. This technique requires the new generation of a real-time PCR machine, but is labor- and cost-effective compared to most other protocols.

- **Quantitative methods**

  COBRA (COmBined Restriction enzyme Analyses) was the first quantitative technique developed in 1997 [6]. This technique is labor intensive, cost-effective and does not require any specific equipment. In this protocol, methylation detection is performed using restriction enzymes. Bisulfite conversion will create a new restriction site or allow retention of a pre-existing site which will be assessed by enzyme digestion after PCR purification. Quantification of methylation is next evaluated using a Southern blot protocol (gel-based detection). The region of analysis is limited to the presence/location of a specific restriction site and the methylation is assessed for only one CpG site.

  Bisulfite PCR followed by MALDI-TOF (Bs-PCR w/MALDI-TOF) [7]. With this approach, a targeted region is amplified using primers containing a T7 promoter. Next, the amplicon is transcribed *in vitro* to generate single-stranded RNA which is then cleaved at multiple sites using RNAse specific for a nucleotide (*e.g.* RNAse A) and separated using MALDI-TOF according to their mass (base content). A methylation ratio can be calculated by the different fragment masses and intensities that are generated from the methylated and unmethylated sequence. The development of such assays can be labor intensive and dependent upon the sequence context (which will be defined by the presence of a cleavage site). However, once an assay is optimized, this technique allows high-throughput analyses

  Bisulfite Pyrosequencing (Bs-pyrosequencing) is currently the most commonly used technique to assess cytosine methylation [8]. PCR amplification is performed on Bs-DNA using primers specific to the region of interest. One of the primers is biotinylated, allowing for the binding of avidin-coated sepharose beads. The amplicon is denatured, and the non-biotinylated strand is washed off. The remaining single strand is then bound to the bottom of the reaction plate, and buffers, sequencing primer and DNA synthase enzyme are added to the reaction. Each nucleotide is then sequentially added to the reaction following a specific dispensation order. If the dispensed base is complementary to the upcoming base in the DNA strand, this nucleotide will be added in the sequence by the DNA synthase, which releases pyrophosphates that are converted into light. The emitted light is proportional to the amount of base incorporated into the DNA sequence. Therefore, the ratio of the signal following the dispensation of a C or a T at the CpG site will represent the level of methylation at this site. This technique requires a pyrosequencer, but is highly quantitative, very reproducible, and easy to develop. Bs-Pyrosequencing can also be used for allele specific methylation, by the use a sequencing primer with a single nucleotide polymorphism (SNP) in its 3′ region [9].

4.3.1.2 Global Methylation of the Genome

CpG methylase assay [10] involves a technique where the genomic DNA is treated with a CpG methylase in presence of S-adenosyl-methionine (SAM) labeled with tritium, which will transfer a methyl group on each unmethylated CpG sites. DNA is next transferred to a membrane and the radioactivity is quantified. The level of radioactivity indicates the degree of global methylation, with higher radioactivity indicative of lower methylation. Although this assay has been used for a long time, due to the use of radio-isotope this technique tends to be avoided if possible.

ELISA-type methods: in these assays, genomic DNA is coated on a plate in the presence of 5-Methyl-Cytosine antibodies. The incorporation of the antibodies is quantified (like any standard ELISA). The development of recent assay kits have decreased the starting amount of DNA, and processing time. The disadvantage of this technique is under estimation of the global methylation since 5-Methyl-Cytosine antibodies preferentially bind to areas of high CG density.

Cytosine extension assay [11] is based on single base pair extension quantification after enzyme digestion of genomic DNA. Two separate reactions are performed, one with a methylation-sensitive enzyme and the other with its isoschizomer (a methylation-insensitive enzyme). Both enzymes produce sticky ends and cut before the cytosine. The incorporation of biotinylated cytosine in the two different reactions is then quantified. This protocol was optimized for a pyrosequencer which is called LUMA [12]. The detection of the methylation is limited to the presence of the restriction site and isoschizomer.

Analysis of repetitive elements is considered a surrogate of the global methylation assays previously presented [13]. This technique consists of amplification (post bisulfite conversion) of repetitive elements like LINE1 or ALU, which are normally highly methylated, analyzing a potential hypomethylation of the genome. This protocol has been developed using different methods previously presented [14–16], though it is not as labor intensive, and requires less starting DNA than other methods. It is important to consider that repetitive elements methylation cannot be directly compared to other global methylation assays, and that there may be different mechanisms which regulate the methylation of repetitive elements and for the whole genome (global) that can be also dependent on pathology and/or the tissue/cell type. Repetitive elements may not provide an accurate representation for specific genomic regions of interest.

4.3.2 DNA Hydroxymethylation

5-hydroxymethylcytosine (5hmC) which is referred to as DNA's sixth base was first discovered in bacteriophage DNA in the 1950s. 5hmC has been a fascinating discovery as it was found present in mammalian embryonic stem cells and in cerebellar Purkinje cells [17, 18]. 5hmC is known to be derived from 5mC by the action of TET family enzymes [18].

The role of 5hmC is still not clear, but is thought have the following effects:

- to affect the annealing of methyl-binding protein to the DNA;
- to induce demethylation through the DNA base pair repair mechanism;
- initiating chromatin modification via the recognition of protein specific interactions.

An important point regarding 5hmC is the absence of conversion into uracil, during bisulfite treatment, creating the possibility of some regions of the genome being mislabeled as methylated when in fact they may be hydroxymethylated. Currently there are only a few techniques to discriminate 5hmC from 5mC:

Detection by restriction enzyme: 5hmC and 5mC are recognized by the same enzymes, but 5hmC can be protected against the enzymatic digestion by adding a protective group [*e.g.* glucosyl, or glucose]. This difference could be assessed by PCR amplification, with cleaved DNA not amplified. However, this method is limited to the recognition site of the enzyme.

Detection by antibodies: similar to the ELISA methods for global methylation, antibodies for 5hmC are available and can be used to determine the global content of 5hmC in the DNA. Up to now, no chromatin immunoprecipitation has been performed using these antibodies, but it could shed light on identifying regions affected by the 5 hydroxymethylation. One issue of using antibodies is the analysis is limited to area with dense representation of the 5hmC alteration.

GLIB (GLucosylation, perIodate oxidation, Biotinylation): A new method was recently described [19]; each 5hmC are conjugated with a molecule of glucose, which enables the subsequent addition of biotin molecules following this, streptavidin is used to pull down the DNA which can be analyzed by microarray or sequencing.

4.3.3 Genome-Wide Methods

Increasingly, more and more epigenetic studies are performed at a genome-wide scale [20]. To date these projects have mainly focused upon DNA methylation and histone modification.

- *Methylation microarray after bisulfite treatment* is currently the most used genome-wide application. In this protocol, BsDNA is amplified and put in contact with probes recognizing one CpG site each. A single base extension with labeled nucleotides will define the ratio of methylation of each CpG sites. However, this method has several drawbacks: firstly, the microarray's targets are limited to the available probes defined by the company, and are not always related to any functional annotations; secondly, the use of bisulfite treatment (as previously described) does not allow the discrimination of 5hmC. The most popular arrays have been the Illumina Golden Gate, the Infinium 27K and the Infinium 450K.
- Several genome-wide assays use restriction enzymes, and one the first methods was *MCA* (Methylated CpG Island amplification) [21]. Enrichment for

methylated DNA is performed by digestion with methylation sensitive and methylation-insensitive restriction enzymes, followed by ligation of adaptors and PCR amplification. The resulting amplicons, which are representative of the methylated fraction, are labeled and co-hybridized in a microarray platform or sequenced.

- With *RRBS* (*Reduced Representation Bisulfite Sequencing*) [22], a restriction enzyme digest is followed by purification of the fragments (within a range of specific lengths), thus enabling the enrichment of sequences containing CpGs. DNA fragments are next bisulfite converted, amplified, and finally sequenced. This labor intensive technique allows the widespread study of methylation across the genome (not exclusively in the promoter region) and works with a very low amount of DNA (less than 100 ng).
- *ChIP-Seq and ChIP-on-chip* are two methods using a chromatin immunoprecipitation as the first step. In this protocol, DNA is precipitated using antibodies specific to 5mC (MeDIP), methyl-binding proteins or histone modifications. The precipitated DNA is next used for a whole genome sequencing analysis (ChIP-Seq) or a microarray (ChIP-on-chip). Currently this is among the most expensive methods, yet these protocols offer an in-depth screening assay. The disadvantages with antibodies are the need for a high density of the target, and the possibility of relatively low specificity creating potential false positives. Moreover, these methods do not allow for the direct measurement of a methylation ratio.

4.3.4 Tips and Tricks to Develop a Methylation Assay

- *Bisulfite conversion efficiency* is an important aspect in 5mC assessment. Incomplete conversion may lead to false estimation of the methylation (particularly true with homemade bisulfite treatment and methylation specific amplification methods such as MSP, and Methylight).
  - One assessment can involve the ratio of converted BsDNA by evaluating an amplification product bearing a C outside CpG sites, and using primers independent of the conversion (without any cytosine in their sequence) [4]. The conversion of cytosine in the amplicon can be checked by probes, melting curve analysis or even sequencing.
  - Another assessment is by investigating the complete conversion of an individual DNA strand for methods which look at individual CpG sites (Bs-sequencing, Bs-PCR w/ MALDI-TOF, and Bs-Pyrosequencing). Failure of complete conversion will result in the presence of cytosine outside of CpG sites, visible in Bs-sequencing and Bs-PCR w/MALDI-TOF. This is available in Bs-Pyrosequencing by adding a C in the dispensation order (at the position of potential unconverted C).
- *Primer design* is one of the most important features of methylation assay optimization [23]. There are several rules to follow, according to the experiment to perform.

  - If the assay is using a methylation-specific amplification (MSP, Methylight), primers should contain one or more CpG site in their 3′ region to ensure a specific amplification of the (un)methylated sequence. Freeware are available for primer design (*e.g.* Methprimer).
  - For an assay using a methylation independent amplification, primers should avoid having CG in their sequence or these dinucleotides must be limited to one or two in the 5′ region. When CpG sites in the primers cannot be avoided, the Cytosine of the dinucleotide (or its complementary depending on the primer direction) should be replaced by a mismatched base independent of the methylation status (A instead of C/T or T instead of G/A).
  - The salt adjusted melting temperature (Tm) should be higher than 60°C, to enable a PCR Tm at 60°C.
  - The 3′ region containing a T derived from the conversion of cytosines not included in a CpG site will ensure strict amplification of the BsDNA.

- *Linearity of detection:* once the bisulfite conversion and the primer design are optimal, the ability to assess the different degree of methylation needs to be checked. A methylation scale should be created by pooling (in a dilution scale) unmethylated DNA with completely methylated DNA. Whole genome amplification can get rid of all methyl groups and complete methylation can be obtained with methylase treatment. The methylation scale provides a useful tool to confirm the accuracy of the primers and overall assay.
- *Reproducibility* must be confirmed by performing at least duplicates of the bisulfite treatment (not only the PCR!). Correlation between replications must to be evaluated to ensure the best assay quality and accuracy of results (for example, a difference between the bisulfite replicates less than two times the standard deviation).

4.4 Epigenetic Studies Involving RNA: Gene Expression

One important way to examine any phenotypic differences across individuals is to investigate biological function of specific genes by measuring gene expression. Isolating RNA can provide valuable samples and important factors must be considered when planning expression experiments. Selection of the tissue and bodily fluid samples should be considered due to the nature of cell-type specific expression of certain genes. Additional transcriptional features such as: the presence of multiple splice variants and isoforms, or other transcripts that may span the region of interest (*e.g.* antisense RNAs) must be thought of prior to experiments.

4.4.1 Types of RNA

Translational RNA: Messenger RNA, transfer RNA, and ribosomal RNA work together in the process of protein synthesis by translating the genetic code into

protein. Messenger RNA (mRNA) carries the protein sequence information to the ribosomes, while Transfer RNA (tRNA) is a small RNA chain (~80 nucleotides) that transfers specific amino acids for protein synthesis during translation. Ribosomal RNA (rRNA) combines with proteins in the cytoplasm to form ribosomes (the nucleoprotein). 18S, 5.8S, 28S and 5S are four the rRNA molecules found in eukaryotic ribosomes.

Non-coding RNA: Not all RNAs encode for protein structures. Many do not and these are referred to as non-coding RNAs (ncRNAs). Non-coding RNAs can have regulatory functions, and can act to down-regulate gene expression. This regulation can be direct or through the targeting of other repressive modifications which will affect gene expression (*e.g*. targeting methylation). Some types of NcRNAs are long non-coding RNAs (lncRNAs) which are classed as greater than 200 nucleotides, as well as various short ncRNAs including small interfering RNA (siRNA, ~21–22 nucleotides), microRNA (miRNA, ~22 nucleotides), small nucleolar RNAs (snoRNAs, ~80–300 nucleotides), and PIWI-interacting RNAs (piRNAs, ~26–30 nucleotides). Taft et al. have written a comprehensive review of ncRNAs and their regulatory role in diseases [24].

Antisense RNAs are transcribed in the opposite direction of one or more endogenous mRNA transcripts which are in the sense orientation. Antisense RNA can act to either activate or down-regulate gene expression by binding to the endogenous sense mRNA [25]. By definition the antisense transcript shares the same sequence of the sense counterpart and raises the need for strand-specific consideration for this common region for expression analysis.

4.4.2 Methods for Gene Expression

Expression analysis is most commonly used to examine the function of a gene(s) at the transcriptional level in a specific tissue or cell type, and for certain instances it is important to investigate the expression patterns in a strand or parent of origin specific manner.

4.4.2.1 Overall Gene Expression

Classical methods for gene expression include Northern blotting and ribonuclease protection assay (RPA). **Northern blots** generate a membrane from gel electrophoresis of RNA samples. Radio-labeled probes specific to the transcript are used to visualize RNA expression. **RPA** is a technique for quantifying and detecting RNAs with the use of radio-labeled complementary *in vitro* transcribed strand-specific probes. The probes and target RNA are hybridized and then treated with a ribonuclease mix which degrades all remaining single-stranded RNA. RNA which is hybridized to the probe will be protected from digestion and is visualized on an

Table 4.1 Methods to study gene expression

Technology	Northern blot and RPA	Expression arrays	Quantitative real-time PCR
Type of RNA assessment	Semi-quantitative and quantitative	Quantitative	Quantitative
Typical starting amount of total RNA	As low as 1 μg, (ideally 5–10 μg)	As low as 100–200 ng	as low as 50 ng (ideally 100–200 ng)
Sample size	Smaller scale	High-throughput	Moderate throughput
Benefits	Cost effective, not difficult to develop, capable of strand-specific analyses	Thousand of genes examined, little sample used	Cost effective, easy to develop, capable of strand-specific analyses
Limitations	Labor intensive, amount of RNA used	High cost	Labor intensive
Key requirements	Radioactivity	Array core facility, bioinformatics analysis	Real-time Thermocycler

acrylamide gel. Some important considerations for these well established classical methods are (Table 4.1): these assays are not ideal for large sample sizes, require a large amount of starting RNA material, and the laboratories must be capable of working with radioactive isotopes, although recent modifications to both protocols allow for non-radioactive labeling using (Digoxigenin).

Generation of cDNA. For some expression methods (see below) mRNA must be first converted to cDNA by reverse transcription. Some essential points for cDNA synthesis are outlined below in the Sects. 4.3.4 and 4.4.3.

Gene expression arrays [26] allow for a high-throughput gene expression profile which measures the expression of thousands of genes in one sample. Microarrays are glass slides coated with transcript specific probes. The RNA sample is fluorescently labeled and hybridized to the microarray slide which is processed and laser scanned. The normalization of the RNA sample for quality control is done using reference RNA (fluorescently labeled differently than the RNA sample). There are various commercially available platforms of expression arrays and some companies offer custom made arrays, for the researcher to choose their favorite genes of interest. Using low amounts of starting RNA, this technology generates vast amounts of data which requires bioinformatics analysis.

Quantitative PCR (qPCR) quantifies gene expression on a smaller scale than the arrays while requiring a small amount of starting material (this is ideal for human samples that are often precious). Unlike classical PCR, which provides an end point measurement, this reaction is done in real-time providing analysis as the reaction progresses. qPCR is monitored with the use of a fluorescent reporter molecule, which accumulates with the product amplification at each cycle. There are two common detection methods when performing real-time qPCR: non-specific detection using DNA intercalating dyes and specific detection with probes designed to a

specific target region (gene of interest). Real-time probes are labeled with a quencher dye and a reporter dye, and are available with different chemistries (hydrolysis or hybridization). Probe-based real-time PCR can be useful for qPCR of mRNA, allele specific discrimination (SNP assays), and microarray validation experiments. qPCR provides an amplification plot containing the point of amplification threshold, and the cycle number this threshold is crossed (Ct). There are different algorithms used to quantify the levels of expression [27, 28].

4.4.2.2 Allele-Specific Gene Expression

Definition of Imprinting in Relation to Expression

The aspects of genomic imprinting are described in more depth in Chaps. 6 and 9. Imprinted genes exhibit monoallelic expression which is specific to the parent-of-origin. A disruption in imprinting (loss of imprinting) could result in either a gain in expression on the normally silent allele causing biallelic gene expression or a loss of expression on the normally expressed allele which would result in an absence of expression.
The study of imprinted genes represents a challenge for different reasons:

- The nature of genomic imprinting requires the discrimination of parental origin which can be addressed by using a SNP located in the coding region (exons).
- Non-coding RNA transcripts are found in most imprinted clusters; the majority of these are antisense to reciprocally imprinted protein coding genes. The ability of strand-specific detection is often essential to address overlapping transcripts.
- Some imprinted genes have multiple transcriptional variants/ isoforms and not all variants are necessary imprinted or expressed in the same tissues (*e.g. GRB10* [29] and *IGF2* [30]).

Selection for Informative Individuals

In the example of imprinted genes, whose function is specific to the parent of origin (inheritance) it is important to discriminate between the maternal and the paternal chromosomes. When using human samples, allelic discrimination is limited to the availability of informative single nucleotide polymorphisms (SNPs). Genotyping of the samples is performed on the DNA to assess the heterozygosity of specific SNPs (defining informative individuals) for a gene. Information on the location of SNPs can be found in genome browser databases (e.g. NCBI and UCSC). If possible, multiple SNPs should be assayed for a specific gene to increase the number of informative samples from the sample pool. Although many SNPs have been well characterized in the literature, it is ideal to validate the presence of a SNP by sequencing. Both genome-wide SNP arrays and gene-specific SNP assay kits are also commercially available.

Methods for Detection of Allele-Specific (Parent of Origin) Expression

All of these methods require the region containing the SNP to be amplified via PCR [31]. These methods can be performed on both DNA for genotyping and RNA for measuring transcriptional levels.

Some SNPs generate a restriction site which can be recognized by an enzyme. The amplicon can be digested with a restriction enzyme, creating a difference in fragment sizes which distinguishes the parental alleles (digested or undigested).

If the SNP does not generate a restriction enzyme site or a more quantitative method is desired, sequence based methods are ideal (*e.g.* pyrosequencing across the SNP).

SNuPE assays (single nucleotide primer extension assay) identify and quantify allelic variants with the use of a labeled (fluorescent or radioactive) primers specific to one or each of the SNP variants in a primer extension step [32]. SNuPE can be modified to measure allele specific transcriptions using qPCR analysis on cDNA [33, 34].

An additional quantitative technique for allelic-specific detections is **Hot-Stop PCR** which involves the addition of a radio-labeled PCR primer in the final amplification cycle [35].

4.4.3 Tips and Tricks to Consider for Expression Analyses

- Knowing the type RNA of interest is important when planning experiments as certain RNA kits do not allow for the isolation of small RNAs.
- RNA quality can be assessed on a gel by measuring the ratio of 18S/28S or by using a more accurate technique like a bioanalyzer (which also provides important quantity values and requires less RNA). Stringent RNA quality assessment must be performed prior to experiments. Possible degradation and contamination will impair the quality and validity of the experiments [36].
- Since transcriptional variants partially share the same sequence and may have a different expression pattern it is important to find a specific SNP to distinguish the individual variants.
- Choosing a reverse transcriptase primer for cDNA synthesis: cDNA synthesis kits provide OligoDT, random hexamers, or a mixture of both for priming oligos.
  - OligoDT offers a more specific priming to poly(A) mRNA tail (as well as polyA rich regions) giving greater assurance that the mRNA is intact.
  - Random hexamer is recommended for total RNA especially if the transcription of interest does not have a polyA tail or is unknown.
- A gene-specific primer is needed to identify the orientation when characterizing a new transcript or when measuring expression of a gene that has an additional

transcript spanning the region in the opposite orientation (antisense). In order to perform RT-PCR in a strand-specific manner (*e.g.* orientation specific), the use of a primer specific to the gene of interest is used during the initial cDNA synthesis instead of random hexamer and oligoDT primers. Therefore, two separate cDNAs are generated: one with a forward gene specific primer and one with a reverse gene specific primer.

- *Controls:* All experiments are only as good as the controls they have. Without the proper controls, the analysis is next to impossible to interpret.
  - Whenever possible the primers should be exon spanning to allow specific amplification of the RNA instead of any possible contaminating gDNA.
  - During the cDNA synthesis, up to half of the reaction must be kept as the negative control (RT minus) with water added in place of the reverse transcriptase enzyme to detect possible gDNA contamination.
  - A housekeeping gene provides the needed normalization step to accurately measure the levels of cDNA samples.
    - Commonly used housekeeping genes include: GAPDH, β-actin, α-tubulin, HPRT, and 18S.
      [Note: OligoDT cannot prime 18S rRNA eliminating the possibility of 18S being used as a housekeeping gene.]
    - It is important to confirm that the housekeeping gene has ubiquitous expression in the tissue type studied.
- **Replicates** (biological and technical) are essential due to possible well to well variation, pipetting error, or poor efficiency of cDNA synthesis.
- **Criteria for primer design and target amplicon**
  - Primers should be around 15–30 base-pairs in length and the melting temperature (Tm) of both primers should be similar or equal to each other. Ideal melting temperatures are around 58–62°C (low temperature can result in non-specific annealing).
  - Confirm that the primers are specific to the targeted region by running the sequence around the primers and probe through a genome database software. Primers with low specificity can amplify other regions of the genome. Avoid repetitive sequences and runs of identical nucleotides.
  - The ideal size for the amplicon length is 50–150 bp, and should not exceed 400 bp. Avoid heavily repetitive sequences by checking the target sequence in repeat masker software.

4.5 Epigenetic Studies Targeting Chromatin

Epigenetic control of gene expression can involve the chromatin structure, through proteins called histones that together with the DNA form the repeating nucleosomal units of chromatin. Modifications to the histone N-terminal tails are known to be somatically heritable, have a role in regulating gene expression and occur

post-translationally through methylation, acetylation, phosphorylation, ubiquitination and by others methods. Various histone modifications have been identified in many regions throughout the genome. Ongoing genome-wide screens are being performed in order to understand the possible link of histone marks with gene expression and chromatin conformation [37, 38].

4.5.1 Nucleosome Occupancy

Nucleosome occupancy reflects the condensation of the chromatin structure, which can affect gene transcription. Nucleosome positioning in a specific locus can be assessed by treating cells or tissue samples with a DNA methylase (*e.g.* CpG Methyl transferase M.SssI), which will add a methyl group on cytosines that are not at the level of the histones core [39]. It results in a difference of methylation pattern depending on the presence of histones and this difference can be assessed using DNA methylation methods. Due to the nature of this protocol, DNA derived from these experiments can be used only for this type of analysis.

4.5.2 Histones Modifications

Most histone modification analyses are through immunoprecipitation [1] where cells are incubated with antibodies targeting a specific modification after crosslinking the histones to the DNA (chemical fixation of the nucleosome). The chromatin immunoprecipitation (ChIP) protocol allows for the enrichment of DNA associated with histone modifications which can then be analyzed by sequencing (ChIP-Seq), or microarray (ChIP-on-chip). The results of previously performed ChIP assays are available in different web databases (*e.g.* UCSC) for various tissue types.

4.5.3 Chromatin Conformation

The chromosome conformation capture assay is a technique used to identify and quantify possible physical interactions that are occurring between any two genomic loci enabling the study of *in vivo* genomic organization and interacting chromatin segments over vast regions of up to several hundreds of kilobases in size [40]. The captured interactions could be specific interactions (*e.g.* between genes and regulatory elements), or random collisions between loci [41]. In the chromatin conformation protocol, cells are first crosslinked which leads to stabilization or capture of the DNA-DNA, as well as DNA-protein interactions. The DNA is then digested with a restriction enzyme and the fragments are ligated, creating a mixture interacting fragments. Sequence analysis of the area of ligation allows the identification

of the interacting chromatin. A variant of this technique was described by Lieberman-Aiden and colleagues, which enables the genome-wide analysis of the chromatin conformation [42].

4.6 Recommendation for the Method Section in Communications and Publications

Providing detailed information such as experimental controls, replicates, chromosomal location and primer details (including the original sequence for DNA methylation assays) are essential for the experimental reproducibility by the scientific community (Table 4.2).

Table 4.2 Key requirements for information to be provided in publications on epigenetic epidemiology studies

Experimental analysis	Key requirements for publication	Examples
Cohort samples	Collection	Cohort details
	Isolation protocol	Kits used, time until sample processing
	Samples	Sample/cell type
	Storage	Storage temperature and duration
Epigenetic study focused on DNA	Target sequence	Accession number, probes filtering for arrays
	Primers sequence	Unconverted and converted sequence
	Reproducibility	Coefficient of variation
	Replication	Number and type of replicates
	Controls	Number and type of controls
Epigenetic study focused on RNA	Target sequence	Accession number
	Primers and probe sequence	Sequence or reference number
	Variant assessment	SNP reference and location
	Replication	Number and type of replicates
	Controls	Housekeeping gene, RT minus
Epigenetic study focused on chromatin	Chromatin preparation	Tissue/cell type, isolation details, cross-linking
	Primers, probes and target sequences	Accession number, and assay conditions
	Antibody information	Reference, dilution
	Replication	Number and type of replicates
	Controls	Type of controls (*e.g.* negative control for IP, antibody specificity control, chromatin conformation ligation template control)

4.7 Conclusion and Perspective

An in-depth knowledge of the relevant methods is essential for defining the experimental hypotheses and aims, as well as making the most efficient use of biological samples and cohorts.

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Chapter 5
Biostatistical Methods in Epigenetic Epidemiology

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Abstract Epidemiologic studies involving epigenetics have, to date, focused mostly on DNA methylation, though studies investigating other epigenetic measures such as loss-of-imprinting and micro-RNAs are becoming increasingly popular. Analysis of data arising from such studies can be complicated by issues such as multiple comparison or lack of clarity about the goals of analysis. In this chapter, we provide an overview of key considerations in the analysis of such data sets, as well as a review of modern statistical techniques that have been used in the analysis of epigenetic data. We distinguish between exploratory studies and confirmation studies, providing guidance on statistical analysis in each case. In particular, we indicate when the control of familywise error rate or of false discovery rate is to be preferred. We provide an overview of unsupervised and supervised multivariate analysis, guidance on the analysis of microarray data, as well as guidance on study design considerations.

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List of Abbreviations

ANOVA	analysis of variance
CART	classification and regression tree
ChIP-on-chip	chromatin immunoprecipitation with combined with microarray technology
CIMP	CpG Island Methylator Phenotype
COBRA	combined bisulfite restriction analysis
FA	factor analysis
FDR	false discovery rate
FWER	familywise error rate
GWAS	genome-wide association studies
HOPACH	hierarchical ordered partitioning and collapsing hybrid
LDA	linear discriminant analysis
LINE	long interspersed nuclear elements
LOI	loss of imprinting
LOOCV	leave-one-out-cross-validation
MCAR	missing completely at random
MeDIP	methyl-DNA immunoprecipitation
miRNA	micro-RNA
mRNA	messenger-RNA
MSP	methylation-specific PCR
PCA	principal components analysis
PCR	Polymerase chain reaction
RPMM	recursively partitioned mixture model
SAM	significance analysis of microarrays
SNP	single-nucleotide polymorphism
SVM	support vector machine

5.1 Introduction

Epidemiologic studies involving epigenetics have, to date, focused mostly on DNA methylation, though emerging work is beginning to address the role of histone modifications, microRNA (miRNA) expression, and loss of imprinting (LOI) in exploring disease causation. The primary reason for the focus on DNA methylation is that DNA methylation has been easier to measure than other epigenetic phenomena. Measurement of each of the many possible histone modifications requires a specific immunoprecipitation assay followed by microarray analysis or sequencing [1], and is thus expensive, especially when the number of combinations of possible histone marks is considered. In contrast, the measurement of DNA methylation requires only bisulfite modification followed by microarray or (pyro)sequencing, though sequencing is complicated by the problem of aligning modified DNA. So-called *global* DNA methylation is actually a measure of methylation of a particular repetitive element sequence, LINE1, a long interspersed nuclear element. Micro-RNA

expression is an emerging area of epigenetic research, requiring more or less the same biotechnology as messenger RNA (mRNA) expression, though normalization of miRNA arrays may require special considerations [2, 3]; aside from these, tools for the analysis of mRNA expression can be used for the analysis of miRNA microarrays. Finally, as discussed below, LOI data typically have statistical properties similar to DNA methylation data, aside from missing data considerations. Specifically, both DNA methylation and LOI assay data arise as percents or proportions, and their interpretations are roughly the same, so that statistical analysis of LOI data proceeds in a manner similar to that used for DNA methylation.

In the following chapter, we will focus primarily on the analysis of DNA methylation, with some discussion on variations applicable to the analysis of miRNAs and LOI. In addition, we will focus on single-locus assays and microarrays, since the cost of next-generation sequencing prevents its routine usage in most epidemiologic investigations to date. The assays and microarray platforms available for measuring DNA methylation of other sequences are numerous [4]; popular examples include Methylation-specific PCR (MSP) [5], COBRA [6], PCR followed by pyrosequencing [7], MethyLight [8], Sequenom [9], Illumina methylation arrays [10–12], MeDIP [13], and CHARM [69] . A more comprehensive list is provided in a recent review by Peter Laird [4]. The first five of these are examples of labor-intensive approaches that are most appropriate for candidate gene studies or validation of microarray results, i.e. studies involving a small number of loci, while the latter two admit high-throughput analysis. MSP produces a dichotomous value for each interrogated locus (gene), unmethylated (coded as 0) and methylated (coded as 1). COBRA, pyrosequencing, MethyLight, Sequenom, and Illumina methylation arrays produce interval scaled values between 0 and 1 (or 0 and 100) for each interrogated locus. MeDIP, on the other hand, produces fluorescence intensity values measured at regularly spaced intervals, typically with very dense coverage of the genome, and is typically reduced to peak intensities associated with p-values. MeDIP arrays are similar to the ChIP-on-chip arrays also manufactured by Roche to assay chromatin modification, and represent similar data complexity and expense. Therefore, they are uncommon in epidemiologic studies, and consequently their analysis is beyond the scope of this chapter. Note that because ChIP-on-chip assays are one of the few popular methods for studying chromatin modification, and due to the complexity and expense of the assays, chromatin modification is still relatively impractical to interrogate in a population-based study.

Most modern genetic and epigenetic studies involve the interrogation of multiple genes, CpG sites, or miRNA sequences; this multiplicity can lead to a number of test statistics, e.g. one for each interrogated locus, thus requiring a method for addressing multiple comparisons. The proper type of adjustment depends on the primary objectives of the study: whether the study is meant to be *hypothesis testing* (a “confirmation study”) or *hypothesis generating* (an “exploratory study”). The former requires rigorous control of Type I error probability (the probability of making any false claim) while the latter admits less stringent methods that control the *false discovery rate* (FDR, the rate of purported discoveries that cannot subsequently be reproduced). These two quantities are quite different, as will be discussed in detail below. A study can be designed to have multiple phases, wherein earlier phases are

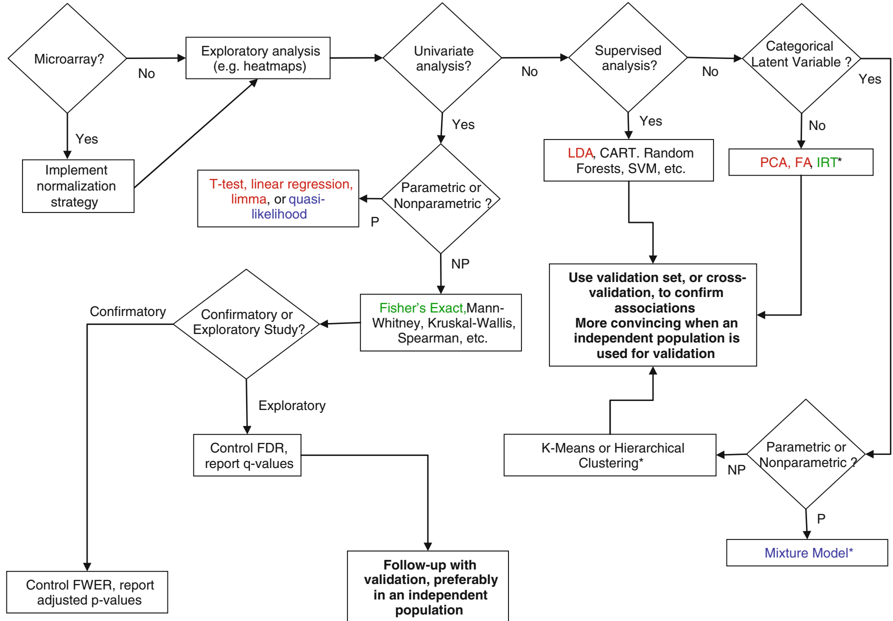

Fig. 5.1 Schematic diagram summarizing the principles and methods discussed

hypothesis generating and later phases are designed to test those hypotheses that arise from earlier phases. For the purposes of this chapter, different phases can be considered distinct studies. However, note that some studies are structured to confirm hypotheses in a population that is independent of the one used in the hypothesis-generating phase, also frequently employing a different assay, while other studies use the same population for both exploration and confirmation, using a different (usually more labor-intensive) assay for the latter. Use of independent populations is often more biologically convincing than the latter strategy, and has been adopted as the standard in genome-wide association studies (GWAS).

The remainder of this chapter is organized as follows. First, general statistical considerations in analyzing epigenetic data are presented. Next, an overview of statistical procedures is given, subdivided into three categories: *univariate* or *locus-by-locus* analysis, *unsupervised* multivariate analysis, and *supervised* multivariate analysis. These sections are followed by a discussion of special considerations for the analysis of microarray data. The subsequent section presents an overview of procedures for adjusting for multiple comparisons and their selection based on overall study objectives. The concluding sections briefly discuss study design considerations and software available for conducting analysis. Figure 5.1 presents a schematic diagram summarizing the principles and methods discussed in this chapter.

5.2 General Statistical Considerations in Analyzing Epigenetic Data

A variety of methods exist for analyzing genetic and epigenetic data, with the choice depending on the anticipated products of the analysis, as will be discussed below. However, a primary consideration is the distribution of the data to be analyzed. Crude versions of MSP produce dichotomized values (0 = unmethylated, 1 = methylated), while other platforms produce continuous values that can typically be scaled to lie between 0 and 1. In principle, some platforms, such as MethyLight, can produce values that are arbitrarily large, lying beyond 1 on any reasonable 0–1 scale, though such values can be considered problematic from the standpoint of interpretation. Thus, for MSP, care should be taken to use methods such as logistic regression that respect the dichotomous nature of the methylation measurement, while the others require techniques that respect the distribution properties of the methylation variable. An important point of consideration is the possible non-normality: because the methylation measurement can be influenced by a high fraction of methylated (or unmethylated) targets, their distributions can be bimodal with modes near 0 and 1, or else profoundly skewed. Consequently, methods with strong normality assumptions, such as t-tests, linear regression, and moderated versions of these techniques, should be avoided unless normality can first be confirmed with an appropriate distribution diagnostic. Nonparametric methods such as the Wilcoxon/Mann-Whitney and Kruskal-Wallis tests can often be used with great success, though they are difficult to extend to more complex regression models. When a need for a regression model arises, parametric approaches may be unavoidable, but several options are still available. One option is to model the methylation variable with a beta distribution rather than a Gaussian distribution, since the beta distribution can adequately capture the bimodality and skewness that is not uncommon with methylation data. This can be done explicitly with specialized maximum-likelihood procedures (e.g. [14]), or else employing quasi-likelihood (e.g. [15]). In either case, the following relation is enforced:

$$\mathrm{var}(Y) = E(Y)[1 - E(Y)]$$

for all methylation variables Y. This relation arises from the properties of a beta distribution, and is identical to the relation arising from a binomial distribution, with which the beta distribution shares many mathematical properties. Note that the beta distribution has been well-motivated for DNA methylation arrays on Illumina platforms [14, 16]. A somewhat simpler approach is to use an arcsine transformation $Y \rightarrow \arcsin(\sqrt{Y})$, which is the *variance-stabilizing transformation* for a beta distribution, i.e. the transformation that removes the dependence of the variance of any beta-distributed variable on its corresponding mean, thus making a Gaussian approximation more plausible; however, this approach is reasonable only when the methylation variables do not display marked bimodality. Another popular transform is the logit (e.g, [70]).

Micro-RNA expression data, generated using identical technology as that used for mRNA expression, will often present as lognormal variables, so that the log

expression values typically arising from these platforms can safely be analyzed as if they were Gaussian. However, it nevertheless is prudent to confirm distribution properties! LOI data typically present as bimodal interval-scaled data bounded between 0 and 1 [17], i.e. they are similar to the methylation assays discussed above. However state-of-the-art assays commonly used to measure LOI are limited by their inability to measure samples that are homozygous for the reporter SNP used to identify parent-of-origin [17]. Consequently, LOI data sets can have a substantial portion of missing data. Assuming that the genotype of the reporter SNP has no biological association with its corresponding (possibly unmeasured) LOI state, the missing data mechanism can be considered to be *missing completely at random* (MCAR), thus the missing values can be safely ignored in data analysis without biasing conclusions [18]. However, in the design of such studies, the anticipated proportion of data missing due to homozygosity must be considered in power calculations.

Choice of analysis is often driven by the objectives of the study. Baker and Kramer [19] prevent an overview of methods appropriate for each of various objectives in the context of microarray analysis, and Siegmund and Laird [20] provide an overview with a focus on DNA methylation. However, many of the same general principles apply to the analysis of smaller-scale studies, i.e. studies considering a small number of features (loci), such as candidate gene studies. These more focused studies will typically employ methods that are simpler, with fewer complications, than those used for the large-dimensional measurements typical of microarrays, but the overarching issues are similar. Broadly speaking, there are three possible approaches: *univariate* or *locus-by-locus* analysis, *unsupervised* multivariate techniques, and *supervised* multivariate techniques. In locus-by-locus techniques, analysis focuses on the univariate associations between methylation and phenotype ("differential expression" when the phenotype is dichotomous). These result in an association test statistic for each individual interrogated locus. Alternatively, using unsupervised multivariate methods, one can attempt to reduce higher dimensionality to lower dimensionality through the use of principal components analysis (PCA) or cluster analysis, obtaining estimates (or, more formally, *predictions*) of low-dimensional latent structure, subsequently assessing associations between phenotypes and these latent variables. Finally, using supervised multivariate techniques, one can directly model a phenotype on the entire multivariate realization of DNA methylation response. Many general variations of the latter two classes of analysis are presented in great depth in Hastie et al. [21]. In the sections below, we present an overview of each of these three classes of techniques.

5.3 Univariate or Locus-by-Locus Analysis

Univariate analysis is usually the most straightforward. In this type of analysis, each locus or feature is analyzed more-or-less separately from the others. For dichotomous methylation values Y such as those arising from MSP, Fisher's Exact test or its large-sample equivalent, the Pearson chi-square test, can be used to quantify an

association between methylation and a categorical phenotype Z (e.g. labels for two distinct groups), while a simple t-test or Mann-Whitney test can be used to quantify associations between Y and a continuous phenotype Z (e.g. age). Similarly, a Mann-Whitney or Kruskal-Wallis test can be used to quantify an association between continuous Y (e.g. % methylation) and categorical Z, while the Spearman correlation coefficient can be used to quantify an association between continuous Y and continuous Z. Note that these nonparametric tests are robust to violations of distributional and other modeling assumptions. In principle, t-tests, analysis of variance (ANOVA), and Pearson correlation could be used instead of their nonparametric analogs, but care should be taken to verify any distributional assumptions of both phenotype and methylation; the arcsine transformation can sometimes be used to transform methylation measures to variables that are more plausibly Gaussian.

When more than one locus is interrogated, it is essential to adjust for multiple comparisons. Broadly speaking, there are two classes of multiple comparison adjustments, those that control *familywise error rate*, or FWER, and those that control the *false discovery rate* [22, 23, 71], or FDR. Familywise error rate is akin to Type I error and represents the probability of any test resulting in significance under the assumption that all hypotheses are truly null; applicable multiple comparison procedures include many older, familiar methods such as Bonferroni-adjustment. On the other hand, FDR, which represents the fraction of significant tests for which the hypothesis is truly null, is increasingly being used to address multiple comparisons in the analysis of microarrays. We will discuss these options in more detail below.

5.4 Unsupervised Analysis

When analyzing data from microarrays, or even more focused candidate-gene studies where a moderate number of loci are being interrogated, it is sometimes advantageous to characterize the correlations among the assayed loci, both for pattern recognition and for dimensionality reduction (i.e. separating signal from noise with the objective of characterizing a large number of loci using a much smaller number of derived measures). The objective of unsupervised analysis is to reduce the dimensionality of a data set by seeking latent patterns or structure. Broadly speaking, unsupervised procedures can be sub-divided into those that seek continuous latent variables and those that seek categorical ones. The former type includes principal components analysis (PCA) and factor analysis (FA), while the latter includes mixture models and nonparametric clustering. A thorough treatment of these methods is beyond the scope of this text, but given extensive consideration in Haste et al. [21]. However, we briefly provide an overview. We assume that the methylation measurement is represented as a d-dimensional vector of continuous values $\mathbf{y}_i$, where i indexes subjects (or specimens) 1 through n.

The goal of PCA and FA is to represent a large number of continuous measures by a much smaller number continuous measures that capture the majority of the true signal present in the data. PCA and FA have been used in a number of recent methylation

studies (e.g., [24–26]). In PCA and FA, it is assumed that an F-dimensional vector $\mathbf{u}_i$ is related to $\mathbf{y}_i$ by the following linear equation:

$$\mathbf{y}_i = \Lambda \mathbf{u}_i + \varepsilon_i \quad (5.1)$$

where Λ is $d \times F$ matrix of parameters and ε_i is a d-dimensional vector of zero-mean uncorrelated errors (constraint 1). Usually the F *factors* or *components* of $\mathbf{u}_i$ are also assumed to be independent (constraint 2). In this model, $\mathbf{u}_i$ "explains" the correlation among the features of $\mathbf{y}_i$, because all such correlations must be induced by their common dependence on $\mathbf{u}_i$. For PCA, $d=n$ and ε_i is identically zero, in which case Λ can be obtained by concatenating rescaled eigenvectors of the matrix $\mathbf{Y} = (\mathbf{y}_1, \ldots, \mathbf{y}_n)$. On the other hand, FA assumes $F << n$, in which case there are numerous procedures for obtaining Λ, including maximum likelihood [27, 28] and the principal factor method which employs an eigen-decomposition similar to that used for PCA [28]. Note that the matrix Λ can be right-multiplied any orthogonal $f \times f$ matrix $\mathbf{Q}$ (corresponding to a geometric rotation, which alters the interpretations of Λ) to obtain a new $d \times F$ matrix satisfying constraints 1 and 2 but with a different interpretation. When $F = n$ and all eigenvalues are unique, then $\mathbf{Q}$ is limited to the set of reflections and permutations (essentially maintaining original interpretations aside from sign considerations), so that PCA yields an essentially unique solution in applied settings. Recent work has demonstrated the utility of factor-analytic techniques to account for confounders [72]. However, when $F < n$, there are nontrivial choices of $\mathbf{Q}$, leading to the familiar "rotation problem" of factor analysis [28], where factor interpretation can be subjective. Nonlinear versions of equation (1) are also possible. For example, an item response theory (IRT) model, a form of FA appropriate for binary variables (i.e. instances where the entries of $\mathbf{y}_i$ are binary), has been used to model methylation [29, 30]. Alternatively, one can substitute a nonlinear relation for $\Lambda \mathbf{u}_i$ in (5.1) [31], although this approach has not appeared in any DNA methylation study to date. Once Λ and $\text{cov}(\varepsilon_i)$ have been estimated, the latent vectors $\mathbf{u}_i$ can subsequently be estimated (or, formally speaking, *predicted*), and associations between overall methylation (represented by the prediction $\hat{\mathbf{u}}_i$) and a phenotype can be assessed through common regression techniques. Note that the number F must be determined in advance; various methods [27, 28] exist, many of them based on the comparison of appropriate goodness-of-fit statistics, but are beyond the scope of this text.

One fundamental problem with PCA and FA is the difficulty in interpreting the factors $\hat{\mathbf{u}}_i$, especially when the dimension F is much greater than 1. As an alternative to methods employing continuous latent variables, one can instead use a method that infers a categorical latent variable. Such methods are commonly referred to as *clustering*. Parametric clustering is typically conducted using a *mixture model* [32], where each subject i is assumed to belong to one of K classes (where K is assumed fixed), and that conditional on membership in a particular class k, $\mathbf{y}_i$ is assumed to have a distribution f_k belonging to a known family, usually normal, lognormal [33] or beta [14]. In other words, the patterns within the data set are assumed to be driven by a fixed number of classes, within which each of the individual features comprising $\mathbf{y}$ is identically distributed (and usually assumed to be independent). As a nonparametric

alternative, one can use *K-means clustering*, where the K classes are determined only by their mean. Examples of the use of mixture models in the DNA methylation literature include [34–37, 73], while K-means clustering was used in Shen et al. [38].

The most familiar form of nonparametric clustering to most geneticists is *hierarchical clustering*. In hierarchical clustering, the data set of n subjects is repeatedly partitioned based on dissimilarity (*divisive* clustering), or the n subjects are agglomerated based on similarity (*agglomerative* clustering), yielding a *dendrogram*, or "tree", that conveys a sense of similarity by analogy with phylogenetics. The dendrogram can then be "cut" in such a way that K distinct classes are obtained, each class represented by a branch on the tree. Dissimilarity between two methylation vectors $\mathbf{y}_i$ and $\mathbf{y}_h$ is measured by *metric* $\langle \cdot,\cdot \rangle$. The most common choice is the Euclidean metric, which posits

$$\langle \mathbf{y}_i, \mathbf{y}_h \rangle = \|\mathbf{y}_i - \mathbf{y}_h\| = \left[\sum_{j=1}^{d} (y_{ij} - y_{hj})^2 \right]^{1/2},$$

(i.e. the conventional understanding of "distance" as the bird flies), but the Manhattan metric

$$\langle \mathbf{y}_i, \mathbf{y}_h \rangle = \sum_{j=1}^{d} \left| y_{ij} - y_{hj} \right|,$$

could also be used (with an analogy to distance provided by the number of square city blocks required to traverse). In order to conduct hierarchical clustering, dissimilarity between two *sets* of vectors ("linkage") must also be specified. Common choices are *complete linkage*, based on maximum distance between pairs of vectors, each from one of the two sets; *single linkage*, based on minimum distance, and *average linkage*, based on average distance. A variant of average linkage is *Ward's linkage* [39], which tends to produce compact, spherical clusters. There are also techniques based on medians [40]. Methods for selecting the optimal cut point, equivalent to selecting the number K of classes, include the *Hierarchical Ordered Partitioning and Collapsing Hybrid* [41], or *HOPACH*, and *Dynamic Tree Cutting* [42].

A common problem in clustering is the choice of K. Methods for choosing K are discussed extensively in Hastie et al. [21], but typically involve varying the choice of K and comparing an appropriate goodness-of-fit statistic for each separate fit. Thus, clustering can become computationally intensive when K is unknown and larger than 3. An alternative in the mixture model setting is *Recursively Partitioned Mixture Model* (RPMM) [14], which obtains the value of K simultaneously as the mixture model is fit, leading to greater computational efficiency. An additional benefit to RPMM is the arrangement of classes in a hierarchy similar to that obtained from hierarchical clustering. Mixture models in general and RPMM in particular have been shown empirically to perform better than non-parametric alternatives for DNA methylation data [14, 33, 43].

Note that the motivation for clustering is inherent in the idea of the *CpG Island Methylator Phenotype* [44–46], or CIMP, which was the explicit motivation for clustering in Shen et al. [38]. Thus, from an interpretation standpoint, clustering may represent an alternative that is superior to FA and PCA. Following the choice

of K and completion of the clustering procedure, subjects are assigned to clusters, either explicitly in nonparametric algorithms such as K-means and hierarchical clustering, or implicitly using empirical Bayes procedures subsequent to the fitting of mixture models. The latter allows "fuzzy" clustering, where a subject can be allocated fractionally to more than one of the K clusters. *Fanny* is another nonparametric clustering procedure that also allows fuzzy clustering [40]. Once study subjects have been assigned to clusters, associations with a phenotype can be assessed by common regression techniques where class membership is included as a categorical variable, or, more simply, by Fisher's Exact Test, t-test, Mann-Whitney test, ANOVA, or Kruskal-Wallis test.

In both types of unsupervised multivariate analysis, regression can be used to investigate the association between a phenotype and overall methylation, represented in reduced form by either a latent vector or a class membership. Simple testing procedures that compare distributions across categories can be used with cluster analysis, permitting exact inference through Fisher's Exact test or by permutation. Permutation tests are conducted by first choosing a test statistic T appropriate for the desired hypothesis, followed by the determination of a null distribution for the test statistic by shuffling the cluster assignments among the subjects and recalculating T, with the latter operation repeated a large but fixed number of times. For example, a test of association between a continuous phenotype (e.g. age) and K methylation classes is obtained by choosing T to be the ANOVA F-test statistic or the Kruskal-Wallis test statistic; recalculating T a large number of times, each time shuffling the K methylation classes with respect to their phenotype; and calculating the p-value as the proportion of shuffled data sets that produce values of T larger than the one calculated from the data in their original configuration. In contrast, PCA and FA obligate the use of a regression method, rendering the selection of an appropriate test statistic T, as well as its interpretation, slightly more difficult when the number of chosen factors, F, is greater than 1. Note that in either case, the testing of associations between a phenotype and the latent variables obtained by unsupervised analysis constitutes a form of analysis closely linked with the naïve Bayes procedure described below, and thus may require validation in an independent data set if the study objective is confirmation.

5.5 Supervised Analysis

In contrast to unsupervised multivariate analysis, the goal of supervised analysis in the context of DNA methylation is to predict a phenotype Z on the basis of a DNA methylation profile $\mathbf{y}$ obtained from a microarray or candidate gene study with moderate number of interrogated loci. This is sometimes called *classification*, with the understanding that classification of $\mathbf{y}$ is "supervised" by Z. Examples of supervised analysis in DNA methylation studies are numerous (e.g. [26, 35, 36]). In this context, there is usually a distinction made between a *training set* used to build a model and a *test* or *validation set* used to verify the properties of the trained model. (Note that

the formal determination of the relative size of the training and validation sets is complex, dependent on numerous factors such as the multivariate structure of **y** as well as the nature of the association between **y** and Z, though typically 1:1 or 2:1 training:test ratios are used.) To distinguish validation from training, for exposition, it often useful to imagine a single additional observation of paired phenotype and methylation $(Z^*, \mathbf{y}^*)$, which has been excluded from the data set used for modeling. In this section, we will assume that Z is continuous or Bernoulli to simplify notation, although the case of a polytomous Z is not much more difficult.

A simple example of a supervised analysis is the regression of Z on **Y**, which provides predictions of the form $E[Z^* \mid \mathbf{y}^*]$. Another simple example is *Naïve Bayes* [32, 47], where unsupervised analysis is first used to cluster **y** into K classes; with $C(\mathbf{y})$ denoting the class to which **y** belongs, $E[Z \mid C(\mathbf{y})]$ can easily be computed by regression or $2 \times K$ table, producing $E[Z^* \mid C(\mathbf{y}^*)]$ from the resulting fitted model. For dichotomous Z, *Linear Discriminant Analysis* (LDA) seeks a vector β of coefficients such that $\Pr(Z = 1 \mid \mathbf{y}_i^T \beta > 0)$ is maximized. On the surface, LDA seems similar to logistic regression, but common estimation techniques require more stringent normality assumptions on **y** than does logistic regression. A closely related procedure is the *Support Vector Machine* [21, 48], or SVM, which provides computational efficiency when the dimension d of **y** is large. *Kernel Machines* [49] relax the strong linearity assumptions of the SVM.

Another common procedure is the *Classification and Regression Tree* [50], or CART, where the data set is recursively partitioned in such a way as to maximize the distinctions between $E[Z \mid R]$ among the partitions R of the data set $\{\mathbf{y}_1, \ldots, \mathbf{y}_n\}$. The output is a dendrogram, similar to that produced by hierarchical clustering, the branches of which represent the data set partitions R and their corresponding predictions of Z. Typically, each branch is determined by a single feature of **y** (i.e. a single DNA methylation measurement). In principle, one could partition all the way to single observations, though the utility of such a model is limited; consequently, there are various strategies employed for *pruning* the tree. As in the choice of K for clustering, these typically involve the comparison of model fit statistics (e.g. BIC). $E[Z^* \mid \mathbf{y}^*]$ is obtained by using $\mathbf{y}^*$ to traverse the tree to a desired depth, arriving at a prediction for Z^*. For large dimensions d, the enhancement provided by *Random Forests* [51] can be used. The Random Forest algorithm iterates through a large number of CART analyses, using a bootstrap (i.e., sampling the n subjects with replacement) for each individual analysis. In addition, for each node of each individual tree, only a subset of the dimensions of **y** are considered for partitioning, determined by randomly sampling (without replacement) a fixed number of components. For each iteration, both *variable importance* and *prediction error* are assessed by comparing $E[Z^* \mid \mathbf{y}^*]$ to Z^* in the "out-of-bag" sample (i.e. among subjects not selected in the bootstrap). Prediction error is simply the average of a reasonable comparison statistic ("loss" function or "risk function"), while variable importance is assessed by the change in loss before and after shuffling the variable in the out-of-bag sample.

The Random Forests algorithm reflects a general principle in supervised analysis: a need to assess the predictive performance of the trained model. If there is a natural

separation between the training and test sets (e.g. temporal or geographic separation of populations), then the comparison of $E[Z^* \mid \mathbf{y}^*]$ with Z^* among the subjects of the test set provides a convincing assessment of the performance of the classifier in question. On the other hand, if no such natural separation exists, a partitioning into training and test sets can be made randomly. Often, in this context, such partitioning is done repeatedly. Cross-validation techniques randomly partition the data set into several subsets, each of which functions as a test set for an individual analysis that leaves that subset out. An extreme form of cross validation is *Leave-One-Out-Cross-Validation* (LOOCV), where the partition consists of n singletons. Marsit et al. [44], provides an example of various supervised analyses, where 15 dichotomous methylation variables are used to classify solid tumors into one of four types.

One drawback of supervised analysis is that (except for Naïve Bayes), no insight is provided on the correlation of the features of $\mathbf{y}$. For DNA methylation studies, such insight may have biological value. Another drawback is the possibility, when d is large, of *over-fitting*, where spurious associations between $\mathbf{y}$ and Z are apparent only because of the large dimension of $\mathbf{y}$; this is a greater problem for microarrays than for candidate gene approaches. To some extent, validation strategies protect against this phenomenon, but validation is most biologically convincing when there is a natural separation between the training and test sets.

5.6 Special Considerations for the Analysis of Microarray Data

Almost all of the univariate and multivariate techniques described above are applicable to the statistical analysis of DNA methylation microarray and miRNA microarrays. However, there are some additional considerations that are specific to the analysis of these high-dimensional, high-throughput data. The first is normalization. There is now a vast literature on normalization of mRNA microarrays [52], some of which may apply to normalization of miRNA arrays, though there is some controversy on the subject [2, 3]. There is also some controversy on whether explicit normalization should be applied to Illumina DNA methylation arrays. Although Illumina makes normalization tools available in its software, the company is neutral on the position of whether they should be used. Some authors have elected to use ad-hoc methods based on bisulfite conversion efficiency [26], while others have elected not to normalize [34–37]. Recent work has focused on accurate normalization of DNA methylation microarrays [53, 70].

Assuming that decisions on normalization have been made, data exploration is often facilitated by the construction of a *clustering heatmap*, a color image of the data matrix $\mathbf{Y} = (\mathbf{y}_1, \ldots, \mathbf{y}_n)$ where rows represent individual subjects, columns represent individual features, and the value of y_{ij} (methylation extent or miRNA expression) is indicated by color. Furthermore, hierarchical clustering is used to cluster the columns and cluster the rows, so that similar array profiles are grouped together and similarly expressing loci are grouped together, allowing for patterns to be recognized by eye. Frequently the dendrograms resulting from the hierarchical clustering are also shown.

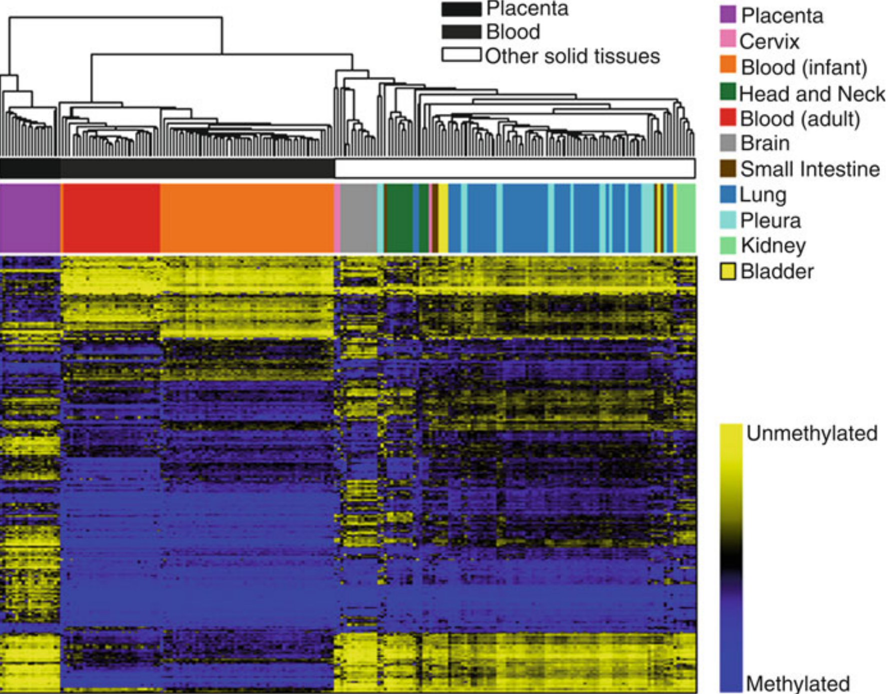

Fig. 5.2 Example of a Heatmap showing data from a DNA methylation microarray (Reproduced from [34])

Figure 5.2 shows an example of a heatmap constructed from DNA methylation data obtained from several different types of non-pathological tissues [34].

Locus-by-locus analysis is often conducted by *moderated* t-tests and ANOVA procedures, available through methods such as *Limma* [52]. These use a Bayesian formulation to motivate a procedure for estimating variance components, resulting in slightly more efficient tests. Because of the large number of loci tested, and the potential for substantial correlation between loci, a Bonferroni adjustment for multiple comparisons can be inefficient. Consequently, control of FDR is typically preferred over the control of FWER. However, the more liberal procedures based on FDR imply a subsequent confirmation or validation of a small number of loci, at least philosophically. This is often done with a follow-up study or study phase, often with a distinct assay of the more labor-intensive variety, with the goal of confirming the biochemical results as much as the epidemiologic associations. This issue is discussed in more detail below. When there is reasonable confidence in the microarray platform, an entire array can be used to test a formal hypothesis. When this occurs, data reduction strategies such as those used in Christensen et al. [34] may sometimes be employed. In this study, CpGs were clustered based on their methylation profile across specimens; subsequently, for each specimen, methylation measurements

were averaged over each cluster, resulting in a small number of average methylation values, each somewhat analogous to LINE1 methylation (a biological average of methylation of a specific repeat sequence). These averages were then tested for association with other variables such as age, with appropriately rigorous multiple comparisons adjustment based on FWER. A similar, more comprehensive study [74].

Microarrays imply a high dimension d of $\mathbf{y}$. Therefore, computationally efficient methods are needed for some of the techniques described in the previous section. For example, RPMM may be preferred over conventional mixture model techniques. In addition, when d is extremely large (e.g. the Infinium array produced by Illumina), preprocessing of the array may be needed, selecting a moderate number of loci to be used in subsequent analysis. One simple approach selects the M most variable loci (with a predetermined choice of M), though this method may inadvertently choose biochemically noisy loci that are not necessarily predictive of phenotype. Consequently, alternatives such as semi-supervised analysis [54, 55] can be used to select predictive loci. These methods employ cross-validation strategies to protect against over-fitting. A semi-supervised version of RPMM [43] has recently been demonstrated to have good performance.

A final consideration in the analysis of microarray data concerns the type of locus on which to focus analysis. A number of studies have found that the association between DNA methylation and phenotype may vary according to the local genomic geography of the target locus (e.g. CpG Island status) [34, 56]. There may be value in stratifying analysis based on such considerations.

5.7 Multiple Comparisons and Study Objectives

Univariate analysis, whether of the low or high dimensional variety, whether employing simple nonparametric tests of association or moderated linear models, will produce a number of test statistics (one for each feature), thus requiring an adjustment for multiple comparisons. The fundamental problem can be illustrated by a simple example. Suppose a study is designed to investigate the association of age with DNA methylation at 100 different CpG sites. Even if none of the 100 sites has a true association with age, approximately 5 of the 100 univariately generated p-values will nominally fall below 0.05, thus misleading the naïve researcher.

As mentioned above, the choice of adjustment depends on the goals of the study. Confirmation studies demand rigorous control of the familywise error rate, i.e. the overall probability of any false conclusion, because their objective is to test an overall hypothesis about a set of loci. FWER, akin to Type I error, i.e. Pr("significant"| truly null), represents the probability of any test resulting in significance under the assumption that *all* hypotheses are truly null. On the other hand, exploratory studies do not demand such rigor, since their design implies a subsequent confirmation step (whether or not it is actually conducted in the context of the study). Errors are therefore tolerable, and the objective is to control the *rate* of such errors. False

discovery rate, which represents the fraction of significant tests for which the hypothesis is truly null, is increasingly being used to address multiple comparisons in exploratory studies, especially those using microarrays. FDR is based on the probability that an individual locus, already having been deemed significant, represents a truly null association, i.e. Pr(truly null | "significant"). Note the difference in denominator between the formulation of FWER and FDR! Methods based on FDR are inappropriate when the intention is confirmation, i.e. the measurements represent a "final" data set that will not be followed up with a validation phase. Confirmation studies generate data with the purpose of providing definitive evidence for or against a hypothesis, and therefore do not represent an exploratory analysis with the goal of "discovery". Consequently, focus on FDR is inappropriate for such studies.

A great many classical methods for controlling FWER, such as those of Tukey/Kramer [57], Scheffé [58], and Duncan [59] are ultimately based on Gaussian statistics, and therefore are appropriate only for Gaussian outcomes or large sample sizes. The classical and straightforward method of Bonferroni and its more powerful variants [60, 61] are popular alternatives, although they have been widely criticized as extremely conservative (i.e. having actual FWER much lower than their nominal value). In the classical approach, the association measured at a single locus is deemed significant at level α if its nominal (unadjusted) p-value is less than α / M, where M is the number of loci being interrogated. A less conservative approach, proposed by Holm, first ranks the loci by p-value, assesses significance for the lowest p-value by comparing it to α / M; if the first test is rejected, the second-lowest p-value is assessed by comparing it to $\alpha / (M-1)$, etc., continuing until the first failure to reject. This approach is related to a testing procedure proposed by Simes [61], where the j'th ordered p-value is rejected if it is less than $j\alpha / M$, and is equivalent to a conservative approach based on the control of FDR, described below. When the methylation variables are independent of one other, Bonferroni adjustment typically represents "the best one can do", in that its true FWER is close to the nominal value α. However, when the methylation values are substantially correlated, as is typical, Bonferroni procedures may be overly conservative and therefore inefficient. In these settings, permutation-based adjustments offer a remedy. These are beyond the scope of the current chapter, but are covered extensively in Westfall and Young [62]. An alternative to permutation-based adjustments is the use of unsupervised techniques to reduce dimensionality before assessing an association with phenotype.

Control of FDR typically utilizes the methodology of *q-values*. Nominal p-values arising from individual tests are converted to their corresponding q-values, which have the following interpretation: for a given threshold q_0, the set of $M(q_0)$ features having q-value less than q_0 (the "discoveries") will contain approximately $q_0 M(q_0)$ features whose associations are in fact null. The practical interpretation is that q_0 represents the proportion of loci that will fail a subsequent attempt to independently validate the $M(q_0)$ most significantly associated loci. Note that, via Bayes' Rule,

$$\Pr(null \mid significant) = \frac{\Pr(significant \mid null)\Pr(null)}{\Pr(significant)} = p_0 \frac{\Pr(null)}{\Pr(significant)},$$

the latter equality justified with an understanding that a fixed p-value threshold p_0 determines significance, and therefore Pr(*significant*) is obtained empirically from the set of generated p-values as the fraction of significant results at threshold p_0. Consequently, a critical quantity in the construction of q-values is the proportion $\pi_0 = \Pr(null)$ of truly null loci on the entire array, and various methods have been proposed for its estimation [23, 63]. Note that Simes' modification of Bonferroni adjustment is equivalent to the construction of q-values with $\pi_0 = 1$, i.e. no truly associated loci. It is important to note that q-values do not provide a method for testing a formal hypothesis in the way that p-values have been used traditionally. Their use is more appropriate when the objective is to select a small number of loci for subsequent validation, where p-values appropriately adjusted for multiple comparisons and control of FWER can be used more efficiently to test a scientific hypothesis. Traditionally, q-values between 0.05 and 0.15 have been considered "significant", though the final determination of significance rests on the subsequent validation, and the selected q-value threshold should really reflect the investigator's tolerance for failure in validation.

A final note is in order on the use of microarrays for confirmatory studies. The analysis presented in Christensen et al. [34] used a multiple comparisons adjustment based on FWER, but was reasonably powered with a moderate sample size because of its data reduction strategy: for each subject, methylation measurements were averaged over CpGs clustered into a small set of classes. This strategy is more efficient than testing each CpG separately with subsequent adjustment by control of FWER. For example, suppose that in a particular study, M CpG sites have similar DNA methylation distributions and are similarly associated with a phenotype Z; then, for each individual CpG, the test statistic for its association with Z will have standard error $\sqrt{M}$ times the standard error of the test statistic for association with the average over the M CpG sites. However, both statistics will have similar effect size. If the individual statistics are not strongly dependent, there will be an additional cost in efficiency after adjusting for multiple comparisons. For example, in the limiting case of independent test statistics, adequate correction for multiple comparisons would effectively require, for each CpG, a critical value corresponding to the nominal level α / M. However, we note that the use of externally derived clusters might be more convincing than the data-driven clustering used in this study, though the latter is no worse than the typical two-stage analyses used with unsupervised analysis and subsequent testing of associations between a phenotype and predicted latent variables. Recent work has explored this topic in detail [74]

5.8 Study Design

In addition to proper microarray design considerations, such as randomizing chip arrangement with respect to target phenotype as much as possible [64, 65], careful attention should be paid to epidemiologic study design. Again, an important consideration is whether the study is designed for exploration or for confirmation. If the data are to be used for exploration, control of FDR is the key consideration, and methods such as *Significance Analysis of Microarrays* [66], or SAM, *PowerAtlas*

[67], or the methods of Liu and Hwang [68] should be used. If the objective is to confirm associations for M specific loci, then power and sample size considerations can be addressed conservatively by assuming Bonferroni correction, i.e. using standard methods for univariate analysis with control of Type I error set to α / M, where α is the desired overall FWER.

For the more complex multivariate models, design considerations can be difficult both analytically and computationally, and may require ad-hoc methods such as simulation. For such models, it may be useful to pose the problem in a simpler framework in order to approximate power. For example, if the design of a study where classification is a goal, two classes with specific prevalences may be assumed, and the problem thus converted to a conventional two-sample comparison.

5.9 Useful Software

The R Project for Statistical Computing (www.R-project.org) offers open-source statistical software with user-updatable libraries, called *packages*. In addition, it serves as the foundation for the *Bioconductor* suite of bioinformatic tools (www.bioconductor.org). Many of the procedures mentioned in this chapter are available through R or Bioconductor packages. The R functions *fisher.test*, *wilcox.test*, and *kruskal.test* allow for nonparametric testing that can be easily programmed in a loop. The R function *glm* can be used to fit quasi-likelihood models appropriate for beta distributions. The R function *hclust* and package *cluster* offer tools for hierarchical clustering, and the function *heatmap* constructs heatmaps. The Bioconductor package *limma* fits moderated versions of linear models. The R packes *qvalue* calculates q-values for controlling FDR. The packages *mclust*, and *RPMM* fit mixture models, *tree* and *rpart* fit CART models, *kernlab* implements SVMs and kernel machines, and *randomForests* implements Random Forests. The R package *samr* implements SAM power analysis as well as many of the univariate test options presented above.

SAS has fewer tools available specifically for the analysis of high-dimensional data arising from microarrays. However, some of the simpler procedures described above are available. Univariate tests can be conducted using the *ttest* and *npar1way* procedures (the latter supporting nonparametric tests). Regression can be conducted using the *reg*, *glm*, *mixed*, or *genmod* procedures; note, however, that the *glm* procedure is not analogous to the *glm* function in R, which is more similar to the *genmod* procedure. Some more complex procedures are also available; e.g. unsupervised multivariate analysis is supported by the *factor* and *cluster* procedures. SAS macros for some more sophisticated procedures can be found by searching online.

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Chapter 6
Epigenome Changes During Development

Gavin Kelsey

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Abstract Epigenetic modifications accomplish the functional compartmentalisation of our genome. They ensure a high level of compaction of our DNA in a manner that nonetheless allows genes vital to given cell types to be expressed appropriately whilst sequestering away silent genes. The stability of epigenetic modifications provides long term memory in phenomena such as X-chromosome inactivation in females and genomic imprinting, but epigenetic states must also be dynamic as they are intimately involved in establishing the gene expression programmes that define cell lineage and are required to register changes in the environment. In this chapter, I shall describe the major epigenomic events that occur during mammalian development,

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from the specification of germ cells, to how the epigenome differences of the gametes are resolved at fertilisation, and how epigenomic events contribute to and reinforce lineage determination events. The advent of genome-wide profiling technologies is providing us with an unprecedented opportunity to investigate the scale of epigenomic changes during development and differentiation and how epigenomes are altered in disease.

Abbreviations

A^{vy}	*agouti viable yellow* allele
Bi-Seq	bisulphite whole genome sequencing
BMP4	bone morphogenic protein 4
c-Kit	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
CARM1	co-activator associated arginine methyltransferase 1
Cdx2	caudal type homeobox 2
Cfp1	CXXC finger protein 1
ChIP-Seq	chromatin immunopreciptation combined with next generation sequencing
Dnmt1	DNA methyltransferase 1
Dnmt3a	DNA methyltransferase 3a
Dnmt3b	DNA methyltransferase 3b
Dnmt3L	DNA methyltransferase 3-like
DMR	differentially methylated region (of an imprinted gene)
EHMT2	euchromatic histone methyltransferase 2
ELF5	ETS-related family transcription factor 5
EOMES	eomesodermin
ES	embryonic stem
Ezh2	Enhancer of zeste homologue 2
GCs	germ cells
H1T2	histone 1 variant T2
H1LS1	histone 1 variant LS1
H2	histone H2
H2A.Z	histone 2a variant Z
H2AR3me2	di-methylated H2A arginine 3
H3	histone H3
H3.3	histone 3 variant 3
H3K4	H3 lysine 4
H3K4me1/2/3	mono-/di- or tri-methylated H3 lysine 4
H3K9ac	acetylated H3 lysine 9
H3K9me1/2/3	mono-/di- or tri-methylated H3 lysine 9
H3K18ac	acetylated H3 lysine 18
H3K27me3	tri-methylated H3 lysine 37
H3K36me3	tri-methylated H3 lysine 36

H4K20me3	tri-methylated H4 lysine 20
H4	histone H4
H4R3me2	di-methylated H4 arginine 3
IAP	intracisternal A particle
ICM	inner cell mass
ICR	imprinting control region
Igf2	insulin-like growth factor 2 gene
iPSCs	induced pluripotential stem cells
KAP1	KRAB (Krüppel-associated box)-associated protein 1
KDM1B	lysine (K)-specific demethyase 1B
KDM2A	lysine (K)-specific demethyase 2A
Klf2	Krüppel-like factor 2
LTR	long terminal repeat
MeDIP-chip	methylcytosine immunoprecipitation combined with microarray hybridisation
NIH	National Institutes of Health
NLRP2	NLR family Pyrin domain containing protein 2
NLRP7	NLR family Pyrin domain containing protein 7
PcG	Polycomb group
PGCs	primordial germ cells
Pparα	peroxisome proliferator-activated receptor alpha
PRC2	Polycomb group (PcG) repressor complex 2
Prdm1(Blimp1)	PR domain containing 1
PRDM9	PR domain containing protein 9
Prdm14	PR domain containing protein 14
Prmt5	protein arginine methyltransferase 5
RdDM	RNA-directed DNA methylation
Sox2	SRY (sex determining region Y)-box 2
Suv39h1/2	suppressor of variegation 3-9 homolog 1 & 2
TE	trophoectoderm
Tet1	ten-eleven translocation 5mc-hydrolase 1
Tet3	ten-eleven translocation 5mc-hydrolase 3
TNP1 & 2	transition protein 1 & 2
Uhrf1	ubiquitin-like containing PHD and RING finger domains 1
ZFP57	zinc-finger protein 57
5mC	5-methylcytosine
5hmC	5-hydroxymethylcytosine

6.1 Introduction

Cellular phenotype is the outcome of the gene expression programme of a cell, which may be defined by a combination of the transcription factor repertoire, signalling events and the developmental history of the cell. The memory of earlier

events that impact on cell identity is epigenetic in nature. At a molecular level, epigenetics refers to the range of chromatin modifications, including DNA methylation, post-translation modifications of histones, the stable association of some non-histone chromatin proteins and higher levels of organisation of chromatin and the chromosomes. Collectively, these have become known as the epigenome. During mammalian development, the epigenome experiences a number of major reprogramming events that have the effect of establishing or restricting totipotency and contributing to and consolidating lineage specification decisions. These reprogramming events are best understood in the mouse (and much of the following description refers to what is known in mouse), but it is assumed that similar events occur in all placental mammals, including human. The key reprogramming events occur during germ cell specification and differentiation, in the embryo immediately after fertilisation, and in the preimplantation embryo as the first lineage specification occurs [1–3]. Subsequently, it is assumed that the epigenomic profiles of the various lineages and cell-types are progressively established throughout embryogenesis, as cell lineages arise and undergo terminal differentiation; each cell type will have its own epigenome, which may be further modulated by extrinsic factors. We also expect a lot of the epigenome: that it retains a memory of earlier development decisions *and* that it can respond appropriately to extrinsic signals and adapt to environmental changes. In truth, the epigenome will probably comprise 'hard-wired' regions, which might include imprinted genes that permanently remember their parental origin throughout life [4], and 'plastic' regions, comprising developmentally regulated genes and sequences exhibiting nutritional, environmental or experience-based adaptations. It has also become apparent that that some sequences escape full reprogramming in the germline, leading to the possibility of transgenerational epigenetic inheritance. We are now enjoying an unprecedented ability to map the repertoire of epigenetic features across the whole genome, either on high resolution microarray platforms or, increasingly, by next generation sequencing based approaches [5, 6]. In this chapter, I shall consider how the epigenome changes during development in mammals to provide a framework for understanding its vulnerability to factors that can cause disease.

6.2 Epigenetic Reprogramming Events in Mammalian Germ Cells

To consider the epigenomic journey experienced by the organism during normal development, let us start with the germline. In mammals, germ cells arise in the gastrulating embryo from a cohort of cells that has initiated a somatic cell programme. Germ cells will undergone two major epigenetic reprogramming events before mature gametes are produced. The first of these is to reverse the epigenotype of the embryonic cells from which the germ cells arise in order to restore a pluripotent state similar to the cells of the inner cell mass (ICM) of the blastocyst. The second is during the differentiation and maturation events that result in production

of the distinctive male and female gametes. Knowing the extent of DNA methylation and other epigenetic modifications in the oocyte and sperm genomes and the processes by which they are established is essential for a full understanding of the possibilities of transgenerational inheritance of epigenetic states and their possible associated phenotypic (mal)adaptations.

6.2.1 Germ Cell Specification and Erasure of the Somatic Epigenome

In the mouse, primordial germ cells (PGCs) are specified from cells destined to form embryonic and extra-embryonic mesoderm situated in the proximal epiblast of the egg cylinder stage embryo at embryonic day (E) 6.0–6.5 [7]. A small number (~40) of cells becomes specified, in part in response to BMP4 signalling, and are visible by prominent expression of the protein Fragilis; PGCs themselves will develop from a subpopulation of this initial pool and are distinguished by expression of the pluripotency factor Stella [8]. A number of epigenetic modifiers play dominant roles in PGC specification, including the PR/SET domain zinc-finger protein Prdm1 (also known as Blimp1) [9], which forms a repressive complex with the arginine methyltransferase Prmt5 that methylates histones H2a and H4 on the arginine 3 residue [10]. As well as suppressing the ongoing somatic cell programme, Prdm1 initiates the germ cell specification programme, including induction of expression of the pluripotency factors Nanog, c-Kit, Sox2 and Klf2. A second PR domain zinc-finger protein Prdm14 is induced independently of and cooperates with Prdm1 [11]. Amongst the factors Prdm1 and Prdm14 repress are Glp, which cooperates with the H3K9 methyltransferase EHMT2 (G9a), the *de novo* DNA methyltransferases Dnmt3a and Dnmt3b, and Uhrf1, which is required to target the maintenance methyltransferase Dnmt1 to replicating DNA. Ezh2, a component of the Polycomb group (PcG) repressor complex 2 (PRC2), is also up-regulated. This combination of changes results in PGCs exhibiting reduced levels of H3K9me2, but a compensatory increase in the repressive mark H3K27me3 [12, 13], and having a DNA demethylating environment. PGCs can be distinguished from surrounding somatic cells at this stage on the basis of their distinctive chromatin state by immunofluorescence for appropriate histone modifications.

From their site of specification in the proximal epiblast, PGCs migrate towards the genital ridges, which develop into the embryonic gonads, arriving around E10.5. During migration, PGCs proliferate such that they number ~8,000 (per embryo) by the time they reach the genital ridges. They continue to proliferate until ~ E13.5, when they enter mitotic arrest (in the male germline) or meiotic arrest (in the female germline), by which time each gonad contains ~25,000 germ cells (GCs). From E11.5, post-migratory PGCs undergo further, dramatic epigenomic changes. The most intriguing of these is global demethylation of DNA (Fig. 6.1a), which has been observed for repetitive sequences and some single copy genes, including imprinted genes [15, 16]. Because DNA demethylation has been observed to occur in a very

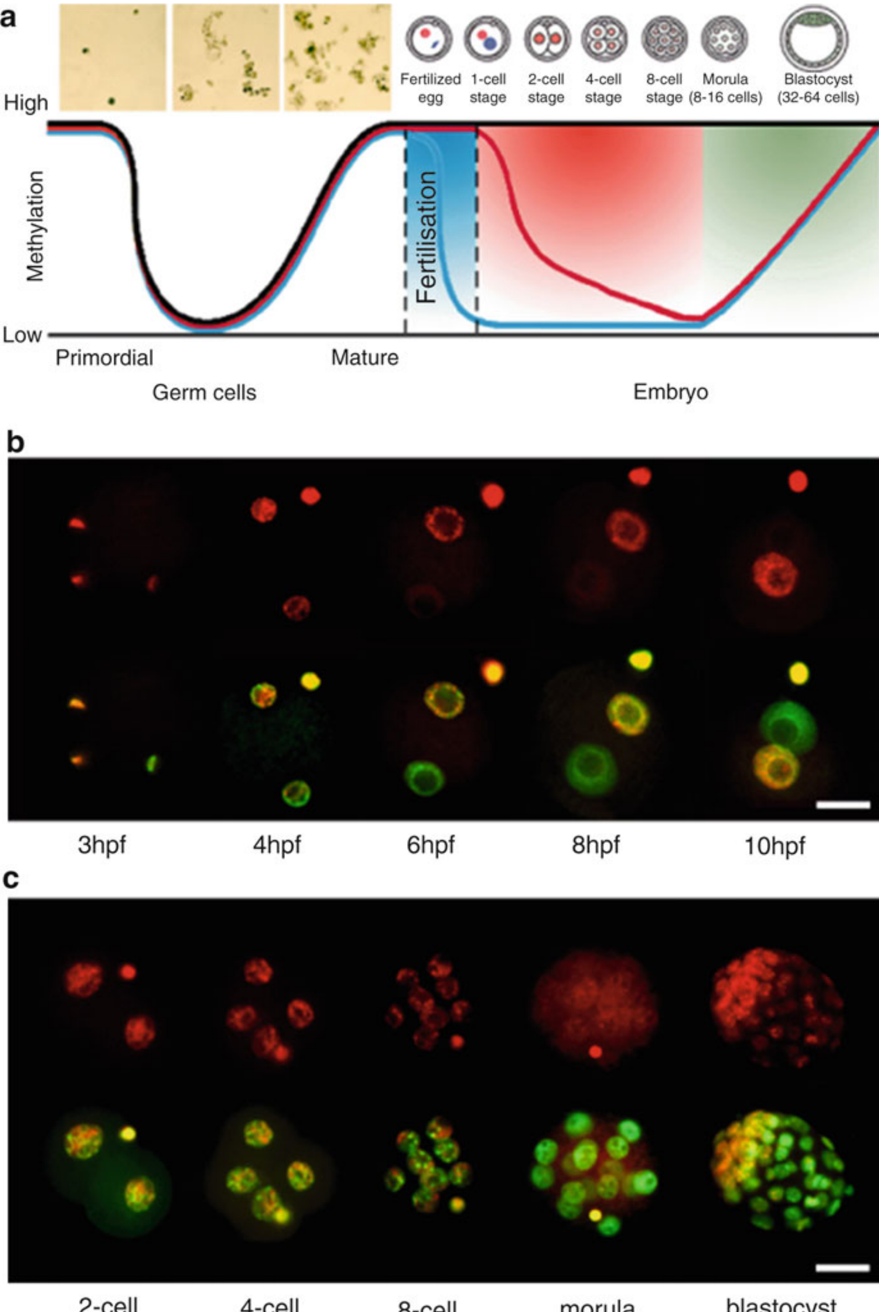
narrow time window (in a single G2 phase of the cell cycle), it is assumed to be in part an 'active' process, rather than simply by dilution of existing DNA methylation patterns owing to the failure to maintain methylation at replication. The mechanism of such dramatic DNA demethylation is not fully understood: it is thought to involve deamination of methylcytosine followed by base excision repair (BER) [17], but other mechanisms have not been discounted. After the onset of DNA demethylation, there is also substantial remodelling of chromatin, as revealed by immunofluorescence, with transient losses in signal for the linker histone H1 and the repressive marks H3K9me3 and H3K27me3, but long-term reduction in H3K9Ac, H2AR3me2 and H4R3me2 [13].

Recent whole genome shutgun bisulphite sequencing (Bi-Seq) of GCs at E13.5 has underscored the comprehensive nature of demethylation [18], although the depth of sequencing in that analysis was not able to detail methylation at individual loci. It is thought, however, that not all sequences are fully demethylated: repetitive elements of the intra-cisternal A particle (IAP) retrotransposon class have been found to be substantially resistant to demethylation [16, 18]. This observation provides a basis for some degree of transgenerational inheritance of epigenetic states. From this nadir, DNA methylation is re-established as GC differentiate (Fig. 6.1a), but follows quite different trajectories in male and female GCs. The dynamics of, and factors involved in, re-establishment are best understood for imprinted genes and repetitive sequences [1], although it might be assumed that other genomic elements that become methylated obey the same principles.

6.2.2 Establishment of Gamete-Specific Epigenomes

In female GCs in the mouse, *de novo* methylation occurs in oocytes arrested at the diplotene stage of meiotic prophase I. It initiates after birth as oocytes have been recruited into growth and is dependent on oocytes attaining a specific size [19, 20].

Fig. 6.1 Methylation reprogramming during mouse development. (**a**) The dynamics of DNA methylation, starting from PGCs as they enter the genital ridge through to blastocyst stage embryos is illustrated. The diagram depicts methylation levels generally of the maternal (*red*) and paternal (*blue*) genomes and of imprinted genes (*black*), which are resistant to demethylation in the zygote. Examples of PGCs at E11.5, 13.5 and 14.5 stained for the PGC marker alkaline phosphatase are pictured above. (**b**) Genome-wide DNA methylation dynamics illustrated by indirect immunofluorescence of fertilised oocytes using an antibody to 5-methylcytosine. Active demethylation of sperm-derived chromosomes during the first cell cycle is revealed by loss of DNA methylation (*red* signal) from the male pronucleus (green in lower merge panel) but not the female pronucleus. The lower panels show a merge (yellow) between the 5-methylcytosine (*red*) and DNA stain (*green*). hpf, hours post fertilisation. Scale bar 25 μm. (**c**) Passive demethylation, thought largely to affect the oocyte-derived chromosomes, is visualized from the 2-cell stage to the morula by reduction in 5-methylcytosine staining (*red*). *De novo* methylation initiates by the blastocyst stage and preferentially in the ICM. The lower panels show a merge (*yellow*) between the 5-methylcytosine (*red*) and DNA stain (*green*). Scale bar 25 μm. (Reprinted from Santos and Dean [14]. © Society for Reproduction and Fertility (2004), reproduced by permission)


a
Fertilized egg
1-cell stage
2-cell stage
4-cell stage
8-cell stage
Morula (8-16 cells)
Blastocyst (32-64 cells)
High
Methylation
Low
Fertilisation
Primordial
Mature
Germ cells
Embryo
b
3hpf
4hpf
6hpf
8hpf
10hpf
c
2-cell
4-cell
8-cell
morula
blastocyst

Full methylation of imprinted sequences may only be completed in fully-grown germinal vesicle stage oocytes, shortly before ovulation. Therefore, the whole cycle from erasure to full resetting of epigenetic marks in the female germline spans the period from germ cell specification in the embryo to pre-pubertal oocyte growth and perhaps beyond; the extent to which this applies in human is not known. Methylation of imprinted sequences and repetitive elements depends upon the *de novo* methyltransferase Dnmt3A and its co-factor Dnmt3L [21–23]; the second *de novo* activity, Dnmt3B, is not highly expressed and is dispensible in oocytes [24]. Recent genome-wide analysis reveals that ~7% of CpG islands (CpG islands are associated with many gene promoters) are highly methylated in mature oocytes in the mouse and are similarly dependent for methylation on Dnmt3a and 3L [25]. Many of these methylated CpG islands retain significant methylation in preimplantation embryos and may thus influence gene expression patterns after fertilisation. Oocytes appear to be unusual (together with embryonic stem cells), in exhibiting a substantial amount of cytosine methylation outside of the normal 'symmetrical' CpG dinucleotide context [26]. This might reflect the fact the DNMT3A does not exclusively methylate cytosines in a CpG context, its high level of activity in oocytes and the absence of replication-dependent maintenance which is only active at CpG sites. It is not clear whether non-CpG methylation has any biological significance. What other factors contribute to *de novo* methylation in oocytes is not fully clear. That the interaction of Dnmt3L and Dnmt3A with chromatin is inhibited by the presence of methylation at H3K4 [27, 28] indicates an important interplay between histone modification state and DNA methylation and is consistent with methylation being directed towards transcriptional inert sequences (H3K4 methylation is a marker of active genes). Consistent with this possibility, methylation of some imprinted sequences is disrupted in oocytes lacking the H3K4 demethylase KDM1B [29]. In addition, however, methylation of some imprint control regions (ICRs) has been shown to depend on transcription through ICRs in growing oocytes [30]. Studies of human imprinted gene syndromes, including biparental complete hydatidiform moles, in which conceptuses lack methylation at imprinted genes determined in oocytes, have identified mutations in two members of the NLRP family of inflammation-related proteins, NLRP2 and NLRP7 [31, 32], but the mechanism of their involvement in DNA methylation establishment is not known.

DNA methylation in the male germline, in contrast, occurs in GCs (referred to as gonocytes or prospermatogonia) arrested in G1 of mitosis. At imprinted sequences and repetitive sequences, *de novo* methylation initiates in fetal gonocytes from E14.5, but may continue until birth and beyond [1, 33]. Genome-wide remethylation is completed in primary spermatocytes, prior to entry into meiosis, although there is evidence that some loci may become methylated late in spermiogenesis [34, 35]. As in oocytes, Dnmt3A and Dnmt3L are both required, but Dnmt3B also plays a role at one of the four imprinted genes known to be methylated in sperm [23, 36]. The mechanisms targeting DNA methylation in male GCs are less well understood, but might be anticipated also to involve interactions with permissive histone modifications [37]. One mechanism, which might be specific to male GCs, is the involvement of a specific class of small RNAs, the Piwi RNAs, in a process called

RNA-directed DNA methylation (RdDM) [38, 39]. This mechanism appears to be directed particularly towards methylation of transposable elements, but also operates at at least one imprinted locus [40].

The outcome of these distinct *de novo* methylation processes are radically different DNA methylation landscapes in the mature oocyte and sperm [24]. Differences in DNA methylation between male and female gametes are exemplified by the germline differentially methylated regions (DMRs) of imprinted genes. Imprinting is controlled by such differences, which manifest as regions of dense CpG methylation that are fully methylated in one germline but not the other; currently, a substantially larger number of DMRs are known that acquire methylation in oocytes than in sperm [26]. These methylation differences, which are faithfully maintained as a memory of parental origin of the alleles in embryos, will determine parental-allele specific expression states, in many cases monoallelic silencing, of the associated genes. The extent to which other sites of differential methylation in gametes impact on gene expression patterns in embryos is not known, but should also emerge from genome-wide methylation studies [25, 41]. One reason for the late onset of methylation is that methylcytosine is the base most prone to mutation: deamination of a methylcytosine results in conversion to thymidine and occurrence of a T:G mismatch. Repair of this mismatch may restore the correct C:G base pair but could also lead to replacement by a T:A pair and an ensuing C→T transversion. Because of this vulnerability, it is suggested that remethylation is left as late as possible during gamete development to minimise the fixation of these transversion events [42, 43]; nevertheless, C→T transversions are the most frequent mutation events in the human genome. There is evidence of natural variation, both intra- and inter-individual, in DNA methylation in human sperm samples [44]; in addition, sub-fertile males appear to have increased incidence of DNA methylation defects at imprinted loci [45, 46]. Variations in sperm methlyation patterns could have an influence on offspring phenotype, if such differences were maintained beyond the genome-wide demethylation experienced by sperm-derived chromosomes in the preimplantation embryo (see Sect. 6.3.1).

There are also changes in chromatin architecture during germ cell differentiation and maturation to accommodate the requirements of meiosis and, in the mature sperm, genome compaction. Both male germ cells and growing oocytes contain variants of the canonical histones [47]. Disruption of genes for the H3K4 trimethyltransferase PRDM9 and the H3K9 mono- and di-methyltransferase EHMT2 cause meiotic arrest in oocytes [48, 49], suggesting that H3K4me3 and H3K9me1/2 are required for chromosome structures for chromosome synapsis and/or recombination, or that these activities are essential for the correct meiosis-specific gene expression. Otherwise, there is little change to oocyte histones during meiosis, except for deacetylation of H3 and H4 at the stage of germinal-vesicle breakdown and the resumption of meiosis prior to maturation. These, and other chromatin modifiers such as the H3K9 trimethyltransferases Suv39h1 and 2, are also required for progress through early meiosis in male GCs [48–50]. Subsequently, during spermiogenesis, chromatin becomes highly condensed through a sequence of events including replacement of testis-specific linker histones H1T2 and H1LS1, replacement of histone

by the transition proteins TNP1 and 2 and, finally, in spermatids their replacement by protamines [47] to complete the compaction of DNA in the spermatozoa. However, sperm chromatin is not devoid of histones, but up to 15% remain (depending on the species). Histone retention is locus-specific, including at imprinted loci and genes with functions in early embryogenesis, and retained histones carry activating and repressive modifications, such as H3K4me3 and H3K27me3, suggesting that developmentally important genes may be poised for activation in the early embryo [51, 52], if these histones and their associated modifications are maintained during the global replacement of protamines by histones in the zygote. Understanding the extent of epigenetic information in sperm and its persistence during embryogenesis is fundamentally important in considering how environmental effects experienced by males can be transmitted to influence offspring phenotype [53].

6.3 Epigenetic Reprogramming Events in Mammalian Embryos

Fertilisation brings with it a new wave of reprogramming of the epigenome that seeks to achieve a homogenisation of the differences between sperm- and oocyte-derived chromosomes, preparation of the DNA for the process of zygotic genome activation, the restoration of totipotency and preparation for the first lineage specification. The major reprogramming events have been elegantly demonstrated by immunofluorescence studies with antibodies directed against methylcytosine (Fig. 6.1b), specific histone modifications and the associated histone modifier complexes [54, 55]; in addition, methylation of a few genes has been analysed directly by bisulphite sequencing [25, 41].

6.3.1 Reprogramming of Paternal and Maternal Genomes After Fertilisation

The first of these events is the decondensation of sperm chromatin and replacement of protamines with histones stored in the oocyte, which occurs in the male pronucleus before formation of the zygote is complete with the fusion with the female pronucleus. This replication-independent deposition of histones results in the chromatin of the sperm-derived chromosomes being enriched in the variant histone H3.3 and depleted in modifications such as H3K9me2, H3K9me3 and H3K27me3, which are apparent in the female pronucleus. The differences in histone modifications between paternal and maternal genomes can be detected at last until the four cell stage [55]. Such differences are prominent at the highly condensed regions of pericentromeric heterochromatin, but whether they also reside at single copy sequences in euchromatic regions and whether they influence how the two parental alleles of

genes are expressed at that stage is not clear. Some imprinted genes have been examined and found to exhibit monoallelic expression in preimplantation embryos dependent on the activity of PcG proteins, but this might depend on the pre-existing DNA methylation differences inherited from the gametes [56].

Soon after protamine replacement, there is a rapid and comprehensive reduction in DNA methylation of the paternal pronucleus, which has been demonstrated by antibody staining and by bisulphite sequencing of specific genes [57–59]. It occurs prior to DNA replication, so is thought to be an active process. The activities responsible for this demethylation have long been sought, and have included members of the transcription elongation complex [60], whose mechanism of action is unclear at this time. Recent studies have identified 5-hydroxymethylcytosine (5hmC) as a possible intermediate [61]. 5hmC has been detected in the zygote by immunofluorescence and one of the enzymes that convert 5mC to 5hmC, Tet3, is present in the fertilised oocyte [62]. 5hmC can be detected at least until the 8-cell stage, suggesting that it remains on the paternal genome without further metabolism, but diluted by DNA replication [62, 63]. What the subsequent fate of this 5hmC is and the extent to which it has an impact on gene expression and on determining other epigenetic modifications is still unknown. 5hmC was first identified as a minor modification in DNA in neuronal tissue and mouse ES cells [61, 64]; more recent studies suggest that it can act as an epigenetic mark it is own right beyond a role as a by-product of cytosine demethylation, as Tet protein activity is required for normal ES cell self renewal and early lineage decisions [65–67].

Remarkably, the female pronucleus appears to be protected from active demethylation in the fertilised oocyte, and the reasons for this are unknown at present, although one oocyte-derived protein, Stella, has been identified without which sequences from the maternal genome also become demethylated [68]. However, there is a substantial reduction of DNA methylation signal overall during the first embryonic cleavage divisions, suggesting that the maternal genome also experiences demethylation, and this is assumed to be a ‘passive’ process, owing to the absence of maintenance methylation at DNA replication: 5hmC appears to be absent from the maternal genome and the Tet3 enzyme rapidly disappears after fertilisation [62]. Indeed, the maintenance methyltransferase Dnmt1, which has been concentrated in the oocyte cytoplasm during oocyte growth, appears to be largely excluded from the nucleus of preimplantation embryos [69, 70]. Thus, by the time the embryo comprises 16–32 cells (morula stage), genome-wide DNA methylation levels have reached a minimum. This picture has depended largely upon 5-methylcytosine (5mC) staining studies and, until recently, the number of single copy genes examined for methylation was limited. It is clear, however, that some sequences do faithfully maintain CpG methylation whilst the genome as a whole is losing methylation and the classic example are the DMRs of imprinted genes [69]. Recent genome-wide analysis of methylation in mouse blastocysts has, however, revealed a greater diversity in the behaviour of gametically-derived DNA methylation and identified additional gene promoters that retain at least some degree of methylation from oocytes throughout preimplantation development [25, 41]. What governs which sequences maintain or lose methylation is not fully understood, nor whether

the existence of gamete-derived CpG island methylation influences early lineage decision events. In addition to the dependence on Dnmt1 and its associated factors [69, 71, 72] a number, but not all, DMRs also require the presence of the zinc-finger protein ZFP57: mouse embryos lacking Zfp57 fail to maintain methylation at a number of DMRs [73] and homozygous mutation of ZFP57 has also been found as a common cause of 'maternal hypomethylation syndrome' in humans, in which patients have loss of methylation of a variable number of imprinted DMRs [74]. ZFP57 is a member of the Krüppel-like family of transcriptional repressors and an interacting protein, KAP1, has been found to be necessary for maintenance of methylation of endogenous retroviral elements (IAPs) in early mouse embryos [75]. The mechanisms by which ZFP57 and KAP1 interact directly or indirectly with Dnmt1 to ensure methylation are not known.

6.3.2 Establishment of New Epigenomes with the First Lineage Specification Events

From a low point in the morula, DNA methylation begins to be restored by the blastocyst stage (Fig. 6.1c), but differences in methylation level between the incipient embryonic lineage, represented by the ICM and extra-embryonic lineage, represented by the trophectoderm (TE) soon become apparent, with the TE showing reduced 5-methylcytosine staining [54]. This global difference likely reflects generally reduced methylation of repetitive sequences in TE [76]. Concomitant with the difference in DNA methylation between ICM and TE, gross differences in some histone modifications have also been observed, including acetylated H3K9 and methylated H3K27 [77]. The ICM and TE lineages are defined by distinct networks of pluripotency and lineage-determining transcription factors and there is considerable interplay between epigenetic modifiers and these factors in the preimplantation embryo (as discussed extensively in [2]). This is exemplified by the co-activator associated arginine methyltransferase 1 (CARM1), which is active against the R2, R17 and R26 residues of histone H3. Blastomeres of the 8-cell embryo expressing higher levels of CARM1 are destined preferentially for the ICM [78]. Of the genes directly or indirectly regulated by the action of CARM1, the caudal-type homeobox 2 gene *Cdx2*, whose expression is reduced in blastomeres with higher CARM1 levels [79], is one of the major determining factors of TE [80]. Epigenetic mechanisms are seen to reinforce lineage specification decisions. The ETS-related family transcription factor ELF5 cooperates with CDX2 and another TE determinant eomesodermin (EOMES) to stabilise the TE decision and once ELF5 is activated the cell fate choice becomes fixed. ELF5 is epigenetically regulated as its promoter is silenced by DNA methylation in the embryonic lineage [81]. Similar epigenetic fixation of lineage decisions can be seen for the pluripotency factor Stella, which is expressed in the ICM but becomes repressed and methylated in the epiblast [82]. Individual blastomeres are not thought to be irreversibly committed initially to either ICM or TE fate but the expression of factors such as ELF5 and their epigenetic silencing form a barrier to switching and consolidate cell fate choice.

Recent genome-wide investigations of promoter DNA methylation performed by MeDIP-chip on DNA from preimplantation (blastocysts, E3.5) and post-implantation embryos (E6.5 and E9.5) reinforce the notion that DNA methylation is used to control the activity of genes in a lineage-specific manner [41]. That study confirmed that trophoblast and pluripotency related genes, including *Elf5* and *Stella*, had become *de novo* methylated in the epiblast by E6.5; it also identified that many germ-cell specific gene promoters are already methylated in blastocysts. In addition, several genes specific to differentiated tissues, including brain, eye and the haematopoietic lineages, were found to be methylated in the epiblast, suggesting that the promoters of these genes must become demethylated specifically in their respective lineages later in embryogenesis. This was indeed found to be the case by testing promoter methylation of selected genes during adult haematopoietic stem cell differentiation into B cells (and has been corroborated in an independent analysis; [83]). Therefore, epigenetic mechanisms and factors are intimately involved in regulating lineage-specifying genes, in reinforcing cell fate choices by disallowing the expression of genes inappropriate to a given lineage and, through demethylation events, in allowing activation of differentiation-specific gene sets.

6.4 Tissue-Specific Epigenomes: Plasticity and Stability

The advent of genome-wide methods to map DNA methylation and histone modifications is providing full epigenomic profiles of specific cell types, how they develop during differentiation and how epigenomes are altered in disease. A goal of current international effort, for example, as coordinated by the International Human Epigenome Consortium and NIH Roadmap Epigenomics Mapping Consortium [84], is to generate standardised reference epigenomes for a common set of epigenomic marks in a defined set of human tissues and cell lines. With the explosion of epigenome mapping studies in the last couple of years it is impossible to give a comprehensive account, so I will restrict the discussion to a few examples to illustrate general principles and some new insights that have emerged.

6.4.1 Epigenome Changes During Differentiation

Embryonic stem (ES) cells have been used widely as a proxy for the ICM to understand the gene expression programmes and epigenomic profiles associated with pluripotency. In an early application of next generation sequencing to chromatin immunoprecipitation of specific histone modifications (ChIP-Seq), Mikkelsen and colleagues profiled mouse ES cells in comparison to neural progenitor cells and embryonic fibroblasts [5]. The power of sequencing based-approaches is the unambiguous way in which data on histone modifications, DNA methylation and mRNA expression can be correlated. In this way, it was possible to detect how the patterns

of enrichment for an activating mark (H3K4me3) and repressive mark (H3K27me3) were able to discriminate genes expressed, poised for expression or stably repressed. This, and other studies (e.g., [85]), suggested that genes poised for expression, in particular, were found to be marked by 'bivalent' chromatin, a paradoxical combination of the active and repressive modification, with bivalent marks resolving into active or repressed states in parallel to changes in gene expression in differentiated cells. In addition, ChIP-Seq was able to confirm the distributions of H3K36me3 as a marker of active transcription units, and H3K9me3 and H4K20me3 as markers of constitutive heterochromatin at centromeric satellite sequences, telomeres and other repetitive element classes. The reference epigenome of ES cells is an essential asset in seeking to determine the extent and fidelity of reprogramming of differentiated somatic cells to induced pluripotent stem cells (iPSCs) [86, 87].

In a systematic investigation of a single cell type, human CD4+ T lymphocytes, Zhao and colleagues mapped the distribution of 39 histone modifications (18 acetylation states and 19 methylation states) in comparison with gene expression data [88]. Although a large number of different combinations was found, a common pattern comprising 17 modifications was detected at over 3000 promoters. Genes with no or low levels of expression were depleted in histone acetylation and marked with H3K27me3 and/or other repressive histone methylation marks (H3K9me2, H3K9me3, H4K20me3). Expressed genes had the 'backbone' set of 17 modifications; additional modifications were associated with the most highly expressed genes, with the number of histone acetylations strongly associated with expression. Different acetylated histones were distributed differentially at promoter regions and gene bodies. Enhancers also appeared to have distinct and limited combinations of modifications, with more than 20% characterised by presence of the variant histone H2A.Z, as well as H3K4me1, H3K4me2, H3K4me3, H3K9me1 and H3K18ac modifications. This diversity of histone modification patterns seems bewildering and leaves one wondering what marks to focus on if one wished to compare epigenomic profiles in case-control studies for a disorder of interest and future insight into the dependencies of histone modifications will be important. To consider how histone modifications differ between cell types, Ren and colleagues mapped a limited number of histone methylation states in five human cell lines. The study found that the chromatin state at promoters was relatively invariant, but that there were highly cell-type-specific histone modification patterns at enhancers that correlated strongly with cell-type-specific gene expression [89]. Similar genome-wide histone modification and transcription factor binding analyses have been applied to *in vitro* differentiation systems, such as adipogenesis in both human and mouse models [90]. One outcome of the study was the identification of novel, stage-specific alternative promoters and distal enhancer elements, which were inferred from changes in chromatin signatures during differentiation (Fig. 6.2).

Whereas histone modification status emerges from such studies as being potentially dynamic, DNA methylation has traditionally been thought of as a more stable mark associated with long-term gene repression. CpG islands, short C:G-rich regions that are associated with a large proportion of gene promoters, are generally viewed as being constitutively unmethylated, except for the rare cases of those in

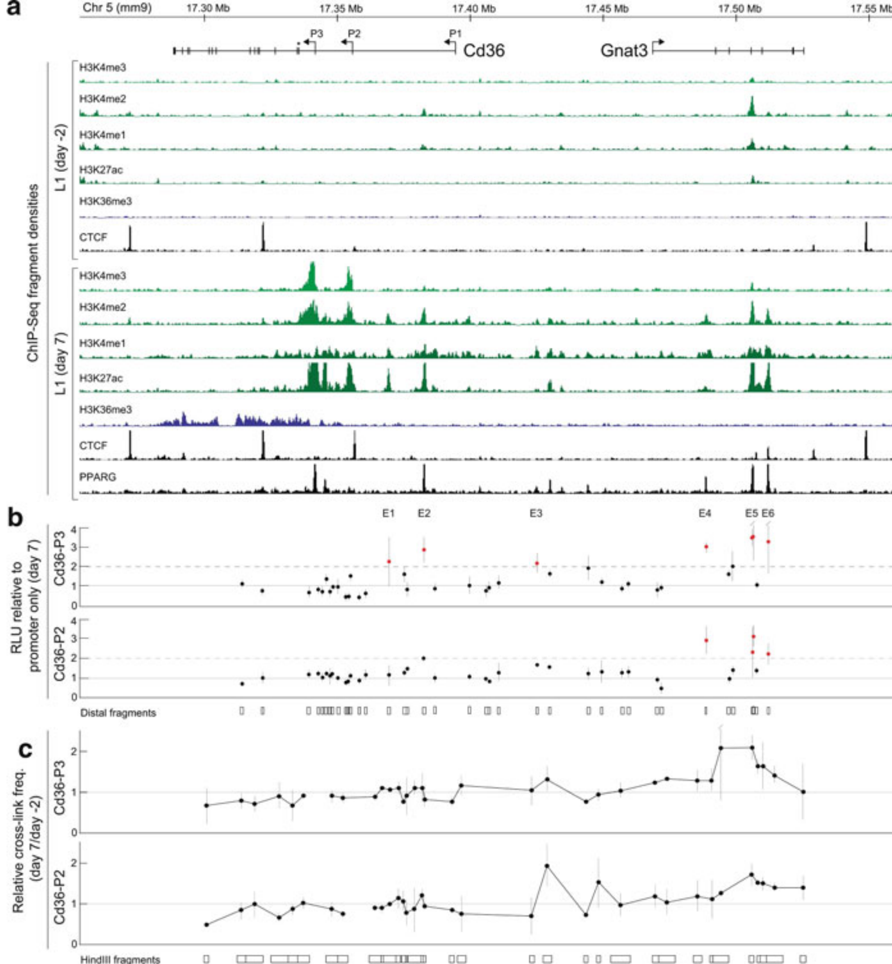


Fig. 6.2 Chromatin changes during adipogenic differentiation. Chromatin state maps of the ~300 kb *Cd36-Gnat3* locus on mouse chromosome 5 mouse L1 preadipocytes (day −2) and mature adipocytes (day 7) obtained by ChIP-Seq. Profiles for modified histone H3K4me1, me2 and me3, H3K27ac1 and M3K36me3 are shown, as well as binding sites for CTCF and Pparγ. Of particular note, enrichment peaks for H3Kme3 reveal the recruitment of new promoters of *Cd36* (P2 and P3) during differentiation and enrichment in H3K36me3 over the *Cd36* gene body is a marker of transcription elongation. Pparγ is a transcriptional regulator during adipogenesis and CTCF is a multifunctional DNA binding factor that has chromatin insulator function. (Reprinted from Mikkelsen et al. [90]. Copyright (2010), with permission from Elsevier)

imprinted genes and those on the inactive X chromosome in females [91]. Aberrant CpG island methylation is a common phenomenon in tumours, in which it is one mechanism by which tumour suppressor genes are inactivated. Several studies have now revealed that around 10% of CpG islands are methylated normally in a

tissue-specific manner [92–94]; in many cases, tissue-specifically methylated CpG islands demarcate alternative and tissue-specific promoters. CpG island methylation is strongly inhibitory to promoter activity, and erasure of methylation for promoter de-repression is likely to require substantial chromatin remodelling events, but absence of methylation by itself is not a strong predictor of expression level (e.g., [42]). Some observations have indicated that the less CpG-dense regions flanking CpG islands, for which the term 'CpG island shores' has been coined, exhibit tissue-specific differences in methylation that more closely correlate with gene expression status [83, 95]. Although DNA methylation has long been suspected as having a role in controlling gene expression programmes during differentiation, very few studies, up to now, have looked globally at DNA methylation status during differentiation of a specific lineage. One recent study has now done so during haematopoietic differentiation in the mouse, comparing methylation at 4.6 million CpG sites in eight sorted populations ranging from multipotent progenitors to progressively restricted progenitors of the lymphoid (T-cell), and myeloid (granulocyte and macrophage) lineages [83]. In this system, reduced methylation is associated with myeloid rather than lymphoid differentiation. Numerous methylation differences were found between the various populations, with differences at CpG islands shores generally predominating except, interestingly, in the earlier differentiation stages. As noted above, CpG island shore methylation correlated more closely with gene expression.

There is assumed to be a considerable interplay between DNA methylation and histone modificadtions. DNA methylation is inversely correlated with H3K4me3 at promoters and CpG islands [42, 93, 96], and this is consistent with the observation that the *de novo* methylation co-factor Dnmt3L is inhibited from binding histone H3 when lysine 4 is methylated [27], so has some mechanistic basis. Other factors are likely to contribute to the general protection of CpG islands against DNA methylation. A protein bound at the majority of CpG islands, Cfp1, interacts with the H3K4 methytransferase Setd1 and CpG islands lose H3K4me3 if Cfp1 is ablated [97]. An H3K36me2 demethylase, KDM2A, is also constitutively bound at unmethylated CpG islands, which might help to ensure a chromatin environment non-permissive for DNA methylation [98]. Cell-type-specific gain of methylation at CpG islands is thus likely to involve destabilisation of binding of several protective factors. Conversely, interactions between Dnmt proteins and Ezh2, a component of the PRC2 complex that deposits the repressive mark H3K27me3, have been found [99, 100]. Interestingly, a number of genes identified with a bivalent chromatin signature in ES cells, and thus targets of the PRC2 complex, undergo aberrant DNA methylation in human cancers [101, 102].

6.4.2 Longevity of Epigenetic Marks

A key question is to what extent the epigenome is 'hard-wired' and fixed in perpetuity once established. Certainly, there is an elegantly simple mechanism by which

DNA methylation can be inherited at each DNA replication cycle, because Dnmt1 is present at the replication fork and methylates the nascent DNA strand at hemimethylated CpG sites. It is assumed that DNA methylation is maintained by default, unless active mechanisms remove it (as in PGCs and the zygote) or prevent access of Dnmt1. This model is likely to be simplistic, as multiple factors, such as the Uhrf1 protein, are required to target Dnmt1 to newly synthesised, hemimethylated DNA at replication [103, 104], and these dependencies offer additional levels of regulation (or scope for error) that could allow methylation states to change. Histone modification states are also seen to be mitotically heritable, as in the blocks of constitutive heterochromatin at centromeres and telomeres. But how histone modifications are propagated at DNA replication is still hotly debated – whether there is semi-conservative inheritance of the dissociated core nucleosomes to the daughter strands that may then template modifications of the newly incorporated histones, or whether there needs to be wholesale re-establishment of histone modifications [105]. Persistence of chromatin states might also involve a cross-talk between DNA methylation and histone modifying factors, for example, Dnmt1 may contribute to maintenance of H3K9 methylation [106].

It is certainly the case that some epigenetic marks are extremely stable, such as the methylation of CpG islands on the inactive X chromosomes of females or in imprinted genes. In the case of X-inactivation, the choice of which chromosome to inactivate and methylate is taken randomly at a cellular level in female embryos around implantation and then all daughter cells maintain this choice throughout the lifetime of the organism. CpG island methylation is just one of the large set of epigenetic properties that differentiate active and inactive X chromosomes, and these epigenetic marks are likely to reinforce one another. For imprinted genes, methylation differences of the two alleles are determined in the parental gametes and these two states persist as a faithful memory of parental origin in all tissues (it is believed) and throughout life. Again, the parental alleles are also differentiated by multiple histone modifications that may act in concert with DNA methylation [107]. DMRs generally extend several kilobases and comprise hundreds of CpGs fully methylated on one parental allele and fully unmethylated on the other [26], so effects on allelic expression may require large scale changes in methylation. However, if lost in somatic tissues, there is no mechanism by which to re-impose methylation imprint marks except by passage through the germline [108]. It might be the case that these represent one extreme for which robust mechanisms have evolved to ensure the maintenance of methylation, because of the extreme adverse effects on X chromosome balance or imprinted gene expression dosage should the mechanisms fail. Nevertheless, there is genetic and molecular evidence that X-inactivation weakens with ageing [109, 110], and a substantial number of genes on the human X chromosome partially escapes inactivation, and this appears also to vary between individuals [111]. Several reports have indicated a relaxation of *Igf2* imprinting with ageing, particularly because of its association with cancer (e.g. [112]). One study reported considerable inter-individual variation in DMR methylation and found that skewed methylation clustered in families, suggesting a genetic or shared environmental component [113]. In contrast, another group found that imprinted genes showed

relatively little change in methylation associated with aging in adults [114]. There may be technical reasons for these apparent discrepancies, including the tissues analysed, methods used and whether the limited number of CpG sites assayed in any one study is representative, and more extensive analyses are warranted. In addition, the inability to assay expression in the most relevant tissues may also explain why imprinted gene expression is sometimes found to correlate poorly with DNA methylation [115]. Beyond X-inactivation and imprinting, twin studies have indicated a drift in DNA methylation patterns even in genetically identical individuals with age: older monozygotic twin pairs exhibited greater differences in DNA methylation profiles [116]. But, again, other investigations suggest a limited number of methylation changes associated with ageing: in microarray analysis of skin biopsies, DNA from older individuals was characterised by a specific hypermethylation pattern, however, this affected less than 1% of the markers analysed [117]. It may be anticipated that additional, carefully designed genome-wide studies with emerging epigenomic techniques will lead to a consensus on the degree to which our epigenomes age with us, the factors that promote change and the genomic loci most vulnerable.

6.5 Transgenerational Epigenetic Effects

There is a legitimate interest, but also some confusion or controversy, over the existence of transgenerational epigenetic inheritance. Epigenetic inheritance could be interpreted from variation in a phenotype present in one generation influencing the penetrance of the phenotype in subsequent generations in the absence of any DNA sequence change, or because environmental conditions (in the broadest sense) present in parents have an impact on subsequent, unexposed generations, bearing in mind that the germ cells that will give rise to grandchildren are developing in a female exposed during pregnancy [118, 119]. Evidence for transgenerational effects in humans and mice comes from the study of metastable epialleles, such as *agouti viable yellow* (A^{vy}) [120, 121], dietary challenges particularly before conception [53], exposure to environmental toxins, such as endocrine disruptors [122, 123], and epidemiological studies showing disease outcomes in grandchildren of affected populations [124, 125]. It is important to set such observations against the knowledge of epigenomic reprogramming in germ cells before concluding whether they satisfy criteria for transgenerational epigenetic inheritance.

As we have seen earlier in this chapter, one of the prerequisites of germ cell development is to erase the pre-existing epigenome of somatic cells to return to a state of pluripotency. However, it is also evident that germ cells are specified and differentiate in the developing embryo, so they could be vulnerable to adverse conditions *in utero*. Gametes have their own epigenomic landscapes, so there is the potential that modified or aberrant epigenetic marking of gametes can be transmitted to the embryo, providing that such alterations can survive the next phase of genome-wide epigenetic reprogramming in the preimplantation embryo and are

not further corrected as lineages are specified. Epigenetic mechanisms are energy consuming processes, so could be affected by the metabolic status of the cell and nutritional conditions of the organism [126] and, indeed, it is expected that there will be at least acute gene expression responses to nutritional changes that depend on epigenetic mechanisms. Finally, exposing pregnant females to diets that significantly influence one carbon metabolism that provides the methyl groups for methylation reactions could affect the maintenance of DNA methylation patterns potentially at loci common in offspring and their progeny (depending on the timing of exposure), because the germ cells for the next generation are developing in the exposed embryos and because maintenance depends universally and ultimately on the activity of Dnmt1.

Amongst the most extensively characterised examples of epigenetic inheritance is the A^{vy} allele in mice [120]. A^{vy} is a naturally occurring allele of the *agouti* locus in which an IAP element has inserted upstream of the *agouti* gene resulting in its misregulation (ectopic expression). IAPs are normal targets of epigenetic silencing but, in this case, methylation is variable between individuals resulting in variation in ectopic *agouti* expression, which manifests in coat colour phenotype, with genetically identically littermates ranging from completely yellow, through mottling to fully agouti (brown). Importantly, the range of coat colour phenotypes amongst offspring is influenced by maternal coat colour. Direct assessment of methylation at the IAP long terminal repeat (LTR) shows not only a strong inverse correlation between coat colour and degree of methylation, both in mothers and progeny, but also that DNA methylation in oocytes and sperm is related to coat colour and methylation type of parents [120, 121]. Embryo transfer experiments revealed that the variation was intrinsic to the A^{vy} allele and not a consequence of physiological differences between the different classes of females [120]. It is probably best to regard this example as a case of incomplete erasure of a somatic methylation pattern in germ cells and, indeed, IAPs are known to resist partially the genome-wide reprogramming in germ cells and early embryos [16, 18]. Intriguingly, however, sperm-derived methylation at the A^{vy} allele is largely erased in the zygote (consistent with absence of paternal transmission), but so is oocyte-derived methylation by the time of implantation [121], suggesting that additional epigenetic marks are responsible for the probability of re-establishing the methylation type of the mother. Owing to the sensitivity of the A^{vy} system, it has been used as a model to study dietary influences on epigenotype. For example, supplementation of the diet of wild-type female mice with methyl donors increases the methylation of the A^{vy} allele of their a/A^{vy} progeny [127]. However, there are conflicting reports whether these acquired methylation changes are passed on to subsequent generations [128, 129].

Analogous metastable epialleles may exist throughout the genome and influence expression of genes in their proximity, including in humans. A recent study identified such epialleles in humans: methylation level was similar across tissues of an individual but varied even between monozygotic twins, demonstrating the early and stochastic nature of methylation establishment at these loci. Moreover, it was found that in a sub-Saharan African population, which experiences profound seasonal fluctuations in food quality and availability, levels of DNA methylation varied in

individuals according to the season of their conception [130]. It was noted that methylation at these loci may be particularly sensitive to this effect, and more so than the imprinted loci tested. Although this observation establishes the principle of nutritionally conditioned epigenetic variation, it will be important to determine whether such epigenetic differences contribute to physiological differences and whether there is any vestige of methylation level in subsequent generations. Perhaps the most extensively analysed example of the impact of extreme maternal nutrition on health outcomes in offspring in humans is the Dutch hunger winter. DNA methylation analysis of several imprinted genes has detected differences at some, but not all, loci in individuals born to women pregnant at the time, with the alterations differing according to whether malnutrition was experienced in the first or third trimester. However, methylation was determined in whole blood DNA of offspring 60 years after the famine (by necessity) and the differences were marginal, so it is not possible to conclude whether they correspond to epigenetic alterations that were induced during gestation or are a consequence of later developmental or physiological differences [131, 132].

Transgenerational effects through the paternal line should be easier to interpret, because exposed fathers merely transmit genetic material and have no other direct influence on development of offspring. Sperm carry epigenetic information in the form of DNA methylation patterns, in as much as these survive early embryonic reprogramming, and the observation that sperm chromosomes retain some modified histones non-randomly adds impetus to investigate the possibility of paternal epigenetic transmission. In this context, it was recently reported that gene expression alterations can be detected in offspring of male mice fed a low protein diet for 3 months before mating [53]. Changes in hepatic DNA methylation were detected, exemplified by a modest but reproducible reduction in methylation at an upstream enhancer for the *Pparα* gene that correlated with gene expression (generally, promoter methylation was unaffected). Gene expression changes found in livers of offspring differed from those of the treated males, but the study did not investigate effects in the next generation. At this time, it is not clear how nutritional intervention in male mice resulted in gene expression changes in offspring, but it appears not to be explained by developmental compromise or immaturity of sperm. Also, there did not appear to be a simple relationship between altered DNA methylation profiles in liver of offspring and sperm, as the *Pparα* enhancer was highly methylated in sperm of control and dietary restricted males alike. Nevertheless, it would be important to ascertain the ontogeny and tissue-specificity of DNA methylation alterations and examine whether the effects noted are treatment specific (and potentially adaptive) or reflect a particularly vulnerable set of loci that could be deregulated by any adverse condition. Of alternative epigenetic marks as potential means of transmission, ChIP analysis detected differences in H3K27me3 at an unrelated locus and differences in mRNA loading of sperm were reported. Although not able to provide an explanation for the effects observed, the study does illustrate that we do now have the techniques to conduct sensitive, genome-wide studies into transgenerational inheritance. The most extensively investigated example of a transgenerational effect through the male germline is after the exposure to an endocrine disruptor, the

fungicide vinclozolin. Exposure of pregnant female rats by a course of intraperitoneal injection during the time of gonadal sex determination in embryos has been shown to cause impaired fertility in male offspring until the F4 generation, and a variety of other effects [119–122]. Microarray analysis of embryonic testis RNA from the F1 to F3 generations detected altered gene expression profiles, and although these tended to normalise with generation, ~200 transcripts remained deregulated in the F3 generation (being the first fully unexposed generation). Such gene expression changes could in part be a reflection of impaired spermatogenesis, although in the F3 generation no histological abnormality was detected in the embryonic testis. Altered expression of epigenetic modifiers including DNA methyltransferases was highlighted [133]. Recent genome-wide studies in sperm of F3 progeny identified a number of genomic loci displaying altered DNA methylation patterns, a number of which were validated in independent assays [123]. Further analysis is needed to follow the history of these alterations in sperm in the previous, exposed generations, as well as to investigate whether these loci transmit altered methylation to the somatic tissues and germline of their progeny.

In conclusion, there is a need to exercise caution and we should, perhaps, be more careful with definitions. An epigenetic change in offspring or grandchildren on account of parental exposure may be easy enough to explain from existing knowledge of the reprogramming events in germ cells and the timing of establishment of the final epigenetic profiles of gametes. This is not to say that such altered epigenetic patterning is unimportant, and can indeed have significant phenotypic or disease outcomes in offspring, but the expectation is that most epigenetic alterations should be reprogrammed in the germline of the unexposed generation and not permanently fixed. The term transgenerational inheritance should be reserved for transmission of an altered epigenetic state perpetuated across multiple generations in the absence of the inducer, for which there is evidence in plants [134]). In particular, it seems premature to entertain the possibility that there is an adaptive process at work, in the sense of Lamarckian-type transmission of acquired epigenetic states.

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Chapter 7
The Role of Epigenetics in the Developmental Origins of Health and Disease

Karin B. Michels and Robert A. Waterland

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Abstract The Developmental Origins of Health and Disease (DOHaD) hypothesis suggests that the prenatal and early postnatal environments shape the future probability of physical and mental wellbeing and risk of disease. A wealth of epidemiologic data supports the associations between maternal malnutrition, intrauterine growth retardation, and birthweight, and the risk of chronic disease including cardiovascular disease, hypertension, type 2 diabetes mellitus, obesity, neuropsychiatric

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disorders, and cancer. While the mechanisms underlying these observations remain unresolved, the DOHaD model assumes a developmental plasticity that allows adaptive regulation of the embryonic, fetal, and/or early postnatal metabolism in response to nutritional and environmental perturbations. Establishment of the epigenome coincides with vulnerable phases in development and provides one potential mechanism for long-lasting responses to transient environmental stimuli. Future studies in epigenetic epidemiology will seek to understand the role of various epigenetic mechanisms in DOHaD.

7.1 The Developmental Origins of Health and Disease

The search for the origins of chronic diseases has shifted focus toward the earliest phases of the life course. While classic epidemiology has targeted lifestyle patterns of adults at various ages, the importance of early life for lifelong health has been increasingly recognized over the past decades. Following the seminal work of Rose [1], Forsdahl [2], and Barker [3, 4], the period from conception to birth as well as the first few years of life are considered critical in the imprinting of disease susceptibility. This shift in thinking and research gave birth to the "Developmental Origins of Health and Disease" (DOHaD) hypothesis.

Epidemiologic studies support the hypothesis that chronic diseases have their roots in early life. Barker's work linked low birthweight to a number of cardiovascular diseases (including ischemic heart disease), hypertension, cholesterol levels, stroke, and impaired glucose tolerance [4–8]. His findings have been confirmed by other groups in different populations [9–11]. Data from the Dutch Famine in 1944/45, when food rations dropped below 1,000 kcal/day for 6 months, suggest an increased risk of obesity among offspring of mothers exposed to the famine during the first and second trimester [12], glucose intolerance if exposure peaked during late gestation [13], and schizophrenia if conception occurred during the famine [14]. Other maternal characteristics such as maternal weight and malnutrition also increase the risk of coronary heart disease in the offspring [15].

However, there is trouble at both ends of the birthweight spectrum. Like low birthweight, high birthweight is also associated with adult obesity [16]. Similarly, women are more likely to become obese in adulthood if their mothers were obese prior to pregnancy and/or had very high or very low gestational weight gain [17]. Gestational diabetes increases the risk of childhood and adult obesity in the offspring [18]. Moreover, a high birthweight is associated with an elevated risk of several cancers. Numerous epidemiologic studies support the association between a high birthweight and an increased risk of premenopausal breast cancer [19, 20]. In addition, a high birthweight has been linked to childhood leukemia [21], childhood brain tumors [22], and testicular cancer [23].

7.2 DOHaD Mechanisms

The fetal origins hypothesis suggests that perturbations at a critical period of development induce persistent alterations with potentially lifelong consequences. These epidemiologic observations led Hales and Barker to suggest a "thrifty phenotype" hypothesis, which proposes that poor fetal nutrition and growth leads to metabolic reprogramming of the glucose-insulin metabolism [24]. This adaptive developmental plasticity allows the fetus to adjust to and survive adverse environments. A limited supply of transplacental nutrients compels the fetus to channel the nutrients to the most vital organs, namely brain and heart, at the expense of other organs, which may remain underdeveloped and compromised in growth and function [25]. Moreover, permanent insulin resistance may be induced during development and reduce basal metabolic requirements; this permits survival under sub-optimal prenatal and predicted postnatal conditions [26]. While the environmental conditions *in utero* are transient, they induce permanent changes in metabolism and alter chronic disease susceptibility. Indeed, environmental perturbations may have a long-lasting impact at times of greatest plasticity during growth and development.

The potential benefits of such so-called 'predictive adaptive responses' depend on the accuracy of the prediction; the cost of inaccurate predictions is high [26]. If developmental conditions that induce intrauterine growth retardation are followed by a resource-rich postnatal environment, high plasma glucose levels will coincide with insulin resistance, greatly increasing the risk for metabolic disease in later life [26]. This "mismatch" between predicted and actual postnatal environment may explain profound long-lasting implications for chronic disease among individuals prenatally exposed to the Dutch Hunger Winter, which lasted only 9 months and was followed by normal nutritional availability [27]. Individual variation in sensitivity to mismatch and in turn to disease susceptibility is likely due to a variety of factors including genetic variation and the degree of developmental plasticity [25].

7.3 Potential Critical Periods for Developmental Epigenetics

The molecular mechanisms underlying DOHaD are not well understood. Developmental plasticity allows a specific genotype to create alternate phenotypes depending on intrauterine and early postnatal conditions, which may induce lasting changes in chronic disease susceptibility. Among the various potential biologic mechanisms underlying developmental plasticity [28], environmental influences on developmental epigenetics are receiving increasing attention [29]. Epigenetics describes the study of non-genetic mitotically heritable states in gene expression potential [30]. Epigenetic regulation is achieved by a layer of marks added onto the DNA that govern selective access to the genetic information and are perpetuated in differentiated tissues. The specific molecular mechanisms that function interactively

Fig. 7.1 Sources of interindividual variation in the epigenome. Environmental influences on the epigenome are likely most important during establishment of the epigenetic marks in prenatal and early postnatal development. (Reprinted with permission from [31])

to heritably regulate chromatin conformation include DNA methylation, various modifications of the histone proteins that package DNA in the nucleus, and auto-regulatory DNA binding proteins [31]. Interindividual variation in epigenetic states provide a basis for epidemiologic studies (Fig. 7.1).

Many studies of epigenetics in DOHaD have focused on imprinted gene regions. Genomic imprinting is the epigenetic silencing of one allele of specific genes by DNA methylation in a parent-of-origin-specific manner. Imprinted genes normally transcribe and express either the maternal or the paternal allele, while the remaining allele is silenced by DNA methylation of CpG islands in the promoter region. Loss of imprinting results in the aberrant biallelic expression of an imprinted gene. In humans, approximately 100 imprinted genes have been identified. Since most imprinted genes play a role in intrauterine and early-life growth, they have been proposed as good candidates to translate early nutritional and environmental influences into fetal development. Moreover, loss of imprinting of fetal growth genes, in particular *IGF2*, has been associated with childhood disorders such as Beckwith-Wiedemann Syndrome [32, 33], Silver-Russell Syndrome [34], and Wilms' Tumor [35, 36], as well as with adult-onset diseases [37, 38]. However, imprinted genes have not convincingly been found to be particularly susceptible to early developmental influences in the few informative human studies that have been conducted [39].

Importantly, the epigenome is established at crucial developmental time points that coincide with vulnerable periods of adaptive plasticity. In the mouse model, the parental epigenome is largely erased during gametogenesis, followed by epigenetic reprogramming as primordial germ cells differentiate into oocyte and sperm [40]. After fertilization, the non-imprinted gene regions in the zygotic genome undergo another round of epigenetic reprogramming that restores totipotency. This permits cell fate commitment of the first cell lineages during the preimplantation phase facilitated by a genome-wide *de novo* methylation [41–44] (discussed in more detail in Chap. 6). These dramatic waves of epigenetic reprogramming likely make early embryonic

development a critical period during which nutritional, environmental, and metabolic factors affect the developmental establishment of epigenetic regulation.

To understand the role of epigenetic mechanisms in DOHaD, defining the window of susceptibility is crucial. Based on the mouse model, the time point of *de novo* methylation differs for imprinted and non-imprinted genes and for the female and male germline [42] (discussed in more detail in Chap. 6). The DNA methylation signature of non-imprinted genes may be most amenable to environmental stimuli after fertilization, when the totipotent blastocyst becomes remethylated prior to implantation. Nutritional and metabolic factors early during the first trimester therefore may have the greatest potential to disrupt the introduction of cytosine methylation. The timing of remethylation of imprinted genes is less clear. By extrapolation from the mouse model, cytosine methylation of the differentially methylated regions (DMRs) of one of the two parental chromosomes is established at different time points for different imprinted genes [45–47]. In the mouse, maternal imprints are established at some point between oocyte development and ovulation [47], and paternal imprints are completed by the time spermatocytes enter meiosis [48]. Whether the establishment of imprinting marks is similar in humans remains to be established.

While stimuli in the periconceptional environment may affect several tissues if epimutations emerge prior to cell differentiation, perturbations during late gestation are more likely to induce tissue-specific epigenetic changes [31]. Further, epigenetic development is not limited to prenatal life; for example, the early postnatal period appears to be a critical period for establishment of DNA methylation in the brain [49].

We have previously proposed two mechanisms to explain environmental influences on the developmental establishment of DNA methylation [31]. First, an imbalance in dietary methyl donors and/or activity of DNA methyltransferases may affect developmental establishment of DNA methylation. Metastable epialleles show large interindividual differences in DNA methylation and gene regulation and appear particularly labile in response to environmental stimuli during developmental establishment of the epigenome. While most transposable elements in the mammalian genome are silenced by CpG methylation [50, 51], some are metastable and can also affect expression of neighboring genes [52].

Secondly, nutritional or environmental stimuli may alter transcriptional activity during periods of *de novo* DNA methylation, which may permanently alter epigenetic regulation and corresponding phenotypes. Genes actively transcribed during *de novo* methylation are protected from methylation and remain hypomethylated [53]. Interference with active transcription renders these promoters susceptible to *de novo* methylation and alters their function [54].

The placenta's critical role in nutrient transfer from mother to fetus makes it particularly vulnerable to adverse intrauterine conditions. Whereas induced epigenetic changes in the soma persist to influence later phenotype, maternal nutrition may also induce epigenetic changes in the placenta, affecting nutrient transport and fetal growth [25]. Imprinted genes are highly expressed in the placenta, which may make them vulnerable to variation in maternal nutrition [55, 56].

7.4 First Clues from the Agouti Mouse

Seminal experiments with the agouti viable yellow (A^{vy}) mouse model support the importance of maternal nutrition for developmental epigenetics [57]. The *agouti* gene codes for yellow pigment in fur. Transposition of an IAP retrotransposon upstream of *agouti* resulted in the A^{vy} metastable epiallele. DNA methylation of the retrotransposon exhibits spontaneous interindividual variation, controlling expression of the *agouti* gene and therefore the coat color of A^{vy} mice. Moreover, supplementation of mouse dams during pregnancy with the dietary methyl donors and cofactors folic acid, vitamin B12, betaine, and choline shifts the coat color distribution of A^{vy} heterozygous offspring from yellow to brown [58] by increasing DNA methylation at the A^{vy} locus [57].

Similarly, supplementation of dams with the phytoestrogen genistein resulted in an analogous coat color shift also mediated through A^{vy} hypermethylation [59]. Maternal methyl donor supplementation studies in another murine metastable epiallele model, the *axin fused* mouse, essentially corroborated the findings in the A^{vy} model [60], indicating that epigenetic regulation at metastable epialleles is generally susceptible to early environmental influences. Putative metastable epialleles were recently identified in humans [61]; as in the mouse models, these human loci showed dramatic and systemic interindividual epigenetic variation that was influenced by maternal environment around the time of conception.

7.5 Epigenetic Epidemiology Data on DOHaD

Data from the Dutch Famine suggest that individuals prenatally exposed to the famine had somewhat lower DNA methylation of the IGF2 genes six decades later compared to their unexposed siblings [62]. In this study of 60 same-sex sib pairs, the authors examined five CpGs in the *IGF2*DMR0 and found in average 2.7% lower methylation among individuals exposed to the famine *in utero*. Whether this small difference in methylation has any functional consequence remains unclear, in particular since the authors did not examine IGF2 expression levels. Methylation differences of even smaller magnitude were observed for some other genes including *IL10, GNASAS, INSIGF, LEP*, and *MEG3* [63].

The association between an epigenetic difference assessed in adulthood and a prenatal exposure does not allow causal inference about the induction of that change by the prenatal factor, unless the change is already present directly after the exposure period [28, 31]. Of course, collecting relevant samples in humans to test such concepts is logistically challenging.

A few studies have considered prenatal exposures and gene-specific DNA methylation at birth. Self-reported high maternal folic acid supplementation (defined as an average 1,200 μg/day or more) was associated with 2.4% lower methylation at the *H19* differentially methylated region (DMR) in umbilical cord blood leukocytes if initiated before pregnancy and 3.7% less methylation at that locus if initiated

during pregnancy, compared to non-users; no difference was found for the *IGF2*DMR0 [64]. A study from the Netherlands reported a 4% higher methylation level in one CpG of *IGF2*DMR0 in the blood of 17-month-old children whose mothers took 400 μg folic acid during pregnancy compared to those whose mothers took no folic acid supplements; no difference was found for four other CpGs, however, and *IGF2* expression levels were not examined [65]. Prenatal exposure to tobacco smoke was associated with marginally lower methylation in AluYb8b retrotransposons (but no difference in LINE-1 methylation) and slightly higher methylation in *AXL* and *PTPRO* in young children [66]. Higher maternal bone lead, but not cord blood lead levels, were associated with marginally lower methylation at *Alu* retrotransposons in cord blood DNA of Mexican infants [67].

A number of studies have explored the epigenetic profile of newborns with low birthweight or intrauterine growth retardation (IUGR). Using a DNA methylation microarray approach, Einstein and colleagues compared cord blood samples from five IUGR and five normal pregnancies and identified methylation differences at a restricted number of loci [68]. A few small studies identified differences in methylation or expression of selected imprinted genes in placenta and cord blood of IUGR or low birthweight compared to normal-weight infants [69–72] and in selected non-imprinted genes [73–75]. High birthweight has been associated with increased promoter methylation of the glucocorticoid receptor gene in human placenta [68–72, 76]. Overall, differences in methylation in these studies were small.

In one recent study, CpG methylation of 68 CpGs in five candidate genes was assessed in umbilical cord tissue from healthy neonates in two prospective cohorts [77]. DNA methylation of one CpG was consistently associated with adiposity at age 9 years but statistical analyses were not adjusted for multiple comparisons.

In summary, it remains unclear whether small differences in DNA methylation at birth associated with intrauterine exposures have functional relevance and are maintained into adulthood. No data are available on perinatal perturbation and epigenetic modifications, which translate into adult disease. Whether the intrauterine environment affects adult disease susceptibility in humans through epigenetic mechanisms remains to be established.

7.6 Intrauterine Exposures and Transgenerational Epigenetic Inheritance

DOHaD is mostly concerned with intrauterine experiences that affect adult disease susceptibility. Much interest in epigenetics has centered around the possibility of transmitting effects of environmental exposures across generations [78]. There has been considerable confusion in the literature about the difference between intrauterine exposures that affect the epigenetic profile in the offspring and transgenerational epigenetic inheritance [79, 80]. If environmentally induced epigenetic changes are inherited transgenerationally, then by definition the putatively inherited epigenetic change must be present in both the contributed gametes and the soma of the offspring.

For epigenetic marks to be inherited, they would have to escape erasure during developmental reprogramming of the epigenome. Such incomplete erasure has been described for epigenetic silencing of the A^{vy} allele [81]. IAP transposons seem to resist developmental reprogramming in the mouse, and their influence on the expression state of neighboring genes may be passed on to subsequent generations [82]. Genomic imprinting is another example of transgenerational epigenetic inheritance in mammals including humans. Following clearance of imprinting marks in primordial germ cells, parents-of-origin-specific allelic methylation and expression of imprinted genes is transmitted from parent to offspring in every generation. The molecular marks insuring such memory remain to be identified. It has been suggested that histone modifications or other marks, perhaps microRNAs, at imprinted gene loci are only incompletely erased and provide signals for *de novo* methylation at DMRs [40].

Whether transgenerational inheritance of acquired epigenetic changes exists in humans remains elusive. These issues are discussed in more detail in Chap. 6.

7.7 Outlook

Future epidemiologic studies exploring the role of epigenetics in DOHaD may target the effect of maternal nutrition on the epigenome of the newborn. Furthermore, studies exploring the time periods of DMR establishment in human gametes will aid our understanding of the window of susceptibility. The NIH Roadmap Epigenomics project and other international epigenome mapping projects will provide important insights into the establishment of the epigenome in humans [83].

Studies on the epigenetic epidemiology of DOHaD have to rely on loci with large interindividual epigenetic variability in easily obtainable tissues of physiologic relevance, preferably at various ages [31]. Metastable epialleles are candidates because their epimutations make them particularly labile to environmental influences during development, accommodating change and contributing to plasticity. Since developmental establishment of DNA methylation at metastable epialleles occurs probabilistically, interindividual variation at these loci is particularly large and thus informative in epidemiologic studies [61]. Moreover, metastable epialleles are less tissue-specific than other epigenetic marks, making them logistically easier to study [57, 60].

7.8 Conclusions

The concept of DOHaD arose from epidemiologic studies. Developmental plasticity allows adaptation of the fetal metabolism to transient nutritional and environmental experiences, which can result in lasting changes in chronic disease susceptibility. While our understanding of the underlying mechanisms is rudimentary, alterations

in the epigenome are likely contributors. We must emphasize, however, that epigenetics is only one of several potential mechanisms explaining developmental plasticity [28]. A better understanding of the mechanisms contributing to DOHaD may make it possible to reduce risk of disease by both preventive strategies targeted at early life, and corrective approaches designed to normalize malleable molecular mechanisms set askew by early adverse exposures.

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Chapter 8
Epigenetics and Assisted Reproductive Technology*

Nicole Banks and James H. Segars

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Abstract With increasing use of assisted reproductive technology (ART), concern has arisen over possible risks and long-term health implications of ART. Studies in animals have revealed epigenetic alterations associated with ART, including altered DNA methylation after embryo manipulation, ovarian stimulation, and in vitro embryo culture. Of concern, studies in humans have suggested an increased prevalence of imprinting disorders in children born after ART, particularly Beckwith-Wiedemann syndrome (BWS) and Angelman syndrome (AS). Moreover, children

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conceived using ART and affected by BWS or AS are more likely to have methylation abnormalities as the underlying molecular cause of the syndrome compared with affected offspring born after natural conception. Fortunately, both BWS and AS are very uncommon and the absolute risk remains low. Large prospective cohort studies of children conceived using ART are needed to better delineate long-term effects of potential epigenetic alterations possibly associated with ART.

Abbreviations

AS	Angelman Syndrome
ART	Assisted reproductive technology
BWS	Beckwith-Wiedemann syndrome
CpG	Cytosine-phosphate-guanine
DMD	Differentially methylated domain
DMRs	Differentially methylated regions
ICR	Imprinting control region
IVF	In vitro fertilization
ICSI	Intracytoplasmic sperm injection
IUI	Intrauterine insemination
LOS	Large offspring syndrome
PWS	Prader-Willi syndrome
PP	Proximal promoter

8.1 Introduction

Since the birth of the first "test-tube baby" in 1978, the use of assisted reproductive technology (ART) has grown exponentially. From 1990 to 2001, the annual number of ART cycles increased by 246% [1]. In 2007, 138,198 cycles of ART were performed in the United States alone, with the birth of 54,656 infants [2]. With increasing use of ART, concerns over possible risks and long-term health implications have also risen.

Animal models have suggested that embryo culture media can affect non-genomic inheritance, lending biologic plausibility to the concern that ART may increase the risk for imprinting disorders [3–5]. The first study to draw attention to a potential link between ART and imprinting disorders was a report by Cox et al. in 2002 [6] of two children born with Angelman syndrome (AS) after intracytoplasmic sperm injection (ICSI). Since 2002, several studies have suggested a higher prevalence of Beckwith-Wiedemann syndrome (BWS) and AS among children born after ART. In this chapter, we examine the current evidence regarding the possible association of imprinting disorders with ART.

8.2 Imprinting

Genomic imprinting is an epigenetic process which regulates gene transcription and may lead to expression of only one allele of a particular gene in a parental-specific manner. Epigenetic mechanisms are reviewed elsewhere in this book in detail, but two genomic mechanisms relevant to published studies involving ART are DNA methylation and histone modification. Sex-specific methylation patterns in gametes occur at DNA sites called differentially methylated regions (DMRs). Gene imprinting was first studied using mouse nuclear-transplant studies in which mouse embryos created with two female pronuclei or two male pronuclei failed to survive in utero [7–9]. Embryos derived from female pronuclei showed near-normal embryos, with abnormal extraembryonic tissue; conversely, embryos derived from male pronuclei showed poorly developed embryonic tissue, but robust development of extraembryonic tissue [7, 9–11]. These studies suggest that for normal embryogenesis to occur, both a maternal and a paternal genome were required. Currently, more than 80 imprinted genes have been identified in mammals, approximately half of which occur in humans [12]. Most imprinted genes are involved in embryonic and extraembryonic growth and development. Ten syndromes are known to be caused by imprinting defects in humans, including the most common conditions BWS, Prader-Willi syndrome (PWS), and AS.

ART interventions have the potential to affect epigenetic processes at multiple stages of gametogenesis and embryo development (Fig. 8.1). In particular, epigenetic reprogramming of DNA methylation occurs during germ cell and preimplantation development when ART could theoretically disturb the normal conception process [13, 14] (reviewed in [15]). Based on data in animal models (see below) there is concern that ART procedures performed on human embryos may also be vulnerable to manipulations. ART manipulations of conception include ovarian stimulation, in vitro maturation of oocytes, the use of ICSI, the use of immature sperm, in vitro culture of embryos, and cryopreservation of both embryos and gametes. A key factor is the *timing* of the manipulation relative to the erasure and establishment of imprinting marks. For instance, oocytes are vulnerable because re-establishment of methylation marks occurs just before ovulation, thus ovarian stimulation in the course of an ART cycle could conceivably affect oocyte imprinting. Likewise, imprinting marks undergo erasure actively in the male haplogenome and passively in the female haplogenome during early embryogenesis, but there is remethylation of the zygotic genome around the time of implantation. Thus, the timing of embryo manipulations at ART coincides with demethylation and remethylation normally occurring in the embryo [15].

A second equally important consideration is that some cells in the embryo appear to be more susceptible to alteration in imprint marks during in vitro manipulation. For example, the placenta exhibited a greater loss of imprinting than did the embryo in a mouse model of in vitro culture [16, 17]. This observation is noteworthy given the transgenerational effects of altered epigenetic marks in the placenta in animals ([18] and references therein) and their relevance to human diseases [19, 20]. Concern

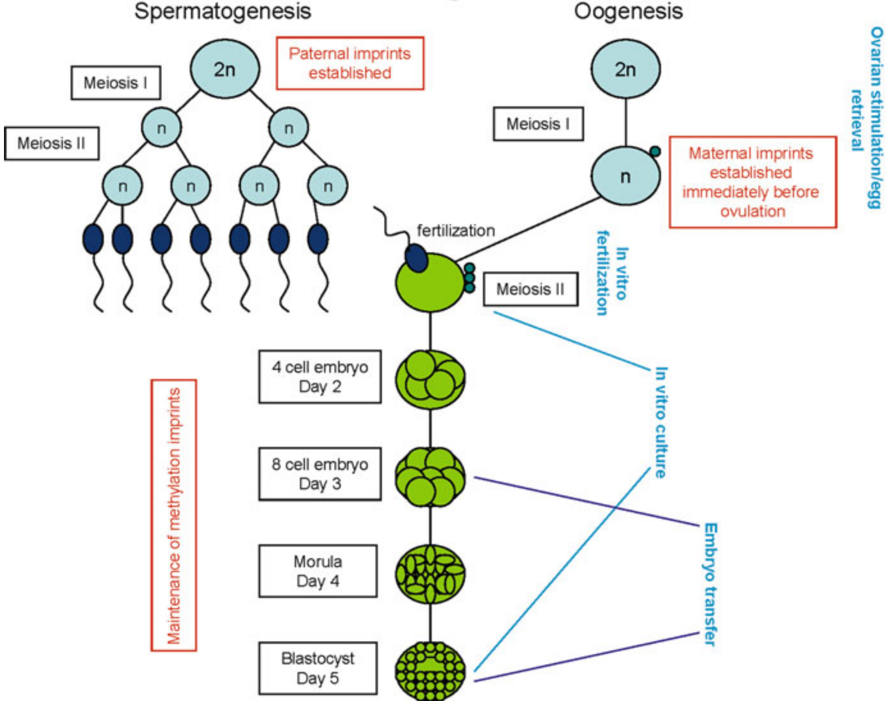

Fig. 8.1 ART influence on gametogenesis and preimplantation development. Steps in meiosis are shown schematically for sperm and oocytes. Maternal imprints finalized before ovulation. Normally, paternal imprints are established very early in spermatogenesis, and are not vulnerable to influences of ART. Following fertilization, imprint marks undergo differential demethylation for each haplogenome, parternal and maternal, and remethylation of the zygote genome occurs around the time of implantation

that ART might alter placental epigenetic marks might seem irrelevant since the placenta is discarded at birth, but even transiently altered placental function theoretically could affect future generations [21]. Nonetheless, the evidence in humans is scant and the concern is inferred, based on results in animal studies. Next, we discuss the biologic plausibility and expand the discussion and examine the evidence for effects of specific ART procedures on imprinting.

8.3 Biologic Plausibility

Many animal and some human studies have been conducted to address how various ART manipulations might affect gene imprinting. Strong evidence from animal studies indicates that in vitro culture can affect epigenetic inheritance. There is

evidence that hormonal stimulation and/or ovarian stimulation may also lead to methylation abnormalities. In addition to DNA methylation and histone modification, other mechanisms of imprinting might be affected by ART manipulations, but evidence for effects of ART on other mechanisms of epigenetic inheritance is sparse, particularly in human offspring. Finally, a few recent studies in humans suggest more widespread altered methylation patterns in children born after ART and support the notion of biologic plausibility (reviewed in Ref. [21]).

8.3.1 In Vitro Culture and Culture Media

The role of in vitro culture in abnormal gene imprinting has been investigated in several animal studies. Cattle born after in vitro culture of embryos are known to develop large offspring syndrome (LOS). Cattle affected by LOS are large at birth, have skeletal anomalies and organomegaly, and suffer from neonatal respiratory distress and increased rates of sudden neonatal death [22]. Sheep cultured in vitro with LOS showed hypomethylation leading to expression of *IGF2R* [23]. Zaitseva et al. [5] performed a study on mouse embryos and found significantly higher methylation levels in two cell embryos cultured in vitro, compared with embryos produced in vivo. The same study also showed a greater degree of demethylation in male pronuclei from in vivo-derived embryos compared to embryos cultured in vitro. This result differed from female pronuclei, which showed no difference in methylation whether developed in vitro or in vivo. The sex-based difference supports the hypothesis that altered patterns of methylation following in vitro culture could arise during culture.

Animal studies have also revealed that different embryo culture media differentially affect imprinting patterns of imprinted genes. One study of mouse embryos detected loss of methylation at *H19* in embryos cultured with Whitten's culture media, but not in embryos developed in KSOM+AA media [3]. Despite hypomethylation of *H19* in Whitten's culture media, no difference in DNA methyltransferase activity or distribution of *DNMT1* proteins was observed. Another study of growth-related imprinted genes in mice compared embryos produced in vivo to embryos cultured in either M16 media or M16 media with fetal calf serum [4]. Significant differences in gene expression were detected between the three groups. Collectively, these studies indicate that embryo culture media has the potential to affect gene imprinting.

8.3.2 Hormonal Stimulation

Ovarian stimulation is a very common practice in infertility treatment and is generally used in most in vitro fertilization (IVF) and ICSI cycles. In a study comparing two cell mouse embryos from superovulated mice and nonsuperovulated mice, a

higher incidence of methylation abnormalities in embryos was detected in mice that received ovarian stimulation [24]. This observation suggests that hormonal stimulation of the ovaries may affect gene imprinting. Market-Velker et al. [25] studied superovulation in a mouse model using embryos fertilized in vivo to examine effects of in vitro manipulations. In comparing superovulated mice versus control mice, loss of methylation was observed on the maternal allele at *SNRPN*, *PEG3*, and *KCNQ1OT1*. This effect was dose dependent and statistically significant at the higher dosage of hormone stimulation. Gain of methylation was present in the *H19* gene, but again in a dose-dependent manner that was significant at the "high dose." Interestingly, both the maternal and paternal *H19* alleles were perturbed by superovulation suggesting that maintenance of imprinting after fertilization may be affected. The authors [25] suggest that superovulation affects oogenesis in two ways, first by disrupting the acquisition of imprints in growing oocytes, and second by altering maternal gene products required for imprint maintenance during preimplantation development. Consistent with this observation, a human study suggested that hormonal stimulation of the ovary affected gene imprinting leading to a loss of methylation at *PEG1* and a gain of methylation at *H19* in superovulated immature human oocytes [26].

Fauque et al. [27] studied the effects of superovulation, IVF, and embryo culture media (M16 versus G1.2/G2.2) on the methylation status of the *H19* imprinting control region (ICR) and *H19* proximal promoter (PP). The study also tested gene expression level of *H19* in the blastocysts for the various conditions. The authors showed that methylation defects occurred only in the IVF group and the defects were more common when the embryo was cultured in M16 medium. Moreover, superovulation significantly disrupted gene expression of *H19* in blastocysts. The culture media again affected the degree of disruption, with *H19* gene expression higher in blastocysts cultured in G1.2/G2.2 media [27]. Taken together, these studies suggest that hormonal stimulation of ovaries may affect oocyte imprinting.

8.3.3 ICSI

Use of ICSI has increased dramatically in recent years and the proposed indications for ICSI are rapidly expanding. The ICSI procedure per se has not been definitively shown to play a direct role in imprinting disorders. Use of immature sperm is unlikely to affect paternal allele imprints because the imprints are normally established early in spermatogenesis [28, 29]. However, it is possible that abnormal spermatogenesis per se is associated with epigenetic anomalies. A study analyzing methylation patterns of *H19* in sperm found an association between low sperm counts and methylation abnormalities [30]. Specifically, 0%, 17%, and 30% of normozoospermia, moderate oligozoospermia, and severe oligozoospermia respectively showed altered *H19* methylation patterns. Another study found that 14% of infertile men had abnormal paternal imprints in their sperm and 20% had abnormal maternal imprints [31]. Kobayashi et al. [32] collected 78 aborted samples from IVF/ICSI procedures and examined seven autosomal imprinted genes and the X-linked *XIST* gene and matched parental sperm.

The study found 17 cases of abnormal DNA methylation [32]. Seven of the 17 cases (41%) had identical alterations in the parental sperm, suggesting that some imprinting errors associated with ART were inherited from the father. Thus, it does appear that abnormal spermatogenesis is associated with epigenetic changes and these changes may be heritable, but no direct effects of ICSI have been detected.

8.3.4 Embryo Manipulation and General Effects

Physical embryo manipulation has been shown to affect DNA methylation status in the mouse model. Embryo manipulation limited to blastocyst collection and culture for 1.5 h or less was correlated with a loss of methylation at the maternal *KvDMR1* locus [17]. The same study found that in vitro culture of embryos from the two cell stage to blastocysts resulted in further methylation abnormalities.

A recent human study analyzed DNA methylation patterns of patients delivered after IVF versus natural conception. Katari et al. [33] studied the methylation patterns of 10 children born after IVF and 13 children conceived in vivo. The investigators used a custom-designed methylation bead-array platform with probes for 1536 cytosine-phosphate-guanine (CpG) sites located in the promoters of more than 700 genes to compare DNA methylation of placenta and cord blood of the subjects and controls. The study found that in vitro conception was associated with lower mean methylation at CpG sites in placenta and higher mean methylation levels in cord blood. Of the CpG sites where mean methylation levels differed in placenta, 63% showed reduced methylation in children born after IVF. For cord blood, 77% of discordant CpG sites showed increased methylation in post-IVF children. The study also attempted to examine gene expression by selecting a sampling of genes for analysis of transcription activity and found that methylation differences in post-IVF children were associated with differences in gene expression at imprinted and non-imprinted genes. The authors suggest [33] that their findings could be explained by pre-existing differences in the gametes of couples undergoing IVF treatments, or alternatively, the differences could reflect changes caused by global patterns of DNA methylation and gene expression due to ART. The study does not address what ART procedures may be the cause of global DNA methylation changes.

8.3.5 Cryopreservation and Vitrification

Currently there are very limited data regarding the possible effects of cryopreservation of embryos or gametes on epigenetic imprints during ART. A recent study in a murine model focused on H19/Igf2 differentially methylated domain (DMD) reported that in vitro culture resulted in epigenetic changes in embryos, consistent with findings of previous investigators, but the process of embryo vitrification significantly augmented the loss of methylation of the H19 DMD [34]. Again, the

placenta appeared to be more affected than the embryo. Despite these data to suggest the process of cryopreservation alters imprint marks in animals, human offspring resulting from cryopreserved embryos have a better neonatal outcome than human offspring after a "fresh" ART cycle ([35], and references therein). The methods of embryo cryopreservation at ART have undergone considerable refinement in recent years and it is worth noting that procedural methodology involved in cryopreservation could greatly influence findings, therefore more studies are needed.

8.3.6 Histones

Very little evidence is available regarding histone modification and ART. One study examined histone modification in murine embryos, but found no difference in histone modification patterns between in vitro fertilized and in vivo fertilized embryos [36].

8.3.7 Current Understanding and Limitations of Existing Knowledge

There is overwhelming evidence from well-designed, rigorously controlled studies of ART procedures in "normal" animals that the processes associated with ART can influence gene imprinting, leading to specific syndromes and phenotypes. Furthermore, evidence in animals clearly demonstrates that different cell types have a differential susceptibility to alteration of imprinted genes. The stress of ART (Fig. 8.2) can be considered similar to environmental stresses that are well-described, but in general, understanding of the mechanisms mediating altered expression on imprinted genes as a direct result of ART procedures are not as advanced as understanding of the mechanisms of chemical or environmental toxins. Understanding of the molecular and cellular processes involved in re-programming, and more specifically the fidelity and preservation of the imprinted marks during their erasure and re-establishment, is incomplete.

While there is no logical reason to assume the human embryos would be immune to the effects of in vitro culture, there are clear species-dependent differences in vulnerability to the alteration of imprint marks. In general, the evidence in humans is not as compelling as in other species, and several additional conditions may confound the relationship. For instance, unlike experiments in animals, ART is not generally conducted on normal controls (other than oocyte donors), and some data suggest the diagnosis of infertility might be associated with altered imprinting. Analysis of imprinted genes in groups such as oocyte donors or "natural cycle" IVF would be of interest, since such studies might control for factors such as infertility and ovarian stimulation, respectively. Based on current evidence, there *does* appear to be an effect of in vitro culture or ART procedures upon imprinted genes. More specifically, a slight effect on a number of imprinted genes has been detected in some ART offspring [33], although the degree of the risk and consequences of this

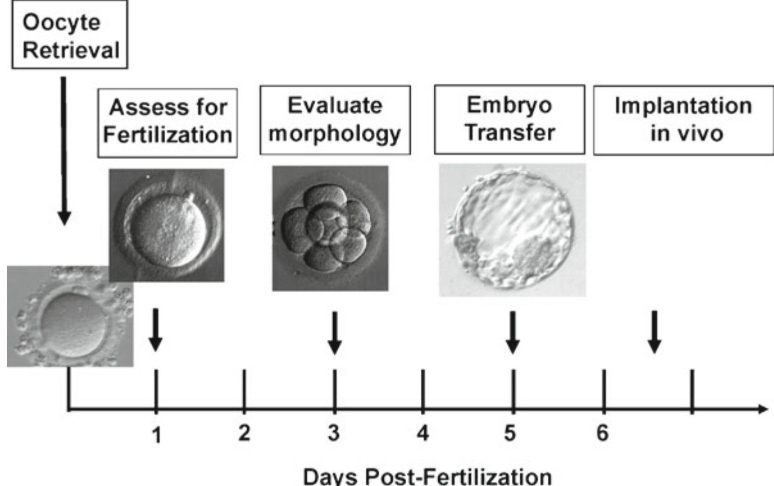

Fig. 8.2 Stages of early embryo development during assisted reproduction. Oocytes are retrieved following stimulation and are evaluated in the laboratory. Following fertilization by either addition of sperm (in vitro fertilization) or ICSI, fertilization is assessed on day 1 following oocyte retrieval (*first down arrow* from the *left*). On day 2 or 3 of culture embryos are re-assessed for development (*second down arrow*), and either transferred to the endometrial cavity, or maintained in culture until day 5, when blastocyst development has occurred (*third down arrow*). Embryos are transferred to the endometrial cavity and implantation occurs in vivo (*fourth down arrow*). Epigenetic imprints are normally completed in oocytes just prior to retrieval (day 0). Epigenetic imprints in male gametes normally occurs in the gonadal ridge and have been completed well before use of sperm for fertilization (evening of day 0). Imprinting marks undergo erasure actively in the male haplogenome and passively in the female haplogenome during early embryogenesis (day 1–5). The timing of embryo manipulations at ART coincides with re-programming of epigenetic marks that occur during early embryo development

level of alteration in imprinting marks is not clear [21]. Two questions before the field are: how often are epigenetic marks altered due to ART, and what, if any, are the long-term consequence of any alterations that may occur?

Based on the analysis of specific diseases, such as Beckwith-Wiedemann Syndrome (BWS) as discussed in the next section, the *absolute level of risk* for profoundly affected offspring is very low. Comparison of ART offspring with BWS, to spontaneously occurring BWS cases where ART was not performed, reveals there is often similarity in the mechanism involved, but notably, loss of methylation of the maternal allele is more frequent in the ART-conceived offspring [15]. A significant current limitation in the level of evidence is that most studies in humans have reported differences in gene imprinting, but have not proven the case by showing altered levels of mRNA transcripts and/or protein expression. Another limitation is that studies have focused primarily on DNA methylation, and other possible epigenetic mechanisms, such as miRNAs, have not been assessed.

In the following sections we examine the association of ART with specific syndromes. Because of the rarity of imprinting disorders that arise spontaneously, many reports are either retrospective observational or case-control design. Accordingly, the

strength of association between ART and the specific condition in question often hinges upon appropriate control groups, absence of recall and ascertainment bias, appropriate control for confounding factors, and adequate statistical power. Unfortunately, many studies have weaknesses that bring into question many of these important considerations, and thus weaken the level of certainty between the specific condition and ART.

8.4 Beckwith-Wiedemann Syndrome

Beckwith-Wiedemann syndrome is a congenital overgrowth syndrome that occurs in approximately one in 13,700 births [37]. Children with BWS exhibit macroglossia, macrosomia, midline abdominal wall defects, neonatal hypoglycemia, as well as other developmental anomalies [38]. BWS is also notable for an increased risk of developing embryonal tumors in childhood, most commonly Wilm's tumor, rhabdomyosarcoma, and hepatoblastoma [39].

BWS is a multi-genic disorder with both known genetic and epigenetic causes. Paternally expressed genes *IGF2* and *KCNQ1OT1* and maternally expressed genes *H19, CDKN1C*, and *KCNQ1* at the 11p15 imprinted region are implicated in BWS [40]. *H19* and *IGF2* are usually jointly regulated by *DMR1* to assure that *H19* is only expressed from the maternal allele and *IGF2* is only expressed from the paternal allele. One cause of BWS involves hypermethylation at the maternal *DMR1*, leading to silencing of the maternal *H19* and bi-allelic expression of *IGF2* [41]. Another cause implicates the imprinting center *KvDMR1,* which controls *KCNQ1OT1* (also known as *LIT1*), *KCNQ1*, and *CDKN1C* expression. Loss of maternal methylation at *KvDMR1* leads to biallelic expression of *KCNQ1OT1* and decreased expression of *CDKN1C*. Approximately 50–60% of spontaneous BWS cases are due to loss of maternal methylation at *KvDMR1* [41]. There is an increased incidence of BWS in monozygotic twins, with the majority of twins being discordant for BWS [42, 43]. The most common defect among these discordant twins was an imprinting defect at *KCNQ1OT1* [42, 43]. Because of this, it has been hypothesized that *KCNQ1OT1* is especially vulnerable to imprinting errors during the preimplantation developmental period, a stage frequently involved in manipulation during ART [43].

Concerns for an association between BWS and ART first arose with the publication of three case series from BWS registries in the United States and Europe in 2003 [44–46]. DeBaun et al. [44] reported a case series of seven offspring with BWS after ART (two IVF and five ICSI). The authors calculated the prevalence of ART in the BWS cohort as 4.6%. This included data after 2001 only, because there was no formalized data collection within the cohort regarding ART prior to that year. Compared to the 0.76% total live births after ART in the general population in 1999, this suggested a six-fold increased risk of BWS with ART. Five of six patients tested for the molecular basis of their disease were found to have an imprinting defect (Table 8.1), specifically hypomethylation of *LIT1* (*KCNQ1OT1/KvDMR1*).

Similarly, Maher et al. [46] reviewed 149 sporadic BWS cases in Birmingham, United Kingdom, and found six BWS children were born after ART (4%),

Table 8.1 Beckwith-Wiedemann syndrome in ART: prevalence and molecular defect

Author	Study type	Number of BWS cases	Prevalence of ART in cases (%)	Prevalence of ART in general population (%)	Percentage of BWS patients with *KvDMR1* hypomethylation[a]
Debaun et al. [44]	Case series	65	4.6	0.76	83.3
Maher et al. [46]	Case series	149	4	1.2	100
Gicquel et al. [45]	Case series	149	4	1.3	100
Halliday et al. [47]	Case-control	37	10.81	0.67	100
Lidegaard et al. [55]	Cohort	0	0	1.3%	0
Chang et al. [51]	Case series[b]	341	5.6	NR	NR
Sutcliffe et al. [52]	Survey	209	2.9	0.8	100
Rossignol et al. [49]	Cohort[c]	11	27.5[c]	NA	100
Bowdin et al. [53]	ART cohort	4	NA	NA	25
Doornbos et al. [54]	Survey	71	5.6	0.92	100
Lim et al. [48]	ART case-control	24	NA	NA	96

NA not applicable based on study design, *NR* not reported

[a]Number of patients with *KvDMR1* hypomethylation/number of tested patients

[b]BWS registry

[c]Cohort with known imprinting defect at *KvDMR1*

significantly higher than the estimated 1.2% ART use in the general population. Two of the patients were tested for *KvDMR1* methylation and both showed a loss of methylation on the maternal allele. The third case series by Gicquel et al. [45] reported a series of 149 BWS cases in France. Six children from that cohort were born after ART (4%), as compared to 1.3% of the general population. Based on these data, an odds ratio (OR) of 3.2 (95% CI, 1.4–7.3) was calculated for the risk of BWS after ART. All six patients were tested for the molecular defect causing BWS and all demonstrated hypomethylation of *KvDMR1*.

One case-control study from Victoria, Australia, studied 37 cases of BWS and 148 non-BWS matched controls [47]. Using data from 1983 to 2003, all cases of BWS in Victoria were identified and for each BWS case, four live-born controls were randomly selected from babies born within 1 month of the index case with maternal parity of one and maternal age within 1 year of the BWS mother. In that study, four BWS patients were conceived via ART (10.81%) and only one infant from the control group was conceived via ART (0.67%); OR = 17.8 (95% CI, 1.8–432.9) [47]. Three of the patients born with BWS after ART were tested for and found to have *KvDMR1* hypomethylation, compared with only 46% of children with BWS conceived without ART.

Another case-control study analyzed the molecular genetic and clinical features of 25 cases of BWS born after ART compared with 87 BWS children conceived spontaneously [48]. The control group was composed of patients with *KvDMR1* loss of methylation (IC2 defect). Of the 25 post-ART BWS cases, 24 had loss of methylation at *KvDMR1* (no molecular cause was identified in one post-ART BWS case). A molecular analysis of DMRs (*ZAC, PEG1, SNRPN*, and *DLK1*) showed loss of maternal allele methylation in 37.5% of ART versus 6.4% of non-ART BWS patients with IC2 defects. The authors concluded that wide-spread hypomethylation of DMRs was more common in post-ART BWS cases than in non-ART BWS patients with IC2 defects. Additionally, this study examined clinical differences between post-ART and non-ART BWS patients and found a significant difference in incidence of facial nevus flammeus, exomphalos and neoplastic lesions. Post-ART BWS patients were more likely to have facial nevus flammeus (90 vs. 46%, $p = 0.0004$) and less likely to have exomphalos (43 vs. 69%, $p = 0.029$). Of the controls, no children developed a neoplasm, but two of the BWS post-ART cases developed an embryonal tumor, one developed a hepatoblastoma and the other a rhabdomyosarcoma, a significant difference ($p = 0.0014$) [48].

Rossignol et al. [49] analyzed the methylation status of 40 patients with BWS and known hypomethylation of *KvDMR1* in three additional DMRs but the findings differed from those of Lim et al. [48]. Eleven patients were born after ART and 29 were conceived naturally [49]. The authors found that 27% of ART-conceived patients and 24% of naturally conceived patients had hypomethylation of at least one additional locus. The authors concluded that the involvement of other loci in hypomethylation in BWS patients was not restricted to those conceived with ART. Additionally, a cohort study from Italy and the Netherlands of 149 BWS patients identified 17 with hypomethylation of multiple imprinted loci [50].

Only one of the 17 patients was born after ART, thus the authors concluded that the use of ART was not the cause of multi-local hypomethylation that has been identified in some BWS patients.

Chang et al. [51] reviewed the same BWS registry used by DeBaun et al. of 341 patients and identified 19 children with BWS born after ART (including ovulation induction and artificial insemination). Twelve of the patient's records were obtained and showed five IVF pregnancies, five ICSI pregnancies, and two with ovulation induction (one gonadotrophin with intrauterine insemination [IUI] and one clomiphene citrate with artificial insemination). The authors reviewed the treatment data for each of the 12 patients to determine what ART treatments might be associated with BWS. There were no observed associations between cause of infertility, type of culture media, type of ART procedure, or day of embryo transfer and BWS. The only commonality in treatment among the 12 patients was the use of ovarian stimulation medications [51].

A study from the United Kingdom was published in 2006 based on a survey of infertility treatments in 213 families with children affected by BWS [52]. Eighty-three responses were received and four familial cases were excluded. The survey found that six BWS children were conceived with IVF or ICSI, for a calculated prevalence of 2.9% of ART use in children with BWS, using the 209 eligible surveyed families as the total number of BWS cases. This was significantly higher than the 0.8% prevalence of children in the general population born after IVF/ICSI during the same time frame. Of the 11 children born after any form of ART (one IVF, five ICSI, five ovarian stimulation medications), molecular analysis was performed on eight. The authors found that all eight children tested had loss of maternal methylation at *KvDMR1*. The study [52] concluded that there was an association between BWS and ART, but also observed that BWS occurred in less than 1% of patients conceived by ART.

Another British questionnaire-based cohort study examined all children conceived by ART from two United Kingdom ART treatment centers [53]. The questionnaire was designed to identify children with a possible diagnosis of BWS or AS. The study had a 61% response rate (1,524 responses out of 2,492). From the questionnaire, 70 children warranted further evaluation. Forty-seven agreed to an evaluation and one case of BWS was identified. This patient was tested for and had *KvDMR1* hypomethylation. The authors concluded [53] that there was a higher prevalence of BWS in children born after ART, but the absolute risk was less than 0.1%.

A questionnaire-based study in the Netherlands assessed families with children affected by imprinting disorders born between January 1983 and December 2003 [54]. The questionnaire addressed infertility and ART (including ovulation induction and artificial insemination). Of 71 eligible cases of BWS identified, six patients with BWS were born after ART (four IVF/ICSI, one IUI with donor sperm, one ovarian stimulation). All six cases were tested for and found to have hypomethylation of *KvDMR1*. The prevalence of BWS after IVF/ICSI was 5.6%, compared to 0.92% in the general Dutch population over the same time period for a relative risk of 6.1 for BWS and ART. However, the authors concluded [54] that after correcting

for infertility problems in parents there was no increased incidence of imprinting disorders (BWS, AS, or PWS) in children born after ART.

Two population based studies arrived at similar conclusions. A cohort study in Denmark from January 1995 to December 2001 used the National IVF registry (6,052 singletons) and the National Birth Registry (442,349 singletons) and found no cases of BWS [55]. The authors therefore did not identify an increased risk of BWS with ART. A Swedish population-based study from 1982 to 2001 tracked the presence of congenital malformations based on data from the Swedish Medical Birth Register, Swedish Register of Congenital Malformations, and the Swedish Hospital Discharge Register [56]. Of 16,280 children born after ART, no cases of BWS were identified [56]. Of note, cryopreservation of embryos in Scandinavia has recently increased, and it is helpful to bear in mind that embryo handling procedures and ART practices in general differ greatly between countries.

The consistent finding of hypomethylation at *KvDMR1* in most BWS patients born after ART raised the question whether clinically normal children conceived by ART might have a higher rate of subclinical methylation abnormalities. To address this question, Gomes et al. [57] tested 18 healthy children born after IVF/ICSI for methylation abnormalities in *KvDMR1*. Three of 18 children were found to have hypomethylation at *KvDMR1* (two IVF, one ICSI), compared to zero of 30 natural conception children used as controls. In contrast, Teirling et al. [58] studied 77 ICSI, 35 IVF, and 73 spontaneously conceived clinically normal children for methylation abnormalities at ten separate DMRs. Nine of the DMRs, including *KvDMR1*, showed no difference in methylation indices between ART and natural conception children. The *MEST* DMR showed a higher methylation index in IVF compared to ICSI and natural conception children. The authors concluded that children conceived by ART do not have a higher degree of imprint variability, but did note that the sample size was too small to exclude rare imprinting disorders [58].

In summary, several studies have examined the association of BWS and ART with conflicting findings. Seven studies among children with BWS showed a higher prevalence of ART use than in the general population (Table 8.1). Findings from large cohort studies were mixed. Two large national studies did not identify any cases of BWS. The third study reported a higher prevalence of BWS with the use of ART. Because BWS is a rare disorder, a large cooperative international study would be required to further study the prevalence of BWS in naturally conceived children and children born after ART. In particular, some findings may be subject to recall or ascertainment bias, because BWS may be underestimated in the general population but more frequently diagnosed in ART-conceived children. Virtually all (90–100%) of BWS cases after ART have imprinting aberration, specifically hypomethylation of the maternal allele at *KvDMR1* (Table 8.1). This is significantly greater than the 50–60% of natural conception BWS patients with the same defect. At this time, there is conflicting evidence whether widespread (generalized) hypomethylation occurs in children born after ART. Overall, the data suggest an association between ART and BWS but the prevalence in ART children is low at approximately one in 4,000.

8.5 Angelman Syndrome

Angelman syndrome is a neurogenetic disorder with a prevalence of approximately 1:12,000 in the general population [59]. It is characterized by severe mental retardation, frequent laughter, seizure disorder, and gait disturbances [60]. The genetic defect in AS is failure of maternal gene expression in the brain of the *UBE3A* gene, located at 15q11-13 [60, 61]. This deficiency can be caused by deletion of maternal *UBE3A* (68%), mutation (13%), uniparental disomy (3%), or imprinting defect causing under-expression of the maternal allele (6%) (Table 8.2) [15, 62]. Imprinting defects as a cause of AS are estimated to occur in 1:300,000 newborns [6].

Cox et al. [6] first raised concerns about AS and ART in 2002 by their report of two cases of AS after ICSI (for male factor infertility). Both affected children were tested and found to have hypomethylation of chromosome 15q11-13. This finding was alarming because of the rarity of AS due to an imprinting defect (1:300,000). A third case of AS after ICSI was reported in 2003. This patient was found to have a complete absence of methylated maternal pattern for the *SNRPN* locus [63]. The case was unique as the father had a normal semen analysis, but the mother and grandmother of the patient had significant reproductive difficulties.

Ludwig et al. [64] reported in 2005 that 20% of AS patients were born to subfertile couples. Using a questionnaire sent to 270 members of the German Angelman Syndrome Support group addressing infertility issues and treatments, the study identified 16 of 79 respondents as having subfertility. Of these 16 cases, four children were found to have imprinting defects. Three of the 16 cases were after ICSI, and only one post-ICSI AS child had an imprinting defect as the cause. The relative risk of an imprinting defect in a child with AS born to a subfertile couple was 6.25. Couples with subfertility who received infertility treatment to conceive had a relative risk of 12.5 of having an imprinting defect as the cause of their child's AS. The authors concluded [64] that imprinting disorders and subfertility may have a common cause and that superovulation, not ICSI, may further increase the risk of imprinting defects in offspring.

The previously mentioned British survey by Sutcliffe et al. [52] queried 384 families with children affected by AS. Eighty-one surveys were returned (21% response rate) and 75 sporadic cases of AS were identified. Three of these cases were born after ART (IUI, donor sperm artificial insemination). The patient born after IUI

Table 8.2 Molecular defect of *UBE3A* in Angelman syndrome

Molecular defect	Approximate percentage
Deletion of maternal *UBE3A*	68
Intragenic mutation on maternal allele	13
Imprinting defect	6
Uniparental disomy	3
Unknown/normal genetic testing	10

to a couple who had previously used IVF was found to have hypomethylation of the maternal *SNRPN* locus. A nationwide survey in the Netherlands [54] found 12 of 63 children with AS (19%) were born to parents with fertility problems, significantly more than the 5.6% of children in the general population. Three of the children were conceived with the use of ovulation induction medications (4.8%), compared to 0.39% of the general population. No cases of AS children born after IVF/ICSI were identified. The authors concluded [54] that there was not an increased risk of imprinting disorders with the use of ART after controlling for subfertility. A survey study of 2,492 children born after ART in the United Kingdom also failed to identify any cases of AS from the 1,524 responses [53]. The authors estimated the incidence in that population of AS from an imprinting defect to be 1:750,000 and determined that the study was not adequately powered to find a 50-fold increased risk.

A small prospective study of 92 healthy children born after ICSI analyzed methylation patterns at chromosome 15q11-13 [65]. A normal methylation pattern was found in all of the children studied. Another study published in 2008 assessed methylation patterns at chromosome 15q11-13 in 53 children born after ICSI, and also found no methylation abnormalities [66]. Both studies pointed out that due to the rarity of imprinting defects and AS, they lacked sufficient power to detect an increased prevalence of such defects.

In summary, seven reported cases of AS after ART were identified, and of these, five suggested an association of ART with altered imprinting. The rarity of an imprinting defect cause in AS, coupled with the high proportion of post-ART AS children with such an imprinting defect, suggests an association between AS and ART. Further study is needed to better examine the potential link between AS and ART.

8.6 Retinoblastoma

Retinoblastoma is a rare childhood malignant tumor of the retina. It occurs in approximately 1:17,000 children [67]. Retinoblastoma is most often caused by a mutation of the tumor suppressor gene *RB1* combined with a loss or deletion of the second allele [68]. Approximately 40% of cases are familial. Hypermethylation of the RB gene has been shown to play a role in retinoblastoma [69, 70]. A case series from the Netherlands reported five children with retinoblastoma conceived with ART between November 2000 and February 2002 [71]. The authors calculated an increased risk of 4.9–7.2. As methylation status was not studied, the authors could not comment on whether imprinting defects were involved. A national cohort study in Denmark of all singleton births from 1995 to 2001 found no cases of retinoblastoma among 6,052 singleton children born after IVF, followed for an average of 4.1 years [55]. The finding of five cases of retinoblastoma post-IVF is concerning due to the rarity of the disease; however, no other studies to date have shown any association between retinoblastoma and ART.

8.7 Conclusion

ART has been associated with an increased prevalence of the imprinting disorders BWS and AS in published reports. Epigenetic alterations due to ART are biologically plausible based on animal studies that have shown DNA methylation defects after embryo manipulation, ovarian stimulation, and in vitro embryo culture. Furthermore, a higher proportion of children born post-ART with BWS or AS have imprinting defects as the cause of the condition, compared to affected BWS or AS children not conceived using ART. Imprinting disorders are exceedingly rare, complicating study of the subject. The existing literature is limited by under-powered studies, and reliance on retrospective observational analyses and survey based studies, both vulnerable to bias. Despite these limitations, multiple case series and case-control studies have detected an association between BWS and ART, especially hypomethylation of *KvDMR1*. While not as extensively studied, five cases in the literature of AS caused by sporadic imprinting disorder raises concern due to the rarity of AS. Some recent reports suggest a generalized alteration in DNA methylation associated with ART, which, given the rarity of both BWS and AS, could obfuscate the findings. More studies are needed to explore whether infertility or any facet of ART treatment may increase the likelihood of diseases associated with imprinting.

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Chapter 9
Imprinting Disorders of Early Childhood

I. Karen Temple, Jill Clayton-Smith, and Deborah J.G. Mackay

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Abstract Imprinted genes are exceptional in that one allele is silenced (imprinted) in a parent of origin specific manner, making the two parental alleles functionally different. Imprinted genes are known to play a vital role in fetal growth and normal metabolism and most of the medical conditions caused by aberrant imprinting result in problems with growth, neurodevelopment and glycaemic control. There are eight known human disorders; Beckwith Wiedemann syndrome (fetal overgrowth, umbilical abnormalities and macroglossia), Silver Russell Syndrome (poor fetal and post natal

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growth and short stature), Prader Willi syndrome (hypotonia, developmental delay, excessive appetite and obesity), Angelman syndrome (severe developmental delay, no speech and ataxia), Transient Neonatal Diabetes (neonatal diabetes and low birth weight), Temple syndrome (hypotonia short stature and early puberty), Wang syndrome (hypotonia, developmental delay and small thorax) and Pseudohypoparathyroidism type 1B (hypoparathyroidism). At any of the imprinted loci involved in these conditions, abnormal gene expression is due to three mechanisms: epigenetic loss or gain of DNA methylation within control regions, uniparental disomy and structural chromosome abnormalities. The known prevalence of each condition depends on the distinctiveness of symptoms and thus ease of diagnosis and the susceptibility of the different loci to genetic and epigenetic aberrations; the reasons for the difference in susceptibility at different loci is not yet known. This chapter describes the imprinted loci and the medical consequences of aberrant imprinted gene expression.

Abbreviations

AS Angelman syndrome
BWS Beckwith Wiedemann syndrome
DMR Differentially methylated region
ICR Imprinting control region
PHP1B Pseudohypoparathyroidism type 1B
PWS Prader Willi syndrome
SDS Standard deviation scores
SRS Silver Russell syndrome
TND Transient Neonatal Diabetes
TS Temple syndrome
WS Wang syndrome
UPD Uniparental disomy

9.1 Introduction

Genetic imprinting is often considered a perplexing and complex phenomenon with limited practical application to mainstream paediatric diagnosis. However, the exquisite molecular mechanisms conserved across placental mammals to maintain tissue- and stage-specific expression of imprinted genes argue powerfully for their importance in normal human development.

9.1.1 Genetic Imprinting

Imprinted genes are exceptional in that one allele is silenced (imprinted) in a parent of origin specific manner, making the two parental alleles functionally different

(for review see [1]). The best-characterised mechanism of genetic imprinting is DNA methylation, where gene expression is regulated by allele-specific methylation of cytosine nucleotides. In imprinted loci critical DNA sequences are ‘marked’ by cytosine methylation in the developing sperm or oocyte, forming primary differentially methylated regions (primary DMRs). These primary DMRs subsequently evade the normal resetting of DNA methylation that occurs in the embryo soon after fertilisation, and remain ubiquitous and permanent in the somatic DNA. There are more maternally derived genomic imprints reported than paternal ones. The DMRs function in conjunction with various associated molecules and processes to alter expression of genes within the influence of the imprint, although the pattern of expression is often complex, differing for each imprinted locus, and can be highly isoform and tissue-specific.

Imprinted gene products, which comprise <1% of the genome (http://igc.otago.ac.nz/home.html) include factors that play key roles in fetal growth and development, from RNA transcription regulators to growth factors. The kinship theory of genomic imprinting predicts that imprinted gene expression is balanced to promote optimal growth of the fetus, and specifically that paternally-expressed genes are fetal growth promoters while maternally-expressed imprinted genes are growth restrictors [2].

Critically, their functional hemizygosity makes imprinted genes susceptible to both genetic and epigenetic mutations. Errors in imprint establishment, erasure or maintenance can lead to aberrant gene expression and disease. Similarly genomic deletions, duplications or uniparental disomy (UPD) that involve imprinted regions can have a similar impact on gene expression. The relative proportion of each subtype of genetic or epigenetic aberration differs for each condition and this has a significant impact on recurrence risks for families. For example if Transient neonatal diabetes (TND) is due to an inherited paternal duplication at 6q24 then subsequent children, born to the father, will have a 50% risk of developing TND. However if the condition is due to uniparental disomy of chromosome 6, the risk to other relatives is very low.

9.2 Human Imprinting Disorders

There are eight recognised human disorders associated with imprinting aberrations (genetic and epigenetic) although it is likely that more will be reported as new imprinted loci are discovered; Angelman syndrome, Prader Willi syndrome, Beckwith Wiedemann Syndrome, Silver Russell syndrome, Transient neonatal diabetes (type 1), Temple syndrome, Wang syndrome and Pseudohypoparathyroidism type 1B. The main clinical features are shown in Table 9.1.

Taken together the conditions have a prevalence of at least 1 in 5,000 but the accurate figure for each disorder is only an estimate and likely to be higher than stated (Table 9.1). For example, one of the studies quoted on the prevalence of Angelman syndrome is from Steffenburg et al. in 1996 [3] who clinically evaluated prepubertal children in Sweden with developmental delay and epilepsy and identified four patients from a total population of 49,000 with clinical features fitting the

Table 9.1 Eight known imprinting disorders with a recognised clinical phenotype

Disease	Prevalence	Main diagnostic clinical features	Additional clinical features (may develop with time)	Frequency of 'epigenetic' aberration	Reference
Prader Willi syndrome	1 in 17,500	Low birth weight Hypotonia Hyperphagia Developmental delay	Hypogonadism Diabetes Obesity	<<1%	[35]
Angelman syndrome	1 in 16,000	Severe developmental delay No speech Epilepsy Ataxia	Microcephaly	4%	[4]
Beckwith Wiedemann syndrome	1 in 13,700	Macrosomia/overgrowth Macroglossia Umbilical defect	Increased risk of Wilms tumor Hypoglycemia	60%	[8]
Silver Russell syndrome	1 in 100,000 likely underestimate	Intrauterine growth retardation Faltering growth Short stature	Relative macrocephaly Genital abnormalities Hypoglycemia	50%	[12]
Transient neonatal diabetes	1 in 400,000	Intrauterine growth retardation Neonatal diabetes with remission	Macroglossia Umbilical hernia Developmental delay Diabetes	30%[a]	[38]

Temple syndrome (maternal UPD 14 associated syndrome)	Unknown	Intrauterine growth retardation Hypotonia Scolisosis Developmental delay Early puberty Short stature	Hydrocephalus Cleft palate	Uncertain	[46]
Wang syndrome (paternal UPD 14 associated syndrome)	Unknown	Bell shaped chest Hypotonia Developmental delay	Umbilical defects	Uncertain	[48]
Pseudohypoparathyroidism 1B	Unknown	Hypocalcaemia due to parathryoid resistance (tetany/parasthesia)		>90%+	[54]

[a]50% due to *ZFP57* mutations + Familial cases often have a deletion at *STX16*

criteria for Angelman syndrome. The diagnosis was confirmed molecularly in only two of the cases, but the study was performed before the advent of new technologies to identify epigenetic aberrations and without the benefit of gene sequencing of *UBE3A*. It is thus likely that this is an underestimate, as the features of Angelman can be variable and the differential diagnosis is broad. The prevalence figure used for Angelman syndrome in Table 9.1, 1 in 16,000, is based on an amalgamation of studies that included earlier estimates of a prevalence of 1 in 20,000 [4], almost certainly based on clinical diagnosis only. This is typical of the research on prevalence for these relatively rare disorders. For many of the other conditions the figures used are even less well substantiated.

Among the difficulties in determining the prevalence of imprinting disorders is the clinical presentation of patients, which is heterogeneous and often non specific. Furthermore, a lack of widely-adopted cost-effective high-throughput testing for epimutations, coupled with the expense of single gene sequencing and the lack of uniform methodology or quality control, puts the diagnosis of an imprinting disorder outside normal diagnostic practice. There is a real need for up-to-date surveys of many imprinting disorders, integrating clinical and molecular information, in order to establish their prevalence characteristics and future therapeutic interventions.

The same problems also confound estimation of the relative contribution of epigenetic versus genetic causes for each imprinting syndrome shown in Table 9.1. For example, the older literature on Transient neonatal diabetes [5] underestimates the prevalence of epigenetically-caused disease, due to small case series and a lack of available technology at the time of the investigation. Another significant confounder is somatic mosaicism, which is well recognised in imprinting disorders. For example, paternal UPD 11 in Beckwith Wiedemann syndrome is usually a post zygotic error and involves only a proportion of cells (see below). This causes problems on two levels: firstly, clinical findings may be restricted to certain regions of the body, making diagnosis difficult; secondly, testing of DNA from leukocytes derived from either blood or saliva, may not detect an abnormality that is restricted to non-hematologic tissues. Another possible confounding factor is the potential for epigenetic mutations to change over a lifetime, although this has never been demonstrated in imprinting disorders where most of the mutations demonstrated to date, are primary and stable.

There is some clinical overlap between imprinting disorders, irrespective of the imprinted locus involved (Table 9.1). Most are associated with disordered growth in fetal and early life. Developmental delay and feeding difficulties are features of some of the conditions but not all, and long term health problems include diabetes and obesity. Hypotonia is a significant component of many of the conditions in early childhood and while congenital abnormalities are not common in general, when they occur they tend to involve abdominal wall development, the genitalia, the tongue and palate. Unusual onset of puberty is another finding, but waiting for this clue delays diagnosis, and early management may be key to prevention of later health sequelae. One explanation for this overlap in clinical presentation is that some of the underlying aetiologies for imprinting disorders cause epimutations at more than one imprinted locus simultaneously. This is well recognised in Transient Neonatal Diabetes where 50% of the subgroup with epigenetic mutations at 6q24

also have epimutations at other imprinted loci such as 11p15.5. These cases are often due to mutations in *ZFP57* (see below) and are described as having Transient Neonatal Diabetes and Hypomethylation at Imprinted Loci (TND HIL) [6].

Despite all of these caveats, the diagnosis of each imprinting disorder discussed below can often be made by the astute clinician, while improving technologies such as routine array CGH and pyrosequencing mean that increasing numbers of cases are being recognised. It should also be noted that epigenetic research is fast-moving, and a patient in whom a positive molecular diagnosis was impossible 5 years ago may now be shown to have a well-defined molecular anomaly. An example of this is the identification in 2005 of a molecular anomaly accounting for ~50% of Silver-Russell Syndrome patients [7], where previously only ~10% of cases where amenable to positive diagnosis. For these reasons, a high-quality diagnostic suite, incorporating molecular analysis of DNA methylation, genetic sequencing, array analysis and cytogenetics, would be an invaluable adjunct to clinical diagnosis.

9.2.1 Beckwith-Wiedemann Syndrome (BWS)

The three cardinal clinical features originally described are macroglossia, gigantism at birth, and exomphalos (for review see [8]); additionally there is a predisposition to tumor development. In childhood there is a facial gestalt for BWS with a midline nevus flammeus of the forehead, infraorbital creases, a large jaw, mouth and tongue, and creases or pits in the ear lobes. Congenital abnormalities reported with BWS include cleft palate, visceromegaly, particularly of the kidneys, cardiac malformations and umbilical defects including exomphalos, umbilical hernia or diastasis recti. Weight and height can continue to increase and cross centiles for the first few years but then normalise and adult height is often within the normal range. Hypoglycemia has been reported in up to 50% in the neonatal period although it usually improves in the first few months. Feeding and respiratory difficulties are common, partially related to macroglossia. Intellectual development is usually normal.

Children with BWS are at increased risk of embryonal tumors, the most common being Wilms tumor followed by hepatoblastoma. This means that it is important to consider the diagnosis even if the features are minimal. Different molecular etiologies have a different risk of tumor development but overall the risk is in the region of 7.5% and significantly reduces over the age of 10 years [9]. This makes diagnosis and molecular classification of medical importance as it alters the surveillance programmes. It should also be noted that many individuals with BWS have a mild phenotype, and asymmetric limb hypertrophy may be the only manifesting feature [10]. This is typical for imprinting disorders where the extent of tissue involvement in a mosaic process makes the presentation variable. Indeed, Scott et al. [10] have reported 3% of individuals with isolated Wilms tumor to have a molecular diagnosis compatible with BWS but not associated with any growth abnormalities. This makes it impossible to instigate effective screening programs to eradicate this tumor, but means that 11p15.5 aberrations must always be tested for in such cases.

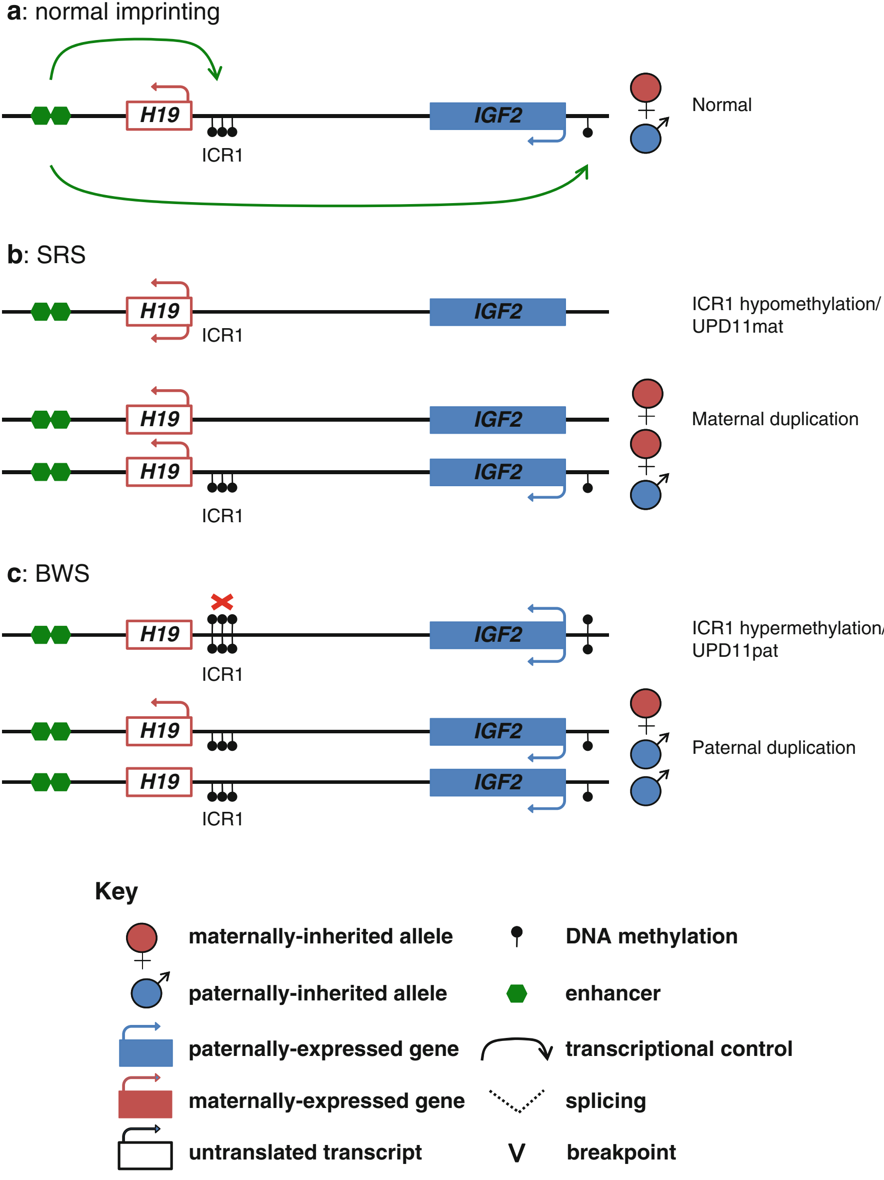
BWS is associated with genetic and epigenetic aberrations at 11p15.5, a region first implicated in patients with paternal duplications, although most such cases also had developmental delay, presumably due to duplication of non BWS genes in the region. 11p15 contains two independent imprinted domains, controlled by the imprinting control regions (ICRs) ICR1 and ICR2. Remarkably, BWS can result from aberrations of either or both. ICR1 is the more telomeric domain and controls two imprinted genes: *IGF2*, a fetal growth factor which is paternally expressed and *H19*, an untranslated RNA that is maternally expressed – see Fig. 9.1. Normally only the paternal allele is methylated at ICR1. The unmethylated maternal allele enables the binding of an insulator protein, CTCF, which prevents common enhancers acting on *IGF2*; hence *IGF2* is expressed only from the paternal allele. Hypermethylation of the maternal ICR1, resulting in maternal IGF2 expression, accounts for 5% of BWS cases.

ICR2, normally methylated on the maternal allele, regulates expression of the maternally-expressed growth restricting gene *CDKN1C* (see Fig. 9.2). ICR2 lies within a non imprinted gene *KCNQ1* and is the promoter for *KCNQ1OT1*, a paternally-expressed RNA whose expression regulates *CDKN1C* in *cis*. Hypomethylation at ICR2, resulting in reduced maternal expression of *CDKN1C*, is the commonest molecular finding (50%) in BWS. In 10% of cases there is a gene mutation in *CDKN1C*.

Partial paternal uniparental disomy (UPD) 11 (20%), usually as a result of a post zygotic error at mitosis, impacts on both domains and results in increased expression of *IGF2* rather than causing a known impact on *CDKN1C* expression.

There is a relation between molecular etiology and BWS features [11]. Most cases with exomphalos have an ICR2 abnormality while Wilms tumor is associated with ICR1 aberrations or paternal UPD 11, both of which result in overexpression of IGF2.

Fig. 9.1 H19/IGF2 Imprinted region on chromosome 11p15. Arrows and symbols above and below the line represent maternal and paternal alleles respectively. (**a**) Schematic of normal imprinting. The paternally-methylated imprinting control region (ICR: *black* "lollipops") lies within the promoter of the untranslated maternally-expressed transcript H19 (*red* unfilled oblong). Repetitive motifs within ICR1 bind the chromatin regulator CTCF in the unmethylated state, constituting a chromatin boundary. Other repeats in ICR1 bind OCT-family transcriptional regulators that are required to maintain the methylated state. The ICR forms a boundary element, regulating the expression of IGF2 (*blue* oblong) under the control of distant enhancer elements (*green* hexagons). The methylated ICR of the paternal allele does not bind CTCF, and therefore the distant enhancers support IGF2 expression. The binding of CTCF to the unmethylated maternal ICR prevents enhancer operation on IGF2, and instead supports expression of H19 (**b**) Mutations and epimutations involving ICR1 in SRS. Inappropriate hypomethylation of the paternal allele of ICR1 makes it behave effectively as a maternal allele, with H19 transcription but reduced IGF2 expression. Maternal uniparental disomy or duplication of the maternal allele of chr11 has a similar effect by causing under-representation of paternal alleles. (**c**) Mutations and epimutations involving ICR1 in BWS. Inappropriate methylation of the paternal allele of ICR1 makes it behave effectively as a paternal allele, with reduced H19 transcription but increased IGF2 expression; a proportion of such cases have microdeletions or mutations within ICR2 disrupting establishment or maintenance of methylation on the maternal allele. Paternal uniparental disomy or duplication of the paternal allele of chr11 has a similar effect by causing under-representation of maternal alleles


a: normal imprinting
H19
ICR1
IGF2
Normal
b: SRS
H19
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Maternal duplication
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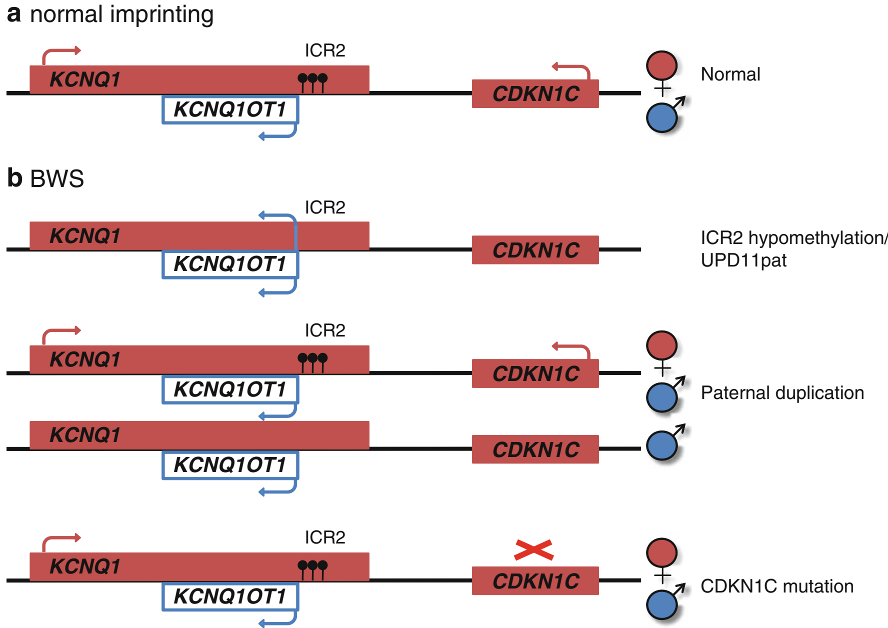

Fig. 9.2 KCNQ1OT1/CDKN1C imprinted region on chromosome 11p15. Arrows and symbols above and below the line represent maternal and paternal alleles respectively. (**a**) Schematic of normal imprinting. The maternally-methylated imprinting control region (ICR2: *black* "lollipops") lies within the promoter of the untranslated paternally-expressed transcript KCNQ1OT1 (*blue* unfilled oblong), which in turn lies within an intron of the maternally-expressed KCNQ1 gene (*red* filled oblong). Paternal expression of KCNQ1OT1 suppresses paternal expression of KCNQ1, and also of the cell-cycle regulator CDKN1C (*red* oblong), such that these proteins are maternally expressed. (**b**) Mutations and epimutations involving ICR2 in BWS. Inappropriate hypomethylation of the maternal allele of ICR2 makes it behave effectively as a paternal allele, resulting in increased transcription of KCNQ1OT1 and reduced CDKN1C expression. Paternal uniparental disomy or duplication of the paternal allele of chr11 has a similar effect by causing under-representation of maternal alleles. Some patients have coding mutations of the maternally-inherited allele of CDKN1C

9.2.2 *Silver Russell Syndrome (SRS)*

This is a condition characterised by intrauterine growth restriction and poor post natal growth with sparing of head growth [12]. Price et al. [13] described five key features:– a birth weight < −2 SD, height < −2 SD, preservation of head circumference, classic triangular facial features with frontal bossing, micrognathia and evidence of body asymmetry. Additional associated features may include patches of increased skin pigmentation and 5th finger clinodactyly, and less commonly cleft palate, genital abnormalities, heart defects and camptodactyly with limitation of peripheral joint mobility. Severe developmental delay is rare but 30% were found to have some mild delay in a recent review of 64 patients with a confirmed diagnosis [12]. The natural history of this condition includes significant feeding difficulties and early motor delay.

Speech problems are a feature in some patients. Postnatal growth is somewhat variable and it is not yet understood why some continue to fall from the centiles in early childhood whereas in others there is no change or an improvement in height standard deviation score (SDS) postnatally [14]. Few studies are available on adult height but of interest some achieve a final height within normal centiles. SRS is an indication for growth hormone treatment but this remains controversial. There is a tendency to central weight gain in older children and adults, and some facial features become less obvious with age. The relatively non specific clinical findings make this condition difficult to diagnose and differentiate from other causes of short stature.

SRS is usually sporadic and for many years after the condition was discovered no consistent cause could be identified. It was not certain that there was a genetic aetiology as the majority of monozygotic twins are discordant for SRS [15] and only a few families are reported with multiple affected members [16]. It is now clear, however, that imprinted genes on chromosomes 7 and 11 are implicated in the SRS phenotype and account for approximately 50–60% of cases with typical SRS.

ICR1 on chromosome 11p15.5 was first implicated in SRS through rare cases with maternal 11p duplication [17] (Fig. 9.1). In 2005, Gicquel et al. [7] showed that up to 40% of cases with classic SRS had hypomethylation at ICR1 (i.e. resulting in silencing of IGF2 and the inverse molecular anomaly to the ICR1 hypermethylation seen in BWS (characterized by biallelic IGF2 expression). This is now recognised as the major molecular cause of SRS, and has been confirmed in many other studies [18, 19]. Mosaic segmental maternal uniparental disomy of 11p15 has also been reported in SRS [20]. Interestingly, epigenetic and genetic mutations at ICR2 are not commonly associated with SRS although there is one reported case with isolated duplication restricted to ICR2 [21].

Kotzot [22] was the first to demonstrate maternal uniparental disomy (UPD) of chromosome 7, the inheritance of both chromosome 7s for the mother with no contribution from the father, in SRS. Maternal UPD7 is believed to account for 5–10% of SRS [23], and is nearly always heterodisomic, leading to speculation that it results from trisomy rescue and therefore that a trisomic cell line may contribute to the phenotype. It can be difficult to differentiate matUPD7 patients from those with other aetiologies [24] although Binder et al. showed a greater likelihood of having reduced postnatal height [14], and Wakeling et al. [12] noted an increased likelihood of developmental delay and a less classical presentation. There are at least three candidate imprinted regions on chromosome 7 at 7p12 (*GRB10*); 7q21 (*SGCE)* and 7q31-2 (*PEG1/MEST)* but there is no clarity on which of these loci may contribute most to SRS in matUPD7.

9.2.3 Angelman and Prader-Willi Syndromes (Chromosome 15q11-13 Associated Imprinting Disorders)

The imprinted chromosome 15q11-13 region contains both maternally and paternally expressed genes, summarised in Fig. 9.3. Interference with expression of the maternally expressed gene *UBE3A* gives rise to Angelman syndrome (AS) whereas a

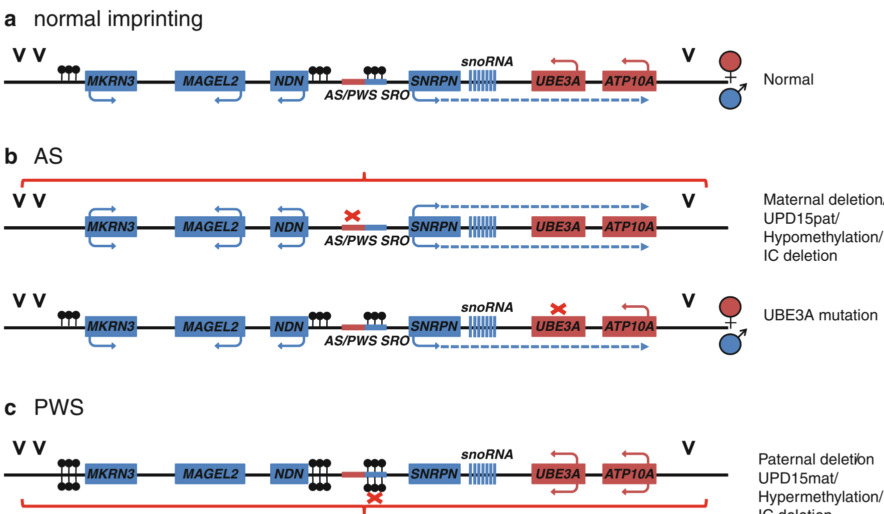

Fig. 9.3 PWS/AS imprinted region on chromosome 15q13. Arrows and symbols above and below the line represent maternal and paternal alleles respectively. (**a**) Schematic of normal imprinting. The maternally-methylated imprinting control region (ICR: *black* "lollipops") lies within the promoter of the SNRPN gene (*blue* filled oblong), which also forms the origin of a long transcript that generates multiple non-coding RNAs. Paternal expression from this promoter suppresses paternal expression of UBE3A and ATP10, such that these proteins are maternally expressed (*red* oblongs) while proximal to SNRPN are several paternally expressed genes (*blue* oblongs) with differential methylation (*black* lollipops) that develops secondarily to the germline methylation of SNRPN. Arrowheads to the left and right of the schematic indicate regions of genomic instability that are frequently observed as breakpoints in chromosomal rearrangements in PWS and AS. The narrow red and blue bars abutting the SNRPN imprinting centre represent the AS/PWS shortest region of overlap (AS/PWS SRO), a minimal region in deletions, and therefore believed to be minimal promoter elements for genes involved in these disorders. (**b**) Mutations and epimutations in PWS. Inappropriate methylation of the paternal ICR makes it behave effectively as a maternal allele, resulting in reduced expression of SNRPN and associated RNAs; some hypermethylation cases are caused by microdeletion involving the PWS-SRO. Maternal uniparental disomy or duplication of the maternal allele of chr15 has a similar effect by causing under-representation of paternal alleles. (**c**) Mutations and epimutations in AS. Inappropriate hypomethylation of the maternal ICR makes it behave effectively as a paternal allele, abrogating transcription of UBE3A; some hypomethylation cases are caused by microdeletion involving the AS-SRO. Paternal uniparental disomy or duplication of the paternal allele of chr15 has a similar effect by causing under-representation of maternal alleles. AS is also caused by coding mutations of the maternally-inherited allele of UBE3A

clinically different disorder, Prader Willi Syndrome (PWS), results if genes which are normally paternally expressed are affected. The expression of imprinted genes within 15q11-13 is controlled by a bipartite imprinting centre (IC) [25]. Part of this IC, termed AS-SRO, is involved with setting of the maternal imprint of genes within the region and the adjoining part, PWS-SRO, affects setting of the paternal imprint. Angelman and Prader-Willi syndrome can result from deletion of 15q11-13, uniparental disomy (UPD) for this region, or from impaired imprinting. Most deletions occur *de novo*, though a predisposing parental chromosomal rearrangement must be excluded.

Uniparental disomy is associated with increasing parental age, and with increased risk if a parent carries a Robertsonian translocation involving chromosome 15. The frequencies of all of the genetic mechanisms and the parental origin of the chromosome involved differ between the two conditions and are discussed further below. An excellent review of genetic testing for AS and PWS was recently published by Ramsden et al. [26].

9.2.4 Angelman Syndrome (AS)

The main characteristics of AS include severe learning disability, ataxia, seizures with a characteristic high voltage EEG, acquired microcephaly and a sociable affect with frequent smiling and laughing [4, 27]. Subtle dysmorphic features include brachycephaly, deep set eyes, a wide mouth which is usually held open and a prominent chin. Infants with AS have significant feeding difficulties. Developmental milestones are delayed and speech rarely develops. Most children achieve walking with a characteristic wide-based, stiff legged gait. The behavioural features of AS include hand flapping, especially when excited, jerky voluntary movements, love of water, sleep disturbance and easily provoked laughter. Though head circumference at birth is usually normal, the majority of children develop microcephaly. Seizures, variable in type and difficult to control, typically develop in the second year of life, lessen in adolescence and return during adulthood. Puberty occurs normally and though final adult height is usually within the normal range truncal obesity may develop, along with joint contractures, spasticity and scoliosis. Life span may be shortened if problems develop secondary to immobility, scoliosis or seizures.

AS results from impaired expression of the maternally imprinted *UBE3A* gene, whose expression is restricted to the brain, and this can be caused by gene mutations, UPD, deletions, and imprinting anomalies.

A small proportion of AS patients have point mutations within the coding region of *UBE3A* [28]. Though the majority are de novo, around 25% are inherited from a phenotypically normal mother and there is also a high risk of maternal gonadal mosaicism. Recurrence risks may therefore be high and study of the extended family is indicated. UBE3A functions as both a ubiquitin protein ligase within the brain, targeting proteins for degradation, and as a transcriptional activator. It is still not clear, however, just how *UBE3A* is involved in the pathogenesis of AS. Animal studies have shown that *Ube3a* is preferentially expressed in neurones where it appears to affect dendritic spine development [29]. It may also affect the number of available synaptic glutamate receptors [30]. Thus, the symptomatology of AS could be due to abnormalities of synaptic development and plasticity.

The majority of AS individuals (70–75%) have a maternally inherited deletion of 15q11-13, while 3–7% have paternal UPD15 [31]. Maternal hypomethylation is present in 4% of AS patients. In 10–15% of these cases, which may be familial, the hypomethylation is associated with small deletions in the AS IC; in the remaining 85–90% the abnormal imprint is presumed to be a chance epigenetic event [31].

An increased incidence of AS with imprinting defects has been reported after assisted conception, possibly arising from the assisted conception process itself or from underlying infertility, though the small number of cases reported makes it difficult to draw any definite conclusions [32]. Imprinting defects frequently occur in mosaic form, in which case the phenotype may be subtly different with more pronounced hypotonia, larger head size, better developed language skills and predisposition to obesity [33].

There are minor phenotypic differences between individuals with AS due to different genetic mechanisms [34]. Those with deletions are more likely to have seizures and microcephaly and usually have hypopigmentation of hair, skin and irides as they lack one allele of the *P* gene, implicated in Type II oculocutaneous albinism. Those with *UBE3A* mutations are normally-pigmented with a lower incidence of seizures and microcephaly. Patients with UPD are the most able and may develop some speech. There is a relatively broad spectrum of severity in individuals with imprinting mutations, reflecting the fact that many are mosaics.

9.2.5 Prader-Willi Syndrome

Prader-Willi syndrome (PWS) is one of the commoner imprinting disorders [with an incidence of 1 in 17,500 (for review see [35]). The high incidence is due to the susceptibility of this locus to undergo deletion and duplication errors rather than epigenetic mutations which make up a low proportion of cases. Its main characteristics are profound neonatal hypotonia and initial feeding difficulties followed by the development of hyperphagia and obesity; moreover, most affected individuals have mild to moderate learning disability. Hypogonadism is usually present and individuals with PWS have specific behavioural features including a tendency to pick at their skin, temper tantrums and obsessive compulsive disorder. Other complications include seizures and scoliosis. Small hands and feet have been noted in some patients and hypopigmentation is a feature in those with 15q11-13 deletions. Characteristic facial features include bitemporal narrowing of the face, almond-shaped palpebral fissures and a downturned mouth. The management of PWS is aimed at weight regulation using behavioural modification techniques, healthy eating and exercise programmes. In recent years, growth hormone treatment has improved the outlook for children with PWS by improving linear growth, reducing obesity and improving muscle mass so that exercise is easier for affected individuals [36]. Lifespan in PWS can, however, be significantly reduced by cardiovascular and respiratory problems occurring as a result of morbid obesity.

Around 75–80% of individuals with PWS have a paternally inherited deletion of the 15q11-13 region (Fig. 9.3). The remaining 20–25% virtually all have maternal uniparental disomy (UPD) for chromosome 15. UPD usually arises due to maternal non-dysjunction of the chromosome 15s during meiosis and is more common in older mothers. Imprinting defects of 15q11-13 occurs in less than 1% of PWS cases.

The 15q11-13 region contains five protein-coding genes and five snoRNA gene clusters with highly repetitive sequences (Fig. 9.3). Sahoo et al. [37] identified a microdeletion affecting only one of the snoRNA clusters, *SNORD116* (HB11-85) in a patient with a fairly typical PWS phenotype and suggested that this may be the critical region for PWS. There were, however, some atypical features including better growth than expected. It seems likely that other genes in the 15q11-13 region contribute to the PWS phenotype and certainly, the hypopigmentation seen in deletion patients is influenced, as in AS, by haploinsufficiency for the Type II oculocutaneous albinism gene. The *SNORD116* snoRNA has been shown to affect splicing of a serotonin receptor but there is no evidence to support this as a mechanism in PWS and the cause of the condition remains unknown.

9.2.6 Transient Neonatal Diabetes (TND)

Transient neonatal diabetes (type 1) (TND1) is a disorder of fetal growth and glycaemic control (for review see [5, 38, 39]). Babies present with hyperglycaemia, typically in the first week of life, and require insulin replacement for the first few months. Insulin levels are often undetectable at birth although ketoacidosis is rare implying that endogenous insulin is being produced. These babies are characteristically growth retarded with a paucity of body fat and may have other associated features including macroglossia and umbilical hernia. Although diabetes remits by 18 months of age these children are susceptible to episodes of hyperglycaemia during childhood, particularly during intercurrent illness, and a high proportion develop type 2 diabetes in later life.

The imprinted locus on 6q24 is one of the least complex human imprinted domains and contains just two known imprinted genes *PLAGL1* and *HYMAI,* where only the paternally inherited allele is normally expressed in the majority of tissues (Fig. 9.4). This expression pattern results from a primary methylation imprint established in the oocyte. The resulting DMR overlaps the main shared promoter of *PLAGL1* and *HYMAI*, and maternal promoter methylation silences the maternal alleles. *PLAGL1* has a second promoter 55Kb upstream from the imprinted promoter which is not imprinted and results in biallelic expression in some tissues including blood and adult pancreas in normal individuals. *PLAGL1* is a zinc finger DNA binding protein with tumor suppressor activity, involved in cell cycle control and apoptosis. Loss of *PLAGL1* expression has been described in a number of cancers. Murine studies have shown that overexpression of *PLAGL1* results in reduced fetal pancreatic beta cell mass [40]. The other imprinted gene, *HYMAI,* is a non-coding RNA gene transcribed in the same orientation as *PLAGL1,* and its function is not known.

TND is caused not by loss of function but by overexpression of imprinted genes. Three mechanisms are recognised: (i) paternal uniparental disomy of chromosome 6 involving 6q24 (35% of cases), (ii) paternal duplication of 6q24 (35% of cases), (iii) maternal hypomethylation of the TNDM1 DMR (in the absence of a genomic rearrangement; 30% of cases). Maternal hypomethylation is usually complete in

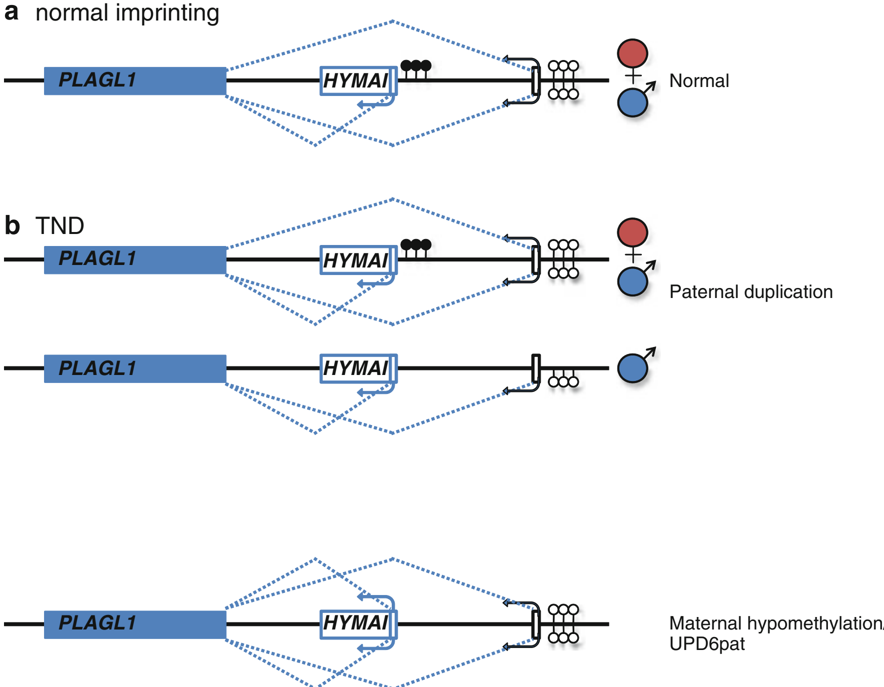

Fig. 9.4 PLAGL1 imprinted region on chromosome 6q24. Arrows and symbols above and below the line represent maternal and paternal alleles respectively. (**a**) schematic of normal imprinting. The maternally-methylated imprinting control region (ICR: *black* "lollipops") lies within an internal promoter of PLAGL1 (*blue* filled oblong), which is also the transcription origin of the untranslated transcript HYMAI (blue unfilled oblong). Maternal methylation of the ICR suppresses expression from that allele, such that PLAGL1 is expressed only from the paternal allele. The upstream promoter of PLAGL1 is unmethylated and supports biallelic expression. (**b**) Mutations and epimutations in TND. Inappropriate hypomethylation of the maternal ICR makes it behave effectively as a paternal allele, resulting in increased transcription of PLAGL1. In some patients this hypomethylation is secondary to homozygous mutation of *ZFP57*. 6q24 duplication of paternal origin has a similar effect by causing under-representation of maternal alleles

blood DNA and its cause is unknown, but as-yet undiscovered genetic mutations in cis may cause the failure of methylation of the maternal allele. In 50% of cases, hypomethylation affects other imprinted loci in addition to 6q24, and in many of these the pattern of loci involved is consistent. Mackay et al. [6] found that these cases were associated with homozygous/compound heterozygous mutation of *ZFP57,* a zinc finger transcription factor expressed in very early embryo development. Therefore this is an example of a genetic cause of global epigenetic aberrations at imprinted loci. Murine studies demonstrated that loss of *zfp57* function disturbs both establishment and maintenance of DNA methylation at a subset of imprinted loci [41].

The clinical phenotype of this autosomal recessive inherited global imprinting disorder has been very variable. While probands have presented with neonatal diabetes,

reflecting the involvement of 6q24, the additional features have ranged from severe developmental delay with brain malformations and early childhood death to normal development with no associated features. Non-probands have been reported with no health issues. The mechanism underlying the relation between *ZFP57* and differential DNA methylation is not known but the presence of a KRAB domain has linked it to the maintenance of the molecular scaffold required for DNA methylation. The cause of the other cases with hypomethylation at multiple loci but presenting with TND1 is not known although monozygous twinning and patients where the parents have received in-vitro fertilisation (IVF) are over-represented in this group [6].

9.2.7 Chromosome 14 Related Imprinting Disorders

The first reports of imprinting disorders in humans associated with chromosome 14 were those of Wang et al. [42] and Temple et al. [43] who reported individual cases with paternal and maternal uniparental disomy of chromosome 14, respectively. The phenotype of both disorders is now well characterised [44]. It has subsequently become clear that the phenotypes are indistinguishable from genetic and epigenetic aberrations confined to 14q32 which variously alter expression of the imprinted genes within the imprinted domain (see Fig. 9.5). Hence it has been suggested that these two conditions should be named after the first authors of the first reports rather than the somewhat cumbersome name of 'uniparental disomy of chromosome 14 associated conditions' [45].

9.2.7.1 Wang Syndrome (Paternal UPD 14 Associated Conditions)

The condition may be diagnosed antenatally with ultrasound evidence of a small thorax, polyhydramnios and large abdominal circumference that can be confused with exomphalos. Babies have normal or relatively high birth weight for gestational age but often have serious respiratory difficulties associated with a bell shaped chest and characteristic chest X-ray findings described as a 'coat hanger' appearance of the ribs. This can be very helpful for diagnosis of the condition. The children may be hypotonic and have abdominal wall defects, most commonly diastasis recti. Dysmorphic features include a high hirsute forehead, blepharophimosis, a small chin and finger contractures. The overall prognosis of this condition is poor and many children die in infancy. The children are reported to have developmental delay but there are relatively few reports of older children.

9.2.7.2 Temple Syndrome (Maternal UPD 14 Associated Conditions)

In contrast this disorder has a good prognosis, and is characterised by pre- and postnatal growth restriction, hypotonia leading to joint laxity, scoliosis which can be severe, and delayed motor milestones [46]. The children may initially be diagnosed

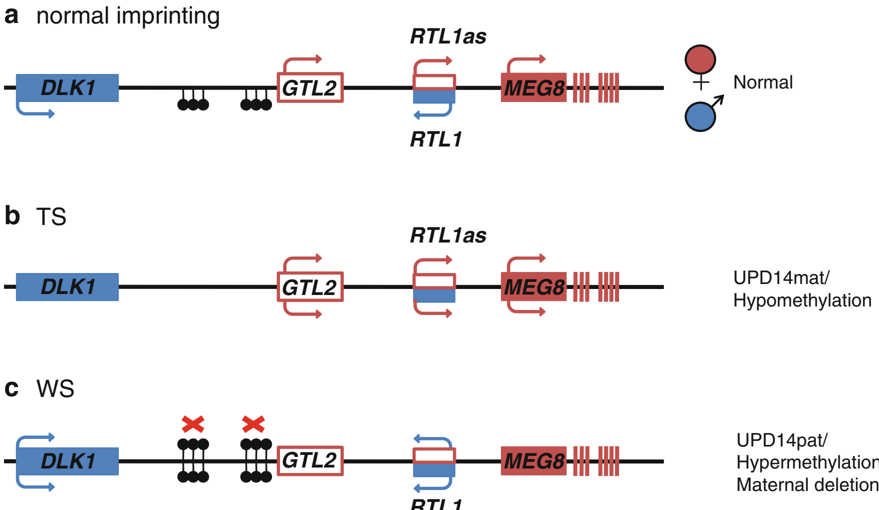

Fig. 9.5 The imprinted region on chromosome 14q32. Arrows and symbols above and below the line represent maternal and paternal alleles respectively. (**a**) schematic of normal imprinting. The paternally-methylated ICR (*black* "lollipops") is intergenic, lies between the untranslated maternally-expressed transcript GTL2 (*red* unfilled oblong) and the paternally-expressed gene DLK1 (blue filled oblong). Differential methylation of the GTL2 promoter is secondary to that of the intergenic DMR (IG-DMR). The IG-DMR regulates parent of origin-specific expression of multiple genes including numerous non-coding RNAs (red lines). (**b**) Mutations and epimutations of the 14q32 ICR in TS. Inappropriate hypomethylation of the paternal ICR makes it behave effectively as a maternal allele. Maternal uniparental disomy or paternal deletion of 14q32 have a similar effect by causing under-representation of paternal alleles. (**c**) Mutations and epimutations of the 14q32 ICR in WS. Inappropriate hypermethylation of the maternal ICR makes it behave effectively as a paternal allele. Paternal uniparental disomy or maternal microdeletion of 14q32 have a similar effect by causing under-representation of maternal alleles. Microdeletions of the IG-DMR and the secondary GTL2-DMR have different effects on placental and fetal development

as possible Prader Willi syndrome (PWS) and there have been several studies diagnosing Temple syndrome in cases negative for PWS [47]. The facial features are however distinct, with a high broad forehead and wide nasal base with prominent lips and a high, narrow or cleft palate. The head circumference is often large in proportion to height, and hands and feet are particularly small. Early puberty is well described although can be normal. The final height is often reduced and can be well below the 0.4th centile.

The imprinted locus at 14q32 (Fig. 9.5) has both maternally (*GTL2/MEG3, MEG8, RTL1as*) and paternally (*DLK1, RTL1*) expressed genes and an intergenic primary imprinting control region (IG-DMR). Hypomethylation at IG-DMR is associated with expression of *GTL2* and *RTL1as* which repress the expression of *DLK1* and *RTL1* in cis. The methylated paternal copy of IG-DMR is therefore associated with repression of *GTL2* and *RTL1as* and expression of *DLK1* and *RTL1* from the paternal allele. A series of microdeletions in rare families has shown that

the Wang syndrome is likely due to over-expression of *RTL1* and the smallest region implicated in either deletions or epimutations (hypermethylation) is the IG- DMR [48], whereas evidence from microdeletion families showed that Temple syndrome was due to a loss of expression of paternally expressed 14q32 domain genes. Kagami et al. [48] hypothesised that deletions involving *RTL1* and *DLK1* had a more severe phenotype with regard to height than those with a *DLK1* deletion alone. This is in keeping with other cases that have been reported with isolated epimutations and a similar phenotype [49]. *RTL1* (retrotransposon-like-1) expression is therefore key in both phenotypes. The paternally-expressed *DLK1* (delta, Drosophila homolog-like1) is a transmembrane signalling protein and regulator of growth. It has homology to proteins in the Notch/delta pathway.

9.2.8 Pseudohypoparathyroidism Type 1b PHP1b

This condition comprises hormone resistance (parathyroid and thyroid stimulating hormone) but only minor physical features of the related condition Pseudohypoparathryoidism type 1A which includes short stature, skeletal changes and variable developmental delay [50–52].

The imprinted *GNAS* cluster (Fig. 9.6) encodes several transcripts with different promoters (reviewed in [53, 54]); the main transcript, Gsα, is biallelic in most tissues but maternally expressed in certain tissues such as the anterior pituitary, the thyroid and the ovary. The protein stimulates adenylate cyclase in response to ligand binding at G-protein coupled receptors. Other transcripts at the locus include (i) *GNASXL,* paternally-expressed in some neuroendocrine tissues, whose protein product XLαs stimulates adenylate cyclase, (ii) *NESP55,* expressed from the maternal allele in some neuroendocrine tissues and encoding neuroendocrine secretory protein 55, a chromogranin-like protein, (iii) *GNAS exon 1A*, a paternally-expressed non-coding transcript originating upstream of exon 1of Gsα , in exon 1A, and E (iv) *NESPAS* another paternally-expressed non-coding transcript transcribed antisense to NESP55 and involved in its silencing.

The tissue-specific imprinting of genes in the *GNAS* cluster accounts for the various clinical presentations and explains why aberrations result in hormone resistance in only some tissues. Imprinted expression is controlled by 3 DMRs; exon 1A DMR (methylated on the maternal allele) regulating the *GNAS* promoter; *GNASXL/NESPAS* promoter DMR (methylated on the maternal allele) responsible for the imprinted expression pattern of both transcripts and the ICR for the locus, and the *NESP55* promoter DMR (methylated on the paternal allele).

PHP1B is nearly always due to a loss of methylation at the exon 1A DMR and no identified mutation in *GNAS*. DNA methylation analysis is therefore the test of choice when diagnosing this condition. In most sporadic PHP1B cases the cause is unknown and there are variable methylation defects at the other *GNAS* DMRs; however, in familial cases, hypomethylation restricted to the exon 1a DMR is often associated with microdeletion of Syntaxin-16 (*STX16*), up-steam of the *GNAS* locus

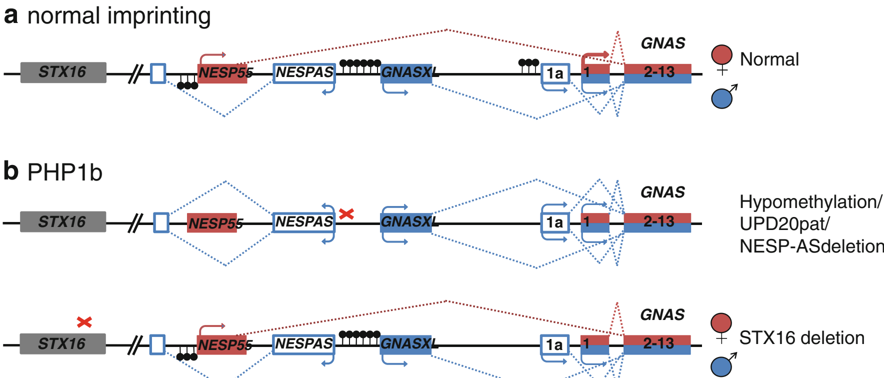

Fig. 9.6 The GNAS imprinted region on chromosome 20q13. Arrows and symbols above and below the line represent maternal and paternal alleles respectively. (**a**) schematic of normal imprinting. The maternally-methylated germline ICR (*black* "lollipops") lies within the promoter of the untranslated paternally-expressed transcript NESP-AS (*blue* unfilled oblong). Secondary differential methylation is established on the maternal allele in the promoter of the adjacent gene, GNASXL (*blue* filled oblong), and on the paternal allele intergenically between NESP-AS and NESP55 (*red* filled oblong). The GNAS exon 1a promoter harbours another ICR whose maternal methylation can be disrupted either in tandem with or independently of that of NESP-AS. Expression of GNAS is biallelic, but predominantly maternal in some tissues. (**b**) Mutations and epimutations in PHP1b. Inappropriate hypomethylation of the maternal NESP-AS ICR makes it behave effectively as a paternal allele, causing paternalisation of all other DMRs in the cluster and leading to reduced GNAS expression. In a small number of cases NESP-AS hypomethylation is caused by microdeletion within the NESP-55 DMR. Paternal uniparental disomy of chr20 has a similar effect by causing effective paternalisation of the region. Maternal deletion of the distant STX16 gene disrupts local chromatin organisation and leads to hypomethylation of the GNAS exon 1a DMR and resultant loss of GNAS expression. Other DMRs in the region are unaffected

[55–57]. This is therefore a poignant reminder that epigenetic aberrations can be related to genetic mutations and can thus be inherited. It is thought that the *STX16* locus harbours a *cis*-acting DNA element necessary for methylation at the exon 1A DMR. It is not yet known whether DNA hypomethylation in some sporadic PHP1B cases are caused by mutations in *cis* or mutations of regulatory genes in trans, and this makes genetic counselling difficult in families with isolated epigenetic aberrations.

9.3 Conclusion

Imprinting disorders are a diverse and fascinating group of conditions, that differ with respect to their relative prevalence. To date all imprinting disorders have been found to be due to a combination of gene mutations, epimutations and cytogenetic errors at specific imprinted loci (Table 9.1). While each imprinting disorder has its

own unique clinical and molecular characteristics, there can be substantial overlap between their phenotypic associations and fetal growth disturbance is the major finding common to many of the disorders. Clinical overlap may be due to common cellular pathways for many of the known imprinted genes but may also reflect the involvement of more than one imprinted locus at a time, so called hypomethylation at multiple imprinted loci. An increasing case literature shows that the clinical and molecular heterogeneity of these disorders currently requires imaginative and insightful diagnosis. Furthermore it is likely that additional phenotypes will be recognised as new imprinted loci are discovered and as diagnosis improves.

The complexity at the molecular level, means that expert interpretation is required when predicting prognosis and recurrence risks for families.

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Chapter 10
The Utility of Twins for Epigenetic Analysis

Richard Saffery, Ruth Morley, and Debra L. Foley

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Abstract Twin studies have played an important role in our understanding of individual variation for over a century. The strength of these studies lies in the capacity to perfectly control for inter-individual genetic variation through the use of monozygotic (MZ) twin pairs. Despite their genetic identity, MZ twins often show phenotypic variability, presumably in response to different environmental exposures. Given that epigenetic mechanisms are widely believed to be the mediators of the influence of environmental factors on the underlying genome, it is not surprising that the use of twins in epigenetic research is increasingly recognised as an important approach to help unravel the complexities associated with human development and disease. In addition, the strategic use of twins in epigenetic studies has revealed the importance of genetic factors and both *in utero* and postnatal environments to the establishment and maintenance of the human epigenome. Finally, twin studies are generating compelling evidence linking epigenetic disruption to the disease-associated risk in humans.

Abbreviations

MZ	monozygotic
DZ	dizygotic
DNA	deoxyribonucleic acid
EEA	equal environments assumption
2D	two dimensional
ANOVA	analysis of variance
ICC	intraclass correlation
RRBS	reduced representation bisulphite sequencing
SLE	systemic lupus erythematosus
RA	rheumatoid arthritis
DM	dermatomyositis
RNA	ribonucleic acid
5MeC	5- methylcytosine
CBMC	cord blood mononuclear cells
HUVEC	human umbilical vein endothelial cells
AIMS	amplification of inter-methylated sites
DC	dichorionic (two placentas)
MC	monochorionic
CGI	CpG island region (enriched for CpG dinucleotides)
3′UTR	non protein coding 3′ untranslated region of RNA transcript
DMR	differentially methylated regions
DRD2	dopaminergic receptor D2
COMT	catechol-O-methyltransferase
MSRDA	methylation sensitive representational difference analysis
PPIEL	Peptidylprolyl isomerise E-like
SMS	Spermine synthase

10.1 Why Study Epigenetics in Twins

In today's post genomic era there has been a shift in our understanding of complex disease to include a more functionally relevant genomics approach, where the dynamic patterns of gene expression are analysed, not in isolation, but jointly as parts of functional clusters or networks. Similarly, more and more studies are integrating both genetic and environmental data into analyses with the aim of providing a more 'holistic' understanding of how diseases arise. The complexities are manifold and require the development of novel methodological approaches. The strategic use of twin samples, especially monozygotic (MZ) twin pairs who share the same genetic material, offers a unique opportunity to circumvent some of the complexities and reduce the required sample size for studies examining the aetiology of complex disease.

Twin studies have played an important role in our understanding of individual variation for over a century. The similarity of MZ and dizygotic (DZ) twins allows an estimation of heritability (the proportion of the total variance in a trait that is attributable to genetic factors). MZ twins are generally considered to be genetically identical, although this is not always true, (see Sect. 9.1 below), whereas DZ twins share on average 50% of their genetic (DNA sequence) variation (0–100% theoretical range). MZ twins exhibit a wide range of concordance rates for complex disease. Whereas MZ twin concordance approaching 100% indicates coinheritance of genetic variants that are likely dominant and highly penetrant, many diseases or traits show concordance rates far lower than this indicating a role of environmental and/or stochastic epigenetic factors in modulating phenotypes associated with underlying genotypes. Given that epigenetic mechanisms are widely believed to be the mediators of the influence of environmental factors on the underlying genome, it is not surprising that the use of twins in epigenetic research is becoming increasingly popular.

Previous studies examining gene expression in MZ twins have identified many hundreds of genes whose within-pair expression discordance [the absolute value of the ratio of (log) gene expression of twin 1: twin 2] is greater than an arbitrarily defined threshold (set above the level of variation attributable to experimental noise associated with a particular array platform) [1, 2]. This supports divergence in gene expression between MZ twins in response to environmental and stochastic factors as the driver of subsequent phenotypic divergence. In accordance with expectations, array based studies have also demonstrated a higher degree of similarity between specific gene expression patterns in MZ than in DZ pairs, highlighting the contribution of genetic factors to overall expression profile [1, 3]. This relationship is even apparent in multiple tissues taken from newborn twin pairs. Nevertheless, MZ pairs also show clear discordance in gene expression profiles at birth, highlighting the importance of the *in utero* environment in determining overall gene expression (and most likely epigenetic) profile [4].

Major molecular mechanisms underpinning gene expression and associated phenotypic discordance in MZ twins are likely epigenetic in nature. Epigenetic status

of a specific genomic region is determined by the sum of genetic, cumulative environmental and stochastic (probabilistic or random) factors. Measurement of epigenetic differences in phenotypically discordant MZ pairs therefore provides a unique opportunity to identify genes sensitive to the environment that are associated with complex disease.

10.2 The Value of Twin Studies in the Understanding Regulation of Epigenetic Profile

Elegant studies in mice have demonstrated that the phenotype in genetically identical animals reared in identical environmental conditions is normally distributed, highlighting the role of probabilistic (stochastic) factors in phenotype development [5]. DNA methylation levels at specific genomic loci in MZ twins are also normally distributed. However, at present the relative contributions of environmental, genetic, and stochastic factors to the establishment and maintenance of epigenetic profile, and its relative stability over time, remain poorly understood.

10.2.1 Twin Studies Highlight the Role of Genetic (Heritable) Factors in the Regulation of Epigenetic Profile

MZ twins share maternal, obstetric and genetic factors, but differences in epigenetic profile will accumulate in response to differences in environmental exposures and stochastic factors, accumulated both *in utero* and postnatally. In contrast DZ pairs share maternal and obstetric factors but the likelihood of sharing specific genetic variations is the same as that of non-twin siblings. Given the large number of genes and/or proteins implicated in the establishment and maintenance of the epigenetic profile, and the demonstrated link between genetic variation and epigenetic profile [6, 7], it reasonable to speculate that DZ twins as a group will be more epigenetically divergent than MZ twins in accordance with gene expression data. A powerful method for estimating the relative contribution of heritable and environmental/stochastic influences on variation in any quantitative trait such as DNA methylation is to compare the degree of within pair discordance in MZ pairs to that in DZ twin pairs. Several studies have now carried out this type of heritability analysis, using twins of varying ages and phenotypes. The underlying assumption of such studies is that genetic factors are the primary driver of cumulative MZ vs DZ disparity. Although highly likely, it is equally feasible that DZ twins as a group exhibit more epigenetic differences than MZ twins because the former originate from different zygotes, potentially already epigenetically divergent. In contrast, MZ twins derive from a single zygotic epigenome.

The major strength of the classical twin study is the lack of confounding by age effects between individuals within a twin pair, and the ability to distinguish between genetic and common environmental effects. However, this is predicated on the equal environments assumption (EEA) that no interaction exists between genes and environment [i.e. no twin-type (DZ versus MZ) differences in response to salient environmental effects]. If this is not the case, then the classical model lacks utility. Both MZ and DZ twins are required to estimate the relative contributions of genetic versus common environmental contributors. Age differences between groups of MZ and DZ twin pairs may be a potential confounding factor specific to epigenetic analyses [8, 9]. As such, care must be taken to match as closely as possible in this regard.

Heritability is an estimate of the proportion of the total phenotypic (outcome) variance in a population that is attributable to additive genetic effects. In twin studies heritability is estimated by comparing the degree of phenotypic similarity between groups of MZ and DZ twins, either as the concordance rate or intra-class correlation. In the context of twins, heritability (h^2) is determined as twice the difference between MZ and DZ concordance rates (correlation; $h^2 = 2(r_{MZ} - r_{DZ})$, where r is the concordance or intraclass correlation between each type of twin [10]. Heritability estimates are population specific unless the environment is constant. Additionally, the contribution of stochastic factors to epigenetic divergence is equally assumed to be constant across populations in these analyses.

10.2.2 Twin Studies Reveal Cumulative Environmental Contributors to 'Epigenetic Drift' Over Time

The cumulative effects of environmental and stochastic variation on changing epigenetic profile were first illustrated by a widely cited study that examined both genome wide and locus specific DNA methylation variation in a small number of young and middle-aged MZ twins [9]. Whereas 3 year-old MZ twins showed relatively few epigenetic differences within pairs, those aged 50 years showed considerable variability within pairs, and this was greater if the twins had divergent lifestyles. A multi-level statistical analysis was performed that generated a single descriptive value for each type of epigenetic measure. For DNA methylation this included (i) the numbers of bands present on a gel (or dots on a 2D gel) between individuals A and B within a pair, and (ii) the absolute percentage of 5-methylcytosine present within an individual. Gene expression differences were measured as the number of genes showing differential expression (adjusted p-values following ANOVA) between siblings. Histone acetylation levels were measured as relative peak heights following high performance liquid chromatography.

The similarity of each descriptive value within pairs was then estimated as the Euclidean squared distance by subtracting their respective values. In order to examine the relation between phenotypic/environmental data and epigenetic variables, categorical principle component analysis was firstly applied to the original questionnaire variables to reduce them to two uncorrelated components termed

'aging' (encompassing age, weight and height) and 'health' (all variables of disease and pharmacological treatments). Mixed models were used to estimate the contribution of each random-effect to the variance of the dependent variable and the variance component procedure was assessed by ANOVA. The relation of single scores generated for questionnaire-derived ageing and health variables to epigenetic data was evaluated by Pearson's test (note that the specific type of test was not described). Using this approach the general conclusion was that epigenetic profile is in constant "drift", although very few young twins were studied and statistical precision was low [9]. In addition, this was a cross sectional study and did not evaluate methylation in the same individuals over time. Finally, the specific epigenetic analyses employed were all low resolution in that they did not examine the distribution of epigenetic differences within the genome, but rather provided a global 'snapshot' of different classes of epigenetic disparity within twin pairs.

The first study to examine epigenetic drift longitudinally in twins involved analysis of buccal cell DNA methylation in 3 genes in 46 MZ and 45 DZ at 5 and 10 years of age [11]. Longitudinal change within individuals was calculated by assessing the correlation in methylation at each age. To assess the relative contributions of heritable and environmental/stochastic components to methylation levels, correlations within MZ pairs were compared to correlations within DZ pairs. Finally, to assess the relative contributions of heritable and environmental/stochastic components to changes in DNA methylation over time, intra-individual change scores were calculated and correlations within MZ twin pairs were compared to correlations within DZ pairs. This study was the first to reveal the extent of epigenetic discordance in MZ twins in early life and highlighted the ongoing instability of methylation levels over time. Importantly different genomic regions were found to show varying levels of epigenetic divergence over time.

An examination of DNA methylation levels at several sites in multiple tissues from newborn twins confirmed that epigenetic drift between genetically identical individuals (MZ twins) begins *in utero* and in a tissue-specific manner. In this study, intraclass correlation coefficients (ICC) were higher in MZ than in DZ twins [12] (Fig. 10.1) supporting previous findings of a role for genetic/heritable factors in the establishment of epigenetic profile.

Epigenetic drift over time has been observed for singletons and it is also possible that maintenance of the epigenetic mark of DNA methylation is under genetic control [13]. However, longitudinal changes in epigenetic marks are most likely to occur in a subset of genes [14], possibly in those with intermediate methylation levels, rather than those that are entirely unmethylated or in a hypermethylated state.

Limited studies are now beginning to utilise the twin model to estimate the relative contribution of genetic and environmental factors in mediating the response of the developing epigenome to specific environmental exposures. An examination of the methylation levels at 3 independent imprinting-associated DMRS (KvDMR1, PEG1 and H19/IGF2) in 59 pairs of twins (including 29 pairs conceived through IVF) did not reveal significant differences at three DMRs between IVF-conceived and naturally conceived twins. However, both sample size and the genomic regions examined were too small for any general conclusions to be made and further analysis in larger groups of twins is warranted [15].

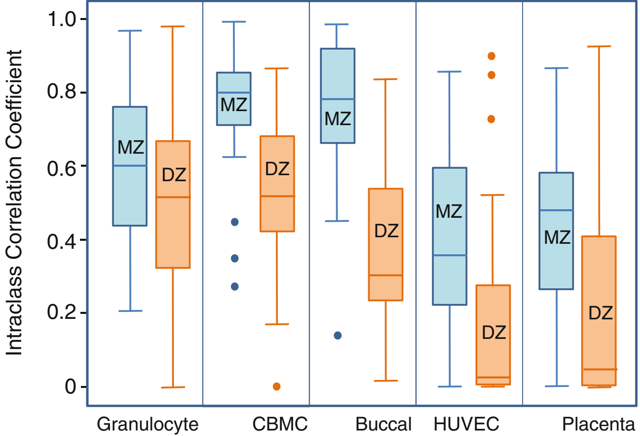

Fig. 10.1 Distribution of intraclass correlations reveals a higher median correlation coefficient for DNA methylation at the IGF2/H19 locus in MZ than DZ twin pairs in five tissues from newborns. The ICC measures the proportion of total variance attributable to within pair variation within MZ and DZ groups of twins. ICC analysis. CBMC- cord blood mononuclear cells; HUVEC- human umbilical vein endothelial cells. (Figure adapted from [12])

10.3 Epigenetics and the Discordant Twin Model

10.3.1 The Assumption of Genetic Identity

Many studies have reported phenotypic differences between MZ co-twins (reviewed in [16]). Discordant MZ pairs have proved very valuable in revealing the contribution of non-genetic variation to disease penetrance, aetiology, or effect of therapies. The underlying assumption in all such studies is that MZ twins are genetically identical. This has been directly disproved in several instances, where either specific point mutations [17–19], uniparental disomy [20], triplet repeat expansion [21, 22], chromosomal mosaicism [23] or heteroplasmy for mitochondrial-encoded mutations [24, 25] or chromosomal aneuploidies (reviewed in [16]) have all been linked to specific phenotypic differences in monozygotic twins. Importantly, differences in both copy number [26] and telomere length [27, 28] have been also described in phenotypically discordant and concordant MZ twin pairs. Nevertheless, such reports are relatively rare and it is likely that non-genetic (epigenetic) variation within MZ twin pairs underpins the majority of observed phenotypic discordance.

10.3.2 Inferring Causation: Epigenetic vs. Genetic Analysis

Since every cell in an individual derives from a single zygote with one genome, genetic studies generally require only a single DNA sample per individual, taken

from any tissue at any age. Cause and effect can be reliably predicted, or excluded, as genomic variation is not considered to be variable over the life course (except in relation to 'rare' somatic mutation). Such rules may not apply to most epigenetic studies where repeated samples from more than one tissue may be desirable. Even if biospecimens are collected very early in life (prior to phenotypic manifestation) unravelling cause and effect is problematic. Despite this and the many other caveats associated with inferring causation in any epigenetic association study (covered in other chapters of this book), several investigators have attempted to link specific epigenetic changes to disease phenotypes. Surprisingly few studies have attempted to utilise the discordant MZ twin pair model, despite the demonstrated utility of this approach in identifying specific risk factors of disease,

10.3.3 Localised Epigenetic Variation in Phenotypically Discordant Twins

Several studies have attempted to identify epigenetic 'causes' of complex human disorders through a low resolution, candidate gene hypothesis examination of epigenetic variation (primarily DNA methylation) at specific gene regions in phenotypically discordant MZ twins (summarised in Table 10.1). The contribution of epigenetic variation to the heritability of complex phenotypes is complex and likely regulated at many levels (Fig. 10.2). Generally speaking, effect sizes have been minimal and there have been few examples of independent replication of identified associations. As such, the biological relevance of such findings remains questionable.

10.3.4 Skewed X-Chromosome Inactivation in Phenotypically Discordant Twins

One of the most widely studied epigenetic phenomena in mammals involves the inactivation of the majority of genes on one X-chromosome in females. This 'dosage compensation' equalises the expression of most X-chromosome genes in males (XY) and females (XX) and usually occurs in a random manner, with roughly 50% of cells in a female showing inactivation of the maternally-derived X, whereas the other 50% show inactivation of the paternally-derived X chromosome. However, in some individuals a skewed pattern of X-inactivation is apparent, with one parental X, over represented in a particular tissue or cell type. In such instances, an otherwise recessive mutation can have profound adverse phenotypic effects. Thus, skewed X-inactivation, or more precisely the chance inactivation of a single functional allele in a physiologically-relevant tissue, has been associated with discordance for several disorders including haemophilia [29], Fragile-X syndrome [22, 30] and Duchenne Muscular Dystrophy [31].

Table 10.1 Non-disease-based epigenetic analyses in twins

Focus	Number of twin pairs	Biological samples	Target/methodology	Analysis methodology	Major finding	Main strengths/ Weakness	Reference
Identification of factors contributing to epigenetic variation	80 twins (age range 3–74 years)	Buccal	AIMS Global 5MeC levels	(see body text)	Increasing discordance in MZ twin epigenetic profile with age. First demonstration of 'epigenetic drift'. Some evidence of increasing drift with increasing cumulative environmental differences	S: multiple marks profiled. Global analysis W: small numbers. No longitudinal analysis	[9]
		Blood lymphocytes Skeletal muscle (n = 14) Abdominal muscle (n = 4)	Histone modifications				
Cataloguing range of interindividual methylation at differentially methylated regions	12 MZ and 14 DZ pairs (age range 27–73 years)	Whole blood	DNA methylation using bisuphite pyrosequencing at two paternal and four maternal DMRs. Gene promoters of *OCT4* and *APC* genes	Box plots to illustrate distribution of methylation. Statistical inference for eight studied regions was done using multivariate analysis of variance (MANOVA). Students *t*-test used to compare means of a particular gene between two groups. Linear regression was calculated to infer the dependence of methylation % on age. Goodness of fit (R^2) was used to quantify this relation. P value calculated by ANOVA. Multivariate discrimination using Fisher's linear discriminant analysis followed by non-parametric Wilcoxon test was used to compare the pairwise methylation differences of MZ vs DZ pairs.	For all DMRs, the median pairwise methylation difference between MZ was small than for DZ at the single gene level. DMR methylation is relatively stable between the ages of 27–72 years at the studied regions. Data are consistent with most methylation being established after fertilization in association with development of methylation mosaicism.	S: sensitive technique W: small numbers of twins and genes tested	[39]

(continued)

Table 10.1 (continued)

Focus	Number of twin pairs	Biological samples	Target/methodology	Analysis methodology	Major finding	Main strengths/ Weakness	Reference
Identification of factors regulating DNA methylation at *IGF2/H19* imprinted region	196 adolescent twins; 176 middle aged twins	Whole blood	Gene specific, bisulphite based Mass Array Epityping of previously described differentially methylated regions (DMRs)	Correlations between methylation at specific sites measured with GOLD [40]. Principle components analysis (PCA) SPSS 11. Heritability calculated using estimates of additive genetic V(A), common environmental V(C), unique environmental + stochastic V(E) obtained from variance components implemented in Mx 1.61 (www.vcu.edu/mx). Association of methylation with body size (BS) performed in variance components setting with BS as outcome measure, component scores for DMRs as variables, and age and sex as confounders.	PCA indicated that one major factor explaining between 53% and 73% of variance at either DMR. Heritability of methylation at individual CpG sites varied from 20 to 74% for H19 DMR and 57–97% for IGF2 DMR. No evidence for an age effect was found between the adolescent vs middle aged groups. Preliminary evidence for an association between H19 DMR methylation and body size ($p < 0.05$).	S: large numbers. Sensitive technique W: focus on single genomic region	[41]
Role of *in utero* environment in specifying methylation at *IGF2/H19* imprinted region in newborns	56 MZ and 35 same sex DZ collected at birth	Buccal CBMC HUVEC Placenta Granulocytes	Gene specific, bisulphite based Mass Array Epityping of previously described differentially methylated regions (DMRs)	Association between methylation at different CpG units, and between methylation and BW or methylation and GA, was measured using Spearman's rank correlation coefficient. The contribution of genetic factors outside the IGF2/H19 DMRs to methylation levels was investigated by fitting separatelinear mixed models for MZ and DZ twin pairs at each CpG unit. Within-twin pair correlation was modelled by including a random intercept specific to each twin pair; the models had no other covariates and were fitted using restricted maximum likelihood. The proportion of total variance attributable to 'between-twin pair' variation was calculated using intra class correlation.	Higher ICC values for MZ than DZ twin pairs. First evidence for epigenetic discordance in genetically identical humans at birth (ie. cumulative effect of environmental/stochastic factors). Additional evidence for a role of genetic factors in regulating the neonatal epigenetic profile. Evidence for tissue-specific effects.	S: multiple tissues from single individuals at birth. Relatively large numbers W: single genomic region	[12]

Annotation of degree of epigenetic metastability	114 MZ and 80 DZ teenage pairs	MZ: White blood cells (n = 19 pairs), buccal epithelia (N = 20 pairs), rectum (N = 18 pairs)	Enrichment of unmethylated DNA fraction and DNA methylation profiling at >12,000 CpG island fragments	Intraclass correlation for each unique genomic region (~6000 unique comparisons per pair; ICC range from +1 > −1 denotes very high > very low correlation). Wilcoxon rank-sum test used to compare variability of CGI vs non-CGI regions. P values corrected for multiple testing by Bonferroni method.	ICC profiles were more similar within different tissues from single individuals than between unrelated individuals. CGI regions less variable than non-CGI regions. ICC for Buccal cell DZ ICC values significantly lower than MZ ICC values; ICC for MZMC significantly lower MZDC pairs. But MZ v DZ ICC differences were tissue specific.	S: large numbers. Multiple technical replicates W: poor platform for methylation analysis	[34]
Effect of assisted reproductive technology on the stability of DNA methylation	29 IVF and 30 non-IVF twin pairs (no zygosity)	Umbilical cord tissue	Methyl-specific quantitative PCR	Fisher's exact test comparing mean methylation at each region between groups	No significant difference between IVF and non-IVF groups however some evidence for increasing variation in the IVF group.	W: limited genomic coverage and small numbers of each twin type. No zygosity information	[15]

5MeC 5- methylcytosine, CBMC cord blood mononuclear cells, *HUVEC* human umbilical vein endothelial cells, *AIMS* amplification of inter-methylated sites, *DC* dichorionic (two placentas), *MC* monochorionic, *CGI* CpG island region (enriched for CpG dinucleotides)

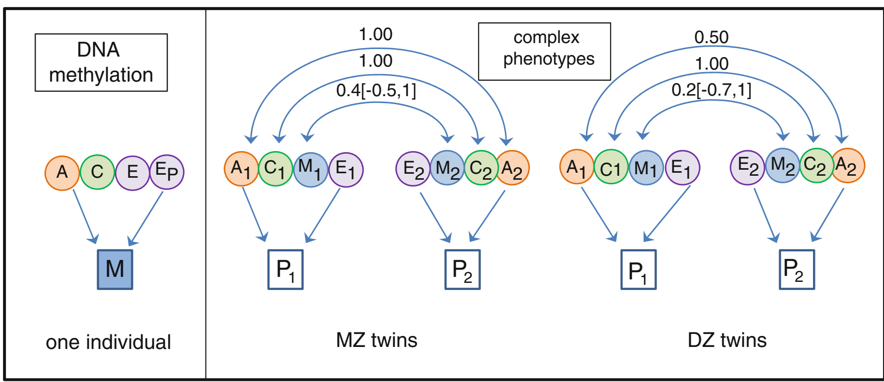

Fig. 10.2 The relation between epigenetic and phenotypic heritability. Proposed contribution of latent variables to the methylation status of an individual at a genomic region (*M*) and to their phenotype (*P*). The left pane represents shows the variables contribution to DNA methylation status at one genomic region in one individual; effects will be specific to age, sex, population (genetic factors), and the tissue sampled, and will also include stochastic factors. Methylation latent factors include additive genetic factors (*A*), common environmental factors (*C*), unique environment (*E*), and heritable and stable epigenetic factors that are not DNA sequence dependent (Ep). The right pane represents the path model in twins, depicting the contribution of DNA Methylation and other factors to the phenotype (*P*) in twin *i* with correlation estimates in MZ (*Left*) and DZ (*Right*) twins of latent variables including additive genetic effects (*Ai*), common environment (*Ci*), DNA methylation (*Mi*), and unique environment (*Ei*). Correlation estimates were obtained from previous genetic [38] and epigenetic studies [34] in twins. In siblings, the correlation in M will be lower than that observed in DZ twins due to age differences and associated increased level of cumulative stochastic change. (Figure adapted from [10])

10.3.5 Genome-Scale Epigenetic Investigations in Twins

Given the relative ease of simultaneously mapping DNA methylation profile across large regions of the genome relative to higher order epigenetic marks, it is not surprising that the limited twin studies carried out in this space have focused solely on DNA methylation (Table 10.2).

10.3.5.1 Medium Resolution (1–2 Sites per Gene in 1–10% of Genes in the Genome)

Recent studies have begun to reveal the true extent of genomic DNA methylation changes in MZ twins that could potentially explain phenotypic discordance. Perhaps the most successful combined application of the twin discordance model and epigenetic analysis has been investigation of the association of DNA methylation changes with immune related disorders, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and dermatomyositis (DM) [32]. A tiered approach to analysis involved

Table 10.2 Examples of epigenetic analysis in discordant twin pairs

Focus	Number of twin pairs	Biological samples	Epigenetic target/ Methodology	Analysis methodology	Major finding	Ref.
Candidate gene approaches						
Schizophrenia (SCZ)	1 discordant + 1 concordant affected	Lymphocytes	DNA methylation of *DRD2* gene by Bisulphite Sequencing	Purely observational No statistical analysis	Discordant twin with schizophrenia shows more similar *DRD2* methylation profile to affected concordant twins than to unaffected co-twin	[42]
Birth weight	12 highly discordant MZ pairs	Buccal cell DNA at age 5	DNA methylation at 2 CpG sites of *COMT* gene by bisulphite pyrosequencing	Not stated	Highly variable methylation concordance rates between pairs. Average within pair methylation discordance was 10.3% at site 1 and 16.1% at site 2. Strong correlation in differences between both sites ($r = 0.87$; $p < 0.001$). No correlation between birth weight and methylation level	[43]
Primary biliary cirrhosis (PBC)	4 discordant MZ, 1 concordant affected	Peripheral blood	DNA methylation by bisulphite sequencing	Not stated	Decreased expression of *CLIC2* and *PIN4* in ¾ PBC affected individuals in discordant pairs. No evidence for DNA methylation changes driving expression.	[44]
Beckwith Wiedemann Syndrome (BWS)	10 discordant MZ 5 control MZ	Skin fibroblasts and/or peripheral blood	DNA methylation by Southern Blotting	Purely observational No statistical analysis	BWS affected individuals show loss of imprinting at *KVDMR1* with biallelic expression of *KCNQ1OT1*	[45]

(continued)

Table 10.2 (continued)

Focus	Number of twin pairs	Biological samples	Epigenetic target/ Methodology	Analysis methodology	Major finding	Ref.
Silver-Russell Syndrome (SRS)	1 discordant MZ pair	Peripheral blood leukocytes	Combined bisulfite restriction analysis (COBRA) and bisulfite sequencing for two H19 DMRs	Purely observational No statistical analysis	Loss of *H19*-DMR in roughly half of cells in the affected twin only with associated decreased expression of *IGF2*	[46]
Caudal Duplication Anomaly (CDA)	1 discordant MZ pair 9 unaffected MZ pairs	Peripheral blood mononuclear cells	DNA methylation of *AXIN-1* by Bisulphite Sequencing	Binomial generalised linear mixed model (lme4 package)	Higher methylation at *AXIN-1* promoter in affected vs unaffected co-twin ($p<0.0001$). Higher methylation in both twins than in controls ($p=0.02$). ICC correlation of 0.76 for all 10 MZ pairs.	[47]
Alzheimer's disease (AD)	1 discordant MZ	Post mortem temporal neocortex	Various epigenetic markers by immunohistochemistry	Two tailed *t*-test of staining intensity	Significantly reduced levels of DNA methylation in temporal neocortex neuronal nuclei in AD	[48]

Body Mass Index (BMI)	16 discordant MZ pairs	Saliva	DNA methylation at 9 DMR regions implicated in growth, by bisulphite based, amplification and primer extension and HPLC. Methylation index calculated as h(C)/h(C)+h(T) where h=peak height on HPLC	Paired *t*-test and Wilcoxon's signed rank test to test for association between phenotype and MI for each discordant pair. Pearson's and Spearman's correlation coefficients calculated to test for associations of intrapair BMI and MI differences	Only small intrapair differences in methylation observed. No significant correlations between intrapair BMI differences and intrapair methylation levels.	[49]
Genome scale (hypothesis free) approaches						
1. Systemic Lupus Erythramatousis(SLE) **2**. Rheumatoid arthritis (RA) **3**. Dermatomyositis (DM)	5 discordant for each disorder −17 SLE sib pairs including 1MZ, 4DZ for validation only	Whole blood: white cell fraction	DNA methylation with Illumina Golden Gate Bead Arrays (>1000 measurements) Validation by Bisulphite Sequencing & pyrosequencing Global 5MeC measurement	Student's *t*-test with FDR correction for multiple testing	No methylation differences associated with DM and RA Consistent methylation changes in SLE twins relative to unaffected co-twins in immune functioning genes Global decrease in 5-methylcytosine in SLE and hypo-methylation of 28 S and 18 S *rDNA* genes	[32]

(continued)

Table 10.2 (continued)

Focus	Number of twin pairs	Biological samples	Epigenetic target/ Methodology	Analysis methodology	Major finding	Ref.
Multiple Sclerosis (MS)	3 discordant MZ pairs,	CD4+ T cells	Next Generation sequencing of genomic DNA, mRNA (50–68 million reads) and RRBS (50–90 million reads covering >two million CpG sites)	RBBS sequence aligned using GSNAP [50]. No statistical testing used to determine significance (or otherwise) of observed within pair methylation differences	Between 2 and 176 significant methylation differences (of 2 million CpGs tested) between MZ co-twins (> 800 methylation differences in unrelated individuals). No evidence for genetic, epigenetic or transcriptome differences underlying MS discordance.	[35]
Bipolar disorder (BPD)	1 discordant MZ pair, 16 unrelated singletons with BPD. Independent set of 14 unrelated BPD singletons	Lymphoblastoid cell lines	MS-RDA with validation by bisulphite DNA sequencing and pyrosequencing	Mann–Whitney *U*-test for analysis of expression and methylation levels. One way ANOVA with cofactors of age and sex for effect of diagnosis controlled for confounders.	10 genomic regions showing differential methylation between co-twins by MS-RDA. 4 of these confirmed by bisulphite sequencing of co-twin DNA. 2 of these (*PPIEL*, *SMS*) confirmed as significant by pyrosequencing in case–control study design (25 BPD cases and 18 controls) with $p<0.05$. Methylation status of *PPIEL* inversely correlated with gene expression levels	[51]

Risk taking behaviour	1 discordant plus 9 MZ control pairs	Peripheral blood	DNA methylation profiling at >12,000 CpG island fragments	Multiple technical replicates. FDR correction for multiple testing. 1.15 fold ($log_2(1.15)=0.2$) used as experimental /technical variance threshold.	No technical comparisons survived FDR correction. Differential methylation of *DLX1*[a] gene (3′UTR) identified specifically in discordant MZ pair ($p<0.0004$)	[37]

[a]Transcription factor involved in the formation of GABAergic interneurons [52].
3′UTR non protein coding 3′ untranslated region of RNA transcript
DMR differentially methylated regions
ICC intraclass correlation
DRD2 dopaminergic receptor D2
COMT catechol-O-methyltransferase
RRBS reduced representation bisulphite sequencing
MSRDA methylation sensitive representational difference analysis
GSNAP [50]
PPIEL Peptidylprolyl isomerise E-like
SMS Spermine synthase

genome scale DNA methylation profiling of over 1500 CpG sites in five pairs of discordant MZ pairs, in combination with gene specific validation in further discordant twins and matched control subjects, and global measurement of 5-Methylcytosine levels. A *t*-test was used to identify array probes differentially methylated between affected individuals and their respective healthy twins. The resulting p-values were corrected for multiple testing using False Discovery Rate methodology [33]. As with most current methylation studies of this nature, an arbitrary mean methylation change between affected and non-affected groups was used as a cut-off (in this instance 10%) to generate a list of candidate genes for subsequent validation.

This approach revealed 49 genes, enriched for immune functioning, showing consistent changes in DNA methylation specifically in SLE. No consistent methylation changes were found in RA or DM. Subsequent validation in matched controls and a further set of discordant twin pairs (again with corrections for multiple testing) confirmed the array result. Interestingly, an examination of global 5MeC levels identified a generalised loss of DNA methylation in the SLE affected individuals, and this was found to be (at least in part) due to a specific decrease in methylation at repetitive 28S and 18S ribosomal RNA genes.

10.3.5.2 High Resolution (1–20 Sites in 10–50% of All Genes)

The most compelling analysis of factors regulating overall methylation profile was obtained through the analysis of three collections of DZ and MZ pairs [34]. This consisted of white blood cells (WBCs) from 19 MZ twin pairs who were dichorionic (each twin had their own placenta) and 20 DZ twin pairs matched for age, sex and blood count, plus buccal epithelial cells from 10 monochorionic MZ and 20 DZ age- and sex-matched. Additionally WBCs and buccal cells were obtained from each of 10 dichorionic MZ and 10 DZ pairs from another independent twin cohort and gut biopsies were collected from 18 MZ pairs from a third twin population. Intra-class correlations for MZ and DZ pairs were calculated for pair wise comparisons of over 6,000 individual data points corresponding to methylation levels at individual genomic regions. These compellingly demonstrated a higher degree of similarity between the MZ twins as a group, relative to the DZ group ($p = 1.2 \times 10^{-294}$). However, in studies carried out in parallel to the human analyses, wide ranging epigenetic differences were also found in nearly identical mouse strains, leading the authors to speculate that a differential zygotic epigenetic profile in DZ versus MZ twins was the primary driver of the ICC differences – not genetic variability between the different individuals [34]. However, this could not be directly tested because of the inability to directly measure epigenetic profile on single celled zygotes.

10.3.5.3 Ultra-High Resolution (Every Site and Every Gene)

The future of both genomic and epigenomic analysis of twins lies in the use of high throughput nucleotide sequencing. Baranzini et al. [35] searched for genetic,

expression and DNA methylation differences in purified CD4+ T cells in a small number of MZ twin pairs discordant for multiple sclerosis (MS). Between 50 and 68 million different sequencing reads of messenger RNAs were counted for each pair. Broadly speaking, this represents the expression 'output' of the original sample. The more reads present for a specific gene as a proportion of the total read number, the more highly expressed it is within the starting cell population. Surprisingly, a diagnosis of MS accounted for only 9.4% of the total variance; 57.3% was attributable to between pair differences, and 26.3% due to day-to-day variation within single individuals. This data alone highlights the highly dynamic nature of gene expression that raises considerable challenges when trying to identify disease-specific gene expression changes. Not surprisingly, no robust gene expression differences could be ascribed to the MS phenotype alone. Despite some evidence for allelic imbalance (where one of an individual's two copies of a gene is expressed more highly than the other) within discordant twin pairs, no such changes were common to all three MS discordant twin pairs examined. An examination of 50–90 million, high-quality reads of reduced representation bisulphite sequencing (RRBS) from CD4+ T cells of the 3 three discordant twins similarly showed little evidence of DNA methylation changes that could be specifically associated with the MS phenotype. Whereas several hundred distinct differences (at specific CpG sites) were apparent between males and females, or between primary and unrelated cancerous tissues, the magnitude of epigenetic differences between MZ twins discordant for MS was at least an order of magnitude lower than non-related individual differences, and ~3 orders of magnitude lower than between primary and malignant tissue. Unfortunately there was no independent validation of observed within pair differences using an alternative methodology, so the robustness of the few observed DNA methylation changes within MZ pairs remains unclear [35].

10.4 Caveats and Cautions

1. There may be epigenetic effects specific to twins that limit their utility as a model to reveal contributors to disease causation more broadly applicable in the wider singleton population. For example, the processes leading to MZ twinning remain to be determined but have traditionally been considered to result from random processes. However, more recent data have suggested that epigenetic factors themselves may be implicated in the embryo splitting that leads to MZ twins [36]. If specific to MZ twins, this would have the effect of confounding any MZ vs DZ comparisons aimed at identifying heritable factors. This could result in differential epigenomes of two newly created MZ embryos independently of later onset of phenotypic discordance.
2. Few studies have attempted to estimate statistical power required to falsify epigenetic hypotheses. The link between the number of technical replicates (independent measures from the same biospecimen, aimed at identifying the contribution of technical 'noise') and the power to detect biologically meaningful

DNA methylation differences in twin pairs has been elegantly examined by Kaminsky et al. [37]. As predicted a larger number of array technical replicates results in a smaller overall level of technical variance at individual array data points. Thus, as few as two technical replicates provided >80% power to detect a 1.2 fold change at ~90% of array probes in only nine twin pairs. This is increased from ~60% of probes with only a single technical replicate. Further power calculations predicted that with two technical replicates and 21, 14 and 6 twin pairs, there was an 80% chance of detecting true DNA methylation difference of 1.15, 1.2 and 1.6 fold, at >95% of data points tested, between groups [37]. One caveat to this calculation is the potential for variation in DNA methylation over time [8, 9]. This would have the effect of increasing population variance in methylation levels at age-sensitive loci. In order to deal with this it would be desirable to use age matched twin pair where possible for identification of epigenetic biomarkers specifically associated with phenotypic discordance. Despite this potential problem, limited attempts at power calculations so far undertaken are encouraging for the application of genome-scale methylation analysis to large twin-based studies.

It is hard to believe that just over 10 years ago the human genome had not been characterised fully, micro RNAs and other non-coding RNAs were considered largely artefactual by some, and epigenetics was considered of 'limited' importance biologically. Ten years from now it is likely that a full characterisation of both genomic and epigenomic data will be within the reach of most researchers; in the case of epigenomic data, this will be contingent on the provision of all manner of tissues from willing donors. In many cases (such as neural tissue in neuropsychiatric disorders), this will need to be done post mortem. It is likely that the level of complexity and variation revealed within individual cells/tissues/organs of any individual, *and* between individuals will be immense, greatly hindering the identification of biologically meaningful differences in epigenetic profile. Despite this, the inherent capacity to control for genetic variation in MZ twin pairs (and to a lesser extent environmental factors) should prove incredibly valuable in helping to unravel the mind boggling complexity of gene:environment:epigenetic interactions likely to contribute to human health and disease.

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Chapter 11
Age-Related Variation in DNA Methylation

Jean-Pierre Issa

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Abstract Methylation is a ubiquitous, naturally occurring modification of DNA in mammalian cells that mediates stable repression of gene expression in epigenetically regulated genes, and plays diverse other roles such as regulation of chromosome structure and silencing of endogenous retrotransposons. DNA methylation patterns are generally stable in the short term but show prominent changes in aging cells and tissues including gains of methylation at previously protected promoter regions and losses of methylation genome wide. These age-related methylation changes remain mechanistically mysterious but are conserved from mouse to man, and are likely caused by infidelity in replication of the epigenome over time. Because of the link between DNA methylation and gene expression, these changes result in a mosaic epigenome in aged cells that could underlie diseases of aging such as cancer and atherosclerosis. Maintaining the health of the epigenome is worth investigating as a strategy to prevent age-related diseases.

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11.1 DNA Methylation and the Regulation of Gene Expression

Methylation refers to the covalent addition of a methyl group and can be done to multiple biological molecules including DNA, RNA and proteins. DNA methylation is normally limited to the cytosine base, primarily in the context of cytosine followed by guanosine (the CpG dinucleotide) in adult cells [1]. The methylation reaction takes place after DNA synthesis, usually in the first few minutes post replication. It is catalyzed by DNA methyltransferases (DNMTs). DNMT3a and DNMT3b are primarily expressed during embryogenesis and in stem cells, and establish initial methylation patterns while DNMT1 is responsible for maintaining DNA methylation by copying the patterns seen on the original strand to the newly synthesized strand [2]. Thus, past embryogenesis, DNA methylation tends to be very stable in adult cells [3, 4].

The impact of DNA methylation on gene expression is highly variable depending on location of the involved CpG sites [5]. Thus, while it is enzymatically a single process, DNA methylation is best understood as multiple biological processes that utilize a common set of enzymes. The prime determinant of the function of a methylated CpG site is its location in the genome. One should distinguish sites located in CpG islands (short stretches of CpG rich DNA) or outside CpG islands. Similarly, one should differentiate promoter sites, genic sites and intergenic sites. Table 11.1

Table 11.1 DNA methylation in different genomic compartments

Location	Normal methylation patterns	Impact on expression	Change with age
Promoter CpG island	<2% of promoters are methylated	Strong silencing	Net increase, though a decrease in a few methylated promoters can also be seen
Non-promoter CpG island	20–30% are methylated	Some are silenced alternate promoters; most have an unclear impact	Net increase, though a decrease in a few methylated CpG islands can also be seen
Promoter non-CpG island	20–30% of promoters are methylated	Associated with lower expression, but can be reversed by transcriptional activation	Variable
Intragenic non-CpG island	Most CpG sites are highly methylated	Weak positive association with expression	Decrease
Intergenic non-CpG island	Most CpG sites are highly methylated	Most are in silenced retrotransposons and have an unclear impact on genes	Decrease

summarizes the methylation compartments, normal methylation patterns and the impact of DNA methylation on gene expression. The most compelling relation is at promoter CpG islands where the presence of methylation is rare, but associated with very stable and irreversible (in adult cells) silencing [1]. Most of the affected promoters are on the inactive X-chromosome in women, on monoallelically expressed genes (e.g. imprinted genes) and on a class of genes expressed exclusively in germ cells [6]. The impact of methylation in other genomic compartments is more variable, from reversible gene silencing (e.g. the IL2 gene promoter in resting lymphocytes [7]) to no clear impact on expression (e.g. intergenic sites, with the possible exception of enhancer sites where methylation remains poorly studied).

The mechanisms that underlie establishment and maintenance of DNA methylation at specific CpG sites remain somewhat mysterious. DNMTs are not known to have particular sequence specificity and it is assumed that the decision to methylate or not a given CpG site depends on co-factors that remain unknown. Recently, the TET family of enzymes was found to catalyze the formation of hydroxy-methylation at sites of cytosine methylation [8, 9]. There is no mechanism of maintaining hydroxymethylation post replication; thus, this reaction may be an intermediate step in catalyzing DNA demethylation. In some cases, strong gene activation is associated with localized removal of DNA methylation, best exemplified at non-CpG island promoters [7]. Thus, there is some dynamic regulation of DNA methylation in adult cells, but overall the patterns are very stable. Indeed, epigenetic processes marked by promoter CpG island methylation such as X-inactivation and imprinting are maintained through numerous cell division cycles in-vitro or in-vivo.

11.2 DNA Methylation Changes with Age

The earliest studies on DNA methylation with age focused on global measurement of 5-methylcytosine, which primarily reflects non-promoter, non-CpG island DNA. Initial studies showed progressive depletion of 5-methylcytosine associated with passaging normal fibroblasts in-vitro [10], a surrogate for age-dependent senescence. Subsequently, similar changes were observed in aging mouse tissues as well as human bronchial epithelial cells [11]. A study in human leukocytes demonstrated a relatively small but statistically significant decline (from ~4.05% to ~3.95%) over seven decades [12]. As expected given the location of most CpG sites, this decline can also be seen in repetitive elements such as retrotransposons, though the small changes noted require a large number of patients and a range of ages of several decades to be reliably detected [13]. It is important to note that genome wide studies have recently shown shifts in global methylation associated with stem cell differentiation[14]. Some studies have also reported shifts in 5-mC content associated with physiologic changes in proliferation (e.g. post-hepatectomy [15, 16]). Thus it remains possible that the shifts in 5-mC content with age (or passage number in-vitro) could be related to small differences in differentiation or proliferation states rather than a true aging defect.

When technology evolved to allow quantitative analysis of DNA methylation at specific sites, it became apparent that human aging was accompanied by small but measurable changes in many genes. Initial studies focused on a handful of genes with promoter CpG islands that were thought to be unmethylated in normal tissues but hypermethylated in cancer cells. In fact, careful studies unveiled a small degree of methylation in normal tissues that increased linearly with age in the Western populations studied. The first gene where this was described was *ERα*, where methylation increases at a rate of 1% every 3 years in human colon [17], but it quickly became apparent that many genes behaved similarly [18]. An unbiased, genome wide study showed that about 70–80% of promoter CpG islands that show hypermethylation in cancer also demonstrate this phenomenon of age-related hypermethylation [19]. CpG island hypermethylation is not a feature of differentiation. When present, it is established in embryogenesis and remains constant post-development [6]. Thus, changes in this genomic compartment truly represent a pathologic event that accompanies aging.

Multiple studies have now confirmed age-related promoter methylation in human tissues. An autopsy study showed that some degree of hypermethylation could be observed in most tissues examined, with significant tissue to tissue variability [20]. In fact, *ERα* methylation is highest in normal liver, followed by colon epithelium, followed by colon stroma, and lowest in normal breast and brain tissues [18]. In the colon, careful crypt by crypt analysis showed considerable variation in age-related methylation, with much higher inter-crypt than intra-crypt heterogeneity [21]. Given that each colonic crypt in older individuals is derived from a single or very few stem cells, the data suggested that age-related methylation was a property of stem cells that is conserved upon differentiation.

Genome wide technologies to study DNA methylation have evolved considerably recently and facilitated studies of aging. Examination of mouse tissues showed the same degree of age-related CpG island hypermethylation in the GI tract as that seen in humans [22]. In addition, age-related hypomethylation was also seen in a few normally methylated promoter CpG islands. Interestingly, methylation changes in mice reach the same degree in 3 years as is reached in 80 years in humans. Thus, methylation changes reflect physiologic age rather that chronologic age. Mouse models also allowed a careful examination of the tissue-specificity of the process. While aberrant methylation could be seen in all tissues, the degree of change varied considerably, with the most prominent changes seen in the GI tract, followed by the spleen, and relatively modest changes in other tissues such as kidneys. Genome wide studies of human tissues also revealed numerous changes (both hyper and hypomethylation) in many tissues [23–26].

11.3 Causes of Age-Related DNA Methylation Changes

In considering causes for age-related changes in methylation, one has to keep in mind that it is bidirectional – i.e. increases at some sites, and decreases at others [22]. Thus, one cannot invoke simple explanations such as changes in DNMTs with

age, which in any case are minor. Unifying features of the process include conservation over evolution and tissue-specificity, with the most proliferative tissues (GI epithelium) showing the highest degree of change. Thus, a simple explanation for the process is replication errors in maintenance of epigenetic states. If one simply postulates that every stem cell replication event is associated with a finite chance of a DNA methylation error at a given CpG site, then methylation may serve as a mitotic clock [21] in the same way as telomere shortening does.

The replication error/mitotic clock hypothesis for age-related methylation does not explain all the features of the process. In particular, there is considerable gene and genomic compartment specificity to the process; many unmethylated promoter CpG islands are completely resistant to age-related changes, and normally methylated genes (e.g. imprinted loci) also show substantial variation in susceptibility to age-related change. Genomic factors linked to this variation include the density of retrotransposons surrounding a given promoter [27] (a high density is associated with fewer changes with age) and the occupancy of the promoter by polycomb group proteins in embryonic cells [26] (with polycomb group targets showing more age-related changes). In addition, baseline gene expression very likely influences the process as well [28]. One can resolve the discrepancy between a random error hypothesis and the observed gene specificity by postulating that the fidelity of transmission of epigenetic patterns can be variable across the genome, in the same way as DNA repair can vary between transcribed and non-transcribed regions [29]. Thus, it is likely that epigenetic errors are repaired (or prevented) more readily in certain regions, perhaps as a result of local features such as transcription factor binding, chromatin characteristics and nucleosome density. Strong evidence for this came from studying a length polymorphism in the RIL gene promoter that creates an extra SP1/SP3 binding site [30]. Individuals who carry the longer allele show less age-related methylation at that site than those with a short allele. The regulation of methylation maintenance and errors remains somewhat mysterious, but there is increasing evidence for such a process. Indeed, the TET family of proteins may play a role in preventing DNA methylation errors [31], and it is possible that their relative targeting to genomic sites determines that site's susceptibility to age-related changes. A "methylation error" hypothesis could account for both hypermethylation and hypomethylation with age at promoter CpG islands. However, because few genes are normally methylated in promoter CpG islands [6], hypermethylation is quantitatively the predominant feature of aging.

The mitotic clock hypothesis, modified to account for uneven methylation maintenance across the genome, is very consistent with both species conservation and tissue-specificity of age-related methylation [22]. It also provides a useful framework to interpret studies on natural variation in the process. Perhaps the single dominant factor in modulating age-related methylation is chronic inflammation. In the colon [32], esophagus [33], stomach [34] and liver [35], chronic inflammation is associated with substantially increased methylation in normal appearing tissues. In a gerbil model of Helicobacter Pylori stomach infection, a methylation increase after infection required chronic inflammation[36], and while bacterial eradication reduced methylation, it did not return to baseline. In a mouse model of inflammatory

bowel disease, inflammation was associated with a marked increase of methylation of genes targeted by polycomb in embryonic stem cells [37]. Thus, inflammation is a universal accelerator of age-related methylation. While it is possible that inflammation interacts with DNMTs or DNA itself, the changes observed are also bi-directional (increases at some genes, decreases at others) just as observed in aging. Thus, it seems more likely that the inflammation effect is to accelerate the mitotic clock through stimulated stem cell proliferation due to cycles of tissue injury and repair.

From a public health perspective, it is important to learn whether environmental exposures and lifestyle factors can also modulate age-related methylation independent of inflammation. A possible player here is dietary habits. One carbon metabolism influences the availability of the methyl donor S-adenosyl-methionine, which could theoretically drive the enzymatic DNA methylation forward [38]. Indeed, gestational exposure to varying levels of folate and vitamin B12 can influence DNA methylation patterns in newborn mice [39]; it is not known whether this extends to diets in childhood or adulthood. Human studies have yielded mixed results so far in this respect, but it may take very large studies of individuals followed for decades to be sure of this. A recent study showed that RBC-folate levels, a measure of chronic exposure, positively correlated with age-related methylation [40]. The top and bottom quartiles in RBC-folate had the same difference in age-related methylation as 10 years of age. On the other hand, folate ingestion was also shown to induce an inflammatory like gene expression profile in the colon [41], and it is therefore possible that the observed association with RBC-folate (which should be validated in other studies) is also related to inflammation. The diet-DNA methylation link and a potential link between environmental chemical exposures and altered epigenetics in humans will only be resolved by extremely precise studies of carefully selected loci in large populations.

11.4 Consequences of Age-Related DNA Methylation Alterations

The consequences of age-related methylation changes remain a matter of speculation. Functionally, changes in promoter methylation, particularly at CpG islands, can clearly impact expression of the gene in affected cells [1]. To fully appreciate the impact of aging, one has first to consider the degree of methylation changes. Quantitative measurements reveal differences in the order of 5–25% methylation (at susceptible genes) between young and old mammals [22]. The impact of this depends on whether methylation is uniformly altered to a small degree in every cell, or greatly altered in a small proportion of cells. This can be tested in two ways – by single stem cell or single crypt analysis, or by high resolution analysis using a technique known as bisulfite cloning-sequencing. Both types of experiments arrive at the same conclusion – that aged cells are epigenetic mosaics, with some cells having a very low degree of methylation at a given gene and others having a very high

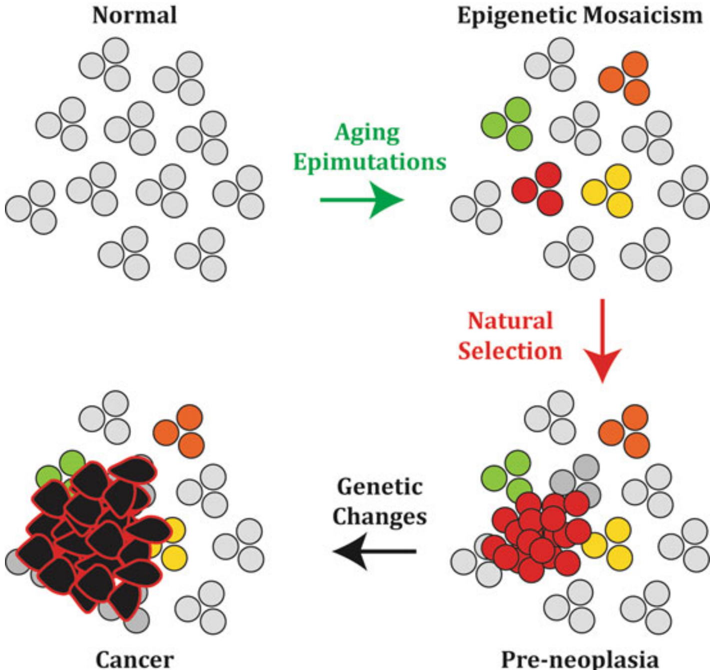

Fig. 11.1 A model of the contributions of age-related DNA methylation alterations to focal diseases such as cancer. In this model, it is proposed that our normal stem cells, represented by clusters of three cells with uniform borders in the top left quadrant, have uniform patterns of epigenetic regulation (represented by the *filled color*). Age-related DNA methylation results in epigenetic mosaicism (*top right*) creating slight stem cell to stem cell variation in gene expression. This variation serves as the engine driving natural selection that promotes the overgrowth of the stem cell that randomly acquires the appropriate gene expression pattern (*red cell cluster, bottom right*). This overgrowth leads to focal problems such as benign tumors (pre-neoplasia) and atherosclerotic lesions. Because of over-proliferation, these cells are also susceptible to genetic damage, either random or carcinogen induced, and the combination of epigenetic priming and genetic damage allows oncogenic mutations (e.g. KRAS mutation) to transform the cells into full malignancy (*black filled cells* with jagged edges, *bottom left*)

degree of methylation at the same gene [21]. These stark differences, which are underestimated when one studies averages of hundreds of thousands of cells, would be expected to result in large differences in gene expression between different stem cells in the same tissue. If one multiplies this effect by the hundreds of genes that show age-related methylation, a picture emerges whereby tissues in older individuals have considerable stem cell to stem cell variability in epigenetic profiles (Fig. 11.1). This variability has been demonstrated at the level of gene expression in intestinal crypts.

Does epigenetic mosaicism impact on the function of aged organs? At first glance, one would speculate that it is unlikely, given the vast reserves we are born with. For several organs, losing up to 90% of the cells has no appreciable impact on function.

However, there are circumstances where this may not be true. Epigenetic mosaicism may constrain the plasticity of stem cells. Indeed, many of the genes affected by age-related methylation are polycomb targets in embryonic stem cells, and are enriched for genes involved in differentiation and organ development [22, 27, 37]. Thus, it is possible that constraints on plasticity compromises the function of some aged stem cells in subtle ways that still could be contributing to declines in tissue function. For example, aged hematopoietic stem cells show skewing in differentiation, with more myeloid and fewer lymphoid progeny [42]; one wonders whether diminished stem cell plasticity and skewed differentiation could contribute to the known functional declines associated with aging.

While whole tissue function may be minimally affected by age-related methylation, the situation is very different for focal changes. Epigenetic mosaicism equates to gene expression variation which, in turn, is a powerful engine for natural selection. Thus, Darwinian mechanisms dictate that a high degree of epigenetic variation would predispose to emergence of cells with a selective growth advantage compared to neighboring cells in a given tissue. The simplest model (Fig. 11.1) would predict that variation plus natural selection results in the emergence of hyperproliferative clonal lesions in affected tissues. This may have different consequences depending on the tissue, the location of the lesion and the nature of existing co-factors. Thus, in tissues exposed to carcinogens, the initial clonal proliferation (and additional selective pressures) might allow oncogenic mutations to transform cells, resulting in full blown malignancy. Keeping in mind that many oncogenic mutations lead to senescence or death of normal cells, the convergence of age-related epigenetic changes and mutations may be the key factor in explaining the dramatic rise in incidence of cancer as humans age. Indeed, a high degree of DNA methylation abnormalities is observed in cancer [43], and often precedes full blown malignancy. In tissues where carcinogen exposure and spontaneous mutations are less prominent, focal proliferation itself may still lead to pathology, as in coronary atherosclerosis. In support of this, aberrant methylation of a few genes such as $ER\alpha$ and $ER\beta$ has been observed in atherosclerotic plaques [44, 45]. It also possible to imagine other situations where alterations in a small percentage of cells could result in significant pathology, such as through induction of a local inflammatory or fibrotic condition. Thus, several age-related pathologies from benign conditions such as loss of skin integrity to more serious diseases such as neurodegeneration could have an epigenetic component.

11.5 Age-Related Methylation and Cancer

Cancer provides an interesting example of how age-related methylation could interact with disease. Neoplastic cells show a dramatic accumulation of aberrant methylation (and other epigenetic alterations) [43]. Increased promoter CpG island

methylation in older cells was discovered by studying normal-appearing tissues adjacent to cancer, and aging changes in normal tissues were suggested to be an early step in carcinogenesis [17]. Indeed, much of the DNA methylation abnormalities in cancer can be traced to aging defects [19], and cancer itself can be seen as reflecting the state of very old cells, if one considers stem cell replication as the ultimate mark of the biologic clock. But do age-related methylation defects cause cancer? Causation is difficult to establish in humans. In mouse models, reducing methylation in normal colon by partial deletion of DNMT1 prevents the development of polyps [46], while increasing methylation by over-expression of DNMT3b increases polyp formation [47]. These data would support the model in Fig. 11.1 that predicts that age-related methylation defects may be rate-limiting for the development of pre-neoplastic lesions or "primed" cells. In human carcinogenesis, cancers are often found to arise in fields of aberrant DNA methylation [48], which is also consistent with the model. On the other hand, it has not been conclusively shown that the degree of methylation abnormalities in normal tissues correlate with the risk of cancer development, or of recurrence of intestinal polyps after resection [40]. Indeed, age-related changes in the colon are ubiquitous in the population, yet the lifetime prevalence of colon cancer is only about 5% in the US. Thus, age-related methylation alterations are clearly not sufficient to bring about neoplasia. As discussed earlier, a useful framework to think of cancer development then would be that age-related methylation subtly changes the physiology of a few stem cells, making them susceptible to neoplastic transformation, perhaps by acquisition of oncogenic mutations [49]. In this model, neither epigenetic changes nor genetic changes in isolation are sufficient for transformation, and the rate limiting step for neoplasia would be the co-occurrence of both processes. Implications for cancer prevention and risk assessment can be hypothesized from this model. Long term epigenetic intervention would be effective for prevention (as seen in mice), but short term studies would not. Conversely, a high degree of age-related changes would predict for the earlier occurrence of pre-neoplastic lesions, but the propensity to acquire genetic changes might be a more effective indicator of cancer risk in older individuals. As we learn more about potential epigenetic interventions and technology to detect rare mutations evolves, these hypotheses could become testable in human studies.

11.6 Conclusions

Aging is accompanied by progressive, proliferation dependent DNA methylation deregulation that creates epigenetic mosaicism in older tissues. These changes may compromise stem cell function and contribute to diseases of aging, particularly focal diseases such as neoplasia. Interventions aimed at reducing age-related methylation deregulation have the potential to attenuate the occurrence and severity of these diseases.

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Chapter 12
Influence of Environmental Factors on the Epigenome

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Abstract In this chapter we will consider the role of environmental factors on the epigenome. The importance of rapidly emerging research into the types of exposures that may alter epigenetic marks is increasingly being recognized. A large portion of epigenetic research to date has focused on epigenetic alterations in cancer and therefore, exposures such as tobacco, alcohol, radiation, arsenic, and air pollution will be covered. Additionally, we review research on other exposures that may affect epigenetic states such as folate and diet, aging, and endocrine disruptors. We will briefly cover environmental exposures and imprinting and development, as well as discuss potential mechanisms for exposures to modify epigenetic states. Appropriate epidemiologic studies will be crucial to understanding the true effect of environmental exposures on the human epigenome and this work is urgently needed in order to better understand the biology of epigenetic alterations. With a more comprehensive understanding of the affects of exposures on the epigenome (including consideration of genetic background), not only will the prediction of the toxic potential of new compounds be more readily achieved, but more personalized disease prevention and treatment strategies may be developed.

12.1 Introduction

The epigenome is the landscape of mitotically heritable changes in gene expression and gene expression potential that are mediated without altering genetic sequence. A role for environmental exposures in epigenetic alteration is increasingly recognized and investigated, and much of the existing evidence implicating a role of environmental factors on the epigenome comes from studies of disease outcomes such as cancer and adverse reproductive/developmental events. Initially, epigenetic alterations were identified in various human tumors and consequently, environmental exposures known to have an etiologic role in cancer such as tobacco, air pollutants, and metals have been studied and implicated in the modification of epigenetic marks. Of course, this has highlighted the need to consider how environmental factors may influence the epigenome in pathologically normal tissues, perhaps representing

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alterations necessary to initiate or predispose to disease phenotypes. The majority of epigenetic alteration work conducted to date has focused on DNA methylation, though emerging work is beginning to address the affect of environmental exposures on histone modifications and microRNA (miRNA) expression. This chapter highlights the current evidence that epigenetic alterations are associated with environmental exposures, focusing on human studies and drawing primarily on studies of human cancer. The potential mechanisms behind environmentally related epigenetic alterations and methods for studying the relation between the epigenome and the environment that are amenable to epidemiologic research are also presented. As the data from human studies has, for the most part, centered on alterations to DNA methylation, this chapter will focus on the examination of DNA methylation and environment.

12.2 Environmental Exposures of Diet and Lifestyle and Epigenetics

12.2.1 Folate, One-Carbon Metabolism, Diet, and Body Mass Index

One-carbon metabolism is the network of biochemical reactions essential to both DNA synthesis and all cellular biomolecule (nucleic acids, proteins, lipids) methylation reactions that involve the transfer of one-carbon groups. A critical nutrient central to one-carbon metabolism is folate (vitamin B_9), which donates its methyl group for homocysteine remethylation to methionine. Subsequently, methionine is the methyl donor for all cellular methylation reactions, most notable for this discussion being DNA and histone methylation via S-adenosyl methionine (SAM), and other B vitamins (B_2, B_6, B_{12}) that act as enzymatic cofactors in the network. Collectively, B vitamins, homocysteine, and methionine are important contributors to the maintenance of DNA integrity and DNA methylation. Figure 12.1 represents the links between diet, one-carbon metabolism, DNA synthesis, DNA methylation, and toxicant metabolism.

In mammals, establishment of somatic cell epigenetic patterns occurs early in fetal development subsequent to genome-wide reprogramming of epigenetic patterns at the multiple developmental stages (fertilization, implantation, blastocyst) when totipotency and pluripotency requirements are dynamic [1]. In addition, DNA methylation is necessary for the mitotic inheritance process of genomic imprinting (monoallelic gene expression which can also be tissue-specific). Embryonic and fetal nutrient availability have great potential to affect the epigenetic reprogramming and patterning phenomena, which has implications for proper development and perhaps even life-long conditioning and health [2, 3]. Transmission of both nutrients and environmental exposures to developing mammals in utero proceeds through the placenta, and maternal folate status is recognizably associated with

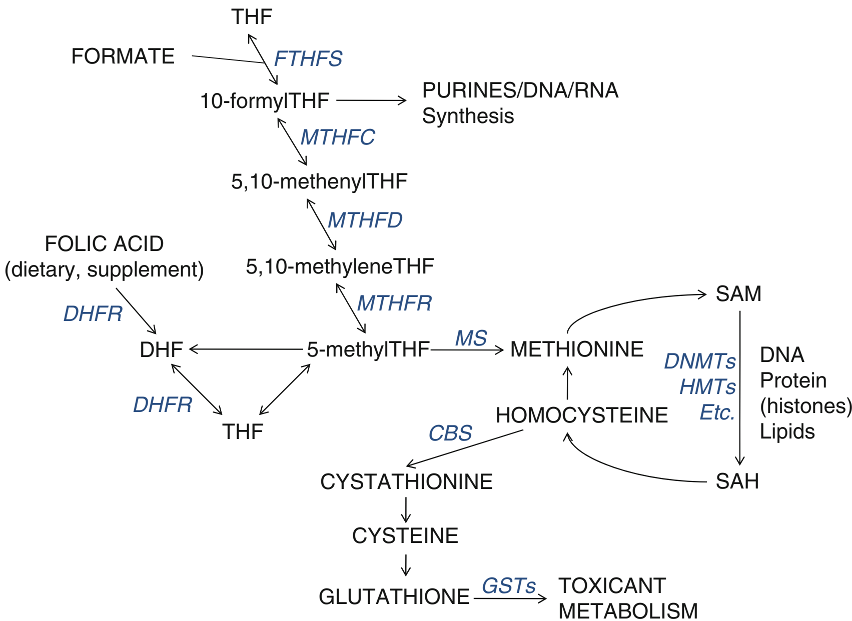

Fig. 12.1 Diet, one-carbon metabolism, DNA synthesis, DNA methylation, and toxicant metabolism

fetal development. For instance, it is well-known that folate deficiency is associated with neural tube defects (NTD) [4] and animal studies have indicated the necessity of sufficient methyl group availability for proper neural tube development [5]. Further, hypomethylation of long interspersed nucleotide elements (LINE) and genomic DNA has been associated with increased risk of NTDs in humans [6]. Beyond this, Waterland and Michels [7] have proposed that epigenetic mechanisms may contribute to the developmental origins of health and disease hypothesis, which states that nutrition and environmental stimuli at critical developmental periods (pre- and postnatal) can induce permanent alterations in susceptibility to metabolic and chronic diseases [8]. Additional details on epigenetic changes during development are available in Chap. 5, and the developmental origins of health and disease hypothesis is covered in Chap. 6.

There have been several animal studies that directly link folate intake, diet, and epigenetic alterations though similar evidence from human studies is only beginning to emerge. Many human studies have, however, described associations between folate, B vitamins, or diet and disease risk and suggested the potential for epigenetic mechanisms to account for these observations. For instance, elevated serum vitamin B_6 and methionine have been associated with reduced risk of lung cancer [9], folate intake has been associated with reduced risk of breast cancer (especially among women with high alcohol consumption) [10, 11] and a protective role for folate in

colon cancer [12] has been consistently reported. In a pooled analysis of 13 prospective cohort studies on folate intake and colon cancer Kim and colleagues reinforced the significant, though modest protective role of folate for reducing risk of colon cancer by approximately 2% per 100 μg/day of folate [13]. A recent work from the Netherlands Cohort Study on diet and cancer investigated risk of colorectal cancer according to methyl-donor (folate, methionine, and vitamins B_2 and B_6) intake and considering genotypes of enzymes in the one-carbon metabolism pathway [14]. These authors did not find significant diet-gene interactions, but did observe a significant inverse association between methionine intake and risk of colon cancer among individuals with common *DNMT3B* (*de novo* DNA methyltransferase) genotypes as well between B_2 intake and risk of colon cancer in individuals with one or less rare allele among the folate enzyme genes *MTHFR, MTRR,* and *MTR* [14]. In addition, a study of serum concentrations of folate and plasma SAM levels in over 330 women showed that body mass index (BMI) was the strongest determinant of SAM concentration, suggesting that nutrient intake and nutrient availability are distinguishable, and that BMI is a potential confounder of folate-methylation associations [15]. Yet, far fewer examples of studies providing direct lines of evidence for associations between folate and/or diet and epigenetic alterations in humans exist [16]. In one such study, Ingrosso et al. demonstrated that global DNA hypomethylation (measured through cytosine extension and Southern blot) was higher in uremia patients with hyperhomocysteinemia, and that folate treatment restored DNA methylation to normal levels [17]. Hypomethylation of non-diseased colon tissue DNA from disease-free controls, measured through ^{3}H-methyl incorporation, was found to be greater in samples amongst individuals with adenomas and colon tumors compared to controls, and this hypomethylation of both colon tissue and leukocyte DNA was associated with risk for colon cancer [18]. Folate status has been inversely correlated with colon tissue DNA methylation extent [19]. In head and neck squamous cell carcinoma (HNSCC) repetitive element methylation extent was associated with folate intake, was reduced amongst individuals with a variant *MTHFR* genotype, and was a significant risk factor for disease [20]. A study of tumor DNA methylation in over 160 breast tumors at over 750 cancer-related genes demonstrated a significant association between methylation profile and dietary folate when controlling for potential confounders such as alcohol intake [21].

Outside of cancer, although there is a large body of literature examining the relation of diet and disease risk, particularly examining nutrients involved in one-carbon metabolism, there is less direct evidence linking these associations through epigenetic mechanisms. As an example, there is a reasonable literature that suggests altered methylation status is associated with cardiovascular disease and this may be linked with folate status [22–24]; however, the causal nature of these associations remains to be shown. Further study in humans investigating the associations between dietary factors and methylation alterations (in normal and diseased states), including potential confounders such as BMI and alcohol are necessary.

Other areas of interest for future study include the potential for differential contribution of nutrient factors dependent upon disease context (healthy vs. diseased), the potential risks of high-dose nutrient supplementation practices, and interactions

between one-carbon network participants. Folate supplementation is associated with a reduced risk of many forms of cancer, though once disease is present it is possible that a relative increase in one-carbon nutrient levels may speed disease progression or invasiveness dependent upon the epigenetic character of the disease-state. Some evidence for this potential comes from our recent study of breast tumor methylation where increased folate intake (controlling for potential confounders) was associated with a methylation profile that was independently associated with increased tumor size [21]. Excessive folate supplementation may have potential adverse effects including masking of B_{12} deficiency, disruption of zinc function, or interfering with one-carbon homeostasis and additional study is required to elucidate these potential effects [16, 25]. The one-carbon pathway links nutrient availability with not only DNA methylation, but also with toxicant metabolism through glutathione and the glutathione transferase (GST) enzyme family (Fig. 12.1). Therefore, interactions between one-carbon pathway participants may modify associations between exposures, pathway participants, and methylation alterations. In fact, a study of reduced GST enzyme activity in a mouse model of Alzheimer's disease and neuronal health has shown that SAM can mediate the activity of GST enzymes [26]. This suggests that reactions dependent upon SAM are necessary for GST activity and that SAM may be a critical mediator of neuronal health [26]. Furthermore, it could be predicted that GST enzyme family genotypes (among other genotypes) will be shown to modify associations between certain exposures and DNA methylation alterations. Hence integrative studies that incorporate genetics (genotype and/or alterations) with measures of epigenetic alterations will allow a more comprehensive understanding of the relation among exposures, epigenetic alterations, and genetic states. However, at this time it is unclear the extent to which genotype may influence epigenetic states. More research is needed to ascertain best practices for assessing folate/methyl availability, perhaps comparing food-frequency-questionnaire data to homocysteine and/or SAM/SAH levels in a prospective manner.

12.2.2 Alcohol

Alcohol is known to interfere with folate absorption in the intestine and hepatic release of folate, and hence, supply to tissues [27]. Although it is a weak mutagen [28], by inhibiting the one-carbon metabolism network, alcohol may contribute to increased risk of disease in an epigenetic fashion. More specifically, ethanol can interfere with several steps of methionine metabolism and can lead to activation of betaine homocysteine methyltransferase to activate a compensatory pathway for maintenance of SAM levels [29]. However, with continued exposure, the compensatory pathway cannot be maintained and liver injury will result. In addition to liver injury, an etiologic role for alcohol in several cancers is well established [30]. For example, alcohol consumption increases colon cancer risk [31], though the association may be modified by dietary folate, and may be specific to LINE-1 hypomethylated

tumors [32]. More specifically, subjects with high folate intake were less likely to develop LINE-1 hypomethylated colon cancers but subjects with high alcohol consumption had a significantly increased risk of developing LINE-1 hypomethylated colon cancers with no association for more highly methylated LINE-1 tumors [32]. In addition, alcohol has a well-recognized etiologic role in HNSCC, and in these tumors, alcohol consumption has been significantly associated with reduced LINE methylation [33]. HNSCC tumor methylation profiles (based on 1413 CpG loci) are significantly and independently associated with alcohol intake [34], and beyond DNA methylation, miR-375 expression levels in HNSCC have been shown to be significantly increased with increasing alcohol consumption [35]. An excess risk for each alcoholic drink per day of about 10% has been reported in multiple meta-analyses of prospective and case-control studies of breast cancer [36, 37], and a study of breast tumor DNA methylation demonstrated a significant, independent association (controlling for age, dietary folate and other variables) between alcohol intake and tumor DNA methylation profile (based on 1413 CpG loci) [21]. In addition to DNA methylation and miRNA expression alterations, ethanol exposure has been shown to selectively acetylate histone H3 at lysine 9 (H3K9) in primary culture of rat hepatocytes [38] and in rats in vivo [39], though studies in humans are necessary. Collectively, these studies suggest that a major carcinogenic mechanism of action of alcohol is interference with epigenetic regulation, in part through disruption of one-carbon metabolism.

12.2.3 Aging

The aging process itself and differences in environment have been hypothesized to influence clinically significant changes in methylation profiles as individuals accumulate varying exposures with age. Epigenetic variation has been hypothesized to cause underlying differences in disease susceptibility among monozygotic twins, and in one study young twin-pairs seemed more epigenetically similar than older monozygotic twins, though there were a limited number of twin pairs studied [40]. An early report from Issa et al. described an association between aging colonic mucosa and estrogen receptor methylation [41]. Cancer is a disease of aging, and initial studies of age-related methylation in normal tissues were motivated in large part by studies of methylation in cancer [42]. Beyond diseased tissues, recent work has shown an overall trend of increased methylation associated with older age in normal human prostate and colon tissues in several genes [43, 44]. Although an increase in promoter methylation with aging is generally accepted, recent evidence from Bjornsson et al. suggests a more complex picture. These authors measured intra-individual global methylation changes over >10 years and found both increased and decreased methylation levels dependent on the individual, with over 50% of participants exhibiting >5% change in methylation in peripheral blood cell DNA over time [45].

In general, trends of global (repeat element) hypomethylation and promoter hypermethylation found in cancer also have been observed in normal tissues with aging [46]. A study from Agrawal et al. recently linked age-related DNA hypomethylation to chronic inflammation via increased reactivity to self-antigens revealed by the hypomethylation *per se* [47]. This is particularly interesting because it links age-related hypomethylation to immunogenicity and inflammation which has been proposed as a mechanism for aberrant hypermethylation. In recent reports of age-related methylation in normal human prostate and colon tissues, several CpG-island-containing genes were reported to have age-related increases in methylation [43, 44]. A study of over 200 non-pathologic human tissues from ten anatomic sites using the GoldenGate methylation array platform (1413 autosomal CpG loci) demonstrated consistent associations between methylation and age at previously reported candidate genes, and in conjunction with other reports, suggested that the relation between aging and promoter CpG methylation is complex [48]. The direction and strength of correlation between age and methylation were largely dependent upon CpG island status, and specifically a propensity for CpG-island loci to gain methylation with age, and non-island CpGs to lose methylation with age was found, a result consistent with [43, 44, 49], as well as with the findings of Tra et al. and Bjornsson et al. who showed bi-modal age-related methylation in normal tissues. Using restriction-landmark genome scanning of over 2000 CpG loci in T lymphocytes comparing newborns, middle age, and elderly people, Tra et al. reported that 29 loci had age-related methylation alterations, with 23 loci displaying increased methylation with age and 6 decreasing with age [50]. In addition Teschendorff et al. have suggested that age-dependent methylation of polycomb-group target genes (genes suppressed in stem cells allowing differentiation) is a hallmark of cancer and is independent of gender, tissue type, or disease state, suggesting a mechanism for aging to predispose to carcinogenesis [51]. The observed pattern of age associated methylation in [48] was also irrespective of tissue-type, suggesting a common mechanism or dysregulation to explain these alterations. Reduced fidelity of maintenance methyltransferases with aging is one potential explanation for age related decreases in methylation; while age related increases in methylation could potentially reflect the accumulation of stochastic methylation events over time. Additional details on aging and changes in epigenetic profiles are available in Chap. 10.

12.2.4 Tobacco

Epigenetic alterations, particularly DNA methylation, have been thoroughly examined in human lung cancer [52] and have been recently reviewed in regards to its contribution to carcinogenesis in rodent lung cancer models and human disease [53]. Of course, the major etiological contributor to lung cancer is tobacco smoking, and this exposure also plays an important carcinogenic role in a number of other solid tumors, such as oral and bladder cancers [54]. The *CDKN2A* gene (an extensively studied tumor suppressor gene encoding p16INK4A), which is involved in

the Rb cell cycle control checkpoint pathway, is one of the most widely described targets of methylation-induced silencing in cancers, and is thought to play an early and important role in tumor development [55]. Methylation silencing of this gene occurs more often in squamous cell lung cancer, and has been associated, in these tumors, with an increasing duration of tobacco smoking [56, 57]. Even in adenocarcinomas of the lung, methylation of the *CDKN2A* promoter occurs more often in smokers [58] and has been associated with a higher degree of background anthracosis [59]. Methylation of the *RASSF1A* gene has been associated with an earlier age at starting smoking in lung cancer patients [60, 61], suggesting that still growing adolescent lungs may be particularly susceptible to tobacco-related epigenetic events [60, 62]. Interestingly, in mouse models of lung adenocarcinoma induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) exposure, tumors exhibit *Cdkn2a* deletion, but also exhibit hypermethylation of *Dapk* and *Rarb* [63–66]. NNK exposure has also been linked to *Cdkn2a* methylation in rat liver adenomas and hepatocellular carcinomas [67].

In oral cancer, methylation of *CDKN2A*, *DAPK*, *RASSF1A*, or *CDH1* (encoding E-cadherin) was significantly associated with smoking, with *CDKN2A* methylation occurring more often in patients who started smoking at a younger age, and *CDH1* methylation occurring in patients with higher pack-years smoked [68]. More recently, profiling of over 750 cancer-related genes with a bead-array approach in 68 tumors of the head and neck denoted that methylation profiles of these tumors were significantly associated with smoking intensity (packs per day) though not significantly associated with smoking duration or cumulative exposure (packyears) [34]. Additionally, in a study of colorectal cancer (Iowa Women's Health Study), smoking variables including age at initiation, duration, and packyears were associated with CpG island methylator phenotype positive tumors [69].

In bladder cancer, which is also associated with tobacco smoke exposures, although to a lesser extent than lung cancer, associations have been observed between smoking and *CDKN2A* methylation, with increased risk of this alteration in current and former smokers, particularly former smokers of less than 10 years [70]. Also in bladder cancer, methylation of any of the *SFRP* genes was associated with having a smoking history [71]. Collectively, these reports suggest that in bladder cancer, less important than intensity or duration is simply the continuous exposure of target cells to tobacco smoke-derived carcinogens, which selects for these alterations.

Building on the mechanistic link between tobacco exposure mediating its cancer risk through epigenetic alterations are a number of studies demonstrating a relation between tobacco exposure and promoter hypermethylation detected in DNA from sputum samples and pre-malignant lung epithelium from heavy smokers [72–78] as well as in non-malignant oral epithelium of smokeless tobacco users [79]. Beyond DNA methylation alterations, recent evidence has suggested that maternal cigarette smoking during pregnancy is associated with downregulation of multiple miRNAs in placental tissues [80].

It should be noted, though, that although tobacco exposure has been associated with specific profiles of tumor methylation including a methylator phenotype, it

does not necessarily lead to global hypermethylation of tumor suppressor genes, suggesting instead a targeted mechanism for the carcinogen's role in epigenetic alteration. One example is *DAPK* methylation which is associated with advanced stage lung cancer, was not associated with tobacco smoke exposure [81]. Promoter methylation of *MGMT* occurs more commonly amongst never-smokers than ever smokers in lung adenocarcinomas [82], and *FANCF* methylation occurs more often in patients with a shorter duration of tobacco use [83]. Methylation of the *ESR1* gene (encoding estrogen receptor-alpha) also occurs at a lower prevalence in NNK-induced lung tumors in mice and rats and in human lung cancer in smokers compared to non-smokers [84]. Together, these results suggest that for some genes, susceptibilities or exposures other than tobacco-smoke may be important in the targeting of these loci for epigenetic alteration.

12.3 Environmental Toxicants and Epigenetic Alterations

12.3.1 Endocrine Disruptors

Exposures to endocrine disrupting chemicals such as diethylstilbestrol (DES) and bisphenol A (BPA) are of particular concern in the context of development. DES is a non-genotoxic carcinogen with developmental stage-specificity, that in the past was administered to women during pregnancy to prevent miscarriages [85]. In utero exposure of mice to DES has been shown to result in the hypermethylation of the developmentally critical (specifically to uterine organogenesis) *Hoxa10* gene [86]. Epidemiologic evidence from individuals exposed to DES during the first 3 months in utero indicates an increase in vaginal clear cell carcinoma incidence and reproductive disorders [87]. In addition, grandchildren of DES exposed women reported higher incidences of rare reproductive disorders; whether this reflects detection bias or possibly implicates a role for epigenetic transgenerational inheritance remains to be clarified [87]. Though the mechanism through which DES establishes altered epigenetic marks capable of transgenerational inheritance remains unclear, a model for the epigenetic effects of DES has been proposed by Ruden et al. drawing similarities between DES and Hsp90, which acts to play a role in modifying H3K4 methylation by increasing the activity of the H3K4 methyltransferase *SMYD3*, thereby altering epigenetic control of various genes [88].

BPA is a monomer used in the production of polycarbonate plastic, a flame retardant, a fungicide and a surface-coating for everyday objects [89], and human exposure to BPA has been increasing in recent years [90]. BPA has been shown to readily cross the placental barrier and accumulate both in the placenta and the fetus [91]. Animal studies have demonstrated that developmental exposure to BPA can alter epigenetic profiles. Briefly, Dolinoy et al. showed that in utero BPA exposure

decreases CpG methylation in agouti mice and that methyl-donor supplementation negated BPA related hypomethylation [92]. In human placental cell lines BPA exposure has been shown to alter miRNA expression levels [93]. More specifically, miR-146a was strongly induced by BPA treatment and resulted in both slower proliferation rate and higher sensitivity to the DNA damaging agent bleomycin [93]. BPA, like DES, has also been shown to alter the methylation status of the *Hoxa10* gene in rodent in-utero exposure models [94], and breast cancer cell lines exposed to BPA showed altered DNA methylation of specific gene promoters [95]. Although awareness of BPA exposure has increased dramatically and some manufacturers have stopped using it, additional study of BPA (as well as emerging substitutes) is necessary to better understand its potential epigenetic effects.

12.3.2 Benzene

Benzene is a ubiquitous chemical, has been shown to be toxic to blood-forming systems, is an established carcinogen [96], and is known to be a cause of acute myeloid leukemia (AML) and myelodysplastic syndromes [97]. Exposed to low dose benzene, gasoline attendants and traffic officers have demonstrated significant leukocyte LINE-1 and Alu DNA hypomethylation as well as gene specific hypermethylation of *CDKN2B* and hypomethylation of *MAGE1* [98]. One preliminary study of benzene exposure using buffy coat DNA from six exposed workers and four unexposed workers has shown some evidence for relatively small changes in methylation [99]. Another preliminary study analyzed miRNA expression in seven exposed-control matched pairs and identified upregulated miR-154*, miR-487a, miR-493-3p and miR-668 in exposed subjects [99]. Additional study of the potential epigenetic effects of benzene exposure is needed.

12.3.3 Radiation

Exposures to ionizing radiation have long been linked to cancer, through DNA damage in the form of large deletions and in some cases point mutation [100–102]. On the other hand, silencing of *Cdkn2a* was detected in lung tumors of rats induced by exposure to ^{239}plutonium [103] and in human lung adenocarcinoma, *CDKN2A* methylation occurred more often in workers compared to non-worker controls of the Russian MAYAK weapons-grade plutonium plant, and the prevalence of this methylation exhibited a dose–response with radiation internal exposure dose [104]. Methylation of *CDKN2A* has also been linked to reactive oxygen species produced by radiation exposures [105]. Murine models of radiation-induced lymphoma have also demonstrated hypermethylation of *Cdkn2b* (encoding p15ink4b [106–108]).

12.3.4 Arsenic

Arsenic exposure has been associated with DNA methylation alterations in non-pathologic as well as tumor tissues and there is some data suggesting that changes in miRNA expression and histone tail modifications are also associated with exposure to arsenic. Although the mode(s) of arsenic's carcinogenicity is not completely clear, there is some speculation about arsenite-generated free radicals and reactive oxygen species lead to genotoxic damage [109]. However, in vitro exposures to inorganic arsenic species have demonstrated dose-dependent increases in promoter region hypermethylation of CpG sites, although not to those resulting in altered gene expression, as well as to the occurrence of genome wide hypomethylation [110–112]. In human bladder cancer, relatively low levels of inorganic arsenic exposure has been associated with methylation of *RASSF1A* and *PRSS3* (but not *CDKN2A*) [70], and in mouse models of methyl- or folate-deficient diets, arsenic exposure through water supply led to hypomethylation in hepatic-derived DNA [113], as well as to increases in chromosomal aberrations in blood lymphocytes [114]. Thus, perhaps a more plausible mechanism for arsenic-related carcinogenicity is via the depletion of S-adenosyl methionine (SAM, the universal methyl donor) due to the metabolism of inorganic arsenic to its methylated forms resulting in altered DNA methylation.

As more studies are conducted a complex picture of dose-dependent DNA methylation alterations with arsenic exposure is beginning to emerge. Chanda et al. have shown that arsenic exposure can result in *CDKN2A* hypermethylation in human blood DNA, but that a subgroup of cases had hypomethylation with high arsenic exposure [115]. More recently, a group of some of the same authors has better quantified the relation showing that exposures to 250-500ug/L of arsenic in drinking water results in global hypermethylation (^{3}H methyl group uptake), but that >500ug/L of arsenic results in global hypomethylation [116]. In another study of peripheral blood DNA methylation by ^{3}H methyl group uptake, Pilsner et al. showed increased DNA methylation to be associated with urinary and plasma arsenic and plasma folate, and that the association between arsenic and methylation was modified by folate in that it was restricted to individuals with high plasma folate [117]. This same group has shown that folic acid supplementation lowers blood arsenic [118], maternal and cord blood pairs have highly correlated arsenic levels [119], and folate deficiency, hyperhomocysteinemia, and leukocyte hypomethylation are associated with arsenic-induced skin lesions [120]. Beyond interfering with one-carbon metabolism and affecting DNA methylation, treatment of human lymphoblastoid cells with sodium arsenite led to global increases in miRNA expression [121]. The genotoxic effects of arsenic may also be mediated by altered chromatin and Ramirez et al. have shown in human hepatocarcinoma cells that treatment with sodium arsenite resulted in global increases in histone acetylation [122]. Thus, it is reasonable to posit that increased expression of miRNAs associated with arsenic exposure may be due to increased histone acetylation. Nonetheless, additional studies are necessary to further elucidate the associations between arsenic and all major forms of epigenetic alteration with particular attention being given to dose and modification by one-carbon metabolism pathway participants.

12.3.5 Other Metals (Nickel, Lead, Cadmium, Chromium)

Although there is currently very little evidence for epigenetic alterations due to exposure to other metals in humans, several studies have suggested that exposures to metals such as nickel, lead, cadmium and others may alter epigenetic marks. For example, nickel exposure has been associated with various histone modifications and exposure of mammalian cells to nickel resulted in decreased histone H4 acetylation [123, 124]. Although it is an established carcinogen, cadmium is a weak mutagen. However, exposure of a rat cell line to cadmium resulted in a concentration-dependent reduction in DNA methyltransferase activity [125]. A study of metal-rich (lead, cadmium, chromium) particulate matter exposure among electric-furnace steel plant workers has shown significant alterations of micro-RNA expression in peripheral blood leukocytes [126]. In the Normative Aging Study, patella lead levels were associated with reduced global DNA methylation (LINE-1 elements) though not Alu repeat regions [127], suggesting a role for lead exposure in epigenetic alterations. Interestingly, a sexually dimorphic global methylation (Luminometric methylation assay for CCGG) level decrease was associated with brain mercury levels in male polar bears [128].

Chromium has industrial applications including chrome plating and stainless steel welding and is implicated in occupationally related lung cancers [129]. Mechanisms of chromium carcinogenicity include adduct formation [130] and DNA strand breakage [131], and some limited evidence suggests the potential for chromium to have epigenetic effects. In cases of chromate-induced lung cancer Kondo et al. showed that although prevalence of *CDKN2A* methylation was similar between chromate and non-chromate lung cancers, chromate lung cancers were more likely to have methylated *CDKN2A* with increasing duration of occupational exposure [132]. An *in vitro* exposure of mouse hepatoma cells to chromium showed transcriptional repression of *Cyp1a1* by local cross-linking of Hdac and Dnmt1 and altered histone marks [133]. However, additional study of this diminishing exposure is necessary to validate potential chromium-induced epigenetic alterations.

12.3.6 Particulates/Air Pollution

Environmental particulate exposure has been linked to increased risk of lung cancer [134] and increased morbidity and mortality from cardiovascular and respiratory illnesses [135], though few studies have addressed the potential for these exposures to alter DNA methylation. In rat lung cancer models of tumors induced by diesel exhaust, 59% were methylated at *Cdkn2a* and 14% at *Esr1*, in those induced by carbon black 45% were methylated at *Cdkn2a* and 14% at *Esr1*, and in those induced by beryllium metal strikingly 80% were methylated at *Cdkn2a* and 50% at *Esr1* [136]. Extending to miRNA alterations, a study using primary bronchial epithelial cells from nonsmokers has shown dramatic alterations in miRNA expression

following treatment with diesel exhaust particles (197 of 313 measured were up or down-regulated more than 1.5 fold), and suggested that the alterations were associated with inflammatory response pathways [137]. In humans, exposure to particulate matter of <10 μm (PM_{10}) in steel plant workers was associated with significantly lower peripheral blood *NOS2* promoter methylation [138]. In addition, this same study reported significantly reduced methylation of LINE-1 and Alu repeat elements in blood DNA associated with long-term PM_{10} exposure [138]. Consistent with this, a recent report examining the affect of ambient particulate pollutants on repeat element methylation in subjects from the Boston area Normative Aging Study found significantly decreased LINE-1 methylation following recent exposure to higher black carbon [139].

Ren et al. have recently provided some insight into a potential mechanism of air pollution associated epigenetic alterations, and evidence for genetic interactions that modify effects of exposure [140]. These authors found that interquartile range increases in $PM_{2.5}$ and black carbon were associated with increases in plasma homocysteine (part of one-carbon metabolism), and glutathione transferase theta genotype (an antioxidant dependent upon homocysteine levels) [140]. This same group of authors also reported that increases in mean air pollution PM_{10} concentrations did not significantly alter fasting or postmethionine-load total homocystiene in non-smokers, but was associated with significantly increased homocysteine levels in smokers, suggesting interactions between exposures may contribute to epigenetic alterations [141]. Another line of evidence for modification of exposure effects on epigenetics by genetics comes from Wilker et al. who have reported that increases in black carbon associated with increased blood pressure are modified by SNPs in microRNA processing machinery genes such as *DICER* and *GEMIN* genes [142].

12.3.7 Asbestos

Exposure to asbestos is the main risk factor for malignant pleural mesothelioma with approximately 80% of cases reporting a known exposure to asbestos [143]. Importantly, in contrast to tobacco smoke and radiation, asbestos is known to be a weak mutagen [144], and there have been several reports of tumor suppressor gene methylation in mesothelioma [145–148]. In patients with mesothelioma, Tsou et al. described a significant association between self-reported asbestos exposure and methylation at the *MT1A*, and *MT2A* gene loci among 28 genes examined [149]. A significant association has been reported between an increasing number of methylated cell cycle control genes and a quantitative asbestos burden measure [150]. This was followed by examination using an array-based approach, which demonstrated that quantitative measure of asbestos exposure was associated with altered methylation at over 100 discrete CpG loci, and that in almost all cases (94%) there was increased methylation associated with increased exposure [151]. Further, overall methylation profiles for mesotheliomas were significantly associated with asbestos exposure burden [151].

12.4 Environmental Exposures and Imprinting and Development

Genomic imprinting involves epigenetic silencing of a single allele distinguishing the maternal or paternal inheritance pattern of a specific locus [152]. Imprinting and its importance in development as well as alterations to imprinting and its effect on disease, including cancer, have been thoroughly reviewed by Feinberg and colleagues [152–154]. Here, we would like to point out some interesting work that suggests that there are environmental exposures that can alter imprinting. The allele-specific expression pattern associated with imprinting is based upon CpG island methylation of differential methylation regions (DMR) which occur in the upstream regions of a number of genes and act as both transcriptional repressive regions as well as insulators to neighboring repressive elements [155, 156]. Alterations to the imprinted status of DMRs has been observed after prolonged culture of mouse embryonic stem cells, leading to a number of phenotypic abnormalities, through both gains and losses of appropriate methylation of these regions [157]. These alterations to the imprinting pattern are believed to be related to exposure to particular media or serum [158, 159]. In humans, it has recently been suggested that assisted reproductive technology (ART) may affect the epigenetics of development thus altering imprinting status. In sheep and cattle, large offspring syndrome has been linked to culture of pre-implantation embryos [160] and in humans, associations between Angelman syndrome [161], and Beckwith-Wiedemann [162] syndrome and in-vitro fertilization have been reported, and are related to changes in imprinting status of specific genes. The exact exposures encountered by these pre-implantation embryos and leading to these epigenetic alterations are unclear. The relation between environmental exposures and imprinting alterations has been examined in a cross-sectional study of patients referred to an academic gastrointestinal practice, and although no associations were observed with exposure to tobacco or alcohol nor with measure dietary factors and NSAID drug use, the authors themselves acknowledge that the study was designed such that it could detect only quite large effects of these exposures on any loss of imprinting [163]. The animal as well as human studies point to the need for additional work to characterize whether alterations in imprinting occur somatically and if these changes are related to environmental exposures, as well as to determine if and how in-utero and neo-natal exposures may affect the epigenetic marks at these imprinted loci. More on epigenome changes during development can be found in Chap. 5, details of imprinting disorders are available in Chap. 8, and assisted reproductive technologies are covered in Chap. 7.

12.5 Mechanisms

Ascribing a mode by which environmental exposures drive or select epigenetic alteration is difficult due in part to the lack of understanding of the mechanism by which *de novo* methylation occurs in disease. In fact, dependent upon context and

exposure, different mechanisms can contribute to epigenetic alterations. Although it is beyond our scope to consider specific contextual details such as relative location (repeat, promoter, other), existing methylation state, nucleosome position, local and regional CpG density and transcription factor binding sites; it is important to recognize that contextual elements may differentially affect the likelihood of exposure-related alterations. For example, a recent report from Rakyan et al. showed that aging-associated hypermethylation was more likely to occur at promoters associated with bivalent chromatin domains [164]. Additionally, distinct mechanisms may be responsible for altered repeat element (global) methylation and altered promoter methylation. Although traditional dogma indicates separate methyltransferase enzymes for maintenance (*DNMT1*) and de novo methylation (*DNMT3A/B*), a recent perspective from Jones and Liang proposes that maintenance methylation in CpG islands may be a cooperative process [165]. When considering the mechanisms of environmental exposure related methylation alterations it is important to remain conscious of the fact that we do not have a complete understanding of *de novo* and maintenance methylation mechanisms. Further, similar to histone modifications, environmental and intrinsic signaling mechanisms that alter miRNA expression levels are poorly understood. Nonetheless, several mechanisms for environmental exposure related epigenetic alterations should be considered.

One mechanism by which an exposure may result in altered DNA methylation is increased reactive oxygen species as a result of inflammatory response. More specifically, it has been reported that 5-hydroxymethylcytosine can be generated by oxidation of 5-methylcytosine [166], and both 5-methylcytosine adjacent to 8-oxoguanine, and 5-hydroxymethylcytosine have been shown to inhibit binding of methyl-CpG binding protein 2, a critical epigenetic regulator that recruits cytosine methyltransferases and histone deacetylases [167]. It is also known that 5-hydroxymethylcytosine is not recognized as 5-methylcytosine by the maintenance methyltransferase DNMT1, and hence, may lead to aberrant loss of methylation during cell replication [168]. Additional base alterations occur via neutrophil and eosinophil peroxidase-derived HOCl and HOBr which can react with DNA to form 5-chlorocytosine and 5-bromocytosine respectively [169]. These halogenated cytosines can be mistaken by DNMT1 as 5-methylcytosine during replication, thus providing a potential mechanism for inflammation-induced aberrant hypermethylation [168].

Another mechanism that has been hypothesized is a spreading process, whereby CpG methylation from within the gene migrates into the promoter due to the loss of protective boundary elements which normally protect promoters from CpG island methylation [170–172]. Alternatively, for example in aging, a spreading process subsequent to a stochastic methylation alteration (in pathologically normal tissue) without functional consequences for gene expression, may allow the initiation of a spreading process that eventually confers an altered expression phenotype. Such age-related drift of normal epigenomes (without prominent changes in gene expression) may confer significantly increased risk of conversion to a pathologic phenotype by enhancing both the likelihood and frequency of methylation events that ultimately result in altered expression or genomic stability. For example, in the

context of acquired "non-functional" CpG methylation in the promoter region of an aged individual, continued stochastic methylation events (e.g. "methylation spreading") increase the chance of methylation induced silencing at that promoter (or silencing of another locus through action at a distance via silencing of other important regions such as enhancers), and hence, progression to a pathologic phenotype. Certainly, this hypothesis is especially plausible for the many diseases of aging. Alternatively, aberrant CpG methylation that silences a gene on a single allele may not appear to have a functional consequence if the complementary allele can provide compensatory expression. As a result, for example, clusters of cellular clones with mono-allelic gene expression could contribute to an increased risk of progression to a pathologic phenotype (e.g. loss of the 2nd functional allele). Future population-based studies addressing the potential of quantifying age and/or exposure associated methylation alterations indicative of disease risk are necessary.

It also remains unclear if changes to chromatin, such as histone H3 Lys 9 methylation occurs prior to or because of CpG island methylation in the promoter, although in tumors these alterations are found together [173–175]. The totality of epigenetic marks (DNA and chromatin) may also be important, especially as the alterations are selected for throughout the carcinogenic process. Several questions remain: is there selective pressure for only some CpG sites to become methylated or must an entire region undergo this alteration? Is the selection based on a binary event, expressing or not expressing the target gene, or may there be reduced expression states important for some periods of disease development? How does this alteration interact with genetic alterations, such as deletion or mutation, in driving carcinogenesis, and is the timing and/or order of the alterations important? These questions not only highlight the need for additional biochemical characterization of the process of *de novo* epigenetic silencing but also suggest how an understanding of the impact of carcinogenic exposure on these alterations may play an important, albeit complex role.

For a number of the exposures discussed above, much is known about their role in the creation of genotoxic damage and thus mutation. For example, tobacco smoke is known to lead to the production of bulky DNA adducts, which are repaired via base excision repair pathways [176–178], but consistent reports have linked tobacco smoke exposure to increasing prevalences of methylation of specific genes, for example *CDKN2A* [56, 57]. These results suggest that there may be a link between DNA damage or DNA repair pathways and methylation in at least this one instance for this one gene locus. These DNA damage events may lead to alterations in the ability of proteins, such as transcription factors or insulating elements, from binding DNA, and may therefore trigger epigenetic alterations at these sites [174, 179]. In addition, genetic alterations in epigenetic regulatory genes may lead to widespread epigenetic alterations. Evidence for this has been shown in mesotheliomas where tumors with deletion of the DNA maintenance methyltransferase gene *DNMT1* had significantly reduced methylation (array-wide) compared to tumors without *DNMT1* deletion [180].

Genomic hypomethylation, in addition to specific gene hypermethylation, is also an important form of epigenetic alteration and a common feature of human tumors, although it is unclear if there is any interplay between these alterations [181].

DNA hypomethylation may lead to the expression of normally silenced transposons, leading to an increase in transposition events and increases in genomic instability [182]. In response, hypermethylation may be occurring as a defense for retrotransposition and the resulting instability, as methylation is thought to suppress homologous recombination [183–185]. An overall decrease in cellular SAM levels through poor dietary folate intake or through alternative use of SAM for carcinogen metabolism (such as that seen with arsenic) may lead to hypomethylation [18, 186–189]. This hypomethylation may also allow activation of normally silenced non-coding RNA sequences, which, as we have discussed previously, may play an important role in the epigenetic programming of specific genes.

Common to all carcinogenic exposures, both mutagenic and non-mutagenic compounds, is their ability to impart selective pressures on cellular clones. Some researchers believe that it is this effect, and not genotoxicity, that provides the carcinogenicity attributable to some exposures, by selecting pre-existing genetic or possibly epigenetic alterations [189]. If *de novo* methylation in the developing tumor occurred stochastically, cells with a silenced tumor suppressor may be selected for growth or expansion due to exposure to specific carcinogens. In time these pressures will allow then for clonal expansion of the epigenetically altered cells into fields which would have an increased susceptibility for tumorigenesis [62, 190].

There is, as we have presented, a growing body of literature beginning to link epigenetic alterations to carcinogen exposures from in vitro work, animal models, and human observational studies. It is also clear that the modes by which exposures drive the clonal selection of genetically or epigenetically altered cells or modulate the epigenetic landscape of individual cells are yet to be elucidated and require further exploration.

12.6 Development of Environmental Epigenomics

The fields of environmental genomics and toxicogenomics are bringing the wealth of knowledge gained through the human genome project to the fields of environmental health and toxicology. This will stimulate novel approaches to understanding the modes of action of various chemical and physical agents. It has also been suggested that collaborative efforts between the various disciplines of environmental science, genomics, systems biology, medicine, and basic biology may hold great promise to elucidate more thoroughly the effect of exposures on human health [191]. In addition to studying the effects at the genotoxic and transcriptional levels, it is important to also consider the role of carcinogenic, teratogenic and developmentally toxic exposures on the epigenome, and on clonal selection, again requiring multi-disciplinary work from in-vitro and model systems to epidemiologic studies of human disease. New technologies now allow for global analysis of epigenetic alterations and these may provide insight into the extent and patterns of alteration in normal and diseased tissue. Notably, the latest generation of CpG methylation arrays interrogates hundreds of thousands of CpG loci, includes all designable RefSeq

genes, and is low-cost enough for application in population-level studies. Next generation sequencing approaches, as their cost decreases and throughput increases will also become increasingly used as tools for annotating the epigenome and its relation to exposures. Mammalian stem cells may also prove extremely useful in bettering the understanding of epigenetics in development, providing insightful models for demonstrating how these pathways become dysregulated in cancer. Finally, appropriate epidemiologic studies, including both case-series and prospective designs, requiring defined and consistent methodologies and data collection will be crucial to understanding the true effect of environmental exposures on the human epigenome. This work is urgently needed in order to better understand the biology of epigenetic alterations and the effects of toxic exposures on these disease-associated somatic alterations. Further, this will lead to better prediction of the toxic potential of new compounds introduced into the environment and allow for more targeted, and appropriate, disease prevention strategies.

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Chapter 13
Epigenetic Epidemiology of Cancer

Angela Risch, Narek Sarkisyan, David Scherf, Hanna Jacobsson, Wolfgang Hagmann, and Christoph Plass

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Abstract Epigenetic changes occur in all types of cancers and include alterations in deoxyribonucleic acid (DNA) methylation, histone modification, or expression changes of non-coding ribonucleic acid (RNA). There is now evidence that epigenetic alterations influence cancer risk and could be developed into useful biomarkers. While it is clear that epigenetic mechanisms can play a key role in mediating environmental influences including dietary folate intake, smoking, other environmental stressors, and age, many of the mechanisms involved remain obscure. There are numerous mechanisms by which genetic and epigenetic factors interact and thus the field of epigenetic epidemiology of cancer faces considerable challenges in tearing apart gene-epigene-environment interactions and in determining how they modulate individual cancer risk. An important question for epigenetic epidemiology of cancer is whether epigenetic changes can be identified in surrogate tissues; e.g., DNA isolated from peripheral lymphocytes, plasma samples, urine, or buccal swabs. The current state of the art of cancer epigenetic epidemiology studies as relating to epigenetic cancer risk factors and markers of diagnostic and prognostic significance is reviewed. In the past, epigenetic work has been carried out in comparative studies of primary human normal/tumor tissue pairs, but recent advances in technologies have made large scale analyses on surrogate tissues within defined cohorts possible. Interindividual variations exist in epigenetic markers, but their origin and relevance needs to be determined. In future studies it will be important to characterize the interaction between environmental, dietary, genetic, and epigenetic factors. Echoing the development in the field of genetics, candidate gene approaches employed thus far will be replaced by genome-wide epigenome analyses in the future. Approaches integrating genetic, epigenetic, and well-documented epidemiological and clinical data are most promising and may improve the currently available risk models for cancer in the future.

Abbreviations

DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
GBM	Glioblastoma multiforme
HCC	Hepatocellular carcinoma
miRNA	microRNA
NSCLC	Non-small cell lung cancer
PDAC	Pancreatic adenocarcinoma
RCC	Renal clear cell carcinoma
RNA	Ribonucleic acid
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
SNP	Single nucleotide polymorphism

13.1 Epigenetic Markers in Tumor Tissue and Surrogate Tissue as Diagnostic and Predictive/Prognostic Markers

Epigenetic questions have reached center stage in cancer research. Here research has focused on the mechanisms that lead to epigenetic modifications (DNA methylation, histone modifications, and alterations of non-coding RNA expression) and how these modifications relate to cancer initiation and progression mediated through their effects on gene expression. However, it has become obvious that epigenetic alterations are also attractive targets for cancer therapy as spearheaded in leukemias. Furthermore, due to the stable nature of modifications, in particular the covalent binding of a methylgroup to position 5 of the cytosine ring (DNA methylation), this epigenetic modification is under intense investigation as a possible target to be developed into a biomarker for early detection or as a marker with prognostic or predictive value. Focus in this review will be on the potential of DNA methylation as a biomarker for risk assessment and early detection with relevance for cancer epidemiology.

13.1.1 DNA Methylation

DNA methylation is probably the most intensively studied epigenetic mark in cancers and is now recognized as a crucial component in cancer initiation and progression. Site specific hypermethylation of the usually unmethylated CpG islands in gene promoters is found in almost every human malignancy and is associated with transcriptional silencing of adjacent genes. Hypermethylation mediated silencing in cancer cells often affects classical tumor suppressor genes involved in DNA repair, cell-cycle control, anti-apoptotic signaling, and genes that regulate developmental pathways. Interestingly, aberrant CpG island methylation in cancer has non-random, tumor-type-specific patterns indicating the presence of molecular mechanisms leading to selected gene silencing. Similarly, global loss of DNA methylation (hypomethylation) in cancer results in aberrant transcription via genomic instability and activation of silenced oncogenes [72, 79].

One of the most promising approaches for the detection of early malignant or even premalignant lesions with the chance of diagnosing early stages of many tumors lies in the development of new biomarkers. Aberrant promoter methylation of tumor suppressor genes is believed to be an early event in carcinogenesis and to occur before the clinical onset of disease, and hence, in principle, holds great promise as early detection marker. Tumor biomarkers are indispensable not only for early diagnosis of cancer but also for prognosis, prediction of therapeutic response, monitoring therapy, or assessment of risk of recurrence after surgery. Given that a number of different cancers frequently harbor hypermethylated tumor suppressor genes together with the observation that tumor-derived genomic DNAs are present in various body fluids, e.g., in sputum [21], urine [33], and bronchial lavage [55], DNA methylation analysis is a promising minimally invasive tool for early diagnosis from

surrogate tissue. The epigenetic biomarkers particularly have the potential to improve the accuracy of diagnosis in targeted sub-populations, such as men at high risk of prostate cancer with a persistently increased level of prostate specific antigen (PSA) and negative biopsies, for the detection of bladder cancer in patients with hematuria, populations with occupational carcinogen exposure, with family history of cancer, etc. Although key studies showed sensitive and specific detection of hypermethylated genes in urine from patients with early stage prostate, bladder, and renal cancer, the sensitivity of detection of methylated genes in urine of those patients varies a lot from 49% to 91% [32]. In cancers of the aerodigestive tract, detection of aberrant promoter methylation of tumor suppressor genes in sputum accurately represents the methylation status of those genes in the tumor. However, disadvantages of sputum analysis are the variability in composition of the specimen (epithelial vs. inflammatory components) and the fact that up to 30% of current or former smokers do not have increased bronchial secretions and will not produce the sputum. Moreover, studies so far have not exclusively examined early stage tumors, for which the sensitivity of detection might be lower. Another important consideration in the development of early detection epigenetic biomarkers is their specificity for tumor DNA compared with DNA from normal or non-neoplastic cells. Studies of gene methylation in body fluids have not examined a sufficient numbers of controls to clearly demonstrate a high specificity for cancer. Differences in sensitivity and specificity between different studies probably derive from different genes analysed, primer design and different methods for methylation analysis.

Recently, cell-free DNA has been identified in the serum and plasma from patients with various cancers [21, 184, 192, 227, 238, 267]. These circulating nucleic acids have demonstrated similar epigenetic alterations and characteristics as those found in the primary tumor providing a potential surrogate marker for diagnosis, monitoring, and prognosis. Their levels appear to be elevated during disease progression. Detection of aberrant promoter methylation of several tumor suppressor genes (*APC*, *GSTP1*, *RASSF1A*, *RARβ2*, *p16*, *BLU*, *FHIT*, *CDH13*, *Pax5α*, *DAPK1*, *MGMT*, *GATA5*, etc.) in blood has also been intensively studied as an early detection and prognostic marker in various cancers, including lung, breast, and colorectal cancer [19, 20, 22, 106, 108, 195, 243]. Although the exact mechanism of the release of circulating DNA remains to be proven [7], an active release of tumor gDNA from highly proliferating tumor cells during its growth into the bloodstream has been proposed [8]. However, the number of such tumor circulating DNA fragments is small compared to the number of normal circulating DNA fragments, making it challenging to detect and quantify them with sufficient sensitivity for meaningful clinical use. Highly sensitive assays will have to be developed to assess gene-promoter methylation in blood [251].

There are already diagnostic assays for routine epigenetic testing used in the clinic; among them tests for hypermethylation of the *MGMT* gene promoter for prediction of response to temozolomide in glioma tissue, *GSTP1* and *APC* hypermethylation in prostate biopsy tissue for detection of cancer, methylation of vimentin in stool-based screen for colorectal cancer, *SEPT9* methylation in serum DNA for colorectal cancer, and *MLH1* methylation for mismatch-repair–deficient colon cancer, etc. [58].

Instead of having one epigenetic biomarker, diagnostic coverage for early stage tumors may require a larger panel of genes. Challenges to the clinical implementation of epigenetic early detection include the need for validation in larger populations with optimized and standardized methodology. Up to now no cut-off point in quantitative methylation values between normal, benign, and cancer DNA is established that could reliably indicate the presence of cancer [32]. The timing of gene methylation during the early stages of cancer must also be better understood.

13.1.2 Histone Modifications and Chromatin Structure

Histones, strongly alkaline proteins found in eukaryotic cell nuclei, which package and order DNA into structural units called nucleosomes, also play an important role in epigenetic gene regulation. In contrast to DNA methylation, the patterns of histone modifications are much more complex. Histones are subject to various post-synthetic modifications, including methylation, acetylation, ubiquitylation, phosphorylation, and sumoylation that play important roles in gene regulation in normal cells. In cancer these patterns can change and lead to altered gene expression. Acetylated histones associate preferentially with transcriptionally activated chromatin and may also facilitate binding of transcription factors to the promoters and disrupt higher order of chromosome structure, promoting transcription. Histone deacetylation is often found to be overrepresented around promoter regions of cancer genes resulting in the formation of a more compact structure to silence genes. Histone modifications can also be used to predict prognosis and response to treatment in subsets of cancer patients. For example, decreased global histone modification levels in tumor tissue (H3K4me2 or H3K18Ac) are predictive for prostate cancer recurrence [213], and global histone modifications of multiple histones have prognostic relevance in glioma patients [147]. A role for global histone deacetylation as a prognostic marker was shown in various tumors including breast cancer (H3K9Ac, H3K18Ac , H4K16Ac), lung cancer (H3K9Ac), and renal cell carcinoma (H3K18Ac) [16, 62, 170]. Low cellular levels of H3K27me3 were also recently associated with poor outcome in pancreatic cancer [155, 259]. Since histone modifications also exhibit abnormal regulation in cancer, and those abnormalities are reversible, they have attracted great interest in cancer research as potential drug targets.

13.1.3 miRNAs

MicroRNAs (miRNAs) are short, single stranded RNA molecules that do not encode proteins but instead restrict the production of proteins by inhibiting translation of coding or messenger RNAs, or by promoting their degradation. The first miRNA was discovered in 1993 in the nematode *Caenorhabditis elegans*, where it was found to regulate postembryonic development [135]. More than 1,000 human miRNAs

have been discovered until now, and this number is increasing rapidly. One-third of all human genes may be regulated by miRNAs and a considerable number of deregulated miRNAs are involved in cancer progression [130]. MicroRNA expression levels differ between normal and tumor tissue and vary among tumor types [186, 209]. During the last decade, a unique set of cancer regulator miRNAs has emerged and they are divided into oncomiRNAs and anti-oncomiRNAs. OncomiRNAs and anti-oncomiRNAs negatively regulate tumor suppressor genes and oncogenes, respectively. Results from expression analysis indicate that specific miRNAs are associated with disease stage and survival in several tumor types, such as chronic lymphocytic leukemia, prostate cancer, breast cancer, lung cancer, and colorectal cancer [34, 78, 211, 244]. For instance, expression levels of miRNA-486, -30d, -1, and -499 in serum are significantly associated with overall survival in non-small cell lung cancer (NSCLC) patients [109]. MiRNA-21 is also overexpressed in sputum of NSCLC patients compared to normal subjects [272]. MiRNAs are stable and differentially present in serum and plasma of cancer patients, as well as in extracellular biological materials such as stool and urine [41, 91, 167] and thus have potential of serving as biomarkers for diagnosis, prognosis, and monitoring of cancer via noninvasive methods. Overexpression of several miRNAs in tumor tissues of various cancer types has already been successfully shown to serve as an independent predictive marker of overall survival. Besides plasma and serum, miRNAs can also be identified in other body fluids such as saliva, urine, and semen. The utility of miRNAs in body fluids as biomarkers for various cancer types is being actively investigated; however, an important obstacle for quantification of miRNAs in body fluids is the relatively low amount of RNA in such fluids, and the lack of a suitable RNA for normalization purposes.

13.2 Epigenetic Mechanisms Play a Key Role in Mediating Environmental Influences on Gene Expression and Tumor Development

Epidemiologic studies have shown that certain environmental exposures are associated with various steps during cancer development. Epigenetic mechanisms play a crucial role in tumor development, also by mediating environmental influences on gene expression (Fig. 13.1). Environmental stress including toxins, as well as microbial and viral exposures, nutrition, and aging can change epigenetic patterns, potentially increase or decrease the levels of transcription, thereby effecting changes in gene activation and cell phenotype (see [95] for review).

13.2.1 One-Carbon Metabolism

The one-carbon metabolism is centered on S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH), and the SAM to SAH conversion frees up methyl

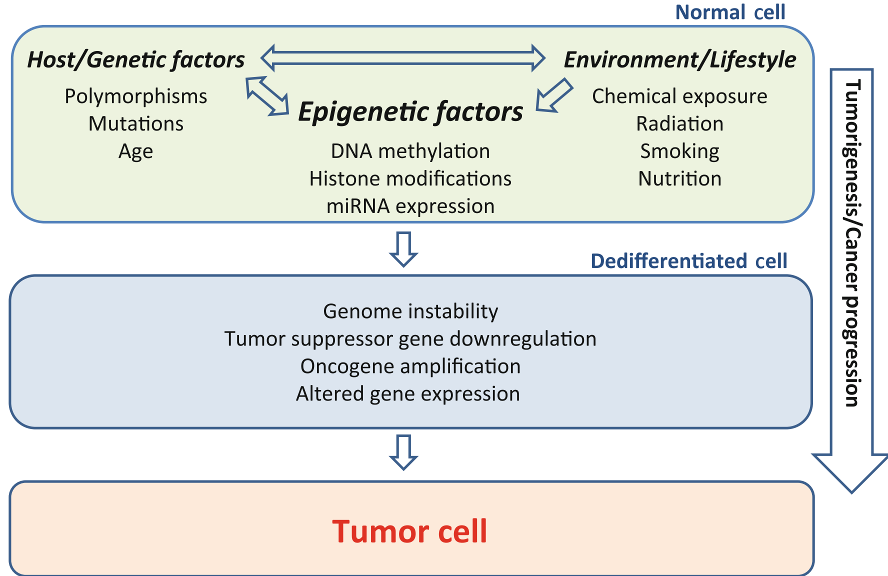

Fig. 13.1 The interplay of host-, environment- and epigenetic factors in tumorigenesis

groups for DNA methylation. This biochemical pathway plays a critical role in DNA methylation and DNA synthesis, and thus facilitates epigenetic and genetic carcinogenic processes. The association between one-carbon metabolism and cancer has been extensively studied and epidemiological studies have shown the contribution of one-carbon metabolism in cancer etiology, prognosis, treatment, and prevention. One-carbon metabolism is not only capable of influencing the pathogenesis of cancer, but it can also be the target pathway for chemotherapy as well as chemoprevention. The major external source of methyl groups comes from folate, which plays an essential role in one-carbon transfer, as well as methionine, and choline in diet. Other vitamins, such as B_2, B_6, and B_{12} are also key factors for one-carbon metabolism. Alcohol is a folate antagonist, and excess consumption of alcohol affects folate absorption and decreases the hepatic uptake and renal conservation of circulating folate. Several case-control and cohort association studies, clinical, and animal studies suggest inverse associations between folate intake and risk of developing various cancers while some more recent studies support a positive association between very high intake from diet with lung, premenopausal and post-menopausal breast cancer and can promote the progression of already existing preneoplasms [60, 145, 183, 208, 230]. Evidence on folate and alcohol intake in relation to methylation in colorectal cancer brings strong support that diet is capable of influencing carcinogenesis through epigenetic pathways by showing that prevalence of promoter hypermethylation of a certain panel of genes is higher in tumors derived from patients with low folate and high alcohol intake compared to those

with high folate and low alcohol intake [252]. Evidence also exists that functional polymorphisms in one-carbon metabolizing genes can modify the DNA methylation status. For example, a study investigating three key genes involved in the metabolism of the methyl group, methylene-tetrahydrofolate reductase (*MTHFR*), methionine synthase, and cystathionine beta-synthase, reported higher DNA hypomethylation in people with the *MTHFR*677TT genotype compared to those with the *MTHFR*677CC genotype in colorectal, breast, and lung cancers [189]. It is also important to mention that unlike genetic changes, epigenetic modifications are potentially reversible, and the concept of modulating DNA methylation by nutrition is a highly attractive proposition in public health.

13.2.2 Smoking, Other Environmental Factors, Age, and Epigenetics

Cigarette smoke contains more than 60 carcinogens and there is a strong link between some of these carcinogens and various smoking-induced cancers, in particular lung cancer, esophageal, oral, pharyngeal, and laryngeal. The mechanisms of actions have been shown to include induction of DNA adducts, gene methylation, mutations and chromosomal translocations. Epigenetic changes, such as methylation patterns of CpG islands, have been linked with smoking, and inactivation of key genes through promoter hypermethylation is particularly believed to be one of the mechanisms by which tobacco smoke promotes the development of lung cancer. In vitro and animal studies showed that carcinogenic components of cigarette smoke induce DNA methylation changes in normal tissue as well as in malignant cells (see also Chap. 12). Human studies to investigate the effect of low-dose benzene, one component of cigarette smoke, on DNA methylation showed that *p15* promoter methylation in exposed individuals is twice as high as that observed in unexposed ones. Also, *MAGE1*, which is usually highly methylated in normal tissue, tends to have lower methylation levels in association with benzene [27], which all together indicates the involvement of benzene in the initial activation of the biological process of carcinogenesis. In a large prospective study it was shown that cigarette smoking is strongly associated with promoter hypermethylation of *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1* tumor suppressor genes in colorectal cancer [143]. In summary, cigarette smoking represents a potentially modifiable risk factor for many cancers, but modulation of epigenetic susceptibility to cancer by cigarette smoke must be further investigated, since the data and results are not comprehensive and coherent between different types of cancers.

A variety of other environmental factors has also been linked with epigenetic phenomena (reviewed in Chap. 12), mainly in model organisms. In order to evaluate the relevance of epigene-environment interactions in human cancer, carefully conducted epidemiologic studies will be required.

Epigenetic investigation of cancer and aging has recently emerged as a crucial area of study to determine the impact of age on epigenetic changes and alterations.

Both genome-wide and specific methylation profiles change with age (see Chap. 11). Age-related differences in tumor methylation have been observed in cancers [124]. Aging is one of the greatest risk factors for cancer and the highest cancer rates occur in people over the age of 70 years. This association between cancer and aging has led many investigators to point to the accumulation of genetic mutations as a primary factor in tumorigenesis. However, a number of investigations have revealed epigenetic, rather than genetic, explanations for loss of gene expression involved in tumorigenesis [129]. In the context of epigenetic epidemiology of cancer it will be of crucial importance to elucidate the mechanisms and patterns of age-related methylation, and to take this phenomenon into account in careful study design or analysis.

13.3 Mechanisms of Interaction Between Genetic and Epigenetic Factors in Cancer (Risk)

Genetic variations as well as epigenetic modifications are known to be involved in tumorigenesis by modifying the expression of cancer genes [18, 25, 121, 203]. Besides the individual impact of these factors recent publications have drawn attention to the interplay between genetic and epigenetic factors [103]. Advanced, sequencing based technologies, now allow the thorough analysis of the dependencies among epigenetic modifications as well as the influence of genetic on epigenetic factors, an example of which are genetic variants in epigenetic genes.

DNA methyltransferases (DNMTs) are the main players in methylating DNA in animals. In humans DNMT1 is mainly responsible for maintaining methylation and DNMT3A/B for de novo DNA methylation. Recurrent mutations recently identified by whole genome sequencing in acute myeloid leukemia highlight the link between genetic alterations and global epigenetic dysregulation [141]. DNMT overexpression has been detected in cancerous tissue and various miRNAs targeting the 3′-UTR of DNMT are downregulated in cancers [253]. Genetic variants in DNMTs are known to modify risk of various cancers and can be linked to deregulation of DNMTs [70, 137, 150, 168, 214, 220, 270]. Epidemiologic studies that combined haplotype and mutagen sensitivity analysis identified *DNMT1* and *DNMT3B* variants which associate with enhanced sensitivity to tobacco carcinogens in cancer-free smokers [139]. In addition to genes affecting DNA methylation further genes involved in histone tail modification are highlighted by other studies. In an association study, consisting of ~4,500 cases and controls, genetic variants in several histone methyltransferases (HMTs) were detected. After genetic and epigenetic follow up in cell lines those HMT polymorphisms could be linked to levels of H3K9 and H3K27 trimethylation [37]. By single nucleotide polymorphism (SNP) analysis in combination with expression, methylation, or ChIP analysis a lot of information could be gained how epigenetic modifications are manifested by germline SNPs and reflected in an expression phenotype [59, 121, 123, 219]. The phenomenon of mitotically inherited germline aberration in methylation has also been described and has been termed “epimutation” [98, 235].

Genetic common variants which have been shown to predispose to cancer by modifying binding of miRNAs either by presence of SNPs in the seeding or the binding site have been detected [128]. Further, a meta-analysis could show that one SNP in pre-miRNA hsa-miR-196a2 was associated with breast cancer risk by potential modification of processing and subsequent expression of the mature miRNA [80].

In addition to genetic factors such as polymorphisms, it could be shown that deregulation of miRNAs by aberrant DNA demethylation can contribute to tumorigenesis either as tumor suppressor or as oncogenes. Loss of DNA methylation in gastric cancer leads to a high level of miRNA-196b [249]. Transfection of neuroblastoma cells with miRNA-152 which targets DNMTs together with all-trans retinoic acid treatment leads to global demethylation, upregulation of the nitric oxide synthase (NOS1), and differentiation [54]. Taken together, there are many examples of how genetic features like mutations or polymorphisms can modify epigenetic events, which eventually could be relevant for the development of malignancies. Hence multifactorial analysis of different regulatory layers, including genetic and epigenetic factors, are necessary to reveal a conclusive picture of disease associations.

13.4 The Potential of Epigenetic Biomarkers in Cancer Epidemiology

As described above, epigenetic changes, in particular DNA methylation, can be detected in body fluids in various cancer types. Aberrant methylation is thus a good candidate biomarker both as clinical (early diagnostic and/or prognostic) marker and for cancer risk assessment. For diagnostic purposes methylation aberrations can be detected in surrogate tissues or body fluids such as in urine for bladder cancer, pancreatic juice for pancreatic cancer, sputum for lung cancer, and stool for colorectal cancer. An impressive panel of genes has already been shown to be aberrantly hypermethylated in tumors, and those same epigenetic aberrations are also being heavily investigated in such body fluids in order to implement specific epigenetic biomarkers as a tool for risk assessment. Epidemiological studies based on biomarkers are crucial to predict and identify high risk populations. For example, in a case-control study it has been suggested that methylation of either *p16* or *RASSF1A* in plasma is associated with an increased risk of lung cancer (odds ratio (OR) 10.2 and 9.9, respectively) [108]. In a bigger cohort study, Belinsky with colleagues observed that aberrant methylation of six genes in sputum is associated with more than 50% increased lung cancer risk [23]. Other case-controls studies revealed nine genes whose methylation status in plasma is a significant and independent predictive factor for colorectal cancer [133], and that epigenetic inactivation of *MGMT* is also a useful tool in risk assessment of that disease [216]. Investigation of epigenetic biomarkers in body fluids for cancer risk assessment in a population-based manner is technically challenging, since only a small amount of DNA is present and thus highly sensitive assays must be performed. However, current rapid technological

progress will make it possible to investigate in more depth the potential of epigenetic biomarkers as risk markers for developing various cancers in big population studies using high-throughput quantitative methylation analysis.

Recently another area in the field of cancer research has emerged, namely to determine epigenetic risk biomarkers in blood, in particular, peripheral blood cells where epigenetic alterations are not expected to be related to the epigenetics of the tumor. Instead, epigenetic variation detected here, if detected before the clinical onset of cancer, may be risk biomarkers and might even be heritable. Challenges to the implementation of epigenetic risk biomarker detection in blood include the need for large population based cohort studies and the fact that those heritable epigenetic risk biomarkers, if so found, might be also mediated by various environmental exposure during the lifetime.

13.4.1 Gene-Epigene-Environment Interaction

Environmental agents can modify gene expression. Epigenetic modifications are the likely mediators of epi-gene-environment interaction because genetic factors can modify the epigenetic response to the environment, and certain epigenetic aberrations can have downstream genetic consequences [75, 115, 248]. There is accumulating evidence that epigenetic alterations may play an important role in determining individual susceptibility to cancer, and that in order to assess the role of epigenetic susceptibility in cancer risk, one should consider the effect of epi-gene-environment interaction in disease etiology. It is imperative to properly design epigenetic epidemiology studies taking into consideration a potential confounding effect of environmental factors (diet, smoking, lifestyle, etc.) and genetic alterations in order to comprehensively elucidate the epi-gene-environment interaction. Conducting epigenetic cancer case-control studies using blood samples one should be aware of the fact that blood might be contaminated by tumor DNA. It is also possible that the composition of cell types in blood is affected by the disease (e.g., due to concomitant inflammation), which may, in turn, affect the epigenetic patterns observed in peripheral blood lymphocytes and could lead to misinterpretation of results.

13.4.2 Transgenerational Inheritance

Although epigenetic states are maintained for the life of the organism, it is rare for these epigenetic states to be passed to the next generation. However, there is strong evidence for transmission of the epigenetic alterations through the gametes to the next generation in the mice. This is known as transgenerational epigenetic inheritance and was first demonstrated at the agouti locus in the mouse by Morgan and colleagues [169]. Transgenerational epigenetic inheritance has then been reported at another murine *Axin-fused* allele [199], at a genetically modified locus [96], and at *c-kit* [201].

Sensitivity to environment, combined with transgenerational epigenetic inheritance, suggests that the diet of a pregnant female could affect not only her offspring's coat color, but also that of next generations. Cropley with colleagues showed that methyl donor dietary supplementation can change the epigenetic state of the A^{vy} allele in the germline and that these modifications are retained through the epigenetic reprogramming during early embryogenesis [50]. Although DNA sequence with epigenetic information can be inherited from parent to offspring in mice, there is little direct evidence for transgenerational epigenetic inheritance in humans.

To summarize, epigenetic biomarkers have repeatedly been shown to have potential for risk assessment, prevention, early detection, diagnosis, treatment, prognosis, and recurrence in cancer epidemiology, and epidemiological studies based on epigenetic biomarkers, behavior, and lifestyle are crucial to predict and identify high risk populations.

13.5 State of the Art

13.5.1 Epigenetic Epidemiology of Colorectal Cancer

Colorectal cancer (CRC) is considered to be one of the major public health concerns and is probably the best studied cancer with respect to epigenetic alterations. This is facilitated by established screening approaches and availability of cancer tissue for epidemiological analysis. Environmental exposure may result in persistent epigenetic changes that can influence cancer development. In the frame of the Netherlands Cohort Study on diet and cancer, the association between early life energy intake and risk of subsequent CRC by promoter methylation of CpG island methylator phenotype (CIMP) genes was investigated [112]. Severe energy restriction during the Hunger Winter was associated with a decreased risk of developing CRC having characterized by CIMP compared to those not exposed (hazard ratio (HR) = 0.65) and exposure to the Hunger Winter was associated with the degree of promoter hypermethylation when further categorize individuals by an index "0–1", "2–3" or "4–7" genes methylated in the promoter region (HR = 1.01, when up to one gene is methylated; HR = 0.83, when 2–3 genes; and HR = 0.72, when 4–7 genes are methylated). Another case-control study within the Netherlands Cohort Study demonstrated that alcohol consumption increases methylation at promoters of *APC-1A*, *p14*, *p16*, *MLH1*, *MGMT*, and *RASSF1A* genes and is associated with methylation-induced silencing in colorectal cancer [252]. Additionally, the degree of promoter hypermethylation of all these genes was higher in cancer tissue from patients with low folate and high alcohol intake compared to those with high folate and low alcohol intake, consistent with the fact that alcohol is a folate antagonist. In another study, within the same Netherlands Cohort Study, Stefan de Vogel and colleagues investigated the association between folate intake, vitamin B2, vitamin B6, methionine, and alcohol and the risk of developing CRC with or without *MLH1*

hypermethylation, absence of *MLH1* protein expression, or *BRAF* mutations. Dietary intake of vitamin B6 was positively associated with CRC in men (RR = 1.54) and the association of vitamin B6 with CRC risk was even stronger with *MLH1* hypermethylation (RR = 3.23) [56]. In a population-based study of 951 cases and 1205 controls, the association of rectal tumors characterized by *CIMP*, *KRAS2*, and *TP53* mutations with dietary and supplemental folate, riboflavin, vitamins B6 and B12, and methionine were examined. Results indicate that folate supplementation effects may differ by gender and might be important in the initiation and progression of CIMP positive rectal cancers in men (OR = 3.2) [52]. A large population-based cohort study, the Iowa Women's Health Study, found that cigarette smoking is strongly associated with promoter hypermethylation of *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1* tumor suppressor genes (RR = 1.88) in CRC [143], whereas no association was found between never-smokers and promoter hypermethylation. Compared with never-smokers, smokers have increased risk of CRC with increased promoter methylation (RR = 1.46). Taking all evidence together there is a strong indication that epigenetic changes may be mechanistically involved in environment-related carcinogenesis of CRC. In two prospective cohort studies (the Nurses' Health Study and the Health Professionals Follow-up Study) it has been reported that synchronous CRC patients have worse survival than solitary CRC patients, and, compared with solitary cancers, synchronous cancer patients showed more frequent promoter hypermethylation in a panel of eight genes [181]. Cui and colleagues investigated loss of imprinting (LOI), a form of epigenetic gene silencing which is found in the tumors and matched normal colonic mucosa of 30% CRC patients compared with only 10% of individuals without CRC, in the insulin-like growth factor II gene (*IGF2*), as a marker of CRC risk [51]. Results of this study indicate that LOI in blood is strongly associated with family history of CRC, compared to those without family history (OR = 5.15), strongly associated with past or present adenomas but no CRC, compared to those without colorectal neoplasia (OR = 3.46), and strongly associated with past or present CRC, compared to those without colorectal neoplasia (OR = 21.7), strongly indicating that epigenetic deregulation of IGF2 expression by LOI is associated with initiation and progression of CRC.

Regarding the field of epigenetic aberrations in CRC, in addition to cohort and case-control studies, it is imperative to mention the breakthrough studies that showed transgenerational epigenetic inheritance in a family with hereditary non-polyposis colorectal cancer (HNPCC). Chan et al. reported heritable germline methylation of *MSH2* associated with cancer development [39]. Germline hypermethylation in *MLH1* observed in two individuals was also found in normal somatic tissue and in spermatozoa of one individual, and there was no evidence of genetic mutations in *MLH1* gene [235], which indicates a germline epigenetic alteration that passes to the next generations. In tumors of patients with HNPCC, also known as Lynch syndrome, with *MSH2* deficiency, Nagasaka and colleagues also reported frequent somatic *MSH2* hypermethylation. In the context of Knudsons's "two hit" hypothesis this likely constitutes an epigenetic "second hit," affecting the wild-type allele. None of the sporadic tumors showed *MSH2* promoter methylation [174].

In comparison with DNA methylation, current knowledge regarding histone modifications in CRC patients is less advanced, but this field is being increasingly investigated. While thus far there is a lack of population-based association studies, aberrant histone modifications in CRC have been reported. CRC patients' DNMT1mRNA levels were significantly different between patients with elevated mRNA levels of SUV39H1 histone methyltransferase compared with those without, and patients with an elevated mRNA level of SUV39H1 had a 2.71 times greater risk of an elevated DNMT1 mRNA level [119], suggesting that SUV39H1 and DNMT1 may work together for transcriptional repression. Overexpression of a putative histone methyltransferase SMYD3 that methylates the lysine 4 residue of histone H3 in colorectal cancer has been also reported [90].

Among the first miRNAs to be studied in CRC were miRNA-143 and miRNA-145, which were both reported to be down-regulated in cancers compared to corresponding normal tissue [164, 257]. The most comprehensive analysis of miRNA expression in CRC performed to date showed that, in particular, miRNA-20a, miRNA-21, miRNA 106a, mi-RNA 181b, and miRNA-203 were found to be over expressed in tumor tissue with high tumor-normal ratios, as well as being associated with poor survival [211, 212]. High expression of miRNA-17-3-p and miRNA-92a was associated with the presence of CRC with quite a promising sensitivity of 89% and specificity of 70% [179]. Landi and colleagues investigated the association between miRNA-binding SNPs sequences and the risk of developing CRC within a hospital-based case-control study [132]. Thirty-seven among 104 candidate genes for CRC showed 57 SNPs in the predicted miRNA-binding sites. Heterozygosity and homozygosity for the variant allele rs17281995 within CD86 was associated with an increased risk of CRC (OR = 1.33 and OR = 2.93, respectively) and an increased risk of CRC was observed for homozygotes for the variant allele rs1051690 within *INSR* (OR = 1.86).

In summary, CRC is one of the best studied cancers in the area of epigenetic population-based association studies, and a role for epigenetic regulation in CRC initiation and progression is reported by numerous studies. The most widely applied screening method for CRC to date is the fecal occult blood test, and the principle of detecting epigenetically altered products shed from a tumor located in the colon and rectum into the stool and blood offers great opportunities for increased diagnostic sensitivity and specificity, and for identifying a population at high risk of developing CRC.

13.5.2 Epigenetic Epidemiology of Upper Aerodigestive Tract Tumors

13.5.2.1 Lung Cancer

Smoking is the most important risk factor for lung cancer. Short-term exposure of mice to tobacco smoke condensate has resulted in epigenetic changes in lung

tissue [191]. In vitro experiments in lung cancer cell lines point to effects of tobacco smoke exposure on expression of methyltransferases [149] and on histone modifications [113, 148]. Promoter hypermethylation and other epigenetic changes have been identified in lung tumors (reviewed in [203]). Examples of tumor suppressor genes upregulated in lung cancer include *p53*, *p16*, and *RB*. Silencing of the *p16* tumor suppressor gene by promoter methylation plays an important role in smoking-related lung cancer, and radionuclides in cigarette smoke may explain the phenomenon of *p16* inactivation by promoter hypermethylation [197]. Inactivation of another tumor suppressor gene, *FHIT*, also occurs very often in aerodigestive tumors, in particular, lung and esophageal cancer, and methylation of the *FHIT* gene promoter is associated with transcriptional inactivation and is strongly linked to cigarette smoking [190]. Belinsky and colleagues showed repeatedly that promoter hypermethylation in *p16* and *DAPK1* is frequently seen in bronchial epithelium from lung cancer current smokers but not in bronchial epithelium from never-smoker lung cancer patients [20, 24]. Aberrant tumor methylation has also been associated with changes in prognosis [236]. Promoter hypermethylation of retinoid X receptors (RXRs) is observed in NSCLC. Methylation did not significantly correlate with prognosis; however, the effect of *RXRG* methylation on prognosis of NSCLC patients was significantly different between never- and ever-smokers [138]. Methylation differences have also been reported between NSCLC tissue from patients with or without COPD [237], and between adenocarcinomas with *EGFR* or *K-RAS* mutations, suggesting specific interactions of genetic and epigenetic alterations in lung tumorigenesis [247]. DNA methylation in sputum is considered a promising early detection [19, 21, 232, 251] and risk marker [23]. A first cohort-based study on dietary factors associated with reduced promoter methylation in cells exfoliated from the airway epithelium of smokers identified multivitamins, folate, and green vegetables as protective [229]. Plasma methylation biomarkers are also showing promise for early detection [13, 68]. Long-term exposure to inhalable nickel and arsenic, which predisposes to lung cancer, has recently been shown to be associated with histone H3K4 dimethylation and H3K9 acetylation in a cross-sectional study of steel workers [36]. Importantly a recent study comparing lung cancer patients and controls from a prospective cohort has shown blood levels of 1-carbon metabolism markers to affect methylation levels in blood cells, but failed to find a clear association between DNA methylation of the five genes investigated and case-control status [255].

13.5.2.2 Head and Neck Cancer

There is evidence from case-only studies that gene specific hypermethylation has also been observed in head and neck squamous cell carcinoma (HNSCC) and has been associated with smoking [122, 157] as well as clinical prognosis [1]. One recent study pointed to age-dependence of an observed association between *p16CDKN2A* methylation and *DNMT3B* polymorphism in HNSCC specimens [71]. Recent evidence from methylation analysis of HNSCC suggests that global

hypomethylation observed in tumors and gene specific methylation is associated in a sequence dependent manner [194]. In a population-based case-control study ($n = 1{,}039$) MIR196A2 genotype was implicated in both susceptibility and prognosis of HNSCC [45].

13.5.3 Epigenetic Epidemiology of Hormone-Related Cancers

13.5.3.1 Prostate Cancer

There is increasing evidence that epigenetic aberrations could be used for risk assessment and early detection for prostate cancer. The best studied epigenetically modified gene in prostate cancer is *GSTP1*, encoding a protein involved in xenobiotic metabolism, protecting DNA against free radical damage. More than 50 independent studies have shown that *GSTP1* is hypermethylated in up to 90% of all prostate cancers, thus making epigenetic modifications a more frequent event than genetic alterations in prostate cancer (reviewed in [17, 187]).

Evidence that epigenetic aberrations may serve as biomarkers for risk or early detection comes from several studies. For example, DNA hypermethylation of commonly methylated genes in prostate cancer was investigated in DNA isolated from whole blood from prostate cancer patients with and without disease progression and compared in age-matched controls. This study suggested that *GSTP1, RASSF1A, APC*, and *RAR beta* are more likely hypermethylated in circulating cell DNA from cancer patients compared with matched controls [205]. Additionally, an increase in methylation for all four genes was seen from the time of diagnosis to the time of relapse but not at the corresponding follow-up visit of patients without relapse. This indicates that the combination of *GSTP1, RASSF1A, APC*, and *RAR beta* methylation could serve as a marker for early detection with potential relevance for prostate cancer recurrence and progression. Interestingly, *GSTP1* methylation has additionally been found in urine, prostatic secretion, and serum, suggesting that GSTP1 methylation is a promising minimally invasive tool for early detection and diagnosis [85, 86].

miRNAs regulate a wide range of biological processes and thereby play a critical role in the pathogenesis of human cancer including prostate carcinogenesis [6, 196]. Several studies have reported that genetic variants within precursor miRNA may influence the maturation of these miRNAs and thus affect a loss or gain of function. This has been reported for miRNA-146, which was shown to be overexpressed in breast, pancreatic, and prostate cancer [256]. A recent case-control study in a Chinese-Han population showed an association between an SNP (rs2910164) in the miR-146a precursor and prostate cancer ($p = 0.03$) [266], a finding also reported for familial breast cancer susceptibility [250]. In contrast, another population based association study in North Indians could not show a significant association with rs2910164 and prostate cancer but instead found that two other SNPs in hsa-miR499 and hsa-mir-196a2 showed significant association with risk [82].

Prostate tumorigenesis depends upon an interplay between genetic and epigenetic changes. In depth investigations of aberrant methylation patterns present in blood or other body fluids on a genome wide scale in prostate cancer patients and investigation of common variants in precursor miRNAs associated with risk are required to clarify the link between epigenetics, genetics, and predisposition to prostate cancer.

13.5.3.2 Breast Cancer

Epigenetic alterations including promoter specific hypermethylation, global hypomethylation, and deregulation of miRNAs have been well studied in breast carcinogenesis (reviewed in [253]). Decreased methylation of downstream target genes of estrogen receptor (ER) alpha is associated with active ER alpha, a risk factor for breast cancer [263]. DNA methylation status of ER alpha targeting genes (ERT) and Poly comb group target genes (PCGT) has additionally been studied in peripheral blood cell DNA in a case-control study where decreased methylation of ERT genes and increased methylation of PCGT genes were associated with breast cancer risk for 7 of 25 genes investigated [262].

Another recent association study observed a relation between gene body methylation of *ATM* and breast cancer risk, suggesting that the methylation variability is highest at ~1 kb downstream of transcription start site [76]. This study conducted a DNA methylation high density tiling array analysis to identify methylation patterns in peripheral blood DNA of 17 breast cancer susceptibility genes in 14 bilateral breast cancer patients and disease-free age-matched controls. Another case-control study performed in Jewish BRCA1/1 mutation carriers with ($n=41$) and without ($n=48$) breast cancer and controls ($n=89$) investigated promoter methylation in several *BRCA1*/*BRCA2* associated genes including *ATM*, and found no significant methylation of *ATM* at the promoter site [127]. This suggests that differentially methylated intergenic regions rather than *ATM* promoter regions may be suitable as a biomarker for breast cancer risk.

Global hypomethylation is an important feature in breast cancer development and has been proposed to contribute to genomic instability and activation of oncogenes [26, 225]. A study by Choi et al., investigated whether 5-methylcytosine at CpG sites and LINE 1 methylation present in leukocyte DNA from 179 breast cancer cases and 180 controls could mirror the overall methylation activity and the association with breast cancer risk [44]. They found a significantly lower methylation level in leukocyte DNA from cases than in healthy controls ($P=0.001$), suggesting that the global genomic hypomethylation may be a risk factor due to genomic instability, as has been seen for many cancer types. This finding provides evidence that loss of methylation may modify breast cancer risk and thus serve as a non invasive method for identification of women at high risk of developing breast cancer.

Three independent studies investigated the association between SNP genotypes in pre-miRNAs and breast cancer risk in Chinese, German, and Italian populations [80]. The first study was carried out in a case-control study of 1,009 breast cancer

cases and 1,093 controls in Chinese women, evaluating the association of four SNPs: rs2910164, rs2292832, rs11614913, and rs3746444, located in pre-miRNA hsa-mir-146a, hsa-mir-149, hsa-mir-196a, and hsa-mir-499, respectively. The SNPs located in hsa-mir-196a2 and hsa-mir-499 were associated with increased risk of breast cancer [110]. In contradiction to this, Catucci et al., found no association between the variants and risk in German and Italian familial breast cancer cases and the common variant of rs11614913 in miR-196a-2 was associated with decreased breast cancer risk in a case-control study conducted in Connecticut, US [104].

Kontorovich and colleagues investigated SNPs within predicted binding sites for miRNAs expressed in breast cancer/ovarian cancer in a case-control study with BRCA1 mutation carriers. They could reveal an association between CC homozygosity for rs6505162 with increased risk for ovarian cancer and heterozygote SNP carriers of rs11169571 had a 2-fold increase of breast and ovarian cancer [127]. Polymorphisms located in miRNAs may change their property through altering miRNA expression or maturation. Since the sequence complementarity of miRNA target regions is crucial for interaction and proper downregulation, variations within the targeting binding sites by SNPs could disrupt the miRNA-mRNA interaction and thus result in diverse functional consequences.

The epigenome of breast cancer has been intensively studied resulting in the identification of several hypermethylated genes that may serve as biomarkers for disease progression, survival, and early detection. Global hypomethylation of repetitive elements as well as polymorphisms in miRNA may serve as risk markers. However, in the future large-scale epidemiological studies are required to clarify if these epigenetic events identified in retrospective case-control studies do indeed have predisposing potential and thus could be used to identify individuals at high risk.

13.5.3.3 Ovarian Cancer

Ovarian carcinoma is often detected at a late and aggressive stage since there are no obvious symptoms at early stages. This makes sensitive screening markers for early detection and risk highly desirable. The Hox gene family has been studied in detail in many different cancers and DNA promoter methylation of *HOXA9, HOXA10*, and *HOXA11* is a frequent event in ovarian cancer [74, 200]. In one study methylation of *HOXA9* was highly significantly ($p < 0.0001$) increased in normal endometrium from pre-menopausal Japanese women with ovarian cancer compared to an age-matched control group [261]. A promising study linking epidemiology with epigenetics was performed using genome-wide DNA methylation profiling in a case-control cohort, analyzing over 27,000 CpG sites in blood derived DNA from 148 healthy individuals, 113 pre-treatment, and 122 post-treatment ovarian cancer cases. Among the CpG sites investigated, 2,714 CpGs, classified as cancer diagnostic CpGs, were found to be differently methylated in blood between pre-treatment cases and healthy individuals. In order to identify CpG sites which may have predisposing features or early detection potential for ovarian cancer, they compared CpG methylation from 70 post-treatment ovarian cancer patients who did not have

recurrent disease at the time when blood was drawn with healthy controls and performed a Surrogate Variable Analysis framework. 84 CpG sites could discriminate between the post-treated cases and healthy controls. The CpGs were further deconvoluted from the diagnostic CpGs and resulted in 18 potentially predisposing risk CpG sites ($P=0.003$). Among them, 11 showed hypermethylation and 7 showed hypomethylation. *TSG101*, a reported tumor suppressor gene and breast cancer predisposing gene, was among the candidates [245]. These results suggest that aberrant DNA methylation patterns detected in peripheral blood cells from cancer patients could indicate a predisposing epigenetic event that could serve as a marker for risk, but careful confirmation of such findings in blood samples collected prior to disease onset will be necessary. DNA methylation as a prognostic and screening marker for ovarian cancer has been shown for a subset of genes where hypermethylation was found in tumor tissue and correlated with DNA methylation patterns in serum; *SFRP1,2,4,5, SOX1, PAX1, and LMX1A* [231]. Additionally, *BRCA1* hypermethylation has been found at elevated levels in serum, plasma, and peritoneal fluid from ovarian cancer patients [114], but the clinical usefulness of such a marker is limited by its lack of specificity.

13.5.3.4 Endometrial Cancer

There are two types of endometrial cancers. Type I is estrogen dependent, positive for both estrogen and progesterone receptors and occurs mainly in pre- or perimenopausal women, while Type II occurs mainly in postmenopausal women and is hormone independent. It is thought that the two types have different molecular mechanisms. Aberrant DNA methylation has been reported to be more common in Type I endometrial cancer than in Type II [264]. A possible explanation for this has been proposed by Zhou and coworkers who observed that Type I endometrial tumors have a 2–4-fold higher DNMT1 and DNMT3B level than in normal endometrium and Type II tumors have a 2–4-fold reduction in relation to normal endometrial tissue [116].

Type I endometrial cancer development depends partly on defects in the DNA repair system. *MLH1* is aberrantly methylated in atypical endometrial hyperplasia, but not in normal endometrium, suggesting that MLH1 may be involved in endometrial carcinogenesis [15].

Endometrial cancer risk factors include hormone exposure, in particular estrogen. The protein expression of the estrogen receptor and progesterone receptor is reduced in endometrial cancer and much effort has been put into elucidating the association between promoter methylation of the estrogen receptor and progesterone receptor and endometrial cancer, however with contradictory results [107, 193, 207, 218]. The relevance of chromatin structure and histone modifications on expression of the ER and PR genes have not been explored in detail and further studies are required to understand this epigenetic deregulation in endometrial cancer.

Epigenetic biomarkers for early detection of endometrial cancer have been suggested by a few studies. Fliegl et al., investigated the methylation pattern of

five genes known to be hypermethylated in tumor tissue (*CDH13, HSPA2, MLH1, RASSF1A*, and *SOCS2*) in DNA isolated from vaginal secretion using the MethylLight methylation detection method. The outcome of this small case-control study (15 cases, 109 controls) revealed that cases had three or more methylated genes and 91% of controls had no or fewer than three genes methylated [73]. However, a larger prospective study would be needed to determine whether these genes have prognostic value, and whether vaginal secretion could be used as a surrogate tissue for aberrant methylation and be applied in early detection screening for endometrial cancer.

13.5.3.5 Cervical Cancer

Aberrant promoter methylation of *FHIT* and *RASSF1A* gene was significantly associated with cervical cancer ($P<0.01$) in a study in a North Indian population with 60 cervical cancer tissue biopsies and 23 healthy control samples with normal cervical cytology [178]. Promoter DNA methylation of *MYOD1, CDH1*, and *CDH13* found in tumor derived DNA in serum from patients with cervical cancer has been associated with poorer disease outcome, indicating that aberrant methylation patterns may be used clinically as a prognostic factor [172].

Human papilloma virus (HPV) infection is the primary cervical cancer risk factor. However, HPV infection alone is insufficient for transforming a normal cell to malignancy, suggesting that other factors such as genetic and epigenetic modifications may contribute to disease progression. The function of the viral proteins HPV E6 and E7 oncoproteins as modulators of cell transformation includes inactivation of tumor suppressors thus making it possible for the virus to avoid cell cycle checkpoints and thereby turning a normal cell into a malignant cell. E6 interferes with and reduces the expression of miRNA-218 and therefore affects downstream targets such as Laminin-5 beta 3 (*LAMB3*), a marker for cervical invasiveness [159, 222]. In a large case-control study with 703 cases and 713 controls in Chinese women, 2 SNPs in pri-miR218 and *LAMB3* were associated with risk of cervical cancer with a dose response effect dependent on the risk alleles for these SNPs [275]. Furthermore, CpG methylation seems to target HPV16 DNA as a cellular defense mechanism against infection. A study by Badal et al., reported that methylation patterns in Long Control Regions (LCRs) and promoter regions of oncogene *E6* decreased with progression of the infection. This study suggests that decreased methylation of promoter and enhancer regions of the oncogenes *E6* and *E7*, and *LCR*, is associated with disease progression [12]. In contrast, another study did not find a correlation between increased methylation and disease progression with significantly higher methylation of the *LCR* region in cervical cancer in comparison to asymptomatic patients [117]. Polymorphisms in methyl group metabolizing enzymes such as MTHFR and MS have been suggested to influence DNA methylation in colorectal, lung, and breast cancer [189] and later also in cervical cancer [120]. This study revealed that hypermethylation of the repair gene *MGMT* was significantly decreased in cervical cancer patients with the *MTHFR* 677 CT genotype. Genetic inter-individual variations in combination with aberrant methylation of crucial genes

involved in cervical cancer development may play an important role for cervical cancer predisposition and progression and thus indicates a great potential for the use of epigenetic markers in a clinical setting and for risk assessment.

13.5.4 Epigenetic Epidemiology in Leukemia

Leukemia is a heterogenous disease with four main disease subtypes: acute lymphatic leukemia (ALL), chronic lymphatic leukemia (CLL), acute myeloid leukemia, (AML) and chronic myeloid leukemia. With the exception of ALL, the risk for development of leukemia increases with age. Several causes for the development of leukemias have been identified. The following sections will indicate advances and strategies to shed light on epigenetic modifications as potential risk factor and as treatment modifier in the different classes of leukemia and the difficulties in designing case-control studies evaluating the role of genetics and epigenetics.

13.5.4.1 Acute Leukemias

Chemical exposure (benzene, pesticides), radiation exposure (therapeutic, nontherapeutic), and chemotherapeutic treatment are known risk factors for developing acute leukemia. Discovery of genetic disorders as predisposition factors contributed to advances in understanding the underlying oncogenic processes. Abnormal karyotypes, caused by translocations and deletions, result in fusion genes, which influence the differentiation of the respective blood cells [40], and are used to determine clinical subtypes of AML and ALL [46, 48]. In addition aberrant silencing by epigenetic mechanisms leads to changed expression patterns interfering with hematopoiesis. The origin of the disease often appears to causally involve a combination of different layers of inherited information and the interplay between these is poorly understood.

Genome-wide association studies find polymorphisms in genes involving the epigenetic machinery as significantly associated with developing acute leukemia, especially during childhood. The translocation t(11;16)(q23;p13.3) of the mixed lineage leukemia (MLL) gene with the CREB-binding protein (CBP), which is a histone acetyl transferase (HAT), is a frequent rearrangement in acute leukemias and associated with poor prognosis [53, 142, 226]. Gene disruption or polymorphisms of epigenetic genes, like miRNAs and HATs, are very common in acute leukemias [28, 146]. Mutations in DNMT3a and EZH2, a polycomb repressor complex member, have been reported in AML [63, 141, 254]. Altogether this leads to the conclusion that DNA methylation and chromatin remodeling are strongly involved in etiology of acute leukemias.

Case-control studies aim to detect risk markers in non-affected tissues using non invasive methods. To avoid a bias, e.g., by clonal selection of epigenetically altered tumor cells, a reference tissue other than blood would be desirable for investigation of epigenetic host factors in acute leukemia. One additional problem is that the results in a blood methylation analysis depend highly on the assay used and the

distribution of blood cell types [265], which can vary. A population-based study determining levels of DNA methylation in a group of patients with a variety of leukemias compared blood methylation of the *bone morphogenic protein 6* (*BMP6*) in patients with peripheral blood monocyte (PBMC) methylation from healthy donors. *BMP6* was hypermethylated only in adult T-cell leukemias, especially an aggressive subtype, [242], which indicates the importance of stratifying for histology. The combination of epidemiologic factors with epigenetic factors appears promising as epigenetic alterations, especially age-dependent DNA methylation changes, have been detected in various cancer-related genes in a mouse model [153], and several studies suggest the potential influence of genetic factors on epigenetics and vice versa.

13.5.4.2 Chronic Leukemias

Chronic leukemia can be divided into the two subclasses: chronic myeloid lymphoma (CML) and chronic lymphocytic leukemia, which is the most frequent leukemia in the western world [228]. Both malignancies are characterized by slow progress during disease and a low incidence rate in children or young adults.

In a large genome-wide association study genetic polymorphisms were connected with CLL and several risk loci were identified [57]. Characterization of 506 CLL patient samples could show the impact of karyotypes, namely deletions in 6q21, 11q22.3, 13q14, and 17p13 (TP53 locus) and a trisomy of chromosome 12 on survival [89]. Additionally, those aberrations could be linked to Immunoglobulin heavy-chain variable-region (IgVh) mutation status which is related to disease development. ZAP-70 expression, which is associated with an unfavorable course of disease, is linked to the IgVh unmutated status [49]. *ZAP-70* promoter methylation is linked to the downregulation of ZAP-70 [47], and additionally ZAP-70 expression was shown to predict methylation levels of important genes, e.g., *Sall1*, *MLL*, *APP*, or of long interspersed nuclear element sequences (LINE-1) which serves as a marker for global methylation [14, 69, 246].

CML is strongly associated with a rearrangement in a 5.8-kilobase stretch of chromosome 22 which occurs in the vast majority of patients [250]. This translocation results in the *BCR/ABL1* fusion oncogene producing a gene product with tyrosine kinase activity. In healthy individuals two active copies of the *ABL1* gene are present. It could be shown in samples of 201 patients that the remaining functional copy in CML patients is not methylated during the chronic stage, is partially methylated in the accelerated, and completely methylated in the blast crisis phase [11].

Chronic leukemias are diseases which seem to be strongly influenced by epigenetic factors, especially DNA methylation. Epigenetic marks clearly determine etiology and course of disease. In the future, genome-wide screens in large cohorts will be powerful tools in identifying epigenetically silenced genes, which influence hematologic diseases. Such insights may also help in the development of effective treatments.

13.5.5 *Epigenetic Epidemiology of Other Tumors*

13.5.5.1 Liver

Hepatocellular carcinoma (HCC) ranks among the most common cancers worldwide, and various risk factors such as hepatitis B virus and hepatitis C virus infection are known to participate in its development [84]. Genome-wide or global hypomethylation, previously identified as a marker for HCC [144], and/or promoter CpG hypermethylation [134, 136, 233, 273] correlate with clinical outcome (survival) in HCC [35]. Specific methylation signatures in HCC exist which are associated with environmental risk factors, survival, and tumor staging; one such methylation signature obtained by using array technology, pyrosequencing, and quantitative polymerase chain reaction comprises various promoter CpG sites and correctly classified tumor and surrounding normal liver tissue, thus a small set of predictor CpG sites may be sufficient for future diagnostic and clinical use [97]. Interestingly, genome-wide DNA methylation profiling using bacterial artificial clone arrays also revealed that few such clones may suffice as an excellent indicator set for HCC risk estimation and prognosis (reviewed in: [118]). Further epigenetic markers linked to HCC include: *LINE-1* hypomethylation in tissue or serum of HCC patients [125] as independent marker of poor prognosis [241]; altered promoter methylation of tumor suppressor genes and oncogenes [126]; histone deacetylase SNPs as potential predictors for tumor recurrence [269]; epigenetic silencing of *Tip30* expression by CpG island DNA hypermethylation is associated with poor prognosis in HCC patients [151]; in rodents, epigenetically silenced *methionine adenosyltransferase* (*MAT1A*) expression is associated with HCC development [163]; and methylation of *dickkopf-3 (DKK3)* was shown to occur early in HCC development and may qualify as prognostic risk factor in HCC [268]. Detection of epigenetically modified biomarkers in serum, e.g., of promoters of tumor suppressor genes [274] are attractive goals for early diagnosis of HCC especially since an epigenetically dysregulated promoter of one gene can influence methylation changes in other genes as shown in a mouse model of HCC [99, 140, 198]. Given evidence that epigenetic aberrations are involved in HCC development (see [94] for review) it appears that investigation of epigenetic markers as possible modulators also of risk of hepatic carcinoma holds promise.

13.5.5.2 Brain

Global DNA hypomethylation is frequent (80%) in primary glioblastomas [31], either in repetitive sequences *(Sat2)* possibly due to DNA methyltransferase 3b (DNMT3b) dysfunction [61] or in single-copy genes such as *MAGEA1* [31, 271]. Mouse models indicate that global hypomethylation is sufficient to initiate tumorigenesis [81]. For glioblastoma multiforme (GBM, reviewed in [160, 173]), promoter CpG island hypermethylation is frequent, e.g., in tumor suppressor (*EMP3*), DNA

repair enzymes (*MGMT1*; [67]), and growth factor genes ($TGF\beta - Smad$ and *PDGF-B*; [30]) and can be associated with poor prognosis [30]. *MGMT* function is lost upon CpG island promoter methylation in brain tumors [67], and this epigenetic silencing is linked to survival time in GBM [66, 92, 93]. *MGMT* promoter methylation is also linked to a hypermutator phenotype during glioblastoma therapy, which has potential clinical implications [177], and its methylation status may change during development of GBM or relapses [29, 161]. In addition, *MGMT* methylation was recently shown to be an independent prognostic factor for overall and progression-free survival in elderly GBM patients [165]. More recently, in secondary glioblastomas *RASSF10* methylation was reported as independent prognostic factor associated with worst-progression-free and overall survival [100]. Further, a direct or indirect link between genetic and epigenetic modifications was recently noted: *MGMT* silencing by hypermethylation facilitates G to A mutations [65, 66], and such a mutated *IDH1* status was found to be an independent prognostic factor in gliomas [206]. Recently, promoter methylation of the suppressor of cytokine signalling gene *SOC3* was found to be associated with outcome in GBM patients [162]. For medullablastoma, aberrant promoter methylation of several promoter sequences also correlated with poor prognosis [77, 171].

In summary, existing evidence links defined epigenetic alterations with brain tumor incidence thus supporting the idea of an association of epigenetics and epidemiology of brain tumors.

13.5.5.3 Stomach

In a recent study, up regulation of *SOX4* was found to be inversely associated with the epigenetic silencing of *miR-129-2* in gastric cancer and inversely correlated with patient survival time (70 cases; [217]). Further, the methylation level in several gastric cancer candidate CpG islands was higher in gastric cancer mucosa suggesting a possible association with gastric cancer risk [154, 175, 176]. Recent studies also linked differential methylation status of *Cystatin M, BNIP3,* and *DAPK* promoters with survival of gastric cancer patients [42, 234].

13.5.5.4 Skin

Many epigenetic alterations are known for skin cancerous tissue (reviewed in [87]). In SCC, hypermethylation of *RASSF2A, MGMT*, and *TSP-1* gene promoters was identified, and *TSP-1* hypermethylation was reported as a bad prognosis factor in vulvar SCC [88]. In malignant melanoma, aberrant CpG island methylation of tumor-related genes is associated with methylation of noncoding methylated-in-tumor (*MINT*) loci, one of which (*MINT31*) was characterized as a predicting factor for tumor progression and survival [240]. In contrast, in cutaneous T-cell lymphomas, methylation profiles of candidate promoters ($p14^{ARF}$, $p16^{INK4A}$, *and* $p15^{INK4B}$) did not correlate with prognosis [131]. In oral SCCs, promoter hypermethylation silenced the

expression of *IKKα*, one of the IkB kinases linked to NFκB-mediated transcriptional activity, and this epigenetic alteration was associated with an unfavorable prognosis [152]. Recent evidence linked microRNAs, which downregulate DNA methyltransferases *DNMT3A* and *DNMT3B*, with survival of skin cancer patients [180].

13.5.5.5 Kidney

In renal cancer, epigenetic silencing of various Wnt pathway genes has been reported. Polymorphisms within the Wnt antagonists of the *Dickkopf (DKK)* gene family have been associated with this cancer, and a specific *DKK2* polymorphism may be a predictor for survival of kidney cancer patients [101, 102]. Other studies on renal clear cell carcinoma (RCC) cases found that clustering is associated with regional DNA hypermethylation suggesting such epigenetic cluster analysis as a prognostic indicator for RCC patients [9, 10]. At present it is controversial whether epigenetic alterations in the von *Hippel-Lindau (VHL)* gene have prognostic value in clear cell RCC [188, 224]. In addition, histone modifications such as histone acetylation as epigenetic regulators of gene activity have been shown to be of prognostic value in kidney cancer [170].

13.5.5.6 Bladder

The candidate tumor suppressor gene *myopodin* is aberrantly methylated in several cancer types including bladder cancer [38] and its hypermethylation was found to be associated with increased recurrence and shorter survival in bladder cancer patients [5]. Also, *polyamine modulated factor-1 (PMF*1*)* was identified to be epigenetically modified in bladder cancer, and its promoter methylation status correlates with tumor progression of bladder cancer [2]. A more recent population-based incident case-control association study analyzing peripheral blood DNA from 285 cases and 465 controls indicated that *LINE1* hypomethylation may be an important if unspecific marker of bladder cancer risk, and the association was stronger among women (OR = 2.48) than men (OR = 1.47) [264]. Further epigenetically modified candidate targets associated with bladder tumor stage or patient survival include *P16INK4a, P14ARF*, and several other promoters [3, 156].

13.5.5.7 Testis

LINE-1 methylation patterns may be associated with cancer risk [166]. Other epigenetic candidate markers for testicular cancer include (reviewed in: [83]): *RASSF1A* (tumor suppressor, promoter frequently hypermethylated in testis cancer) [105]; altered global methylation profiles (seminomas: hypomethylated, practically no CpG hypermethylated, whereas nonseminomas are less hypomethylated and have variable CpG island methylation) [223]; increased expression of *DNMT3A; and DNMT3B* in embryonal carcinoma subtypes [4, 221].

13.5.5.8 Pancreas

Epigenetic epidemiology studies have thus far not been reported for pancreatic carcinoma. However, methylation profiling of CpG island promoter sites in normal pancreas, pancreatic tumors, and cancer cell lines indicate frequent aberrant DNA methylation in pancreatic cancer [239]. Many of these sites were known from other cancer types [64] and include genes for, e.g., cadherins, growth factor binding proteins, imprinted genes, and transcription factors. Another study comparing the genome-wide promoter methylation profile of a human pancreatic cancer cell line (PANC-1) with that of a non-neoplastic cell line (HPDE) also detected highly significant differences in methylation which were validated for some genes in normal and pancreatic cancer tissue [182]. An earlier prospective study with cases and controls found that plasma levels of folate, vitamin B6, vitamin B12, and homocysteine are not associated with the risk of pancreatic cancer [210], which may argue against a role of methylation in epigenetic epidemiology of pancreatic adenocarcinoma (PDAC). However, epigenetic silencers such as polycomb proteins were found upregulated in PDAC tissue which may link them to pancreatic tumor development [158]. In addition, varying expressions of epigenetic modifiers including *DNMT1, HDAC1*, methyl-CpG binding proteins, and miRNAs such as miR-373 are associated with staging or survival in cases of this tumor type [43, 258].

13.6 Outlook

Important technological developments will advance and considerably change the field of epigenetic epidemiology of cancer. The fact that quantitative instead of qualitative methylation measurements are becoming standard even for high-throughput analyses [202] will much refine future analyses. Methylation next generation sequencing approaches, while not yet affordable in high throughput, will provide important new targets.

While the majority of studies to date relating to epigenetic cancer biomarkers have been focussing on CpG island promoter methylation, in the future whole genome methylation and histone modifications will become more prominent. Repetitive element hypomethylation in leukocyte DNA was recently investigated within the Normative Aging Study cohort, and associated with increased incidence and mortality from cancer [276]. While this is interesting the clinical use of this phenomenon is limited due to its lack of specificity. The development of quantitative detection of DNA methylation in minute amounts of DNA [185] will be particularly important in the context of epigenetic early diagnosis markers.

The fact that within case-control studies, sample collection occurs after disease development makes it impossible to determine whether epigenetic associations observed are causal or possibly related to tumor development. Cohort studies will thus be invaluable in future investigations to confirm any associations observed. Population based cohorts with good follow-up allow the investigation of samples

from individuals with tumors that were diagnosed after sample collection. Another important challenge for future investigations will be to identify the doses and time-window in an individual lifetime when particular exposures can lead to epigenetic changes [260]. Given the reversibility of epigenetic alterations and the importance of epigenetic mechanisms for gene regulation, cancer chemoprevention by targeting the epigenome is an obvious goal, and there is evidence that this is becoming a real possibility [111]. In this context especially, the identification of epigenetic risk factors and refinement of currently available cancer risk models has real public health potential.

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Chapter 14
Epigenetic Epidemiology of Infectious Diseases

Toshikazu Ushijima and Hideyuki Takeshima

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Abstract Cancers induced by infectious agents, such as *Helicobacter pylori*, hepatitis viruses, Epstein-Barr virus, papilloma virus, liver fluke, and Schistosoma, are often associated with frequent DNA methylation of tumor-suppressor genes. Chronic inflammation and direct effects of some infectious agents are responsible for methylation induction. Analysis of non-cancerous, thus polyclonal, tissues provides unbiased information on which genes are methylated by specific agents. Gastric mucosae with *Helicobacter pylori* infection show methylation of specific promoter CpG islands. Promoter CpG islands of genes with low transcription levels and trimethylation of histone H3 lysine 27 are susceptible to methylation induction, and those with RNA polymerase II are resistant, even if the genes are not transcribed. Methylation of specific genes is expected to remain even after its inducing agent has vanished and these cytosine methylation fingerprints may prove to be good markers of past exposure to specific agents in molecular epidemiology.

Abbreviations

CGI	CpG island
CIMP	CpG island methylator phenotype
DNMT	DNA methyltransferase
EBV	Epstein-Barr virus
ESCC	Esophageal squamous cell carcinoma
H. pylori	*Helicobacter pylori*
H3Ac	Acetylation of histone H3
H3K27me3	Trimethylation of histone H3 lysine 27
H3K4me3	Trimethylation of histone H3 lysine 4
H3K9me3	Trimethylation of histone H3 lysine 9
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HPV	Human papilloma virus
LMP1	Latent membrane protein 1
MeDIP	Methylated DNA immunoprecipitation
MSP	Methylation-specific PCR
Pol II	RNA polymerase II
PRC	Polycomb repressive complex

14.1 Introduction

Infectious agents are some of the most well-known inducers of aberrant DNA methylation. Infection with *Helicobacter pylori* (*H. pylori*), a bacterial strain causally involved in gastric carcinogenesis, is known to induce aberrant DNA methylation in

gastric epithelial cells [1–3]. Infection with hepatitis C and B viruses (HCV and HBV), both of which are involved in the development of hepatocellular carcinomas, is associated with aberrant DNA methylation in cancer tissues and surrounding non-cancerous tissues [4–6]. Infection with Epstein-Barr virus (EBV), associated with lymphomas, nasopharyngeal cancers, and gastric cancers, is also associated with frequent DNA methylation in tumor tissue. Aberrant methylation induced by an infectious agent is present not in random genes, but in a group of genes affected by the agent [7]. Such gene-specific methylation provides an excellent tool for molecular epidemiology revealing past exposure to specific infectious agents, even if these agents are no longer present.

In this chapter, we will introduce methylation induction by infectious agents, its mechanisms, methylation of specific genes by specific inducers (a methylation fingerprint), and its mechanisms. We will conclude with the potential application to epidemiology.

14.2 Infectious Agents and Methylation Induction

Cancers induced by infectious agents, such as *H. pylori*, hepatitis viruses, EBV, papilloma virus, liver fluke, and Schistosoma, tend to have frequent aberrant DNA methylation of promoter CpG islands (CGIs) of tumor-suppressor and other genes (Table 14.1). In addition, non-cancerous tissues exposed to some infectious agents also show methylation of some genes, many of which are carried over into cancer cells.

14.2.1 The Significance of Methylation in Cancers and in Non-cancerous Tissues

Methylation of promoter CGIs of tumor-suppressor genes is frequently present in cancers induced by an infectious agent. This can be a result of frequent induction of methylation in the infected tissue by the agent, or a result of growth advantage of a cell with methylation of a tumor-suppressor gene and its clonal expansion. If methylation is present in non-cancerous (thus polyclonal) tissue after infection, it is unlikely that a cell with a rare event of methylation of a tumor-suppressor gene, which itself is unrelated to the infection, has been selected and expanded. Therefore, when tumor-suppressor genes or other genes whose methylation can confer growth advantage to a cell are analyzed, use of non-cancerous tissues provides more unbiased information on whether or not an infectious agent is capable of inducing DNA methylation.

Since non-cancerous tissues are polyclonal, high methylation levels of a specific gene means that more cells acquired methylation of this gene in the past. Therefore, high methylation levels can be associated with stronger tissue damage in the past

Table 14.1 Infectious agents and genes whose methylation is associated with an agent

	Methylated genes in cancer tissue	Methylated genes in non-cancerous tissue	Ref
Helicobacter pylori			
Gastric cancer			
	CDKN2A	*CDKN2A*	[2]
	FLNc	*FLNc*	[2]
	LOX	*LOX*	[2, 8]
	THBD	*THBD*	[2]
	miR-124a	*miR-124a*	[8]
	26 genes, including BDNF and IGFBP3	*26 genes, including BDNF and IGFBP3*	[9]
Hepatitis virus B (HBV)			
Hepatocellular carcinoma			
	APC	*APC*	[6]
	CDKN2A	*CDKN2A*	[6]
	RASSF1A	*RASSF1A*	[6]
Hepatitis virus C (HCV)			
Hepatocellular carcinoma			
	APC	*APC*	[5, 6]
	CDKN2A	*CDKN2A*	[6]
	RASSF1A	*RASSF1A*	[6]
Epstein-Barr virus (EBV)			
Gastric cancer			
	CDH1		[10]
	CDKN2A		[11, 12]
	MGMT		[11]
	PTEN		[13]
Papilloma virus (HPV)			
Cervical cancer			
	RASSF1A (unmethylated)[a]		[14, 15]
Head and neck cancer			
	RASSF1A (unmethylated)[a]		[16]
	SFRP4		[17]
Liver fluke			
Cholangiocarcinoma			
	MLH1		[18]
Schistosoma			
Bladder cancer			
	APC		[19]
	CDH1		[19]
	CDKN2A		[19]
	RASSF1A		[19]

[a]Considered to be due to the functional interaction between *RASSF1A* inactivation and HPV infection

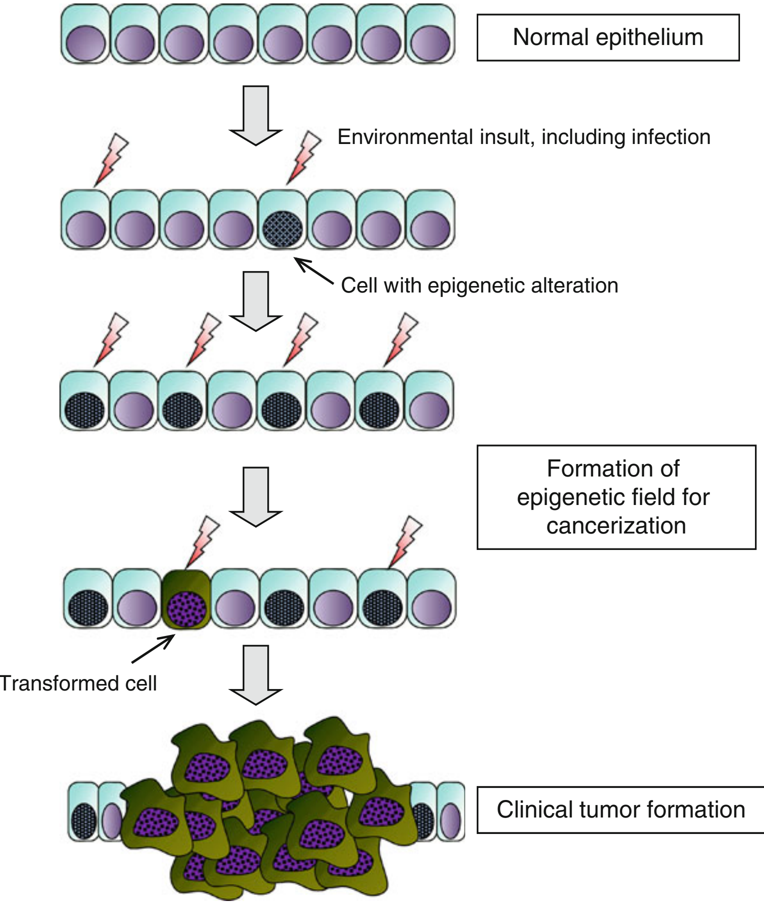

Fig. 14.1 Formation of an epigenetic field for cancerization. Exposure to environmental stimuli, including infection, induces epigenetic alterations in a significant fraction of cells in normal-appearing tissues. When such stimuli continue, epigenetic alterations accumulate in a tissue, and an epigenetic field for cancerization is produced

and accumulation of epigenetic alterations. Indeed, for cancers like gastric cancers and hepatocellular carcinomas (HCCs), methylation accumulation in non-cancerous tissues is known to form a field from which cancers tend to develop (an epigenetic field for cancerization; Fig. 14.1) [20]. Methylation levels in gastric mucosae are associated with risk of developing gastric cancers [20, 21].

14.2.2 Helicobacter pylori Infection

Gastric cancer is one of the leading causes of cancer deaths in the world, and most gastric cancer patients have a history of *H. pylori* infection [22]. *H. pylori* is a

Gram-negative bacterium, and is involved not only in gastric cancers but also in peptic ulcers and specific types of gastric B-cell lymphomas [23]. The infection itself is very prevalent, affecting nearly half of the world's population, although the prevalence is consistently decreasing [24]. *H. pylori* infection usually leads to acute and chronic gastritis, but only a small fraction of the infected individuals develop gastric cancer. Differences in *H. pylori* strains and host genetic backgrounds are considered to be responsible for the diverse clinical outcomes [25].

In gastric cancers, tumor-suppressor genes, such as *CDKN2A*, *MLH1*, *CDH1*, and *RUNX3*, are inactivated more frequently by methylation of their promoter CpG islands than by mutations [1]. Recent genome-wide analyses revealed that in addition to tumor-suppressor genes, hundreds of other genes are also methylated in gastric cancers [26, 27]. The possible association between *H. pylori* infection and methylation suggested by analysis of gastric cancers is solidified by analysis of non-cancerous gastric tissues with and without *H. pylori* infection [2, 9]. Methylation levels of eight specific CGIs in individuals with *H. pylori* infection were 5.4- to 303-fold as high as those in individuals without *H. pylori* infection ($P < 0.0001$). In addition to protein coding genes, microRNAs are also methylated in *H. pylori* infected tissues [8].

The accumulation level of aberrant DNA methylation in gastric mucosae is known to correlate with gastric cancer risk [2, 21]. Among individuals without current *H. pylori* infection, methylation levels in gastric mucosae were lowest in healthy individuals, high in cancer patients with a single gastric cancer, and highest in cancer patients with multiple gastric cancers (both metachronous and synchronous gastric cancers) [21]. The correlation strongly supports that the accumulation of aberrant methylation in gastric mucosae produces an epigenetic field for cancerization.

An animal model is available for gastric cancers induced by *H. pylori* infection [28]. Mongolian gerbils infected with *H. pylori* showed increased methylation levels compared with age-matched non-infected gerbils, demonstrating a causal role of *H. pylori* infection in methylation induction [3].

14.2.3 Hepatitis Viruses

HCCs are also a major cause of cancer deaths and are known to arise from liver tissues that heavily suffered from chronic inflammation, such as chronic hepatitis and liver cirrhosis [29]. As etiological agents, HCV and HBV play major roles, as well as some additional agents, such as excessive alcohol consumption and aflatoxin.

HCC tissues also have aberrant methylation of promoter CGIs of multiple tumor-suppressor genes [5, 6, 30]. Some genes, such as *CDKN2A*, are specifically methylated in HCCs while others, such as *RASSF1A*, are already methylated in surrounding liver tissues with chronic hepatitis or liver cirrhosis [30]. HCCs arising from liver cirrhosis have more methylated genes than those arising from chronic hepatitis, indicating that background tissue damage is reflected in HCCs [30]. When methylation

of 19 genes, mostly tumor-suppressor or tumor-associated genes, was analyzed, HCV-positive HCCs had more frequent methylation than had HBV-positive cancers and virus-negative HCCs [6].

Aberrant methylation of various genes is also present in surrounding non-cancerous liver tissues of patients with HCCs [6, 30, 31]. HCV-positive liver tissues show more frequent methylation than HBV-positive liver tissues and virus-negative liver tissues [6], strongly supporting HCV infection induces aberrant DNA methylation. Non-cancerous liver tissues of individuals with HBV and alcoholic hepatitis also had higher methylation levels than normal liver tissues of healthy individuals, indicating that these agents can induce aberrant DNA methylation in human liver tissues.

14.2.4 Epstein-Barr Virus

EBV infection is highly prevalent in the world, and is known to affect mainly B cells [32]. Its infection is implicated as an etiology of EBV-associated B-cell, T-cell, and NK-cell lymphomas and nasopharyngeal carcinomas. A minor fraction of gastric cancers is also associated with EBV infection, and such cancers are accompanied with strong infiltration of inflammatory cells [11, 33]. Gastric cancers with EBV infection are known to be associated with highly frequent methylation of multiple genes, including *CDH1*, *MGMT*, *PTEN*, and *p16*, demonstrating the CGI methylator phenotype (CIMP) [10–13, 33]. Surprisingly, non-cancerous gastric mucosae of patients with gastric cancers with EBV infection tended to have lower levels of methylation than those of patients with gastric cancers but without EBV infection, most of whom were infected by *H. pylori* [12].

14.2.5 Papilloma Virus

Human papilloma virus (HPV) is associated with cervical cancer, head and neck cancers, oral cancers, and possibly lung cancer [34–36]. In cervical cancers, HPV infection was associated with unmethylated status of *RASSF1A*, possibly due to their complementary functions in cervical carcinogenesis [14]. In contrast, in cervical dysplasia, from which cervical cancers develop, aberrant methylation of multiple genes, including *CCNA1*, *DAPK1*, *HS3ST2*, *PAX1*, and *TFPI2*, was present [37, 38], suggesting that HPV infection was associated with methylation induction.

In head and neck cancers, HPV infection was associated with unmethylated status of *RASSF1A*, again possibly due to their complementary functions in cervical carcinogenesis [16]. HPV infection was associated with *SFRP4* methylation in cancer tissues [17].

14.2.6 Liver Flukes

Liver flukes are parasites that infect the biliary tract, and are known to be a risk factor for cholangiocarcinoma, a rare malignant liver tumor arising from the intrahepatic biliary tract [39]. Like in other cancers, aberrant DNA methylation of tumor-suppressor genes, such as *APC*, *CDH1*, *CDKN2A*, and *MLH1* is frequently observed in cholangiocarcinoma [40]. Especially, *MLH1* methylation is frequently observed in liver fluke-related cholangiocarcinoma in Thailand [18].

14.2.7 Schistosoma

Schistosoma is a parasite that infects the urinary ducts and bladder, and Schistosoma infection is associated with risk of several malignancies, including bladder cancers [41]. Bladder cancers in general also show aberrant DNA methylation in multiple genes [42]. Notably, bladder cancers with Schistosoma infection had more methylated tumor-suppressor genes, including *APC*, *CDH1*, *CDKN2A*, and *RASSF1A*, than those without Schistosoma infection [19].

14.3 Mechanisms for Methylation Induction by Infectious Agents

Infectious agents are inducers of inflammation, which is known to be deeply involved in methylation induction [43, 44]. In addition, direct effects by infectious agents on cancer precursor cells need to be considered.

14.3.1 Role of Chronic Inflammation

The role of chronic inflammation in methylation induction was proposed based on the observation that aberrant methylation of specific genes was present in colonic mucosae of patients with ulcerative colitis [43, 45]. In addition, the infectious agents listed above are all associated with chronic inflammation. This strongly indicates that chronic inflammation is involved in methylation induction.

Direct evidence for the role of chronic inflammation in methylation induction was provided by an animal model of methylation induction by *H. pylori* infection. When inflammation induction by *H. pylori* infection was suppressed by treating Mongolian gerbils with cyclosporin A, an immunosuppressant, methylation induction was markedly suppressed without affecting colonization of *H. pylori* (Fig. 14.2). This showed that it is inflammation, not *H. pylori* itself, that is involved in methylation induction [3].

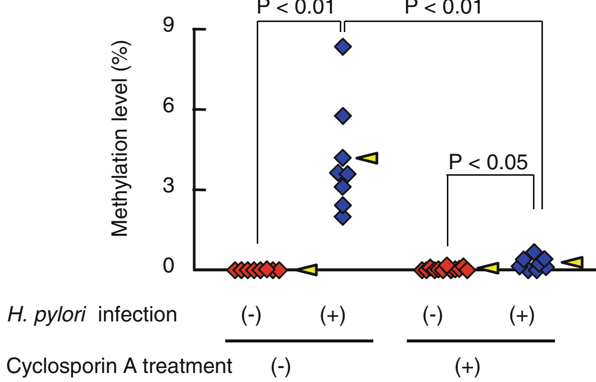

Fig. 14.2 A critical role of inflammation in methylation induction. The methylation level of a CGI (HE6) was measured by quantitative MSP in gastric mucosae of gerbils infected with *H. pylori* with or without cyclosporin A treatment. While a high level of methylation was induced by *H. pylori* infection without cyclosporin A, the induction was markedly suppressed with cyclosporin A. It was confirmed that cyclosporin A suppressed immune reaction, but did not affect colonization of *H. pylori*. Arrow head: mean methylation level in a group (Modified from Niwa et al. [3])

14.3.2 Direct Effect of Infectious Agents

In addition to signals from chronic inflammation to epithelial cells, some infectious agents have a direct effect on methylation induction. Especially, up-regulation of DNA methyltransferases by infectious agents has been reported for multiple viruses. The X protein of HBV (HBx) up-regulates DNMT1 and DNMT3A; the HCV core protein up-regulates DNMT1 and DNMT3B; HPV E7 and adenovirus E1A up-regulate DNMT1; and Kaposi's sarcoma-associated herpes virus LANA protein up-regulates DNMT3A [46–52]. The major EBV oncogene, latent membrane protein 1 (LMP1), up-regulates DNMT1, DNMT3A, and DNMT3B in nasopharyngeal carcinoma cell lines and induces methylation of the tumor-suppressor genes *RARB* and *CDH13* in these cell lines [49, 53]. LMP2A, another EBV latent gene, up-regulates DNMT1 and induces methylation of the tumor-suppressor gene, *PTEN*, in gastric cancer cell lines [11, 13]. In contrast with these viruses, DNMTs were not upregulated in human and gerbil gastric mucosae infected by *H. pylori* [3, 9].

14.4 Methylation Fingerprints Produced by Specific Inducers

It is likely that promoter CGIs of specific genes are methylated by a specific inducer, as described in this section. We here call this specific pattern of methylation "methylation fingerprint," as a mutation pattern specific to a mutagen is termed its

"mutational fingerprint" [54, 55]. The presence of methylation fingerprints was first suggested by analysis of cancer tissues, and was recently shown by analysis of non-cancerous tissues as well [56].

14.4.1 Target Gene Specificity of DNA Methylation in Cancers

The presence of target gene specificity in DNA methylation induction was initially indicated by the presence of methylation of specific genes in cancer cells [57–59]. A pioneering study of 1,184 CGIs using restriction landmark genomic scanning, an early-stage genome-wide scanning technique for differences in DNA methylation, revealed that certain CGIs were more frequently methylated in specific tumor types [57]. Analyses of promoter CGIs of mostly tumor-suppressor genes suggested that some CGIs were methylated at high incidences in specific tumor types [58]. Methylated DNA immunoprecipitation (MeDIP)-microarray analysis of colon cancer tissue revealed that most methylated genes were located within defined genomic clusters [59]. Comparison of 77 HCCs associated with either HBV or HCV revealed that some genes are preferentially methylated in HCCs, depending on the specific hepatitis virus [6].

14.4.2 Strategy to Analyze Methylation Fingerprints in Non-cancerous Tissues

Analysis of methylation in non-cancerous tissues should be conducted using a method with high sensitivity, such as methylation-specific PCR (MSP), because aberrant methylation of a gene is expected to be present only in a minor fraction of cells in a polyclonal tissue. Since methods with high sensitivity can analyze only selected genes, this requires pre-specification of genes to be studied. By a genome-wide methylation analysis of cancer tissues, we can obtain candidate genes methylated also in non-cancerous tissues. Using these pre-specified genes, we can perform a high-sensitivity methylation analysis in non-cancerous tissues [9, 60]. Recent methylome analyses yielded many promoter CGIs methylated in various cancers, including gastric, colon, lung, and breast cancers [26, 61–63]. High-sensitivity methylation analysis of these CGIs in non-cancerous tissues of patients with documented exposure to specific agents will lead to establishment of methylation fingerprints of the agents.

14.4.3 Methylation Fingerprint in Gastric Mucosae Exposed to *H. pylori* Infection

To clarify the presence of target gene specificity in methylation induction by *H. pylori*, methylation analysis of a set of candidate CGIs in gastric mucosae was

Fig. 14.3 The presence of a methylation fingerprint of *H. pylori* infection. Methylation of promoter CGIs of 48 genes was analyzed in gastric mucosae of individuals without and with *H. pylori* infection using a high-sensitivity method, MSP. Although genes 1–8 were not methylated at all, genes 22–47 were consistently methylated in individuals with *H. pylori* infection (Modified from Nakajima et al. [9])

conducted by searching for promoter CGIs methylated in gastric cancers [26]. MSP of 48 promoter CGIs in gastric mucosae with and without *H. pylori* infection suggested that specific genes were consistently methylated in gastric mucosae with *H. pylori* infection while others were not, forming a methylation fingerprint (Fig. 14.3) [9]. The susceptible genes had lower transcription levels in normal

gastric mucosae than had the resistant genes. Up-regulation of DNMTs was not observed in gastric mucosae with *H. pylori* infection.

14.4.4 Methylation Fingerprint in Esophageal Mucosae of Long-Term Smokers

Esophageal squamous cell carcinomas (ESCCs) are cancer types infamous for repeated occurrence, which is considered to be due to accumulation of genetic/epigenetic alterations in the background esophageal mucosae. We isolated 13 promoter CGIs methylated in ESCCs, and analyzed their methylation levels in esophageal mucosae. Methylation levels of 5 genes (*HOXA9*, *MT1M*, *NEFH*, *RSPO4*, and, *UCHL1*) were significantly correlated with smoking duration [60]. Although smoking is not an infectious agent, this finding in non-cancerous esophageal mucosae supports the theory that a specific agent induces methylation of specific genes, leaving a methylation fingerprint.

14.5 Mechanisms for Formation of a Methylation Fingerprint

A methylation fingerprint in non-cancerous tissues is formed because some specific genes are susceptible and others are resistant to methylation induction. Factors involved in such susceptibility and resistance have been studied for the past decade, and include low transcription levels, trimethylation of histone H3 lysine 27 (H3K27me3), and binding of RNA polymerase II (Pol II) (Fig. 14.4).

14.5.1 Low Transcription Levels and DNA Methylation Susceptibility

A low transcription level of a gene was proposed to be involved in methylation induction in the early 2000s [64–67]. Song et al. demonstrated that disruption of promoter activity (thus low transcription levels) of a transfected gene leads to aberrant DNA methylation of promoter CGIs in a cancer cell line [64]. de Smet et al. demonstrated that a gene demethylated by a DNA demethylating agent, 5-aza-2′-deoxycitidine, tends to be re-methylated when it is not transcribed [65]. The majority of genes methylated in cancer tend to have low transcription levels in normal cells [59, 66–68]. Among genes methylated in non-cancerous tissues, genes susceptible to methylation induction had lower transcription levels than resistant genes [9].

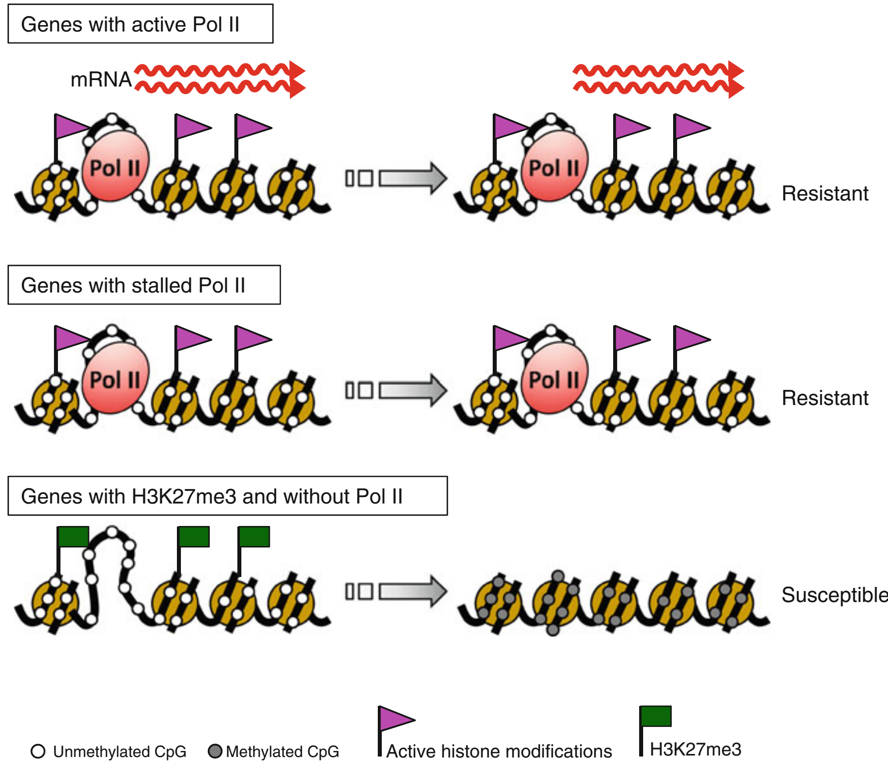

Fig. 14.4 Players involved in induction of DNA methylation in specific genes. Genes with active Pol II, 68% of which have active histone modifications (H3Ac), are resistant to DNA methylation induction. Genes with stalled Pol II, 19% of which also have active histone modifications (H3Ac), are also resistant. On the other hand, genes without Pol II, 90% of which are associated with H3K27me3 modification, are susceptible to methylation induction

14.5.2 Trimethylation of Histone H3 Lysine 27 and DNA Methylation Susceptibility

Histone modifications and DNA methylation depend upon each other. Using a limited number of genes, genes methylated in cancers were shown to be pre-marked by H3K27me3 in embryonic stem cells [69–71] and normal corresponding tissue [71]. This finding was also confirmed by genome-wide analyses [68, 72, 73]. H3K27me3 is recognized by a polycomb repressive complex (PRC) [74–76]. EZH2, a component of PRC2, and CBX7, a component of PRC1, are known to interact with DNA methyltransferases (DNMTs) [77, 78], and H3K27me3 may function as a recruiting signal for DNMTs. Another representative repressive histone modification, trimethylation of histone H3 lysine 9 (H3K9me3), was not associated with DNA methylation susceptibility [68]. Conversely, histone modifications

of active chromatin, such as acetylation of histone H3 (H3Ac) and trimethylation of histone H3 lysine 4 (H3K4me3), are weakly associated with resistance to DNA methylation [68].

14.5.3 Stalled RNA Polymerase II and DNA Methylation Resistance

Even among the genes with low transcription levels, some genes are still resistant to methylation induction. We have demonstrated that binding of Pol II to promoter CGIs (stalled Pol II) is associated with resistance to methylation induction [68]. Multivariate analysis of transcription levels, H3K27me3, H3Ac, and Pol II binding suggested that Pol II binding had a stronger effect on DNA methylation resistance than active histone modifications. Taken together with the fact that transcribed genes are resistant, binding of Pol II, active or stalled, is associated with resistance to methylation induction during carcinogenesis. Molecular mechanisms of how Pol II binding confers resistance to methylation induction remain to be clarified, but protection of CGIs from accession by DNMTs is one possible mechanism.

14.5.4 Role of Genomic Position Relative to Repetitive Elements

In addition, genomic architecture is also involved in gene-specificity of methylation induction. Compared with methylation-resistant genes, methylation-prone genes are located further apart from SINE and LINE retrotransposons [79]. However, since genome architecture does not change according to inducers of aberrant methylation, its role in the formation of a methylation fingerprint of an inducer is limited.

14.5.5 A Model for Formation of a Methylation Fingerprint of an Agent

As a mechanism of how a specific agent induces methylation of specific genes, we speculate that the agent first induces changes in transcription, H3K27me3, and binding of Pol II in its target genes, and that the changes then lead to methylation induction of the genes that acquired a susceptible epigenetic status (Fig. 14.5). Since the first changes in epigenetic status are consistently induced depending upon an agent, DNA methylation is expected to be induced in specific genes, forming a methylation fingerprint. This model is currently being validated using animal models.

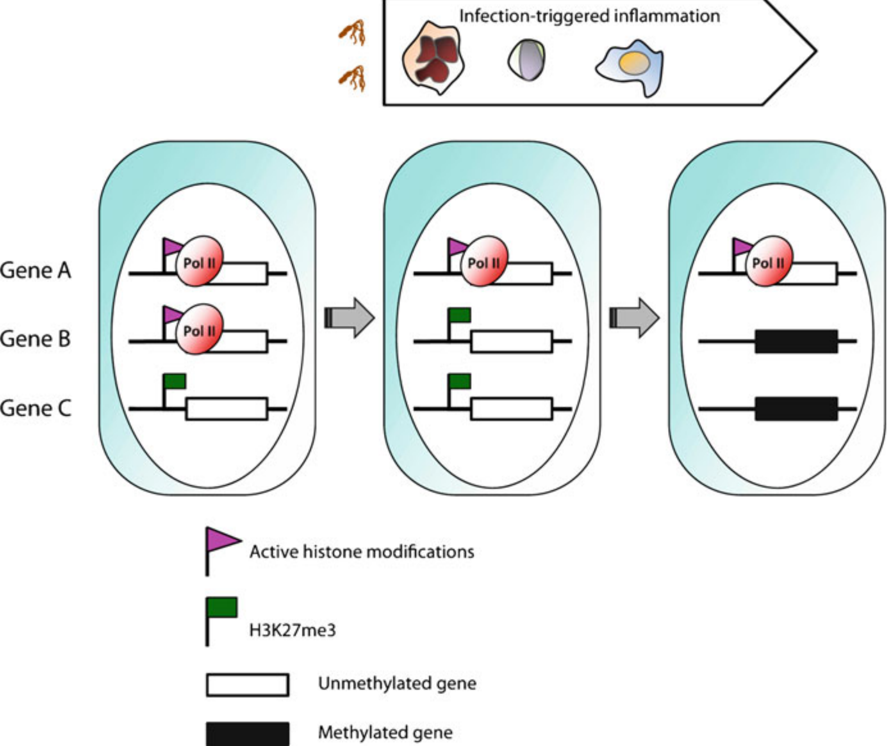

Fig. 14.5 A model for formation of a methylation fingerprint by an infectious agent. An agent is expected to induce changes in epigenetic statuses, including H3K27me3 and binding of Pol II. A gene that acquires a susceptible epigenetic status by exposure to an agent, such as *H. pylori* infection, is expected to become methylated (Gene B). Since the changes in epigenetic statuses are consistently induced by an agent, specific genes are expected to be methylated by the agent. A gene with Pol II (Gene A) and one with H3K27me3 (Gene C) regardless of the exposure to an agent are expected to be resistant and susceptible, respectively, to methylation induction. This model needs to be validated

14.6 Epilogue

Aberrant DNA methylation is induced by various infectious agents, and inflammation is an important element in the induction. Specific genes are methylated by specific inducers, forming methylation fingerprints. The potential application of methylation fingerprints in molecular epidemiology is to identify past exposure to infectious agents. An antibody to an agent usually gradually decreases after elimination of the agent, but a methylation fingerprint is expected to remain forever. So far, the number of known methylation fingerprints of specific agents is limited. However, a strategy to identify the fingerprint of an agent is already established if the agent induces cancer.

Once methylation fingerprints of various infectious agents become available, their use is expected to add new information to the involvement of infectious agents in various human disorders.

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Chapter 15
Epigenetic Epidemiology of Inflammation and Rheumatoid Arthritis

Caroline Ospelt and Steffen Gay

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Abstract The field of epigenetic research has evolved dramatically in recent years and has given valuable insight in regulation and dysregulation of gene expression in health and disease. The knowledge of the mechanisms controlling epigenetic changes increased and in more and more diseases a role for epigenetics could be found. Rheumatoid arthritis is a chronic inflammatory, autoimmune disease which leads to joint destruction but also affects other tissues and organs. Its etiology is not yet clarified, but a combination of the genetic background and environmental factors is proposed to trigger the onset of the disease. Epigenetic changes might be the link between non-genetic risk factors and development of symptoms. Changes in DNA methylation and histone modifications have been detected in patients with RA and have been found to promote inflammation and joint destruction. Future studies will have to show which epigenetic changes are causative factors and which are induced at a later stage by the chronic inflammatory environment seen in the disease. This knowledge holds the potential for new preventive, predictive and therapeutic opportunities in RA.

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Abbreviations

ACPA	anti-citrullinated peptide auto-antibodies
DNMT	DNA methyltransferase
EBV	Epstein Barr virus
H3	histone 3
H4	histone 4
HAT	histone acetylases
HDACs	histone deacetylases
HERV	human endogenous retrovirus
IGF	Insulin-like growth factor
IL	interleukin
MHC	major histocompatibility complex
PBMCs	peripheral blood mononuclear cells
RA	rheumatoid arthritis
SFN	Sulforaphane
SLE	systemic lupus erythematodes
TLRs	Toll-like receptors
Xi	inactivated X chromosome

15.1 Introduction

The classic definition of epigenetics describes epigenetic changes as mitotically heritable changes in gene function that in contrast to mutations do not alter the sequence of the DNA. However, the more epigenetics is studied in health and disease, the more facets and extensions have been added to this definition. Although an intriguing concept, the stable and mitotically heritable changes induced by the environment exclude a number of chromatin modifications and transcriptional regulators which, while short lived, profoundly influence gene function and might initiate changes that are passed on to the next generation of cells. In the current review, we follow the definition of Adrian Bird, describing epigenetic events as “the structural adaption of chromosomal regions so as to register, signal or perpetuate altered activity states” [1]. This definition not only describes the classic epigenetic systems such as methylation of DNA and of histone residues, but also includes more transient histone modifications such as acetylation and phosphorylation. Furthermore, it emphasizes the responsive nature of the epigenome, which is of particular interest in the context of the contribution of epigenetics to the development of diseases. Epigenetics may be the missing link between risk factors such as ageing, environmental toxins or co-morbidities and pathognomonic findings.

15.2 Epigenetics in Immune Function and Inflammation

The initiation of an immune response demands activation of regulatory processes to keep the frail balance between efficient defense and self destruction. Epigenetic modifications have been found to play an important role in the transcriptional regulation of genes modulating and coordinating the immune response at various levels.

The innate immune system gets activated via binding of pathogen-associated molecular patterns, so-called PAMPs by innate immune receptors. The best-studied innate immune receptors at the moment are Toll-like receptors (TLRs) which initiate a fast pro-inflammatory response against invading pathogens. It has long been known that repeated stimulation of TLR pathways, in particular of TLR4 pathways leads to the induction of tolerance against the inducing agent [2]. This mechanism controls the pro-inflammatory response and protects the organism from development of a septic shock. Foster et al. could show that there are two categories of genes that are induced by TLR4 stimulation [3]. The first set of genes is only induced at the first response, becomes then silenced and is not any more expressed after further TLR4 stimulations. This set mainly comprises pro-inflammatory mediators whose prolonged presence in tissues is detrimental and can induce tissue damage. The second set of genes is induced after every TLR4 stimulation. Even more, the magnitude and rapidness of their expression can even increase with repeated simulations. This set includes genes responsible for elimination of pathogens, expression of further immune receptors and activators of the adaptive immune response. After an initial TLR stimulation both sets of genes undergo specific epigenetic modifications such as histone acetylation and methylation which shape innate immune responses, improve the elimination of the pathogen and prevent inflammatory tissue damage.

Not only innate immunity, but also adaptive immunity gets modulated by epigenetic mechanisms. The specific cytokine pattern produced by Th1 and Th2 T cell subtypes for instance could be shown to be regulated by histone acetylation and methylation. While in Th1 cells expression of interferon γ and interleukin (IL)-4 is repressed by histone methylation, Th2 cells have no histone methylation at this locus but instead show hyperacetylation of histones at the *IL-4-IL-13* promoter site facilitating gene expression [4–7]. Also, expression of IL-2, a key regulator of T cell differentiation and survival is regulated by histone deacetylases (HDACs) [8].

Since in addition the expression of a variety of pro-inflammatory cytokines such as IL-1, IL-8 and IL-12 has been found to be controlled by HDACs, it can be assumed that modulation of the histone code is an important general mechanism to contain the inflammatory response [9–11]. Accordingly, disturbances in epigenetic control mechanisms might promote excessive, chronic or self-directed immune responses resulting in chronic inflammatory diseases and autoimmunity.

15.3 Rheumatoid Arthritis

To scale the influence of epigenetic changes on disease development, the case of monozygotic twins with discordant disease is often drawn upon. In celiac disease concordance in monozygotic twins is more than 80%, suggesting a strong genetic component in this disease [12, 13]. In rheumatoid arthritis (RA) the concordance rate between monozygotic twins is about 15% [14, 15]. However, concordance rates are lower among diseases with low prevalence. Heritability, which estimates the genetic contribution, is independent of disease prevalence and is about 60% for RA [16]. Nevertheless, these data suggest a substantial role of non-genetic factors in RA etiology. In the following, we will discuss current evidence on the contribution of non-genetic factors to the development of RA and the role of epigenetic mechanisms.

15.3.1 Epidemiology of RA

RA is a systemic autoimmune disease which simultaneously affects multiple joints and ultimately leads to irreversible damage of articular structures. Destructive arthritis primarily affects small joints of the hands and feet, but larger joints are also affected during the course of the disease. In a murine disease model circulating synovial fibroblasts in the blood were connected to this typical pattern of joint involvement [17]. The most common extra-articular manifestations of RA are subcutaneous nodules, serositis, and amyloidosis.

15.3.1.1 Geography

Worldwide, 0.5–1% of the population is affected by RA. Incidence rates vary between countries; RA was reported to newly affect 9/100,000 individuals per year in France and 45/100,000 individuals per year in the U.S. [18, 19]. However, these differences may be, at least partly, due to methodological differences, making it difficult to compare data from different studies. The prevalence of RA differs between regions and is generally lower in less economically developed countries [20, 21]. While genetic variance, environmental and socioeconomic factors affect disease prevalence, data from epidemiologic studies may reflect underdiagnosis due to lack of access to healthcare. In addition, lower life expectancy may also contribute to the low prevalence of RA in developing countries. Studies measuring disease rates within a country are particularly useful in separating the contribution of genetic and non-genetic factors. High prevalence rates in population subgroups point to genetic components, whereas higher rates of disease in specific geographical areas are indicators of environmental influences. Examples for the first scenario are studies indicating a very high RA prevalence in Native-Americans [22]. A larger genetic contribution can be assumed in these population groups, although social habits and living conditions may also play a role. Since the incidence and prevalence of RA in

Pima Indians has significantly decreased over a 25-year time period, an important environmental contribution cannot be excluded [23]. A Finnish study suggested geographical variation in the incidence of RA in Finland with more than 2.5 fold higher incidence rate in north-eastern compared to north-western Finland [24]. These variances could not be explained by genetic differences or by differences in health care or diagnostics. In central Finland, RA incidence rates declined from 45/100'000 in 1980 to 27/100'000 in 1995. Worldwide the incidence of RA has declined in many countries, e.g. in the United States or Japan [19, 25]. Since the genetic background in a population hardly changes in such a short time-period, these studies support a role of environmental factors affecting RA etiology.

15.3.1.2 Sex

Like most autoimmune diseases, RA is more common in women than in men, however, the ratio changes with age. While in women the RA incidence rate peaks at around 55 years of age and then remains stable, it increases in men up to an age of about 85 years. Accordingly, the sex ratio is highest in the mid-forties with 4:1, declines in the mid-fifties to about 2:1 and levels in women and men over the age of 70 years [19, 26]. A possible explanation is the role of sex hormones on inflammation and immune response, but mechanisms are insufficiently understood. Studies explored the influence of reproductive factors on RA risk, but provided inconsistent results. Some studies identified breastfeeding as possible inducer of postpartum RA flares [27, 28], while others could not confirm these results [29], or even found a protective role of breastfeeding on RA development [30]. Similarly, reports on the influence of age at menarche, use of oral contraceptives and exogenous hormones are inconclusive [30–32].

15.3.1.3 Lifestyle

Occupational risk to develop RA was mainly described in professions where workers were exposed to silica [33]. Also exposure to mineral oils and mineral dust seems to lead to an increased risk of RA [34].

It remains unresolved whether infectious agents may influence the development of RA. One of the most prominent candidates is the Epstein Barr virus (EBV), but studies analyzing the presence of EBV in RA patients were often limited by technical shortcomings and results were conflicting [35–37]. However, RA patients consistently have higher titers of EBV antibodies compared to healthy controls [38, 39]. These may result from a generally dysregulated immune response in RA patients, since a clear link between EBV infection and RA is missing [40]. A variety of other infectious agents, e.g. parvovirus B19, *Mycoplasma pneumoniae* or *Proteus mirabilis* have also been implicated in increased risk of RA, but none provide strong and consistent evidence for a causal role in RA [41].

Studies on the role of dietary factors in RA etiology are compromised by the complexity of diet, making it difficult to single out the possible contribution of

individual foods or nutrients and by the presence of unidentified confounding factors that cannot be corrected for. Conflicting results do not permit conclusions on the role of consumption of fruits and vegetables, dairy products, coffee or alcohol [42, 43]. More consistent evidence links anti-oxidants and RA. High dietary intake of antioxidants was found to decrease the risk for RA in the Iowa Women's Health Study cohort of older women [44], and several studies found lower serum levels of antioxidants in individuals who subsequently developed RA compared to healthy individuals [45–47]. However, no association was found between intake of antioxidants from foods and supplements and the risk of RA in the large prospective Nurses' Health Study [48].

The role of smoking in RA etiology is supported by several studies; two studies have also described an elevated risk in past smokers until at least 10 years after cessation [49–52]. A gene-environment interaction was identified in smokers that carry risk alleles within the *HLA-DRB1* locus. The *HLA-DRB1* gene codes for the β-chain of the major histocompatibility complex (MHC) class II receptor on antigen-presenting cells and is highly allelic diverse. *HLA-DRB1* RA-risk alleles have a common amino acid sequence, which is important in peptide binding and is therefore known as "shared epitope". Depending on the type of risk allele, individuals carrying shared-epitope alleles are 1.5–3 times as likely to develop RA compared to non-carriers. Sub-classification of RA patients into those with anti-citrullinated peptide auto-antibodies (ACPA) and without, revealed that shared epitope alleles mainly influence the development of ACPA in RA patients [53]. With an odds ratio 5.27 compared to shared epitope negative non-smokers, smoking is a strong risk factor for the development of ACPA positive RA in individuals with the shared epitope but not in individuals without any risk alleles [54, 55].

15.3.2 Epigenetics in RA

In RA, the field of epigenetics is only at its beginnings, but already now, interesting data about how epigenetics might contribute to the pathogenesis of RA and how it could be connected to the above mentioned risk factors for RA are emerging. One reason for the failure to identify environmental risk factors for RA in humans might be the focus of studies on the time around disease onset, whereas environmental factors might act much earlier and lead to epigenetic changes that predispose for the development of RA.

15.3.2.1 DNA Methylation

One of the main tasks of epigenetic research nowadays is the mapping of genes that are susceptible to environmentally induced changes in DNA methylation in humans. This knowledge is a prerequisite for the in-depth analysis of epigenetic dysregulation in disease.

Transposable elements:
It is well known that in humans the expression of transposable elements is regulated by DNA methylation [56]. Transposable elements are mobile DNA sequences that can move to different positions in the genome either by transcription/reverse transcription (retrotransposons) or by a cut and paste mechanism (DNA transposons). These remnants of ancient infections comprise around 45% of the human genome and have a sustained effect on human cell biology and evolution by influencing gene expression and genomic organization. However, insertion and/or expression of transposable elements can interfere with normal cellular processes and lead to diseases such as transposon induced coagulopathies. In this case insertion of transposons disrupts gene function. On the other hand, demethylation of transposal elements leads to their transcription which can increase expression of neighboring genes by providing promoter functions or can disturb cell physiology by translation of transposon gene products. The product of the retrotransposon *HRES-1*, which belongs to the family of human endogenous retrovirus (HERV), for instance was shown to suppress CD4 expression on human CD4+ T cells [57]. Most important for autoimmune diseases, transposon products, in particular HERV proteins can induce an antibody response and these antibodies can cross-react with auto-antigens [58]. In patients with the autoimmune disease systemic lupus erythematodes (SLE), expression of the HERV-E clone 4–1 *gag* transcripts correlated with the presence of the anti-nuclear antibodies anti-U1 ribonucleoprotein and anti-Sm [59]. Furthermore HERV-E clone 4–1 transcripts were only found to be expressed in peripheral blood mononuclear cells (PBMCs) from SLE patients but not from healthy controls and their expression could be induced by DNA demethylation with 5-azacytidine [60]. Similar results in RA suggested that the retrotransposable element LINE-1 is expressed in synovial fibroblasts from RA patients but not from patients with osteoarthritis (OA) and its expression *in vitro* was also induced by DNA demethylation [61]. Together these data clearly point to the fact that aberrant expression of transposable elements due to promoter hypomethylation plays a role in autoimmune diseases. Why they are re-activated and how they influence cell physiology however has yet to be clarified.

Imprinted genes:
In addition to transposable elements, imprinted genes are particularly sensitive to changes in DNA methylation. A recent study suggests that loss of imprinting may play a role in the pathogenesis of RA. A subset of RA synovial fibroblasts was found to have loss of imprinting at the *Insulin-like growth factor* (*IGF-2)* gene locus [62]. High expression of IGF-2 was further found to correlate with a low-inflammatory type of RA, suggesting that in a subgroup of RA patients, loss of imprinting of the *IGF-2* gene in synovial fibroblasts leads to higher expression of IGF-2 promoting synovial fibroblast proliferation and an inflammation-independent type of synovial invasion. It is not clear however, at which stage of the disease loss of imprinting occurs and whether this phenomenon really causes a specific disease subtype or is a mere bystander effect that occurs in a subgroup of patients.

Promoter specific changes in methylation:
Expression of transposable elements and loss of imprinting in RA patients both generally point to a hypomethylated state in this disease. And indeed in RA synovial tissues in particular in synovial fibroblasts DNA is hypomethylated which could be linked to decreased expression of DNA methyltransferase (DNMT)-1, the enzyme that promotes methylation of DNA [63]. In addition to global hypomethylation, also specific loci were found to be hypomethylated in synovial fibroblasts of RA patients. Increased production of the chemokine CXCL12 by RA synovial fibroblasts could be shown to be caused by hypomethylation of the CXCL12 promoter in these cells [93]. Decrease in DNA methylation was also found in PBMCs and in T cells from patients with RA [64]. Further analysis of the 'senescent' subset of T cells, namely CD4+ CD28-, which are mainly found in elderly people and in people with chronic inflammatory diseases such as RA, revealed that demethylation leads to the specific gene expression pattern seen in these cells. Due to repetitive stress, these T cells downregulate ERK and JNK signalling pathways which was connected to loss of methylation via downregulation of DNMT1 and DNMT3a [65]. In PBMCs it could furthermore been shown that in RA patients methylation is lost at a specific site in the *IL-6* gene promoter. Lack of methylation at this site was connected to higher levels of IL-6 transcripts after stimulation [66]. Even though most studies with RA patients found global and site-specific hypomethylation, single DNA sites can also be hypermethylated in RA. Takami et al. found higher methylation rates in the promoter of the death receptor *DR3* in synovial fibroblasts of RA patients compared to OA patients. Accordingly, protein levels of the DR3 receptor were found to be lower in RA synovial fibroblasts compared to OA synovial fibroblasts, which might be the explanation for the lack of apoptosis seen in RA synovial fibroblasts after application of the physiologic ligand of DR3 [67]. Even though a direct correlation between promoter methylation status and mRNA levels of DR3 is missing, these data provide evidence that despite of global demethylation single promoter regions might be hypermethylated as also seen in tumor cells [68].

In summary these studies suggest a dysregulated methylation pattern in peripheral blood cells and local cells in the joints of RA patients. However, since biospecimen were obtained from patients with manifested RA, the temporal relation between changes in DNA methylation and RA remains unclear, and it is possible that the methylation changes result from chronic inflammation. Some epigenetic changes as seen in CD4+ CD28- cells might result from repetitive stress of immune cells during chronic inflammation and may evolve during disease and perpetuate the inflammatory process. Since the enzymes transferring methylation marks after mitosis have a much higher error rate compared to DNA polymerases, differences in methylation patterns accumulate with every mitotic cycle; this may explain why the risk of developing a variety of diseases including RA increases with age [69]. Some epigenetic changes may already be induced years before onset of disease, since peri- and postnatal nutrition and environment impacts the epigenome in animal models. Even though some epidemiological studies suggest a connection between incidence rates of RA and year of birth, the underlying events are not clear and studies on the early life origins of RA patients are scarce and some results conflicting [19, 70]. Associations between

being breastfed and risk of RA could be shown in one study, but were not confirmed by another [71, 72]. Whereas no relation between birth weight and Rheumatoid Factor positivity could be found, there seems to be a higher risk of developing RA associated with high birth weight [71, 73, 74].

Another lifestyle factor that might affect methylation is smoking; a recent study detected changes in the methylation pattern in colorectal biopsies in smokers and proposed a potential causal relation between smoking and hypomethylation [75]. In conjunction with the genetic background, smoking-induced hypomethylation may lay the groundwork for the development of various diseases including tumors and autoimmune diseases.

15.3.2.2 Histone Modifications

In addition to DNA methylation, epigenetic regulation also occurs via histone modifications. These modifications are more dynamic and varied and include acetyl, methyl, phosphate and ubiquitin residues which can be placed at different sites on the histone tails. Not only influence the different histone modifications each other, but they are also influenced by DNA methylation creating an intricate system. The combination of different modifications at specific positions at a certain stage of transcription tips the scale for transcriptional repression or activation [76]. The complexity of this system has led investigators who explore altered histone modifications in disease to mostly concentrate on specific modifications at specific sites. Such data can only provide a limited picture of the complex interactions *in vivo*. Most intensely studied are acetylation and methylation of histone 3 (H3) and histone 4 (H4). Histone acetylation is commonly associated with transcriptional activation, whereas deacetylation by histone deacetylases (HDACs) leads to transcriptional repression. In CD4+ T cells of SLE patients global H3/H4 acetylation was decreased which suggests transcriptional repression. Accordingly levels of histone acetylases (HAT) were significantly lower in these patients [77]. However, HDAC2 and HDAC7 levels were also lower in SLE patients than in controls suggesting that not the expression of single HAT and HDAC but the balance between total HAT and HDAC activity may be relevant for global histone acetylation levels. In synovial tissues of RA patients this balance was found to be shifted towards higher levels of HAT with hyperacetylated histones promoting gene expression [78]. In contrast, another study found increased activity and expression of HDAC1 in RA synovial tissues [94]. These discrepancies might be caused by differences in the treatment and the lifestyle of the study patients, since levels of TNF and nutritional factors can influence the expression of HDACs. Also in peripheral blood cells of RA patients HDAC activity was higher than in healthy controls [95]. However, global histone acetylation has not been measured in RA up to now and given the intricate interactions between different histone modifications it is questionable whether a single modification impacts gene expression.

Another hypothesis linking histone modifications to the development of autoimmune diseases suggests that changes in the histone code lead to the appearance of

neo-epitopes which, after cell death, might be recognized by lymphocytes and induce an autoimmune response [79]. In SLE and in chronic inflammatory bowel disease auto-antibodies against the mono-ubiquitinated H2A are regularly found and also auto-antibodies against poly (ADP) ribose, another histone modification, are present in the serum of these patients [80–82]. However, the appearance of these auto-antibodies may be the consequence and not the cause of an aberrantly activated and disturbed immune system.

Histone modifications are much more dynamic than DNA methylation and a variety of external stimuli likely have an impact on the histone code. Sulforaphane (SFN) is an organosulfur compound that is present at high levels in cruciferous vegetables such as cabbage, broccoli and horseradish and is a naturally occurring HDAC inhibitor. Study participants were asked to consume one cup of broccoli sprouts after 48 h of a cruciferous vegetable-free diet and HDAC activity and H3/H4 acetylation was measured in PBMCs [83].Three hours after broccoli consumption a strong downregulation of HDAC activity was detected and H3 and H4 were hyper-acetylated accordingly. This effect was still detectable 48 h after consumption of the broccoli sprouts. SFN is not the only naturally occurring HDAC inhibitor and the authors speculate that long-term intake of such modulators of the histone code may prime cells for an appropriate response to exogenous insults [84]. A combination of different dietary modulators of chromatin remodelling may interact to induce constant subtle changes in the epigenetic state, and this dynamic state may reduce susceptibility to diseases with epigenetic components such as chronic inflammation, autoimmunity or cancer.

Certain histone modifications can be regulated by the activation of the transcription factor NF-κB. NF-κB is a key transcription factor in inflammation and cell growth and its activation leads to expression of a variety of pro-inflammatory cytokines and chemokines [85]. Saccani et al. observed that not all NF-κB target genes are accessible for NF-κB binding and that H3 phosphorylation/phosphoacetylation or H4 acetylation needs to be increased before NF-κB can bind to the promoter of *IL-6*. Their data also suggest that different stimuli induce histone modifications at different gene locations, thereby shaping the resulting inflammatory response [86, 87]. H3 methylation seems to have a crucial role in basal and post-inductional repression of a subgroup of NF-κB inducible genes, tightly regulating gene expression and leaving only a small time window for activation [88]. These examples of NF-κB regulation by histone modifications underline the importance of epigenetics in the regulation of inflammation and indicate that small but lasting changes in the epigenetic code may have a sustained effect on inflammatory and immune responses.

15.3.2.3 Sex Difference

One of the main questions regarding the occurrence of autoimmunity is why women are generally more frequently affected than men. Among different explanations including hormone status in particular estrogen levels and differences in environmental exposures, hypotheses relating to the sex chromosomes have recently drawn

increasing attention. During embryogenesis one of the two X chromosomes in the cells of female mammals gets inactivated. In humans the choice between paternal or maternal X chromosome is randomly, but once made, the choice is permanent for the cells. Silencing is achieved by different epigenetic mechanisms. The future inactivated X chromosome (Xi) expresses a large non-coding RNA from the *Xist* (X inactive specific transcript) gene which binds to Xi and suppresses gene expression. Inactivation of Xi is completed by histone ubiquitination, methylation and loss of acetylation and DNA methylation [89]. The combination of these epigenetic modifications makes Xi silencing irreversible albeit not complete. Only 75% of the genes on the Xi are constantly silenced, 15% are constantly expressed and another 10% are differential expressed between individuals [90]. The fact that there are genes that are constantly expressed at a higher level in females than in males and that the expression of some of these genes also varies between females might be of importance in the development of diseases. Furthermore, Xi chromosome reactivation may play a role. Methylation of the promoter region of the *CD40LG* gene on the X chromosome was diminished in women with SLE and transcript levels of this B cell co-stimulatory molecule were accordingly higher in affected women compared to healthy women, but interestingly also compared to affected men [91]. However, direct proof that silencing *in vivo* was lost at this specific site of Xi in female SLE patients is lacking. Finally, skewed X chromosome inactivation has been implicated in disease development. In this case, inactivation of the X chromosomes is not random, but either the maternal or the paternal X chromosome is preferentially silenced. The cause of skewed inactivation is not clear yet. Although skewed X inactivation has not been observed in RA, SLE, inflammatory bowel disease or multiple sclerosis patients, a higher frequency of skewed inactivation patterns was found in peripheral blood cells of patients with scleroderma compared to healthy controls [92].

15.4 Conclusions and Perspectives

In recent years, epigenetic research has drastically increased and provided interesting and exciting insights in gene regulation and transcriptional control mechanisms. However, we are only beginning to understand how cells react to an altered environment via the intricate network of epigenetic modifications. Depending on the cell type every individual gene has a particular pattern of DNA methylation and histone modifications which change with its state of activation and must be tightly regulated. Environmental factors such as nutrition, toxins, or infections that can interfere with the epigenome may induce epigenetic changes over time that persist even in the absence of the triggering factor and shape response mechanisms.

Some epigenetic changes that are seen in chronic inflammation and autoimmunity may be induced by consistently aberrant activation of certain signalling pathways and perpetuate disease by facilitating accessibility to these pathways like a well-trodden path. As illustrated in Fig. 15.1 epigenetic changes might also be induced before disease onset by seemingly disease-unrelated factors shifting cellular pathways towards a vulnerable state that together with a susceptible genetic

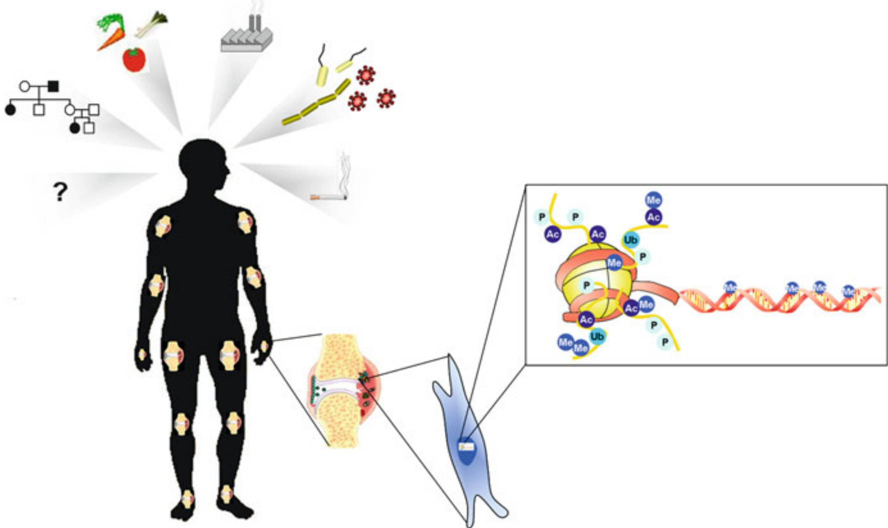

Fig. 15.1 The combination of a variety of different factors such as smoking, infections, pollution, nutrition, genetic predisposition and other factors may lead to changes in histone modifications and DNA methylation in local cells of the joints, thereby supporting the onset of chronic inflammation and autoimmunity (*P* phosphorylation, *Ac* acetylation, *Me* methylation, *Ub* ubiquitination)

background or additional exogenous factors triggers disease. One key question for future research will therefore be which epigenetic changes occur before disease onset and which occur as a result of disease. Answers to this question will not only offer novel insights in disease pathogenesis, but also provide new preventive, predictive and therapeutic opportunities.

Future epidemiologic studies will benefit from additional epigenetic insights and start to integrate epigenetic data. The combination of epidemiologic, genetic, and epigenetic data can be a powerful tool to clarify susceptibility, outcome, and therapeutic response not only in RA but in a variety of chronic inflammatory diseases.

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Chapter 16
Asthma Epigenetics: Emergence of a New Paradigm?

Rachel L. Miller

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Abstract Asthma is a disease that is influenced by environmental exposures, including those that occur prenatally. Recently, epigenetic regulation has been posited as an explanation for how environmental toxicants may induce asthma-related immune responses. However, our knowledge of the epigenetic regulation of asthma lags substantially behind our understanding of the epigenetic regulation of other complex diseases such as cancer. Fortunately new data are beginning to emerge. These include translational data from molecular experiments that implicate epigenetic regulation in T helper differentiation and/or the development of T regulatory cells, important in allergic immune responses. They also include a growing collection of cohort studies that associate epigenetic regulation with several components of the asthma clinical phenotype. So far these clinical studies are small, often

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unconfirmed, and only have started to address key issues relating to tissue specificity. Nonetheless, these studies provide a preview of what future research may reveal and raise the possibility that previously held paradigms for asthma pathogenesis may, and perhaps should, be changing.

Abbreviations

ACSL3	Acyl-CoA synthetase long-chain family member 3
COX	Cyclooxygenase
CCCEH	Columbia Center for Children's Environmental Health
CI	Confidence interval
CpG nucleotides	C–phosphate–G nucleotides
GRs	Glucocorticoid receptors
GSTM1	Glutathione S-transferase mu 1
HDAC	Histone deacetylases
HAT	Histone acetyltransferases
Ig	Immunoglobulin
iNOS	Inducible nitric oxide synthases
IGF	Insulin-like growth factor
IFN	Interferon
IL	Interleukin
LINE-1	Long interspersed nucleotide elements
MS4A2	Membrane-spanning 4-domains, subfamily A, member 2 gene
miRNAs	MicroRNAS
MyD88	Myeloid differentiation primary response gene (88)
nfkb	Nuclear factor kappa B
OR	Odds ratio
PM	Particulate matter
PAHs	Polycyclic aromatic hydrocarbons
RR	Relative risk
Runx3	Runt-related transcription factor 3
Th	T helper
TLR	Toll like receptor
Treg	T regulatory

16.1 Introduction

Our understanding of epigenetic regulation in asthma lags substantially behind our understanding of epigenetic regulation in other complex diseases such as cancer [1, 2]. But experimental work discovering new potential roles for epigenetic regulation in asthma-related immune pathways has grown exponentially in recent years, as

reviewed recently [3–5]. New epidemiological data that link molecular mechanisms or epigenetic biomarkers with asthma-related clinical outcomes are beginning to emerge.

Asthma is a disease that is influenced by environmental exposures, including those that occur prenatally [6, 7]. The longstanding presumption is that the development of asthma is plastic, i.e., not a foregone conclusion based on genetic makeup, and hence modifiable. Asthma also is a disease that exhibits a notoriously variable phenotype [8]. Epigenetic marks that occur prenatally or during other susceptible time periods may modify the clinical manifestations and variable nature of this complex disease. In this chapter, significant advances in the knowledge of epigenetic regulation in response to environmental exposures implicated in asthma will be reviewed. Emerging translational basic science that implicates epigenetic regulation associated with T helper differentiation and/or the development of T regulatory cells that may underlie clinical manifestations of asthma will be mentioned. The last several years have produced many molecular epidemiological reports that associate epigenetic regulation and several components of the clinical asthma phenotype. These studies provide a preview of what future research may reveal and raise the possibility that previously held paradigms for asthma pathogenesis may, and perhaps should, be changing.

16.2 Common Epigenetic Mechanisms

DNA methylation, histone modifications, and production of noncoding RNAs are epigenetic molecular changes that can alter gene transcription without changing the DNA coding sequence. As a result, the host response to environmental exposures, the downstream proinflammatory reaction, or even the therapeutic efficacy of pharmacological agents may be changed if epigenetic modifications ensue. Several animal studies have provided some evidence that the epigenetic state induced by environmental exposures may occur prenatally and influence the phenotype in the offspring [9, 10]. Alternatively, epigenetic modifications may occur postnatally, leading to sustained effects on gene transcription. Another feature of epigenetic regulation is that it may be modifiable [11–13]. There are specific time periods when asthma may be more susceptible to the effects of exposure to environmental toxicants (i.e., gestation, early childhood, adolescence). It has been postulated, but not yet demonstrated, that epigenetic modifications may be contributing to the greater susceptibility that seems to occur during these time periods [3] (Fig. 16.1).

DNA methylation refers to the process whereby methyl groups are added to the fifth carbon of the nucleotide cytosine. This process can suppress gene transcription by either inhibiting transcription factor binding to the recognition sites on CpG nucleotides or by aiding transcription inhibiting protein binding [14]. Importantly, DNA methylation may not be stable and exhibit variation over time [11, 13, 15]. In comparison, post-translational modifications of histones, key elements in the chromatin packaging of DNA, occur by means of acetylation, methylation, and

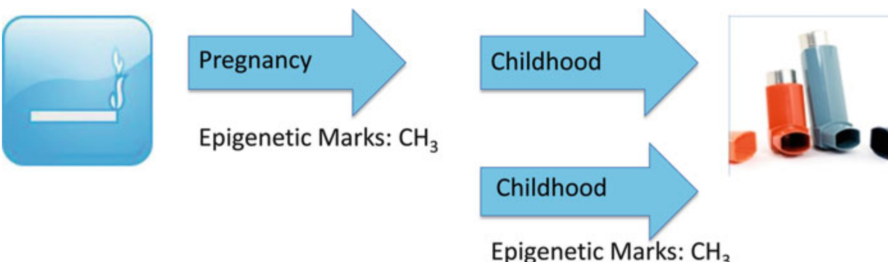

Fig. 16.1 Intrauterine exposures to cigarette smoke, air pollution, diet, and other environmental stressors may induce epigenetic modifications that may affect the risk for developing asthma. Alternately epigenetic changes may occur postnatally and lead to sustained effects on gene expression that as well may affect the risk for asthma development or be associated with its severity

phosphorylation. During acetylation by histone acetyltransferases (HAT), the DNA tightly packaged around the histone core unwinds, activators of transcription obtain access to DNA, and gene expression can then proceed [5]. Histone deacetylases (HDAC), of which there are at least 11 isoenzymes, reverse acetylation and turn off transcription [16]. In general, the extent of histone acetylation is a result of the amount of HAT compared to HDAC activity. The third prominent epigenetic mechanism involves the activity of microRNAs (miRNAs) that function as endogenous inhibitors of translation and thereby regulate protein production. They consist of single-stranded RNA molecules 21–23 nucleotides in length that induce the degradation of target mRNA. The activity of microRNAs' role in asthma or any complex disease is perhaps one of the least understood epigenetic mechanisms. But one exciting study by Mattes and colleagues found that inhaled dust mite allergens increased the expression of specific miRNAs in the airways through TLR4- or MyD88-dependent mechanisms in mouse models. The associated allergic phenotype was inhibited by selective blockade of microRNA (miR)-126 [17].

16.3 Environmental Exposures Implicated in Both Asthma and Epigenetic Regulation

One environmental exposure implicated in both asthma and epigenetic regulation is diet. In the former case, a number of cohort studies have implicated various dietary modifications with greater or reduced risk for asthma, such as supplementation with vitamin D, E, soy, and Mediterranean diet [18–20]. However, the dietary supplement most implicated for inducing epigenetic changes relevant to asthma is folic acid, a source of methyl donors. Hollingsworth and colleagues suggested such an association using elegant mouse models. In their experiments, folic acid supplementation administered during gestation and weaning was associated with greater airway hyperactivity and eosinophilic inflammation, as well as chemokines and immunoglobulin

(Ig) E production in the offspring. They argued that this association occurred due to folic acid's ability to hypermethylate DNA and hence suppress the expression of several genes including *Runx3*, a gene involved in CD4 silencing during T cell lineage decisions [21]. In human cohort work, Whitrow and colleagues assessed the use of supplemental folic acid in women during pregnancy, determined retrospectively by food frequency questionnaire, and found associations with a greater risk of reported physician-diagnosed asthma in children [22]. The strongest association occurred when women took supplemental folic acid later in their pregnancy (30–34 weeks; relative risk (RR) 1.26, confidence interval (CI) 1.08, 1.43), suggesting a possible time window during pregnancy when supplemental folic acid ingestion may induce more extensive epigenetic alterations associated with pediatric asthma. The association between prenatal folic acid supplementation and asthma in children was examined as well in the Norwegian Mother and Child Cohort Study. In Norway, it is recommended the women take supplemental 400 mg of folic acid daily around the time of conception and during the first 3 months of pregnancy. Because the food is not fortified, the authors argued that their assessment of folic acid intake by questionnaire may be relatively accurate. In this cohort, folic acid supplementation only in the first trimester was associated with a slightly greater relative risk of lower respiratory infections (LRTI) (adjusted RR 1.10, 95% CI 1.01–1.20) and hospitalizations for LRTIs (RR 1.28, CI 1.07–1.53) in the child through 18 months of age. Folic acid supplementation anytime during pregnancy was associated with more wheeze at ages 6–18 months (RR 1.07, CI 1.02–1.12). In other models, they adjusted the effects of exposure in the first trimester to exposure both later in pregnancy and in infancy, and the associations with exposure in the first trimester on LRTIs remained significant. In the follow-up nested study within the same cohort, the group reported on maternal folate levels obtained on nonfasting specimens collected during the second trimester of pregnancy (median 18 weeks) and reported that levels were higher among women who reported supplemental folic acid use. An increased risk of asthma at age 3 years for children with maternal plasma folate levels in pregnancy in the highest compared with the lowest quintile, and a trend of increasing risk across quintiles of folate levels also was reported. The authors acknowledged that exposure to folic acid supplements in pregnancy was associated as well with several other characteristics presumed to lower the risk of LTRI, including higher maternal educational level, longer duration of breast feeding, and less smoking, so residual confounding (that for these covariates could lead to a negative bias) could not be ruled out. They also acknowledged that nonfasting plasma samples may be influenced heavily by the most recent intake of folic acid and could have added preanalytical variation [23]. In contrast, Matsui and Matsui determined that folic acid supplementation, indicated by serum levels measured by the 2006–2006 National Health and Nutrition Examination Survey, was associated with a reduced risk for seroatopy and wheeze in individuals age 2 years and older [24]. It is unclear whether the apparent discrepancies among the three cohort studies may be related to exposure misclassification (i.e., from retrospective questionnaires vs measured sera folate levels) or timing of exposure (i.e., prenatal vs postnatal, adult), but some links between folic acid intake and asthma are suggested.

Furthermore, another group determined that periconceptional folic acid administration (400 μg/day) was associated with hypermethylation of the insulin-like growth factor *(IGF)2* gene. In this cohort study, 17-month-old children of mothers who used folic acid had a 4.5% higher relative methylation level of the differentially methylated region of *IGF2* compared to children who were not exposed to folic acid ($p=0.014$). Higher levels of methylation were observed for individual CpG dinucleotides comprising the area, but they were not always statistically significant. *IGF2* methylation of the child also was associated with the maternal (but not child) S-adenosylmethionine blood levels (+1.7% methylation per SD S-adenosylmethionine; $p=0.037$) [25]. Higher methylation levels were associated with decreased birth weight. However, the authors acknowledged that even the statistically significant differences were very small, suggesting that other genes or mechanisms may be involved as well. They also proposed that the extraction of DNA from whole blood composed of multiple cell types may have diluted some of the biological outcomes measured. Still, the paper remains one of the first studies to link periconceptional folic acid supplementation with DNA methylation and a clinically relevant phenotype in the child.

Another environmental exposure long considered a risk factor for the development of asthma in children is maternal smoking [7, 26–28]. One intriguing study by Li and colleagues suggested the mechanism of action on childhood asthma could be transmitted across generations. As part of a case-control study nested within the Children's Health Study, detailed maternal and household smoking histories and histories of other asthma risk factors were obtained by telephone interview. The authors found that a reported history of prenatal smoking was associated with increased risk for asthma diagnosed in the first 5 years of life (odds ratio [OR] 1.5; CI 1.0–2.3), and for persistent asthma (OR 1.5; 95% CI 1.0–2.3). The associations did not differ in children with early transient asthma compared to those with early persistent asthma. Interestingly, grandmaternal smoking during pregnancy was associated with a greater risk of asthma in grandchildren (OR 2.1; 95% CI 1.4–3.2) that remained borderline significant even if there was no report of maternal smoking during pregnancy (OR 1.8; 95% CI 1.0–3.3) [29]. The study was retrospective and did not test epigenetic regulation specifically. Yet this first report of a grandparental effect on asthma may lead to speculation about a contribution of epigenetic regulation to inheritance that would need to be tested rigorously.

16.4 Environmental Exposures Implicated in Asthma Are Susceptible to Epigenetic Regulation

Several environmental exposures implicated in asthma have been shown to induce epigenetic alterations, but perhaps the exposures best characterized in ex vivo or cohort studies are air pollutants. For example, Cao and colleagues documented that diesel exhaust particles induced chromatin modifications in an assay involving exposure of human bronchial epithelial cell lines. Increased histone H4 acetylation

and posttranslation degradation of histone deacetylase (HDAC1) was found. These events in turn were associated with activation of the cyclooxygenase *(COX)-2* promoter [30]. Baccarelli and colleagues recently compared exposure to traffic-related air pollution at multiple time points with repeated analyses for global methylation among a cohort of elderly males as part of the longitudinal Normative Aging Study. Ambient particulate matter $(PM)_{2.5}$, black carbon, and sulfate were measured by stationary site monitoring, and average pollutant concentrations 4 h to 7 days were calculated (moving averages) prior to phlebotomy. DNA methylation in long interspersed nucleotide elements (LINE-1), an indicator of global methylation, decreased in relation to higher black carbon and $PM_{2.5}$ levels, especially during the longer time windows (2–7 days) [13]. Tarantini and colleagues examined two indicators of DNA methylation, Alu and LINE-1, and one measure of asthma candidate gene-specific *(iNOS)* DNA methylation, after short- (after 2 consecutive days off from work in a steel production plant) and long-term (after 3 consecutive days of work) exposure to PM_{10} exposure among electric furnace steel plant workers ($n = 63$). They reported that ambient PM_{10} levels at work sites may be associated with the extent of DNA demethylation of the *iNOS* promoter. Also, PM_{10} levels correlated with global DNA demethylation as estimated in Alu repeated elements and LINE-1 [15].

The relative expression of several candidate miRNAs implicated in oxidative stress and inflammation (miR-222, miR-21, and miR-146a) also were measured in the blood prior to and following 3 days of work among the electric furnace steel plant workers. Levels were compared to individual exposures to fine and course PM and PM metal components (chromium, lead, cadmium, arsenic, nickel, manganese). Among the post-exposure samples, miR-222 expression was correlated positively with lead exposure ($\text{beta} = 0.41$, $p = 0.02$), but miR-21 expression was not associated with individual PM or metals. Some negative correlations between miRNA levels and measures of metals exposure were found [31]. At minimum, the study suggests that changes in miRNA expression may occur, albeit inconsistently, in association with exposure to PM and its metal components, pollutants associated with asthma and other respiratory diseases [32]. Our group at the Columbia Center for Children's Environmental Health (CCCEH) showed that higher levels of polycyclic aromatic hydrocarbons (PAHs), measured prenatally using personal monitors worn by pregnant women, was associated with greater DNA methylation of Acyl-CoA synthetase long-chain family member 3 (*ACSL3*) and other genes (see below) [33]. Finally, Breton and colleagues reported that a history of prenatal tobacco smoke exposure was associated with lower levels of DNA methylation for short interspersed nucleotide element (AluYb8; but not LINE-1) from buccal cells collected from children. Ilumina GoldenGate Bead Array DNA methylation assay of 1,031 gene-specific loci of similar samples yielded 9 candidate genes whose expression differed by prenatal tobacco smoke exposure. Furthermore, when children's data were stratified by the absence or presence of the common GSTM1 null genotype, prenatal tobacco smoke exposure was associated with lower LINE1 methylation in the GSTM1 null children but higher methylation in the GSTM1-present children. This work suggests a novel interaction between genotype and environmental exposure on DNA methylation [34].

16.5 Epigenetic Regulation and Clinical Allergic Disease

Reviewing the epigenetic epidemiology of asthma is plagued by the lack of cohort studies. But significant advances in basic molecular work that provide the biological support for several translational and clinical studies have been made in this area. These include mounting evidence suggesting that immune programming associated with the development of proallergic T helper (Th) 2 cytokine responses (e.g., interleukin (IL)-4) or the counterregulatory Th1 cytokine responses (e.g., interferon (IFN)γ) may be susceptible to epigenetic regulation [35–39]. The most progress to date has been made in determining how DNA methylation of CpG sites within the counterregulatory IFN-γ promoter may confer protection from proallergic immune activation [39]. The IFN γ promoter also has been shown to be susceptible to chromatin remodeling [40]. Susceptibility to proallergic Th2 immune activation in association with demethylation occurs at the IL-4 locus [36, 41] and at the proximal IL-13 promoter [42]. Moreover, epigenetic mechanisms controlling T regulatory (Treg) development are just beginning to be explored. Early results suggest that Treg suppressive function important to allergic sensitization may be dependent on demethylation of *Foxp3* [43, 44].

More clinical studies of epigenetic regulation in clinical asthma now test the etiology of the observed "maternal effect" of atopy. This paradigm refers to the greater predominance of asthma or allergy if the mother, as opposed to the father, is afflicted with the disease. Indeed this pattern has been observed for both asthma and production of proallergic IgE antibodies [5, 8]. To date, one cohort study by Ferreira and colleagues tried to associate such patterns with altered DNA methylation of either AluSp repeat or membrane-spanning 4-domains, subfamily A, member 2 gene (MS4A2) (β-chain of the IgE high-affinity receptor), a key gene in the allergic cascade. Their small study was essentially negative, failing to find differences in AuSp DNA methylation between cases and controls or according to atopic status of the mother or father [45].

As mentioned earlier, our group at CCCEH conducted one of the first proof of concept papers that tried to discover candidate asthma genes that may be susceptible to altered DNA methylation following air pollution exposure. In this case, the approach was to study cord blood DNA derived from high versus low prenatal PAH exposure groups. Genomic DNA was digested with methylation sensitive restriction enzymes, amplified, and aberrantly methylated bands on a gel eluted, reamplified, cloned, and sequenced. Using BLAST search or silicon database analysis of sequenced DNA that focused on promoter and CpG island searches, candidate clones were selected to undergo more intensive evaluations. These validation experiments included bisulfite conversion of DNA and methylation-specific PCR that revealed the 59-CpG island methylation status of *ACSL3* as associated with prenatal PAH exposure. In addition, methylation of *ACSL3* was associated significantly with a parental report of asthma symptoms in children prior to age 5 years [33]. This study is the first to associate a measured prenatal environmental exposure with

altered gene-specific DNA methylation in cord blood and then associate such alterations with the later development of disease. Subsequently, Nadeau and colleagues using a cross-sectional design of age and sex-matched participants, recruited children in Fresno and Stanford California. They compared regional levels of ambient air pollution exposure with peripheral blood DNA methylation of Forkhead box transcription factor 3 (Foxp3), a key transcription factor in Treg suppressive activity and asthma symptom scores. They found that asthmatics living in Fresno where levels of polycyclic aromatic hydrocarbons (PAH), fine particulate matter, and ozone are higher than in Stanford had worse asthma symptoms and higher levels of methylation in the CpG islands in the *Foxp3* promoter and one intronic region compared to nonasthmatics from Fresno and participants from Stanford. An association between the subject-specific estimated annual average PAH exposure in Fresno and the number of methylated CpG islands among Fresno asthmatics was detected as well [46]. While interesting, it should be noted that the study did not adjust the analyses levels for seasonal variation in PAH measures nor control for levels of other pollutants [47].

Other groups have reported provocative experimental results from small human studies, particularly in genes associated with Th polarization associated with allergic airway disease. As an example, ex vivo *IFN-γ* promoter methylation was reduced in CD8+ T cells, but not CD4+ T cells from atopic, but not healthy, children [48]. Kwon and colleagues measured the extent of DNA methylation of *IFNγ* and *IL-4* gene promoters following ex vivo stimulation of peripheral blood mononuclear cells with *Dermatophagoides pteronissinus/Dermatophagoides farinae* dust mite antigens. Among asthmatics, methylation at the *IL-4* promoter (CpG −80) increased following antigen stimulation and was strongly correlated with *IL-4* production. In contrast, demethylation at the *IFNγ* promoter increased following ex vivo stimulation with phytohemagglutinin [49].

Su and colleagues reported on the importance of endogenous HDAC activity in regulating Th1 versus Th2 differentiation. Their approach was to block endogenous HDAC activity with trichostatin A following ex vivo polyclonal stimulation of peripheral blood mononuclear cells derived from children. Not only did trichostatin markedly reduce endogenous HDAC activity, but this result was associated with greater Th2 polarization and increased expression of GATA-3, suggesting that endogenous HDAC activity is necessary for preserving Th1 versus Th2 balance. Finally, the same group compared levels of HAT activity and endogenous HDAC in atopic nonasthmatic ($n=27$) versus atopic asthmatic ($n=18$) children with the extent of bronchial hyperresponsiveness, an indicator of asthma severity. They found significant elevations of HAT activity and reductions in the level of HDAC activity (determined in nuclear peripheral blood mononuclear cell lysates) among asthmatic children compared to atopic nonasthmatic controls. Moreover, these changes were associated progressively with the degree of bronchial hyperreactivity becoming the first study to compare the extent of epigenetic alteration with clinically relevant physiological outcomes [50].

16.6 Pharmacoepigenetics

Given that epigenetic alterations may be reversible, at least in theory, it stands to reason that there may be substantial potential to develop or take advantage of epigenetically-based therapies in asthma. DNA methyltransferases and histone deacetylases have shown promising anti-tumorigenic effects for some malignancies (reviewed in [51, 52]). Inhibitors of enzymes controlling epigenetic modifications associated with inflammation or other immune processes that result in airway disease, specifically DNA methyltransferases, histone deacetylases, and inhibitory RNAs, have therapeutic potential.

One way to consider the therapeutic potential of epigenetic regulation is to consider mechanisms that underlie the efficacy of glucocorticoids. Steroids exert some of their anti-inflammatory properties by inducing the acetylation of anti-inflammatory genes important for the production of cytokines, chemokines, or adhesion molecules. Specifically, glucocorticoid receptors (GRs) bind to their DNA binding site following acetylation [53]. Under other circumstances, such as during suppression of Nfkβ activation, steroids may recruit histone deacetylase-2 (HDAC-2) to activated inflammatory gene complexes [53]. Indeed, alveolar macrophages have been shown to express elevated levels of HAT activity and reduced levels of HDAC activity [54, 55]. One could speculate that therapeutics that modify the relative HAT, HDAC levels or target miRNA expression levels in the airways or their alveolar macrophages may hold promise as novel anti-inflammatory treatments for allergic asthma [17].

16.7 Conclusion

Recent advances have been made in the last few years, particularly in the molecular epidemiological work that associates epigenetic regulation with asthma phenotypes. However, there are several areas where this field has barely begun to tackle important questions. These include translational studies that examine the differential effects of epigenetic regulation according to specific cell types. For example, HDAC activity sampled from human alveolar macrophages was lower than levels sampled from those measured simultaneously in peripheral blood mononuclear cells [54]. Promoter methylation of leukotriene B4 receptor and its associated gene expression also has been shown to vary according to cell type [56]. In addition, the cohort studies conducted to date have been small and possess limited statistical power to adjust for covariates or to measure small effect sizes associated with epigenetic changes. Large prospective cohort studies are needed to examine all of these issues and how they may apply to the complex disease of asthma. Despite these limitations, there is exciting promise that the "bench" will communicate with the "bedside" and direct appropriate translational studies in the molecular epidemiology of asthma epigenetics.

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Chapter 17
Epigenetic Epidemiology of Autism and Other Neurodevelopmental Disorders

Rima Woods and Janine M. LaSalle

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Abstract Mammalian brain development continues postnatally, as neuronal circuits and pathways undergo maturational development in response to experiences in early life. Therefore, epigenetic mechanisms that act at the interface of genetic and environmental information are increasingly recognized for their essential roles in postnatal brain development, and they are thought to play an important role in learning and memory. Here we review some of the emerging examples of human genetic neurodevelopmental disorders with known epigenetic etiologies and use these examples to speculate on the potential epigenetic etiology of the more common

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forms of idiopathic autisms. Understanding the epigenetic and biochemical pathways disrupted by genetic disorders can help direct the field of epigenetic epidemiology in its search for evidence of environmentally altered pathways, including investigations of how environmental factors such as nutrition and toxin exposures may contribute to epigenetic information essential for the higher cognitive functions associated with language and social behavior.

17.1 The Importance of Epigenetic Modifications in the Brain

The epigenomic landscape of the nervous system is dynamic and complex, and equally complex regulatory mechanisms direct the response of the neuronal system to environmental signals. Epigenetic mechanisms, including DNA methylation and histone modifications, are important for proper neuronal function through their regulation of chromatin structure, imprinting, and X-inactivation. The epigenome allows for varying degrees of regulation of the inherited genome, depending on the stimulus presented and the reaction required.

DNA methylation of CpG dinucleotides is orchestrated by DNA methyltransferases which "write" the methylome and methyl binding proteins which "read" the methylome. Despite being mitotically inactive, neurons have high levels of both *de novo* and maintenance DNA methyltransferases [1–4]. This suggests that neurons have a dynamic methylome that is capable of changing in response to stimuli. Both genetic and chemical methods of inhibiting DNA methylation significantly reduces learning and memory in rodents [5, 6], and hypo- and hypermethylation have been linked to various neurological disorders [7]. DNA methylation also plays a key role in imprinting and X-inactivation. Imprinted genes are silenced via DNA methylation based on the parent of origin in an allele specific manner. X-inactivation uses DNA methylation and chromatin modifications to randomly silence one of the two X chromosomes in female cells. Both imprinting and X-inactivation act to maintain proper gene dosage, and errors in these mechanisms can lead to neurological disorders. Maintaining the dynamics of DNA methylation is therefore crucial to proper postnatal neurological function.

The core histone proteins that package DNA into chromatin contain a number of post-translational modifications, which are collectively called the "histone code." The addition and removal of modifying marks on histone tails serves to alter the chromatin landscape and can globally and locally affect the expression of genes. For example, acetylation of lysine 9 on histone H3, a modification associated with gene activation, has been shown to change over time as neurons mature [8]. Histone deacetylases (HDACs) are enzymes that remove acetyl groups from histones and generally serve to inhibit transcription. The clinical use of HDAC inhibitors such as valproic acid (VPA) to treat neurological symptoms in schizophrenia, epilepsy, and mood disorders [9] highlights the importance of histone modifications in the brain.

Epigenetics is important for neuronal development and plasticity, which is achieved through regulation of the chromatin landscape. The level of chromatin

compaction greatly affects accessibility of DNA to various DNA-binding proteins, transcription factors, and cellular machinery. In normal cells, this serves to tightly regulate gene transcription and potentially serve as a molecular basis for memory. In cells with abnormal epigenetic marks, the effects on neurodevelopment can be profound. For instance, the combined contribution of both maternal and paternal epigentically marked genomes is required for normal neurodevelopment [10]. More subtle dysregulation of epigenetic mechanisms have been implicated in a variety of human neurodevelopmental disorders, which are discussed in greater detail in this review.

17.2 Neurodevelopmental Disorders with Known Genetic Causes That Affect Epigenetic Pathways

17.2.1 Disorders Associated with Chromosome 15q11-13 Deletion and Duplication

Among the first characterized "epigenetic" human diseases are two neurodevelopmental disorders caused by deletions of 15q11-13, Prader-Willi and Angelman syndromes (PWS and AS, respectively). Genetic rearrangements of human chromosomal locus 15q11-13 are common in a wide range of neurodevelopmental disorders, as this locus is a hotspot for low copy repeats in humans predisposing to deletions and duplications, as well as parentally imprinted genes [11–13]. The methylation status of a small imprinting control region (ICR), and its presence or absence, is central to the imprinting mechanisms in both humans and mouse models. Human PWS infants are recognized by hypotonia and failure to thrive in infancy, small gonads, small hands and feet, obsessive compulsive and hoarding behaviors, and an insatiable appetite leading to obesity. AS individuals are also hypotonic at birth, but develop distinct features from PWS, including a happy disposition, ataxic gait, absence of speech, and severe seizures.

Prader-Willi syndrome occurs as a result of loss of an unmethylated ICR from the paternal chromosome. Loss of the ICR is caused most frequently by paternal deletion of 15q11-13 (70%) or maternal uniparental disomy (UPD, 25–30%), in which both chromosome 15 s are of maternal origin. The remaining 3–5% of PWS cases are caused by small paternal deletions of the imprinting control region (PWS-IC). Angelman syndrome is caused by the reciprocal chromosomal imbalances as PWS, which are maternal 15q11-13 deletion (70%) or paternal uniparental disomy (<5%). The remaining cases of AS are caused by maternal mutation of the gene *UBE3A*, encoding a ubiquitin ligase E3, or imprinting mutations, which lack the maternal methylation imprint at the PWS-IC, resulting in reduced *UBE3A* expression. Mouse models of AS have implicated a role for *Ube3a* in experience dependent plasticity of the cortex and the maturation of synapses by ubiquitination of Arc [14–16].

Uniparental disomy in PWS and AS is predicted to derive from rare rescue events of trisomy for chromosome 15, and is thus influenced by increasing parental age, particularly maternal [17, 18]. Parental imprinting of the PWS/AS locus is established in the gametes and regulated through a bipartite imprinting control region (ICR), defined from rare small noncoding deletions in PWS and AS patients specifically affecting parental DNA methylation patterns [19, 20]. The maternally inherited AS-ICR is required during oocyte maturation in order to methylate the maternal PWS-ICR [21]. The maternal specific methylation of the PWS-ICR is diagnostic for the majority of PWS and AS patients and is stably maintained in clonal cell populations [22]. Maternal methylation at the PWS-ICR also serves to silence the maternal allele of an exceptionally long and complex transcriptional unit, starting at the PWS-ICR and containing *SNRPN* (encoding a splicing factor), two small nucleolar RNAs (snoRNAs) clusters, a spliced long noncoding RNA, and the antisense to the maternally expressed gene *UBE3A* [23]. The paternal allele *SNRPN* through *UBE3A* locus undergoes extensive chromatin decondensation in postnatal neurons that requires PWS-ICR transcription [24], suggesting that the local highly active chromatin environment of the paternal locus may be a critical feature lacking in PWS.

In addition to the 15q11-13 deletion syndromes, duplications of 15q11-13 are found in approximately 1–3% of children with autism-spectrum disorders, although the actual prevalence of 15q11-13 duplications in the general population has not been established. The phenotype of 15q duplication syndrome is heterogeneous, but it is becoming a clinically recognizable neurodevelopmental disorder characterized by hypotonia in infancy, followed by language and motor delays and frequently, severe seizures. Anxiety, hyperactivity, emotional lability, and sleep problems are also characteristic of 15q duplication syndrome in addition to the social behavioral deficits that enable a diagnosis of autism prior to genetic karyotype analysis (reviewed in [25]).

The chromosomal rearrangements leading to chromosome 15q duplication are due to meiosis U-type rearrangements between low copy repeat clusters in humans, and thus are more frequent in maternal meiosis and impacted by maternal age [18, 26]. 15q11-13 duplications leading to autism are almost exclusively maternal in origin, emphasizing the importance of parental imprinting in all of the 15q [27]. Paternal origin of 15q duplications have been described, but the phenotypes vary widely, including social and language delay, severe cognitive impairment, PWS-like behaviors, and an apparently normal phenotype [27–29]. Preliminary analyses of postmortem brain of 15q duplication samples suggest that transcript levels do not correlate to copy number and parental origin, suggesting that epigenetic dysregulation of the region has occurred [13].

Maturing postnatal neurons appear particularly sensitive to genetic and epigenetic alterations to both coding and noncoding regions of 15q11-13. The absence or duplication of a methylated or unmethylated ICR in 15q11-13 determines the phenotypic outcome, providing an example of loss of epigenetic control of a chromosomal region. Because of the occurrence of 15q11-13 rearrangements in autism spectrum disorders (~1%), further understanding of the epigenetic complexities as well as the functions of the critical imprinted genes is essential to future diagnosis and treatment of autism.

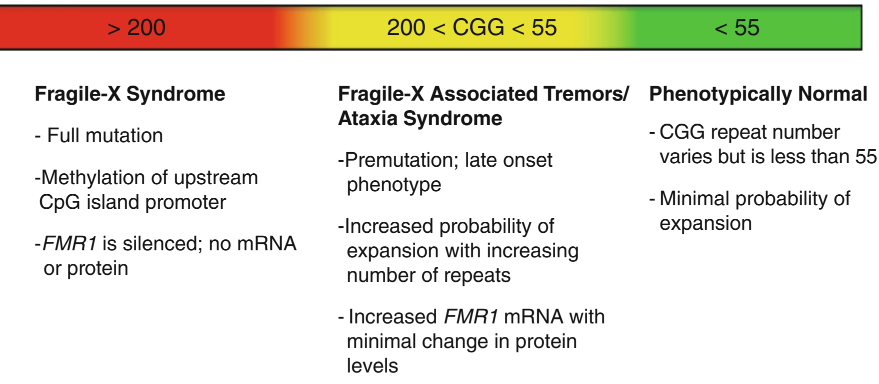

Fig. 17.1 Genotype-phenotype correlation of FMR1 CGG repeat number. The 5′ *FMR1* CGG polymorphic repeat is polymorphic in humans. Individuals with <55 repeats (*green*) are in the phenotypically normal range, those with intermediate expansions (*yellow*) carry a permutation and can develop Fragile-X Associated Tremors/Ataxia syndrome, while those with >200 repeats (*red*) generally silence *FMR1* and cause Fragile X syndrome

17.2.2 Fragile-X Disorders

The epigenetic mechanisms responsible for Fragile-X Syndrome (FXS) were discovered while the field of epigenetics was in its infancy. Fragile X-Associated Tremor Ataxia Syndrome (FXTAS), a closely related disorder involving the same genomic region but with a very different etiology, was discovered a decade later. The relation between FXS and FXTAS clearly shows the variable effects of epigenetic dysregulation in relation to neurological disorders. Aberrant epigenetic silencing through DNA methylation of a promoter causes FXS, whereas FXTAS results from uncontrolled gene expression. Both diseases stem from a genetic expansion of CGG repeats in the same X-linked gene, but their pathogenesis differs in the dysregulation of epigenetics that results from the expansion (Fig. 17.1).

Fragile X Syndrome is caused by aberrant silencing of *Fragile X Mental Retardation 1* (*FMR1*) and the loss of the *FMR1* product, Fragile X Mental Retardation Protein (FMRP). FMRP is an RNA binding protein and is involved in mRNA trafficking during synaptic activity [30, 31] and is important for neurodevelopment and neuronal function. Individuals with FXS often present with intellectual disabilities, autism spectrum disorder, ADHD, anxiety, and seizures [32, 33]. FXS occurs in approximately 1/2500 births [34], and is more often observed in males. *FMR1* is located on Xq27 with a CpG island at the promoter region that is unmethylated in normal individuals [35]. The 5′ UTR of *FMR1* contains a tandem array of up to 55 CGG repeats. The number of CGG repeats is variable in normal individuals, with the most common allele containing 29 repeats [36]. In FXS, the CGG repeat region is greatly expanded to over 200 copies (termed ‘full mutation’) during oogenesis, due to meiotic instability. This leads to methylation of the upstream CpG island, and silencing of *FMR1* [36–39]. Increased levels of repressive histone marks,

including H3K9me3 and H4K20me3, within the repeat region [40] may add an additional layer of silencing by altering the chromatin structure around *FMR1*, further silencing the expanded allele. Individual phenotypic severity varies greatly, as mosaicism and variation between individuals in repeat length and CpG island methylation has been observed. Repeat length mosaicism is due to mitotic instability at the repeat region, and differences in methylation are likely due to a combination of variables including environmental and stochastic factors [33, 37].

FXTAS, a late onset neurodegenerative disorder, is also due to expansions in the CGG repeat region in the 5′ UTR of *FMR1*. FXTAS is caused by a smaller expansion of between 55 and 200 CGGs (termed 'premutation') and is characterized by progressive cognitive decline and memory loss, action tremors, parkinsonism, and generalized brain atrophy [41]. Like FXS, the repeat expansion occurs in the female germline as a result of meiotic instability at the CGG repeat region. Often, a grandparent (who will go on to develop FXTAS later in life) will pass on a premutation to their daughter where it expands during oogenesis to a full mutation, resulting in a grandchild with FXS [39, 42]. Larger premutations result in an increased likelihood of expansion to a full mutation, with 100% likelihood of expansion to full mutation with a premutation of more than 90 CGG repeats [43]. Since FXTAS is an X-linked disorder, females can be less severely affected due to mosaicism or favorable skewing of X-inactivation [44]. It is estimated that 1/3000 males over the age of 50 will develop FXTAS [34].

A hallmark of FXTAS is elevated levels of *FMR1* mRNA in patients carrying premutations but near normal levels of FMRP. The increase in expression correlates with increased number of repeats, with a five-fold increase in expression in patients carrying more than 100 repeats [45]. An RNA toxicity model has emerged for FXTAS, where an increased number of CGG repeats leads to increased *FMR1* expression, possibly due to decreased translational efficiency, which in turn leads to the formation of *FMR1*-containing nuclear inclusions in brain and spinal cord tissues, and the neurological problems associated with FXTAS [46, 47]. A study of 93 male carriers of premutations found a significant correlation between the number of CGG repeats and the age of symptom onset [48]. A CGG knock-in mouse model carrying a human CGG repeat containing 98 CGGs showed inclusion size in neurons increased with age [49], suggesting the late onset of symptoms is due to the gradual accumulation of nuclear inclusions.

The Fragile-X related disorders provide a unique example of epigenetically regulated transgenerationally inherited neurological disorders. While the etiology is based on a genetic mutation (CGG repeat expansion), epigenetic changes to the X-linked gene *FMR1* are responsible for FXS. Aberrant silencing of *FMR1* via promoter methylation causes FXS. FXTAS results from abnormal increased expression of *FMR1* and serves as an intermediary between a normal phonotype and FXS. A premutation resulting in FXTAS often leads to a full mutation and FXS in as few as two generations. Although the mechanism responsible for the CGG repeat expansion is unknown, the vastly different effects of dysregulated epigenetic processes are evident in the two diseases. The Fragile-X related disorders highlight the importance of epigenetic control of gene dosage for proper neurological function.

17.2.3 ICF Syndrome

Immunodeficiency, centromeric region instability, and facial anomalies syndrome (ICF) is a rare autosomal recessive disorder linked to loss of DNA methylation. This decrease in global methylation is thought to lead to loss of heterochromatin and genomic instability at centromeric and telomeric regions. Fewer than 50 patients have been described worldwide. This rare syndrome is divided into two types. Approximately half of all identified ICF cases have mutations in *DNMT3B*, a *de novo* methyltransferase, which are responsible for ICF syndrome Type 1 and are thought to cause the global hypomethylation observed [50]. ICF syndrome Type 2 also shows decreased global methylation but lack DNMT3B mutations. Mutations in a gene coding for the zinc finger protein, *ZBTB24* are associated with ICF syndrome Type 2 [148]. Both types are characterized by facial dysmorphisms, including hypertelorism, flat nasal bridge, and low set ears, immune system deficiencies including decreased immunoglobulin in the presence of normal B and T cell counts, and cytogenetic abnormalities occurring at the centromeric regions of chromosomes 1, 9, and 16. Additionally, delayed development has been observed in 68% of cases, and mild to moderate intellectual developmental disability (IDD) has been observed in 61% of cases. The median age at death is 8 years old, with death usually occurring due to severe and/or recurrent infections [50, 51].

Both ICF Type 1 and 2 patients exhibit hypomethylation of pericentromeric satellite 2 and 3 repeats, telomeric, and subtelomeric regions resulting in decondensation of pericentromeric heterochromatin and telomeric shortening. ICF Type 2 also displays hypomethylation of alpha satellite DNA, indicating additional unknown factors are involved in ICF syndrome that affect methylation pathways. The hypomethylation characteristic of ICF syndrome leads to only a 7% reduction in global methylation, indicating that relatively small changes to global methylation levels can have a large impact on neurodevelopment.

Decondensation of pericentromeric heterochromatin has been observed in both metaphase and interphase chromosomes in ICF cells [52–54], and may be due to hypomethylation. These large-scale changes to chromatin structure likely affect transcription of a wide variety of genes as it is becoming increasingly clear that the spatial arrangement of the chromosomes within the nucleus plays a role in transcriptional regulation. Microarray expression analyses comparing ICF and control lymphoblast cells found both up and down regulation of various genes involved in immune function, development, neurogenesis, and apoptosis [51, 55]. A subset of these genes were examined for altered methylation at regulatory regions, in an attempt to explain the expression changes but no direct correlation was found. It therefore remains unclear if altered methylation patterns at regulatory regions of genes with altered expression profiles play a causative role in ICF syndrome and if the hypomethylation is responsible for the transcriptional changes. It is interesting to note that decreased H3K27 trimethylation, a repressive histone mark, was detected at the promoters of genes shown to be upregulated in ICF syndrome [55]. It seems likely that a combination of low-level changes in methylation along with altered histone marks at the regulatory regions of dysregulated genes contributes to the etiology of ICF syndrome.

In addition to the effects seen at the centromeric and pericentromeric regions, telomere shortening and hypomethylation at telomeric regions has been observed in ICF cells, despite levels of telomerase activity comparable to that found in control cells. The hypomethylation of the telomeric regions is accompanied by increased transcription of telomere RNA (telRNA or TERRA) [56]. These transcripts localize to the telomeric regions and inhibit activity of telomerase [57]. Decreased methylation at the telomeric regions may lead to increased expression of telRNA in ICF cells thereby inhibiting telomerase activity and resulting in shorter telomeres and increased chromosome instability [56]. Additionally, telRNA plays a role in telomeric heterochromatin formation and has been shown to associate with two key players in the epigenetic regulation of chromatin structure – Heterochromatin protein 1 (HP1) and methyl CpG binding protein 2 (MeCP2) [58–60].

ICF syndrome has a very complex epigenetic etiology involving altered DNA methylation patterns, chromatin structure, and histone modifications at dysregulated genes leading to wide scale changes in nuclear morphology, gene expression, and chromosome stability. Although the disorder is rare, it provides insight into the importance of DNA methylation during development, and strengthens the idea that epigenetic mechanisms are interconnected and essential for proper cellular function.

17.2.4 X-Linked IDD and α-Thalassemia Mental Retardation, X-Linked

X-linked mental retardation is the most common cause of intellectual developmental disability (XL-IDD, previously X-linked mental retardation) in males. Female carriers of XL-IDD mutations generally show favorable skewing of X-inactivation and are asymptomatic. Non-syndromic XL-IDD is caused by a number of different mutations, deletions, and duplications on the X chromosome. XL-IDD is associated with a varying degree of intellectual disability, facial abnormalities, and is often present with autism. Chromosomal regions of interest have been identified in several cases including Xp11, Xp22, and Xp28 [61, 62]. No single genetic mutation accounts for more than a few percent of cases of XL-IDD. Bioinformatic analyses of genes on the X chromosome revealed fewer than ten candidate genes and could not identify the genetic cause of XL-IDD in most families examined [63], suggesting a causative role for epigenetics.

Syndromic XL-IDD disorders, which have known genetic causes and an established set of phenotypic characteristics, are valuable tools for exploring the epigenetic mechanisms involved in X-linked neurological disorders. α-Thalassemia Mental Retardation, X-Linked (ATR-X) is a syndromic X-linked neurodevelopmental disorder that results in the dysregulation of epigenetic mechanisms. Over 200 cases of ATR-X have been identified with more than 100 different identified mutations [64]. ATR-X is caused by mutations in *ATRX* (also denoted as *XH2*), which codes for ATRX, a nuclear retained protein involved in chromatin remodeling. Deletions, duplications, missense mutations, and premature stop mutations have all

been identified as causing ATR-X [65, 66]. ATR-X is characterized by severe intellectual disability, developmental delay, genital abnormalities, microcephaly, seizures, and α-thalassemia [67]. ATRX levels increase with neuronal differentiation, and stay elevated in post-mitotic cells [68], much like the elevated levels of MeCP2 observed in post-mitotic, mature neurons. This evidence suggests an obligate role for ATRX in the epigenetic regulation of chromatin structure during neurodevelopment.

ATRX is a member of SNF2 family of chromatin remodeling factors that is targeted to heterochromatin via an interaction with methyl CpG binding protein 2 (MeCP2) [64, 69, 70]. This is of particular interest since MeCP2 is implicated in various other neurodevelopmental disorders. Most of the mutations that cause ATR-X disrupt heterochromatin targeting, highlighting the importance of chromatin structure in neuronal cells. Additional evidence indicates that ATRX interacts with the inactive X chromosome in both stem cells and somatic cells [71]. A possible role for ATRX in X-inactivation involves chromatin remodeling which promotes condensation of the inactive X chromosome, either directly or indirectly by recruiting additional factors, to effectively silence genes subject to X-inactivation.

In addition to its interactions with neuronally important proteins and its possible effects on chromatin structure, ATRX may also have an effect on DNA methylation. Although no change in methylation at imprinted genes has been found, changes in DNA methylation at repetitive regions has been observed including hypomethylation of ribosomal DNA arrays and hypermethylation of a Y-chromosome satellite repeat (DYZ2) in peripheral blood of ATR-X patients [72, 73]. The N-terminal PHD domain of ATRX, which targets it to heterochromatin, is sometimes referred to as the ATRX-DNMT3-DNMT3L (ADD) domain due to sequence and structural similarities with the *de novo* methyltransferases [4]; however, no methyltransferase activity has been observed, suggesting that ATRX does not directly alter methylation patterns but may recruit additional factors which then alter the methylation in a site-specific manner.

Mutations in *ATRX* can have severe effects on neurodevelopment. ATRX has been implicated in multiple epigenetically regulated events, including chromatin remodeling, DNA methylation, and X-inactivation, and interacts with other epigenetically important proteins. The variability in the ATR-X phenotype suggests a variety of factors influence the epigenetic mechanisms involved. As new causal genetic evidence is emerging for ATR-X and XL-IDD, it is likely that more emphasis will be placed on the importance of epigenetic regulation and chromatin remodeling in these, as well as other, neurodevelopmental disorders.

17.2.5 Rett Syndrome and the Spectrum of MECP2 Disorders

Rett syndrome (RTT) is an X-linked dominant disorder caused by mutations in *MECP2*. MeCP2 is at the center of shared pathways in human neurodevelopmental disorders and a central regulator of neuronal responses to activity-dependent DNA

methylation dynamics in the mammalian brain [74]. MeCP2 is a member of the family of methyl binding proteins that bind to methylated DNA [75]. MeCP2 is abundant in mature neurons of the mammalian brain [76–78] and DNA methylation appears to specify its chromosomal location [79, 80]. While originally thought to silence genes with methylated promoters [81, 82], the suggested functions of MeCP2 have increased in the last decade. Structural roles for MeCP2 in compacting and looping chromatin have been observed [83–85], as well as roles in RNA splicing [86], long range modulation of active genes [87], and transcriptional activation [88]. Neurons are exquisitely sensitive to MeCP2 levels and both *Mecp2* loss and gain have reciprocal detrimental effects on glutamatergic synapse strength and numbers [89]. Astrocytes also show alterations due to MeCP2 deficiency that are likely relevant to disease pathogenesis [90, 91]. Many downstream target genes have been characterized, but overall MeCP2 target genes depend on tissue and developmental context [92–96].

MECP2 mutations are the cause of RTT in >90% of the "classic" RTT cases [97]. Classic RTT is recognized clinically as affecting females who have a period of normal infancy, followed by a period of regression. The regressive period occurs between 6 and 18 months of age and is notable by the loss of language and motor skill, autistic features, characteristic hand wringing stereotypies, severe seizures, and autonomic dysfunctions such as breathing, cardiac, temperature, and gastrointestinal abnormalities. Some RTT girls develop Parkinsonian-like tremors and features as they reach adulthood [98]. Some cases of atypical RTT, characterized by infantile seizures, have mutations in the *CDKL5* [99], encoding a kinase predicted to phosphorylate MeCP2 [100], suggesting that post-translational modifications of MeCP2 are necessary for its function.

MECP2 is located at Xq28 and subject to X chromosome inactivation, thus making important differences in disease severity and pathogenesis between males and females. Females with RTT exhibit a mosaic pattern of MeCP2 expression, with some cells expressing the normal copy of MeCP2. Because they only have one X chromosome, males that inherit a RTT causing *MECP2* mutation have severe neonatal encephalopathy with death in infancy, rather than the classic RTT features seen in girls [101]. Exceptions to the severity of RTT-causing mutations in males have been observed with sex chromosome aneuploidy (47, XXY) or somatic mosaicism [102–104].

Chromosomal duplications in Xq28 that include *MECP2* are implicated in 1–2% of XL-IDD cases in males [103, 105–107]. Boys with *MECP2* duplication syndrome have clinical features including severe IDD, progressive spasticity, lack of speech, and immune abnormalities, including increased susceptibility to respiratory infections. In cases where males inherit *MECP2* duplications from their mothers, autism is also a common clinical feature [108].

Unlike the female carriers of *MECP2* mutations, females with *MECP2* duplications have highly skewed X inactivation in favor of the normal single copy allele. Mothers of these patients often have mild neuropsychiatric features including anxiety, depression, and compulsions [108]. These observations suggest that non-RTT causing *MECP2* mutations and altered expression levels may be more common than previously predicted.

MeCP2 seems to play a role in several neurodevelopmental disorders. This is likely due to its requirement during neurodevelopment and its role in neuronal maturation. This overlap is evident in postmortem cerebral cortex samples of individuals with autism and other neurodevelopmental disorders that displayed altered MeCP2 protein levels, suggesting a shared pathway among the disorders [109–111]. Epigenomic investigations of MeCP2 binding compared to DNA methylation and transcription genome-wide are likely going to be required to fully understand the diverse functions ascribed for MeCP2 and the importance of correct MeCP2 levels for the mammalian brain.

17.3 Autism Is a Spectrum of Neurodevelopmental Disorders with Complex Genetic and Environmental Contributors Affecting Epigenetic Pathways

The majority of neurodevelopmental disorders have complex genetic and environmental etiologies involving several different pathways, all of which manifest into an abnormal neurological phenotype. Autism spectrum disorder (ASD) is a prime example. ASD is termed a spectrum disorder due to the large variation in phenotype of affected individuals. ASD is characterized by abnormal social interactions and communication (both verbal and non-verbal), and the propensity for stereotyped and repetitive behaviors [112, 113]. ASD afflicts an estimated 1 in 150 individuals, with males 4 times more likely to be diagnosed than females [114, 115]. The phenotype of afflicted individuals is quite heterogeneous, with only a moderate percentage having known genetic or environmental causes.

Evidence suggests a strong genetic component since the incidence of ASD is strongly familial. Monozygotic twins have a 70–90% concordance rate, dizygotic twins have a 0–10% concordance rate, and siblings have an estimated concordance rate of 6% [116–118]. Conflicting estimates show anywhere between 10–25% of ASD cases are syndromic and have a known genetic or environmental cause [118–120]. Of these, no single genetic cause accounts for more than 2% of cases [120]. Because of the vast heterogeneity in sample populations, genome-wide association studies have had little success in identifying major genetic causes for ASD. Genetic association or linkage has been reported on every chromosome, but most genetic loci have not been replicated in subsequent studies. Chromosomal abnormalities, including duplications, deletions, and copy number variations (CNVs) together account for 5% of ASD cases, with the most common occurring on chromosome 15q11-13 [121–123]. Additional genetic regions of interest include 7q22-36, 16p11, 17q21, 22q11-13, and Xq27-28 [114, 123–125]. The number of autism candidate genes continues to fluctuate, highlighting the individuality of this disorder. It is likely that different genetic abnormalities alter the epigenetic landscape that in turn affects neurologically important pathways, resulting in phenotypically similar outcomes.

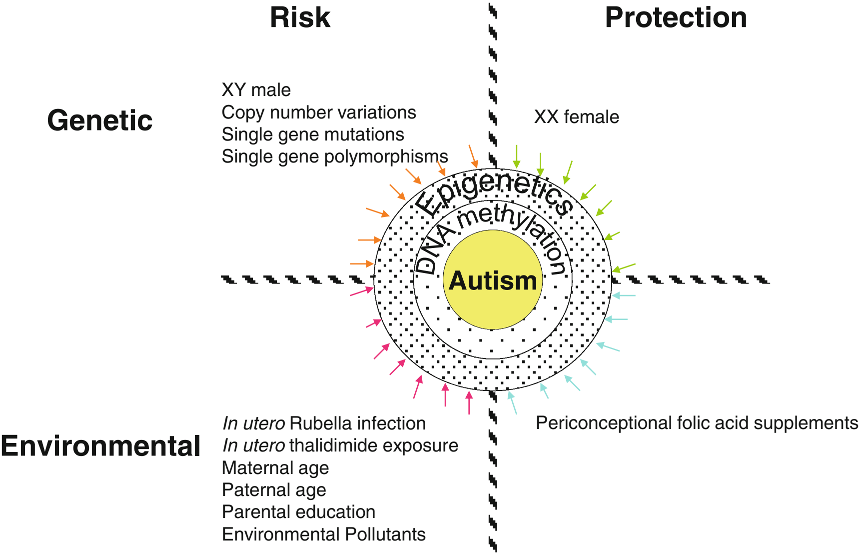

Fig. 17.2 Epigenetic mechanisms act at the interface of genes and the environment. Risk for the complex genetic disorder autism is likely influenced by multiple genetic and environmental risk and protective factors, represented by colored arrows (*orange*, genetic risk; *green*, genetic protection; *red*, environmental risk; *blue*, environmental protection). Epigenetic mechanisms such as DNA methylation (*spotted circles)* act at the interface of genetic and environmental risk and protective factors. For instance, multiple environmental risk factors correlate with global DNA hypomethylation, while folic acid supplementation promotes DNA methylation

Environmental risk factors for ASD have been identified in recent epidemiologic studies, but the understanding of environmental factors in autism is still in its infancy. A recent study of twin pairs estimates that shared environmental components play a larger role than genetic components in susceptibility to ASD [149]. Research to date indicates that a combination of genetic and environmental influences combine to affect neurodevelopment perinatally with many possible outcomes, one of which is ASD. Among the reported risk factors are increased maternal and paternal age, increased maternal education, and premature birth (less than 35 weeks gestation) [116].

Epigenetic mechanisms act at the interface of genetic and environmental risk factors for autism in considering both risk factors as well as protective factors (Fig. 17.2). Evidence for epigenetic alterations in specific gene loci has been accumulating. Deficiencies in *UBE3A* and the $GABA_A$ receptor gene *GABRB3* are common to idiopathic autism, RTT, and AS, suggesting epigenetic overlap in multiple neurodevelopmental disorders [126]. As mentioned above, reduced expression of MeCP2 is observed in 79% of autism brain samples and correlates with increased *MECP2* promoter methylation in males [109], The three 15q11-13 $GABA_A$ receptor genes, *GABRB3*, *GABRA5*, and *GABRG3,* while biallelically expressed in control

brains, show epigenetic dysregulation leading to monoallelic and reduced expression in a subset of autism cortex samples [127]. The gene encoding oxytocin receptor (*OXTR*) was found to have both genetic and DNA methylation alterations in peripheral blood of individuals with autism [128]. In addition, a recent genome-wide methylation profiling of lymphoblastoid cells identified increased methylation of the circadian clock regulator *RORA* and the repair pathway gene *BCL-2* [129]. These studies have increased the evidence for DNA methylation abnormalities in idiopathic forms of autism, but raise many questions about which environmental factors may contribute.

Recently, folate metabolism has been linked to ASD. Genetic mutations in this pathway are expected to have epigenetic effects that are highly relevant to autism epidemiology. Folate metabolism is intertwined with epigenetics in that it produces S-adenosyl-methionine (SAM), which serves as a universal methyl-group donor for DNA, RNA, and histone methylation. SAM is converted to S-adenosyl-homocysteine (SAH) during methylation, and the balance between SAM and SAH is important for methyltransferase activity. Methylene tetrahydrafolate reductase (MTHFR) is an enzyme important for SAH metabolism and is required to maintain the proper SAM:SAH ratio. Dietary intake of B vitamins provides some of the components necessary for folate metabolism, and deficiencies have been linked to neural tube defects [130].

Evidence has emerged that implicates polymorphisms in *MTHFR* (specifically C677T) in some cases of idiopathic autism [131–134]. This polymorphism is associated with a 2.79 fold increase in autism risk. Additional polymorphisms have been found in *MTHFR* at A1298C, and are suggested to increase the risk for autism 8-fold when found in combination with the C677T allele [133]. These polymorphisms reduce enzymatic activity in a dose-dependent manner [135].

These studies of mutations in genes important in folate metabolism were spurred by observations that plasma from autistic patients had decreased levels of SAM and methionine and increased levels of SAH, in addition to altered levels of other metabolites important in folate and one-carbon metabolism. The two-fold decrease in SAM:SAH observed in autistic cases suggests an imbalance in the folate pathway which could affect DNA methylation [136]. SAH levels were significantly elevated in mothers of autistic children, resulting in a decreased SAM:SAH ratio. This increase in SAH was linked to a 7-fold increase in the likelihood of having a child with autism. Additionally, the maternal samples with the highest levels of SAH were shown to have global hypomethylation. It has been theorized that maternal increases in SAH can affect the methylation status of the developing fetus by altering the SAM:SAH ratio *in utero* [137]. A combination of factors most likely influences the prenatal methylation status of children with autism, including the mother's genotype and dietary intake.

A recent work has disputed the importance of polymorphisms in *MTHFR* and instead posits that maternal polymorphisms in *RFC1*, which codes for a carrier protein involved in folate metabolism, are a major contributor to autism risk. Mothers homozygous for *RFC1* A80G were 46% more likely to have a child with autism. Interestingly, the increased autism risk was independent of the child's genotype [138].

This observation, combined with hypomethylation in the mothers of autistic children and the children themselves, supports the idea of environmental epigenetic alterations *in utero* leading to neurodevelopmental disorders. Polymorphisms in genes whose products are important in folate and one carbon metabolism may increase the individual's need for folate and other B vitamins. If these needs are not met, pre- and post-natal abnormalities in neurodevelopment may result.

Of interest is the idea that maternal genetics and environmental exposures can have an effect on the epigenetic neurodevelopment of the offspring. For example, genetic mutations in the mother that lead to increased SAH levels can influence the epigenetic state of the fetus including DNA and histone methylation. This can lead to epigenetic effects which persist after birth. Alternatively, if the genetic mutations is present in the fetus, *in utero* neurodevelopment progresses normally dependent on high maternal dietary intake of folate and input of intermediaries from the maternal folate pathway. Postnatal neurodevelopment, however, does not follow a normal trajectory due to abnormal folate and one carbon metabolism in the offspring, which essentially leads to a build up of post-natally acquired epigenetic abnormalities and possibly leads to neurodevelopmental disorders [139]. A recent epidemiology study has shown a protective effect of prenatal vitamin supplementation taking prior to conception and in the first trimester of pregnancy [140], supporting a potential role of DNA methylation in the protection.

In addition to the nutritional environment of the developing fetus, environmental toxins are expected to contribute to autism risk [141]. Environmental toxins could act through mutations and DNA repair pathways [142] or through epigenetic pathways such as DNA methylation, but an exploration of potential human exposures and autism risk is still in its infancy. Two possible environmental exposures from epidemiological studies of autism include organochloride pesticides from proximity to agricultural fields and air pollution from residential proximity to freeways [143, 144]. Interestingly, human air pollution exposure has been shown to negatively correlate with global levels of DNA methylation at *LINE-1* and *Alu* repeats [145, 146].

In addition to intersecting with genetic pathways, environmental exposures are expected to intersect with nutritional pathways. A unifying mechanism for a combination of diet and environmental exposures has been proposed [147]. Because the one-carbon metabolism and the glutathione (GSH) synthesis pathways are biochemically linked, enhanced need for GSH, an important antioxidant, to conjugate chemicals acts as an added "demand" on the availability of methyl-donors for DNA methylation. Deficiencies in methylation, GSH, and oxidative stress pathways have been implicated in autism [136].

17.4 Conclusions

It is becoming increasingly clear that environmental and genetic factors significantly impact the epigenome. The epigenome is responsible for modulating gene expression in all tissue types, and is especially important in neuronal cells.

The epigenome serves to interpret environmental signals and adjust the neuronal environment and transcriptional activity accordingly. Alterations in DNA methylation, whether large scale or site specific, can significantly affect transcription of neuronally important genes. Alterations in histone modifications can affect the spatial arrangement of chromatin, restricting or allowing access to transcriptional machinery. Understanding the improper progression of epigenetic changes that occurs in neurodevelopmental disorders can shed light on both genetic and epigenetic causes, and hopefully lead to effective treatments.

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Chapter 18
Epigenetic Epidemiology of Psychiatric Disorders

Bart P.F. Rutten and Jim van Os

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Abstract Exciting developments in the field of epigenetics have generated great interest within psychiatric epidemiology to focus on direct and indirect evidence for epigenetic involvement in behavior, mental health, and complex psychiatric disorders. Epidemiologic evidence on epigenetics in psychiatry, however, is currently very sparsely available. With the aim to address the current status of the literature on evidence indicative of involvement of epigenetic mechanisms in psychiatric disorders, we describe a clear role for epigenetic mechanisms in development and aging of the brain, with experiences and environmental exposures particularly during early life having considerable impact on the development of functional abilities of the brain. Besides the psychiatric consequences of classical syndromes of genetic imprinting in humans, findings of twin discordance, parent-of-origin effects, paternal age effects, and sex differences in psychiatric disorders suggest epigenetic involvement in the etiology of psychiatric disorders. The evidence is further strengthened by observations of endurable effects of various environmental exposures during life on risk of psychiatric disorders, and preliminary epigenetic studies showing differential epigenetic profiles in patients with several psychiatric disorders. Findings of these first (and preliminary) epigenetic studies should be interpreted with caution because of small samples sizes, lack of replication, limitations in the etiologic validity of psychiatric diagnoses, and in accessibility of the regions and cell types of the brain at "appropriate" periods during life. Despite the sparse availability, the current evidence for epigenetic involvement in (particularly early) brain development, mental health, and psychiatric disorders appears very promising, and may be used in bringing together inherited and acquired risk factors into a neurodevelopmental etiological model of psychiatric disorders with epigenetics as a plausible key mediating mechanism. Given the dynamic nature of epigenetic regulation of gene expression and the potential reversibility of epigenetic modifications, future well-designed multidisciplinary and translational studies will be of key importance in order to identify new targets for prevention and therapeutic strategies.

Abbreviations

5-mC	5-methyl cytidine
AD	Alzheimer's disease
ADHD	Attention deficit hyperactivity disorder
AKT1	v-akt murine thymoma viral oncogene homolog 1

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APOE	Apolipoprotein E
APP	Amyloid precursor protein
BDNF	Brain derived neurotrophic factor
CB1	Cannabis-1
CDH1	Cadherin 1
Cdk5	Cyclin-dependent kinase 5
COMT	Catechol-O-methyltransferase
DC-MZ	Dichorionic monozygotic
DNMT	DNA methyl transferase
DRD2	Dopamine D2 receptor
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders 4th edition
DZ	Dizygotic
GABA	Gamma-aminobutyric acid
GAD	Glutamic-acid decarboxylase
H4K12	Histone H4 lysine 12
H4K16	Histone H4 lysine 16
HIST1H2AG	Histone cluster 1, H2ag
HIST1H2AH	Histone cluster 1, H2ah
HIST1H2BJ	Histone cluster 1, H2bj
HIST1H2BK	Histone cluster 1, H2bk
HIST1H4I	Histone cluster 1, H4i
HTERT	Telomerase reverse transcriptase
MAPT	Microtubule-associated protein tau
MC-MZ	Monochorionic monozygotic
MeCP2	Methyl CpG binding protein 2
MS	Multiple sclerosis
MTHFR	Methylenetetrahydrofolate reductase
MZ	Monozygotic
NPAS3	Neuronal PAS domain protein 3
NR3C1	Glucocorticoid receptor
OCM	One-carbon metabolism
PBCs	Pregnancy and birth complications
PCR	Polymerase chain reaction
PPIEL	Peptidylprolyl isomerae E-like
PSEN1	Presenilin 1
PTSD	Post-traumatic stress disorder
RELN	Reelin
SIRT3	Sirtuin 3
SMARCA5	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5
SMS	Spermine synthase
SNP	Single nucleotide polymorphism
THC	Δ^9-tetrahydrocannabinol
TRKB	Neurotrophic tyrosine kinase, receptor, type 2
UBE3A	Ubiquitin protein ligase E3A
WHO	World Health Organization

18.1 Introduction

Studies on the environmental and genetic epidemiology of psychiatric diseases have taken important steps forward in estimating heritability rates and identifying associations between a range of environmental and genetic factors and psychiatric phenotypes. Various environmental and genetic factors interact in complex manners throughout an individual's life to contribute to psychiatric disorders. Recent exciting developments in the field of epigenetics suggest that epigenetic mechanisms may mediate sustainable effects of environmental exposures. These ideas have generated great interest within many research disciplines, including psychiatric epidemiology. The "seductive allure of behavioral epigenetics" [1] has prompted psychiatric epidemiologists to focus on direct and indirect evidence for epigenetic involvement in mental health and normal behavior as well as in complex psychiatric disorders, in an attempt to elucidate the role of epigenetic mechanisms and possibly identify new strategies for prevention and treatment of psychiatric disorders [2]. Without attempting to provide a complete overview, this chapter addresses the current status of the literature on evidence indicative of involvement of epigenetic mechanisms in psychiatric disorders. The chapter starts with a summary of the evidence for a role of epigenetic mechanisms in development and aging of the brain and its functional abilities. Next, we exemplify that aberrant epigenetic mechanisms are linked to neuropsychiatric phenotypes by briefly describing psychiatric consequences of classical syndromes of genetic imprinting in humans. Thereafter, we summarize general epidemiologic findings that are indicative of epigenetic involvement in psychiatric disorders and review the more direct epidemiologic evidence (i.e., differential epigenetic profiles) for epigenetic involvement in the most prevalent and severe psychiatric illnesses. We will end the chapter by discussing current research challenges and future perspectives on epigenetic involvement in psychiatric disorders.

18.2 Epigenetic Mechanisms in Development and Aging of the Brain

The brain is the most complex organ of the human body and enables the individual to respond with appropriate behavior to a diverse range of internal and external stimuli, a process which requires dynamic adaptations in the molecular and cellular processes that represent the pathway from sensory perception to behavioral responses. Psychiatric disorders are characterized by mental symptoms and complaints that are accompanied with dysfunction of the human individual in interaction with the social world. The very complex molecular and cellular changes that underlie the development of functional abilities of the brain come together with sensitive time windows at which experiences and environmental exposures co-determine the developmental outcome. Pioneering work in the last decade has uncovered epigenetic regulation of gene transcription as fundamental for normal

development and functioning of the organism, especially in relation to appropriate responses to stimuli [3]. The various epigenetic mechanisms encompass DNA methylation, histone modifications, genetic imprinting, X-inactivation, and non-coding RNAs (described in detail elsewhere) which give rise to tissue- and cell-type specific profiles of gene expression and epigenetic marks during development. Besides robust changes in reprogramming of genomic methylation patterns in germline cells as well as in preimplantation embryos [4], it has become clear that dynamic changes occur in the patterns of DNA methylation, histone alterations, and expression of microRNAs throughout life and especially during development. Brain development involves cellular processes such as cellular proliferation, cellular differentiation, and maturation [5], but also myelination [6] and synaptic plasticity, and accumulating evidence indicates that these processes depend on appropriate epigenetic regulation [7]. Recent experimental animal studies have, for example, established that the functional abilities of memory formation, learning, motivation, and reward are all linked to epigenetic regulation of gene expression [8–10]. Genetic manipulation of Dnmt1 and Dnmt3a in mice has shown that long-term plasticity (which underlies learning and memory) in the mouse hippocampus depends critically on these major DNA methyl transferase enzymes [11]. Experience-driven developmental changes impact at different biological levels, such as membrane depolarization, calcium influx, and induction of transcription factors [12], and recent studies have discovered that sustainable effects of developmental exposures and experiences are mediated (and reflected) by epigenetic alterations [13], as discussed in more detail below. Evidence that humans with mutations in gene encoding methyl CpG-binding protein 2 (MeCP2) frequently show markedly decreased cognitive performances is furthermore in line with this notion. Epigenetic changes are proposed to have crucial impact on early life programming and thereby on neurodevelopmental disorders [14], but also affect age-related changes in the brain. Recent work reports that epigenetic markings are subject to change with advancing age [15, 16]. While earlier work suggested that aging was associated with a global loss of DNA methylation, more recent work indicates that age-related changes are also CpG-island dependent [15–17]. Investigating non-pathologic human tissues at 1,413 autosomal CpG loci associated with 773 genes, Christensen et al. observed highly significant CpG island–dependent correlations between age and methylation: loci in CpG islands gained methylation with age, loci not in CpG islands lost methylation with age [17]. Another large scale study using human brain tissue identified CpG loci, primarily CpG islands, with consistent positive correlation between DNA methylation, and chronological age [18]. In addition, recent findings on an aging mouse cohort kept under controlled environmental conditions throughout live showed that the level of the major *de novo* methylation enzyme DNA methyltransferase 3a (Dnmt3a), which catalyzes DNA methylation, increased with age in the hippocampus [19], and correlated with age-related increase in levels of 5-methyl cytidine (5-mC). The same study showed that caloric restriction, which increases lifespan and prevents age-related alterations and pathology in various animal species [20–23], was able to prevent these age-related changes in hippocampal levels of Dnmt3a [19] and 5-mC [251]. The speculation of a causal involvement of

epigenetic mechanisms in age-related decline of functional abilities of the brain [24] is in line with (1) findings that age-related memory disturbances in mice are associated with altered chromatin plasticity (in particular with dysregulation of H4K12ac) in the hippocampus [25], and (2) the link between sirtuins, i.e., molecules which deacetylase H4K16ac in a Nicotinamide adenine dinucleotide-dependent manner, and life span [26]. Thus, epigenetic mechanisms appear to be fundamentally involved in the neurobiological processes that govern development [14] and aging of the brain [16], and may be involved in the formation of an individuals' functional abilities and personality characteristics, as well as in neurodevelopmental and neurodegenerative trajectories of psychopathology [1, 27].

18.3 Psychiatric Phenotypes in Disorders of Genetic Imprinting

The crucial role of genetic imprinting in brain function is exemplified by the clinical pictures of two syndromes that are caused by aberrations in genetic imprinting: Angelman syndrome and Prader-Willi syndrome [28]. Angelman syndrome results from loss of expression of the maternal gene *UBE3A* at chromosome 15, by either deletion or other genetic abnormalities [29]. The maternal gene is normally expressed while the paternal gene is normally silenced. The characteristic clinical picture of Angelman syndrome comprises neurodevelopmental disabilities, motor abnormalities, seizures, and speech deficits, resembling the clinical picture in patients with deficiencies in 5,10-methylenetetrahydrofolate reductase (*MTHFR*) or in patients with mutations in methyl-CpG binding protein 2 (*MeCP2*) [30]. Prader-Willi syndrome results from the loss of paternal expression of genes (on the same region on chromosome 15 as Angelman syndrome) [28]. Prader-Willi syndrome is clinically characterized by the psychopathological features of mental retardation, obsessive-compulsive symptoms, eating problems (hyperphagia), hypersomnia, and neurodevelopmental delay of motor skills [31]. Thus, aberrations of genetic imprinting in the same region on chromosome 15 can cause two syndromes with overt neuropsychiatric illness phenotypes. The epigenetic changes underlying these syndromes are discussed in more detail in Chap. 9.

18.4 Twin Discordance

Proband-wise concordance rates for psychiatric disorders in classical twin studies are generally spanning a wide range, in the order of 20–80%, while many psychiatric disorders also have clear links to aberrant neurodevelopment, e.g., autism spectrum disorders, ADHD, and schizophrenia, that all show substantial heritabilities. The standard assumptions that greater disease concordance rates in monozygotic (MZ) versus dizygotic (DZ) twins indicate genetic contribution, and concordance rates

well below 100% in MZ twins indicate an environmental component, have been challenged by evidence indicating that (1) gene-environment interactions may contribute to both the "genetic" and "environmental" components [32], and (2) possible effects of stochastic factors exist on biological processes, such as regulation of gene expression, throughout life [2, 33].

While most MZ twins show phenotypic similarity and MZ twin pairs are expected to be concordant for congenital malformations, chromosomal abnormalities, and Mendelian disorders, phenotypic discordance in MZ twins does occur frequently and may arise from various sources such as (1) chromosomal and monogenetic variations, (2) differences in environmental exposures in the intrauterine (differential timing of the twinning process, a differential number of cells allocated to each twin, and differential placental vascularization), perinatal (such as hypoxia) and postnatal environment, as well as (3) epigenetic differences [34]. An indication of epigenetic mediation of environmental exposures during life is provided by whole-genome and locus-specific methylation analyses of DNA from lymphocytes of MZ twins (15 male twin pairs and 25 female twin pairs) showing that approximately one-third of these MZ twins harbored significant epigenetic differences in DNA methylation and histone modification that were more distinct in MZ twins who were older, had different lifestyles, and had spent more of their lives apart [35].

Recent methylation microarray analyses of DNA in lymphocytes and buccal mucosa of 114 MZ twins and 80 dizygotic (DZ) twins confirmed the presence of substantial differences in DNA methylation profiles in MZ twins, while also showing epigenetic metastability of 6,000 unique genomic regions in MZ twins, and greater epigenetic similarity in MZ co-twins than in DZ co-twins [36].

Differences in methylation profiles in buccal mucosa of MZ twins have furthermore been reported for CpG sites in a number of specific candidate genes for psychiatric disorders such as the dopamine D2 receptor (*DRD2*) gene [33] and the catechol-O-methyltransferase (*COMT*) gene [37]. In addition, differential DNA methylation profiles in buccal mucosa have been observed in MZ twins discordant for bipolar disorder (BD) [38] and the behavioral phenotype of risk-taking behavior [39]. A recent methylation microarray analysis of MZ twins discordant for BD observed increased methylation in the affected twins upstream of the spermine synthase gene (SMS) and lower methylation upstream of the peptidylprolyl isomerase E-like gene (PPIEL) [38]. However, very detailed analyses on MZ twin pairs discordant for multiple sclerosis (MS) [40] indicated that the puzzle of explaining MZ twin discordance is far from being solved. Analyses of the genome sequences of an MZ twin pair discordant for MS, and messenger RNA transcriptome and epigenome sequences of lymphocytes from three MZ twin pairs discordant for MS failed to detect reproducible differences in approximately 3.6 million single nucleotide polymorphisms (SNPs) and approximately 0.2 million insertion-deletion polymorphisms between the co-twins [40]. The authors furthermore did not observe reproducible differences between siblings of the three twin pairs in HLA haplotypes, MS-susceptibility SNPs, copy number variations, mRNA and genomic SNP, and insertion-deletion genotypes, or the expression of approximately 19,000 genes. Although the detailed analyses did detect 2–176 differences in the methylation of

approximately two million CpG dinucleotides between siblings of the three twin pairs, it is unlikely that these methylation differences can fully explain disease discordance [40].

Thus, preliminary evidence indicates epigenetic involvement in MZ twin discordance, although the complete puzzle of explaining MZ twin discordance in the various (neuro)psychiatric disorders is far from being solved. Future twin research will be of crucial relevance for the formulation and testing of new etiological models of complex psychiatric traits and disorders integrating the contributions of genetic components, environmental exposures, gene-environment interactions, stochastic factors, and epigenetics [2, 35, 41–43].

18.5 Parent-of-Origin Effect and Paternal Age

Parent-of-origin effects reflect a differential proportion of paternal or maternal disease-causing transmission to offspring. Thus, the risk-increasing effects of alleles for certain complex diseases may depend on their parent of origin. It has been proposed that parent-of-origin effects are either based on mutagenesis, causing de novo spontaneous mutations which would then propagate and accumulate in successive generations of sperm-producing cells, or on genomic imprinting, an epigenetic mechanism in which differential epigenetic modification of genes occurs depending on their parental origin [44]. Parent-of-origin effects have, for example, been described in attention deficit hyperactivity disorder (ADHD), autism, psychosis, and late onset cases of Alzheimer's disease (AD) [45–50]. It has furthermore been described that paternal and maternal ages (at the time point of conception) are risk factors for various psychiatric disorders with neurodevelopmental origins such as schizophrenia and autism. Paternal age effects have been proposed to be mediated by epigenetic mechanisms [51]. In autism, a 10-year increase in paternal age (independent of maternal age) is associated with a 22% increased risk for autism while a 10-year increase in maternal age (independent of paternal) age has been associated with a 38% increased risk for developing the disorder [52], which is in line with other findings reporting associations between parental ages and autism [53–55]. A recent meta-analysis on the association between paternal age and schizophrenia reported that both advanced paternal age (>/=30) and younger paternal age (<25) may increase the risk of schizophrenia, with further analyses indicating that younger paternal age may be associated with particularly an increased risk in male but not female offspring [56]. Given these effects of paternal (and maternal) age on risk of several psychiatric disorders, one could speculate that paternal and maternal age influence general functional abilities of the offspring's brain rather than inducing abnormalities specific for a certain disorder. An interesting recent study on 33,437 children drawn from the US Collaborative Perinatal Project reported that offspring of older fathers were impaired in tests of neurocognitive ability (at an age of 7 years) while advanced maternal age was associated with better neurocognitive abilities [57]. The pattern of childhood behavioral problems in the same population

was also differentially affected by advanced paternal age as compared with advanced maternal age; while advanced paternal age was associated with externalizing behaviors in the offspring but not with internalizing behavioral outcomes, advanced maternal age was significantly protective against externalizing behavioral outcomes, but associated with an increased risk of internalizing behavioral problems [58]. Evidence that, at least for some disorders, both high and low paternal or maternal age were associated with increased risk of psychopathology, e.g., in autism [53] and schizophrenia [56], is in line with the concept of the existence of an optimal (epigenetic-regulated) window of paternal and maternal ages at conception. Thus, parent-of-origin effects and effects of paternal (and possibly also maternal age) on risk of psychopathology in offspring point to a possible role for genomic imprinting in the etiology of psychiatric disorders.

18.6 Sex-Differences in Psychiatric Disorders

The onset, course, and phenomenology of psychiatric disorders often show sexual differentiation [59, 60]. Female sex has consistently been associated with increased risk of depressive disorders [61] and anxiety disorders such as panic disorder, post-traumatic stress disorder (PTSD), and social phobia [62], while male sex is associated with ADHD [63], autism spectrum disorders [64, 65], and substance abuse disorders [66]. Sexual differentiation is furthermore apparent in age of onset, phenomenology, and developmental trajectories toward psychopathology [67, 68]. Various epigenetic mechanisms have been proposed to underlie sexual differentiation in psychiatry. Besides differential exposures to environmental risk factors with sustainable effects on the individual's phenotype, sexual differentiation in many features of psychiatric disorders may be the result of sex hormone-induced differences in the epigenetic status of key genes impacting during sensitive time periods during development [69]. For example, methylation of the promoter of the estrogen receptor-alpha gene has been reported to increase during development [70].

Another epigenetic mechanism giving rise to sexual differentiation is X-chromosome inactivation. X-inactivation is the irreversible epigenetic process that involves silencing of one of the two X-chromosomes in female individuals during early development [71]. X-inactivation is estimated to take place just before the morula stage when the human embryo contains less than 16 cells [72, 73]. Previous studies have shown that the timing of X-inactivation is associated with the timing of chorionic twinning [72]: dichorionic monozygotic (DC-MZ) twinning is estimated to take place at, or before, the morula stage (so, just at or before X-inactivation) and the monochorionic monozygotic (MC-MZ) twinning event occurs after the morula stage (and consequently after X-inactivation) [72]. Consistent with the notion that individuals who are members of DC-MZ twins are separately subject to X-inactivation, patterns of X-inactivation are more identical in MC-MZ twins than in DC-MZ twins [72, 74]. Thus, chorionicity may be used as a proxy-measure of X-inactivation. While it has been proposed that X-inactivation may affect functional

abilities and risk of psychiatric disorders particularly in females [75], only a very limited number of studies in psychiatry have directly measured variations in X-inactivation, although various epidemiological studies have used proxy-measures of X-inactivation. For example dichorionicity in twins and female sex have been considered as a proxy measure of X-inactivation. In contrast to previous twin studies using sex and chorion type in isolation as proxy-measures for X-inactivation [75], a recent study by Peerbooms et al. combined information on sex and chorion type in an improved proxy measure and found no association between this proxy measure for X inactivation and variations in intelligence and behavioral problems in children with a mean age of 10 years, thus indicating no major role for X-inactivation in variations of these traits at this age [76]. However, a recent study by Rosa et al. using more direct measures of X-inactivation by comparing methylation at CpG islands of X-linked housekeeping genes on the active and inactive X-chromosomes in blood and buccal epithelial cells (as an index of X-chromosome inactivation) found evidence that was suggestive for a role of X-inactivation in BD. The index of X-inactivation showed a borderline statistically significant difference for discordant BD twin pairs as compared to controls [77]. Nevertheless, the role of X-inactivation in behavioral traits and psychiatric disorders has only received very little research interest thus far and requires further exploration.

18.7 Enduring Influences of Environmental Exposures

A wealth of epidemiologic evidence indicates that environmental exposures influence susceptibility to disease in later life [14, 42]. A consistent line of evidence has connected exposures to nutritional factors, childhood trauma, minority group position, drugs of abuse, and pre- and perinatal complications to an increased risk of psychiatric disorders such as depression and schizophrenia later in life, especially in genetically vulnerable people [32, 41, 68, 78, 79]. It has been argued previously that the contributions of genetic and environmental factors are unlikely to be independent and that models taking into account gene-environmental interactions may be much more plausible [80–82]. While epigenetic mechanisms have been proposed as a prime candidate for mediating some of these environmental effects, primarily on the basis of indirect evidence and findings in experimental animal studies [41, 42], direct evidence in humans remains very sparse. Based upon the currently available evidence from both animal and human research, we focus here on examples of environmental factors associated with psychiatric disorders, for which evidence suggests epigenetic involvement.

18.7.1 Pregnancy and Birth Complications

Population-based studies have suggested an association between psychiatric disorders and a wide variety of prenatal exposures, including maternal stress [83–85], maternal nutritional deficiency [86, 87], prenatal iron deficiency [88], maternal serum lead levels [89], maternal serum homocysteine levels [90], rhesus incompatibility [91],

low and high neonatal vitamin D [92], prenatal toxoplasmosis [93, 94], specific viral infections [95], bacterial infections [96], maternal pyelonephritis [97], maternal hypertension [98], and maternal use of analgesics [99] and diuretics [98] during pregnancy. As many of these exposures have been found to associate (or induce) epigenetic alterations in cell culture or animal studies, epigenetic mechanisms have been proposed to mediate at least some of these effects [41, 100–103].

There are, however, few true (i.e., corresponding in trimester timing, exposure definition, subgroup-only effects) replications, and credible non-replications, on the associations between pregnancy and birth complications (PBCs) and psychiatric disorders [104–109], such that no definitive conclusions about association (nor mediation) can be drawn at this stage. It is important to realize that while causal relationships and underlying mechanisms can be examined easier in animal research, inferences of animal findings to the human situation are limited [110, 111], which is especially relevant for the domain of psychiatric disorders. Nevertheless, it is attractive to hypothesize that the wide variety of exposures may reflect a single underlying epigenetic mechanism associated with subtle developmental perturbations that increase risk for psychotic outcomes in interaction with genetic risk variants [97]. A recent study suggested that individuals from the Dutch Hunger winter who were exposed to famine prenatally showed hypomethylation at the imprinted *IGF2* gene when compared to their unexposed same-sex siblings six decades after the period of severe famine [112]; an association that was specific for peri-conceptional exposure to famine, thus suggesting crucial relevance of epigenetic mechanisms in very early mammalian development [112]. As famine may also evoke psychological stress (or be associated with other co-variates), these effects may not be solely attributable to impaired nutritional uptake during pregnancy. Several nutritional factors such as deficiencies of vitamin A or D, protein content, essential fatty acids, and folate have been proposed to mediate the increased risk of psychiatric disorders in offspring subjected to famine during fetal development [113]. A large birth cohort study with data on schizophrenia in adulthood indicated that elevated homocysteine levels in the third trimester of pregnancy are associated with an increased risk of schizophrenia in the offspring [90]. A study in which birth interval was used as a proxy of folate levels during pregnancy (as postpartum restoration to normal maternal folate values may take up to 1 year after pregnancy) further suggested an association between folate and schizophrenia [114]. It cannot be excluded that reported findings on the wide range of prenatal exposures in part represent the effects of publication bias, multiple testing in small samples, or genetic confounding occasioned by maternal behaviors associated with genetic risk for broadly defined psychiatric disorders (given that most psychiatric disorders show a familial relationship with schizophrenia [115]). On the other hand, inconsistencies across studies and lack of reproducibility may also be due to differential confounding.

Eight prospective, population-based studies on PBCs and schizophrenia were available for meta-analysis [116]. This meta-analysis reported a wide range of PBCs that were associated with a diagnosis of schizophrenia including (1) complications of pregnancy (bleeding, diabetes, rhesus variables reflecting rhesus incompatibility, rhesus-negative mother, rhesus antibodies, pre-eclampsia); (2) abnormal fetal growth and development (low birth weight, congenital malformations, reduced head

circumference); and (3) complications of delivery (uterine atony, asphyxia, emergency Caesarean section). Pooled estimates of effect sizes were generally around 2. These studies suggest an etiological signal associated with PBCs, confirmed by later studies on this topic [117].To establish a more robust link between an exposure and a complex outcome usually require replication of association across a range of studies using a variety of different designs and approaches [118]. Prospective population-based studies in Denmark and Sweden of groups with high rates of PBCs, such as twins, have been inconclusive [119–121], and studies focusing on subclinical psychotic symptoms and PBCs similarly are not consistent [122–124]. Familial risk may be associated with greater sensitivity for the risk-increasing effect of PBCs on schizophrenia and on brain structural alterations associated with schizophrenia [125, 126], which may be linked to effects of hypoxia [127, 128], that may be epigenetically mediated [129].

18.7.2 Rearing Environment and Childhood Abuse

Adoption studies provide evidence for an association between rearing environment, childhood stress, and an increased risk of psychopathology at a later age. For example, adoptees with a positive family history for psychosis who had been brought up in dysfunctional adoptive family environment displayed an increased risk of psychiatric disorders [130–133]. Conversely, a positive rearing environment may decrease the risk of psychotic disorder later in life, as it has been shown that high-risk children with positive parental relationships have a lower risk for developing schizophrenia [134]. Studies on the effects of an adverse early psychosocial environment on later psychiatric disorder have been re-invigorated by recent prospective epidemiological findings showing that victims of bullying at ages 8 and/or 10 years have a 2-fold risk of psychotic symptoms at age 12 with an even more elevated risk when victimization is chronic or severe [135], and recent surveys in large population samples indicate that childhood adversities (especially when associated with maladaptive family functioning) account for approximately 30% of all psychiatric disorders [136], and are associated with earlier onset of psychiatric disorders, persistence of mood and anxiety disorders [137, 138] and with suicidal behavior [139]. No studies have yet examined the link between childhood stress, epigenetic changes, and the onset of psychiatric disorders in humans. Recent experimental animal research, however, has suggested that the psychosocial environment and stress can mediate changes in gene expression during key developmental periods through epigenetic mechanism with long lasting effects on behavior. Meaney and colleagues observed that postnatal maternal care in rats was apparently associated with epigenetic modification of a transcription factor binding site in the promoter region of the glucocorticoid receptor gene (*NR3C1*) which in turn directly alters gene expression and behavioral phenotypes in the offspring which persist into adult life although experiments lacked proper reproducibility studies with updated techniques [140]. In subsequent experiments methyl supplementation during the same early postnatal period seemed

to reverse the epigenetic modification induced by maternal care, with related gene expression changes and behavioral phenotypes in adult offspring [141]. Subsequent transcriptomic studies by the same group identified over 900 genes in the rodent hippocampus that are stably regulated by maternal care [142], suggesting an even more widespread effect of the early social environment on gene expression through the life course. However, these studies still await robust replication. A recent study in humans suggested epigenetic differences in a homologous *NR3C1* promoter region, comparing DNA methylation in postmortem hippocampus samples obtained from suicide victims with a history of childhood abuse to that seen in samples from either suicide victims with no childhood abuse or controls. In line with the animal findings, abused suicide victims were reported to have increased methylation of the *NR3C1* promoter with concomitant changes in mRNA [143]. These preliminary findings however await replication.

18.7.3 Drugs of Abuse

In recent years, evidence from epidemiological studies and meta-analyses has established that cannabis and other drugs of abuse can be considered risk factors for later psychotic symptoms or psychotic disorders [144–146]. The age (or developmental stage) at which individuals start using cannabis influences this association [147]. Further evidence suggests that gene-environment interactions are likely implicated in the association between cannabis and psychosis [145, 148–152]. For example, the psychotomimetic effect of cannabis is much greater in siblings of patients with a psychotic disorder, who are at increased genetic risk to develop psychotic disorder than in controls [153]. Cannabis use increased the risk for developing psychotic symptoms and schizophreniform disorder only in carriers with the valine158 allele in the gene encoding catechol-*O*-methyltransferase (*COMT*) [148], while the risk-increasing effects of cannabis on psychosis expression were moderated by genetic variation in *AKT1* in another study [150]. The primary psychoactive component of cannabis is Δ^9-tetrahydrocannabinol (THC), which is thought to exert its effects through cannabis-1 (CB1) receptor-mediated signaling [154]. Administration of THC or cannabis elicits long-term molecular and cellular changes in the brains of mice and humans [155–158], as well as on electrophysiological and biochemical measures of neuronal signaling in brain structures such as nucleus accumbens and hippocampus [159] with differential effects by duration of exposure and timing during development [159–162]. The abuse of psychostimulants, such as cocaine and amphetamine, has consistently been associated with major psychotic disorders and substance use disorders. Many drugs of abuse may act via the common mechanism of sensitization and sensitization may play a key role in psychosis. In humans and animals, repeated exposure to psychostimulants induces a sensitized state [163], i.e., an enhanced response to subsequent low dose challenges [164]. Sensitized animals (to different drugs) share a number of neurobiological changes such as long-term alterations of mesolimbic and prefrontal dopaminergic neurotransmission

while also expressing behavioral abnormalities resembling positive symptoms and enduring cognitive deficits such as seen in schizophrenia [163]. In addition, acute exposure to drugs of abuse provokes transient increases in cFos and other members of the Fos family of transcription factors in the striatum [165]. The majority of these *Fos* factors become desensitized during chronic abuse, with the exception of ΔFOSB, which itself stimulates the expression of other genes, such as *Cdk5*. [166, 167] ΔFOSB accumulates in the nucleus accumbens and dorsal striatum – two brain regions important for reward and locomotor drug responses – and remains detectable for several weeks after cessation of drug administration, suggesting a role in the onset of addiction [167]. In mouse models of cocaine response, the upregulation of cFos and ΔFOSB upon acute administration is accompanied by rapid and transient H4 hyperacetylation at the *cFos* and *FosB* gene promoters [168]. Chronic exposure, however, does not result in H4 hyperacetylation but is associated with H3 hyperacetylation of the *FosB* promoter, with no effect on the *cFos* promoter. H3 hyperacetylation of two genes associated with chronic, but not acute, drug use – *Cdk5* and *BDNF* (brain derived neutrophic factor) – was also detected after chronic cocaine administration, and persisted even 1 week after treatment ceased [168].

These findings implicate epigenetic mechanisms in the development of addiction, and particularly in the immediate and sustained behavioral effects of cocaine use [168]. Human studies found enduring changes in gene expression in subjects exposed to psychostimulants. For example, prolonged exposure to amphetamine is associated with long-term reductions of dopamine transporter density in the brain as measured by in vivo imaging studies [169]. Exposure to cannabis, cocaine, and phencyclidine have long lasting effects on intracerebral gene expression, and recent evidence suggests that in fact these drugs may all affect common neurobiological pathways. In a recent post mortem study using human brain tissue samples, exposures to cannabis, cocaine, and phencyclidine were associated with many of these transcriptional changes in the brain [170]. Hierarchical clustering of these transcripts indicated that genes from the calmodulin signaling cluster were predominantly affected in the brains of abusers, which may be of particular importance given that enhanced dopamine release due to sensitization depends on calmodulin signaling [171, 172]. Thus, chronic exposure to drugs may induce a sensitized state with enduring intracerebral changes in gene expression (particularly in dopaminergic neurotransmission), with evidence – at least from animal studies – suggesting a crucial, mediating role for the epigenetic machinery.

18.7.4 Minority Group Position and Social Defeat

Meta-analytic work shows consistency for the association between psychotic syndrome and minority group position across a wide range of approaches, endpoints, settings and cultural group definitions [173, 174], and after adjustment for a range of confounders. The possibility of cultural bias in diagnosis and selective migration has been examined but not found to have a major impact on the association. The association with minority group position is observed in both first and second

generation migrants [173, 174], as well as in minority groups without recent migration [175], indicating that pre-migration factors or migration itself are unlikely to mediate effects. Studies in four different countries have shown that the effect of minority ethnic group on psychotic syndrome depends on the ethnic density of the area the person is living in: the greater the proportion of the own ethnic group in the area, the lower the risk for psychotic disorder [176, 177]. These findings suggest that it is not ethnic group per se that increases risk, but rather the degree to which one occupies a minority position, or stands out in relation to the wider social environment. Additional research suggests that effects associated with minority group position may be mediated by chronic social adversity and discrimination [178], resulting in social marginalization or a state of social "defeat" (chronic experience of an inferior position or social exclusion [179]). Although epigenetic consequences of chronic social "defeat" has not been shown in humans yet, animal research has shown that chronic social defeat stress (by daily experience of defeat by a bigger and more aggressive mouse for 10 consecutive days) induces gene expression changes, particularly of *BDNF*, via a range of epigenetic mechanisms including histone tail modifications and DNA methylation. For example, chronic exposure to social defeat stress in mice significantly downregulated mRNA levels of histone deacetylase-5 in the nucleus accumbens [180]. Chronic defeat stress in mice also induced enduring downregulation of BDNF transcripts and increased histone methylation [181]. Interestingly, chronic treatment with the antidepressant imipramine can reverse downregulation of BDNF transcripts while increasing histone acetylation at the corresponding promoters [181]. Thus, animal research suggests that social stress may be an epigenetically mediated, environmental factor that underlies proxy risk factors such as ethnicity and migration in psychiatric disorders.

18.7.5 Synergism in Environmental Exposures

It has been proposed that psychiatric disorders arise slowly from subclinical symptoms that become abnormally persistent when synergistically combined with various environmental exposures during development that may impact on behavioral and neurotransmitter sensitization [78, 182, 183]. Although various experimental animal studies have indicated synergistic effects of environmental exposures [184–186], data from epidemiologic studies remain sparse. For example, heritable risk for depression and the combined effects of several environmental exposures (over the course of development measured as lower birth weight for gestational age, childhood adversity, and negative life events in adulthood) have recently been reported to have a synergistic impact on the psychiatric phenotype of stress-sensitivity (that is proposed to underlie depressive symptomatology) [78]. While it remains to be established whether epigenetic alterations mediate these effects, future prospective epidemiologic studies with (epi) genetically sensitive designs may further focus on synergistic effects of environmental exposures during development with inherited sensitivity and epigenetic profiles.

18.8 Variants in Epigenetic-Relevant Genes and Psychiatric Disorders

Genetic studies have indicated that variations in genes with crucial relevance for epigenetic mechanisms may increase the risk for developing psychiatric disorders.

18.8.1 MTHFR

The gene encoding 5,10-methylenetetrahydrofolate reductase (MTHFR) is an epigenetically relevant gene as it encodes a crucial enzyme involved in the folate-mediated one-carbon metabolism (OCM), which is essential for purine and thymidylate biosynthesis, methylation of DNA and amino acids, and necessary for reactions forming neurotransmitters [187]. Dysfunction of one-carbon metabolism has been linked to a range of disorders including neural tube defects [188, 189], autism [190], leukemia [191, 192], dementia [193, 194], colorectal cancer [195, 196], cardiovascular disease [197], and congenital abnormalities [198, 199]. Genetic studies on associations between genetic variants in *MTHFR* and various major psychiatric disorders such as schizophrenia, BD, and unipolar depressive disorder have yielded largely inconclusive and often mixed results [200–206]. Given the essential role of *MTHFR* in brain function and neurodevelopment [207, 208], and the fact that family and twin studies have established considerable shared genetic variance between psychiatric disorders [209–212], a recent meta-analysis (on a total of more than 29,000 subjects) tested whether genetic variation in *MTHFR* contributes to the shared genetic vulnerability of schizophrenia, BD, and unipolar depressive disorder [213]. This meta-analysis showed that *MTHFR* C677T was significantly associated with the combined group of schizophrenia, BD, and unipolar depressive disorder (odds ratio = 1.26 for TT versus CC genotype carriers; confidence interval 1.09–1.46) without evidence of modifying effects of psychiatric diagnosis, sex, ethnic group, or year of publication, thus providing evidence for shared genetic vulnerability for SZ, BPD, and unipolar depressive disorder mediated by *MTHFR* 677TT genotype [213].

18.8.2 MeCP2

The gene encoding for MeCP2 is another gene crucially involved in epigenetics. MeCP2 protein selectively binds CpG dinucleotides in the genome and mediates transcriptional repression through interaction with histone deacetylase [214]. Besides being the major cause of Rett syndrome (a rare but fulminant neurodevelopmental disorder in young girls) [215], mutations in this X-linked gene have been found to be associated with a broad array of other neurodevelopmental disorders in

males and females, including X-linked mental retardation [216, 217], severe neonatal encephalopathy, Angelman's syndrome, and autism [214]. Thus, these findings show that variants in the key epigenetic regulator gene MeCP2 impact crucially on brain development and thereby on risk of psychiatric disorders.

18.8.3 Histone Genes

A recent large meta-analysis suggested that common variants located at chromosome 6p22.1 in a cluster of the histone genes HIST1H2BJ, HIST1H2AG, HIST1H2BK, HIST1H4I, and HIST1H2AH are associated with an increased risk of schizophrenia [218]. Although the observed associations may be connected to one or several genes, intergenic elements, or longer haplotypes that include susceptibility alleles in many genes, the strongest association was observed for a region in and near a cluster of histone protein genes, making variants in histone coding genes prime candidates for further analysis, at least in schizophrenia.

18.9 Aberrant Epigenetic Profiles in Psychiatric Disorders

18.9.1 Depression, PTSD, and Suicide

Methylation microarray analyses of whole blood-derived, bisulfite-converted DNA indicated differential methylation profiles in 33 individuals who reported a lifetime history of depression, as compared to 67 non-depressed adults, suggested patterns of increased methylation in genes relevant for brain development and tryptophan metabolism and patterns of decreased methylation in other biological processes [219]. In PTSD, a similar microarray approach indicated differential methylation profiles in 23 PTSD-affected as compared to 77 PTSD-non-affected individuals; genes implicated in immune function were uniquely unmethylated in the affected individuals [220]. In post mortem obtained brain tissue, H3K27me differed in the *TRKB* promoter locus in suicide completers ($n = 20$) as compared to control subjects ($n = 10$) in the orbital frontal cortex but not in cerebellum [221].

18.9.2 Schizophrenia and Bipolar Disorder

Early studies primarily focused on differential epigenetic marks in specific candidate genes in post mortem brain tissue. Such studies reported DNA methylation differences associated with schizophrenia in catechol-*O*-methyltransferase (*COMT*) [222] and reelin (*RELN*) using methylation-specific PCR [223], although studies using

full quantitative methylation profiling methods did not confirm these findings [224–226]. Post mortem analyses of cortical GABA-ergic neurons in schizophrenia have shown increased levels of Dnmt1, which was associated with altered expression of reelin or GAD67 [223, 227]. As discussed earlier, MZ twins discordant for BD had differential DNA methylation profiles in regions upstream of the spermine synthase gene (*SMS*) and upstream of the peptidylprolyl isomerase E-like gene (*PPIEL*) [228]. While DNA methylation upstream of *SMS* did not affect expression of the gene, a strong inverse correlation between *PPIEL* gene expression and DNA methylation was observed. Methylation microarray analysis of frontal cortex tissue from patients with schizophrenia and BD revealed DNA methylation differences of numerous loci, including several involved in biological processes such as glutamatergic and GABA-ergic neurotransmission, brain development, and other processes functionally linked to disease etiology [225].

18.9.3 Alzheimer's Disease

Decreased global levels of DNA methylation were observed in the entorhinal cortex of AD patients using immunohistochemical analyses [229]. Several human post mortem brain studies analyzing the methylation status of promoter regions of candidate genes of AD have yielded mixed and inconsistent findings. Analysis of the *APP* promoter from temporal cortex failed to show a difference in methylation status of the promoter region between control and Alzheimer's disease patients [230]. DNA methylation was decreased in the promoter region of the tau protein gene in the parietal cortex, but its transcription was downregulated [231]. In a postmortem analysis of the frontal cortex and hippocampus, no significant differences between Alzheimer's disease patients and age-matched controls were found in the methylation patterns of the promoters of *MAPT*, *PSEN1*, and *APP* [232]. Another study demonstrated increased methylation within the promoter regions of *APOE* and *MTHFR* in Alzheimer's disease patients when compared to controls, in both post mortem prefrontal cortex tissue and peripheral lymphocytes [233]. No differences in methylation patterns of DNA from blood lymphocytes of AD patients versus controls were observed in promoter regions of the *SIRT3* (sirtuin 3), *SMARCA5* (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a 5), and *CDH1* (cadherin 1) [234]. The methylation status of *HTERT* (telomerase reverse transcriptase) in DNA of blood lymphocytes, however, differed between Alzheimer patients and age-matched controls [234]. Epigenetic analyses in post mortem brain samples furthermore showed that the epigenetic distance from the norm (the median methylation of the control individuals) increased with age, and was higher in people with AD than in healthy controls [233]. The general age-dependent epigenetic drift was also found in lymphocytes of AD patients, although the differences were smaller when compared to brain tissue [233]. Thus, clear and consistent evidence for epigenetic involvement in AD is currently lacking while adequately powered epigenetic analyses are warranted.

18.10 Summary and Perspectives

It is easy to speculate about the role of epigenetic processes in mediating susceptibility to psychiatric disorders, but investigating these modifications at the combined epidemiologic and molecular level is far from straightforward. The first generation studies on epigenetic differences in psychiatric disorders are characterized by a cross-sectional design investigating DNA methylation differences of candidate genes in small numbers of patients ($n = 1–50$) and age- and sex-matched "super-controls" (i.e., healthy controls without any psychopathology). DNA was derived from a range of tissue types (whole blood, blood lymphocytes, or homogenates of various brain regions) and processed with variable laboratory procedures, i.e., bisulfite conversion, methylated DNA immunoprecipitation, and microarray techniques. As these studies harbor various limitations, such as risk of false-positive findings (e.g., due to small sample size and the comparison with supernormal controls), lack of prospective investigations, selection bias, treatment effects, disease effects, lack of primary tissue of interest (the brain), and lack of replication, results from these first generation studies should be interpreted with caution [235]. Nonetheless, the findings do suggest possible differential epigenetic profiles in psychiatric disorders and generate hypotheses for future studies.

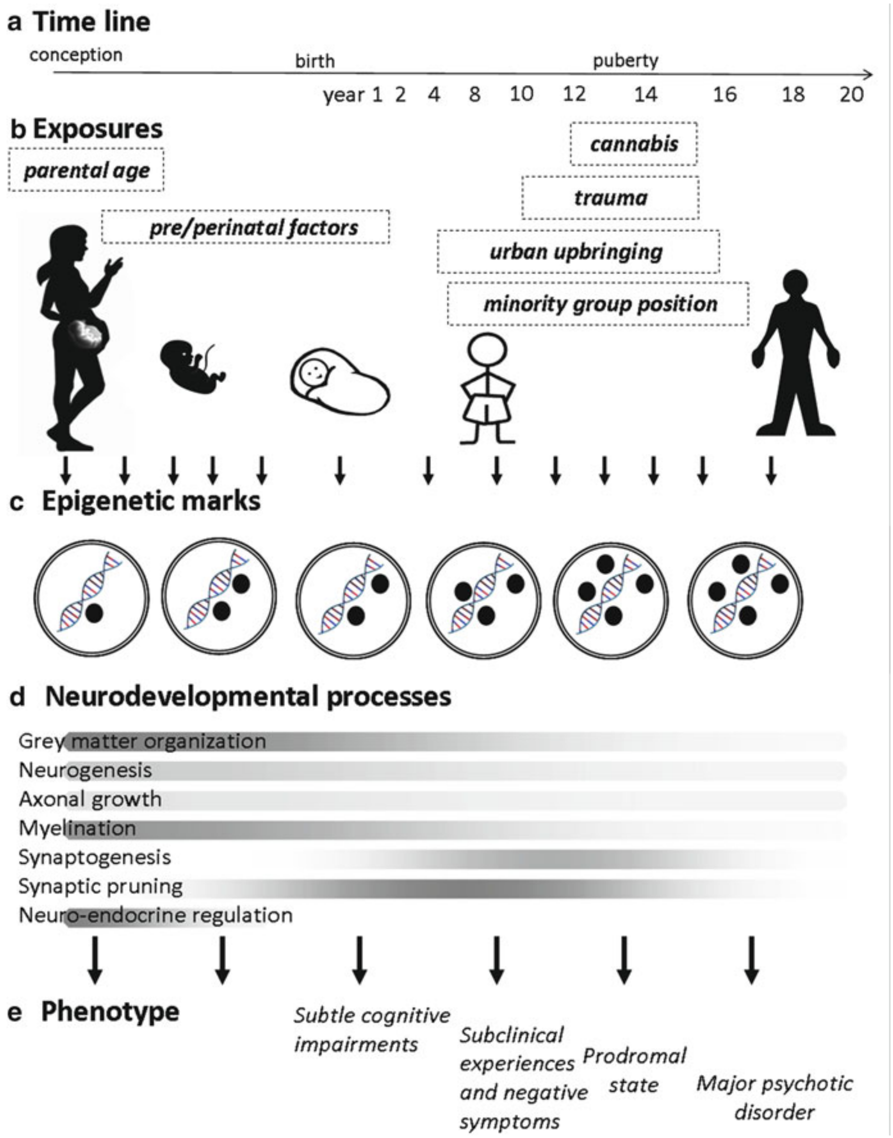
Difficulties in establishing etiological classifications of psychiatric disorders and phenotypes have defined intermediate phenotypes, dimensions of psychopathology, or sub-classes of conventional disease categories which may be more proximal to the actual neurobiological causal factors, and thus more suitable for epigenetic epidemiologic research.

Analyses of DSM-IV classifications of diagnoses in the WHO mental health surveys have established that the temporary presence of any distinct psychiatric disorder predicts subsequent onset of other psychiatric disorders, and that, therefore, "comorbidity" of psychiatric disorders is the rule rather than the exception [236]. In line with this, a diagnosis of schizophrenia has been found to be highly predictive of virtually all other Axis I and Axis II psychiatric disorders in the same person [237]. These findings suggest that dimensional approaches may be useful. Moreover, cognitive alterations, psychosis (hallucinations and delusions), and affective dysregulation that are observed in psychotic disorders (schizophrenia, BD) are also prevalent in common mental disorders (anxiety disorders, depression) [238, 239]. The relative non-specificity of psychiatric disorders extends to familial aggregation and to risk and protective factors. A family history of schizophrenia is associated with the strongest relative risk; however almost any psychiatric disorder in first-degree relatives is associated with an increased risk for schizophrenia. Similarly, nearly 30% of schizophrenia in the population can be attributed to psychiatric family history in general, compared to 6% that is attributable to a family history of schizophrenia specifically [115]. Many psychiatric syndromes/disorders share common risk and protective factors. Common genetic factors, such as polymorphisms in *MTHFR* [213] and *NPAS3* [240], as well as various environmental exposures such as childhood adversity [136] have substantial effects that transcend traditional clinical

diagnostic boundaries. These data suggest that shared genetic, epigenetic, and environmental factors contributed to neurodevelopmental alterations resulting in broad dimensions of mental ill-health [241]. While integrative investigations on genetic data, epigenetic data, and environmental data thus seem pertinent, analyzing these effects in integrative statistical models remains a tremendous challenge [242, 243].

The first epigenetic studies in psychiatric disorders have increased awareness of numerous challenges at the technical level as well. The limited accessibility to high-quality human brain tissue from well-phenotyped patients, in combination with the cell-type-specific and temporal-specific nature of epigenetic programming, poses great challenges for epigenetic studies. Whether more accessible tissue sources (like lymphocytes) for epigenetic profiling will reflect similar pertinent epigenetic changes as the respective tissue of interest (i.e., brain) remains to be explored. There is increasing evidence from other disorders that many epimutations are not limited to the affected tissue or cell type, but can also be detected in other tissues. Furthermore, there is great need for studies that establish the "normal" epigenome, especially for tissues such as brain, and patterns across tissue types and across development. Translational studies that combine findings from (a) carefully conducted epidemiologic studies including longitudinal twin studies [244], (b) molecular biology studies on prospectively collected, easily accessible human tissue (such as blood lymphocytes, buccal mucosa, or germline cells) as well as post mortem brain tissue [245], and (c) experimental animal studies on the effects of environmental exposures during sensitive time periods of development [68, 246] will allow for further exploration on the role of epigenetics in human development, physiology, and pathophysiology. Figure 18.1 illustrates a neurodevelopmental view on the role of epigenetics in the

Fig. 18.1 Neurodevelopmental model of epigenetic involvement in the etiology of major psychotic disorders. A scheme on a neurodevelopmental model of the etiology of psychiatric disorders illustrating an individual's age (Fig. 18.1a), the approximate timing of risk-increasing exposures (Fig. 18.1b), epigenetic marks that can be inherited or acquired over life, as a possible consequences of exposures (Fig. 18.1c), timing of neurodevelopmental processes (Fig. 18.1d), and the temporal sequence of the expression of psychiatric phenotypes (Fig. 18.1e). Exposures depicted in 1b may thus induce epigenetic alterations on the molecular level that may impact on crucial neurobiological processes during sensitive periods of neurodevelopment, and may synergistically give rise to aberrations in mental health, phenotypically expressed by quantitative (and/or qualitative) alterations in psychological functions that in interaction with the individual's social world may result into psychiatric disorder. *Black circles* in 1c represent epigenetic modifications to DNA (e.g., methylated cytosines or histone modifications). The row represents a nucleus of a post-mitotic cell with dynamic changes to DNA modifications which may accumulate within the individual throughout development as a consequence of the synergistical combination of multiple exposures. These dynamic changes in DNA methylation may be associated with aberrations in neurdevelopmental processes such as, for example, myelination (Fig. 18.1d), and the appearance of subclinical psychotic symptoms (Fig.18.1e) that can become abnormally persistent and ultimately lead to the onset of a major psychotic disorder (Fig. 18.1e). While this figure illustrates that development of major psychotic disorders is associated with accumulation of DNA modifications of a gene, scenarios are equally possible where the gene is associated with the removal of epigenetic modifications



etiology of psychiatric disorders. Current scientific approaches can take advantage of recent developments in genome-wide analyses of genetic variations, gene expression, and epigenetic marks that allow for explorative, hypothesis-free investigations.

The exploration of epigenetic mediation of environmental exposures may benefit from testing for gene-environment interactions by investigating genetic variants in epigenome-relevant genes for their interaction with environmental exposures in

large, well-characterized samples of subjects with psychiatric disorders [32]. Furthermore, Mendelian randomization designs [247], longitudinal studies including patients with "at risk" mental states for psychiatric disorders (such as in psychosis [248] and dementia [249]), and the use of twin studies discordant for psychiatric phenotypes and/or environmental exposures [244] may yield important insights in the role of epigenetic alterations in the onset and/or course of psychiatric disorders. Although rodent studies do not allow to make direct inferences about the human situation, animal studies will be very informative in elucidating the role of epigenetics in brain function and dysfunction, as both genetic and environmental factors can be (relatively) well controlled and brain tissue easily obtained [250].

In conclusion, the field of epigenetic research in psychiatric disorders is in a very early phase, but promising preliminary findings encourage carefully designed studies combining epidemiology, clinical science, and molecular genetics.

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Chapter 19
Epigenetic Epidemiology of Type 1 Diabetes

Amanda J. MacFarlane

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Abstract Type 1 diabetes (T1D) results from the immune-mediated destruction of insulin-secreting β-cells that reside in the pancreatic Islets of Langerhans. Genetic susceptibility is necessary but not sufficient for the development of autoimmune diabetes, indicating a key role for risk modification by environmental factors. Epigenetic mechanisms could mediate the effect of specific environmental factors

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and could therefore explain, at least in part, non-genetic susceptibility to Type 1 diabetes. Exposure to variable nutrition or infection in early life, including *in utero* experiences can modify T1D susceptibility, which may occur via epigenetic means. T1D is characterized by autoimmune destruction of pancreatic insulin-secreting β-cells and their aberrant development and function of β-cells; these pathogenic mechanisms are also subject to epigenetic regulation. Furthermore, the diabetic state, characterized by fluctuations in insulin and glucose, can influence genomic methylation profiles and gene expression, which can potentially impact susceptibility to co-morbidities of T1D. This chapter identifies potential epigenetic mechanisms that may modify T1D risk and describes study designs that can be implemented to determine the role played by epigenetics in disease pathogenesis.

Abbreviations

AdoMet	S-adenosylmethionine
AdoHcy	S-adenosylhomocysteine
BMI	Body mass index
CBS	Cystathionine β-synthase
ChIP	Chromatin immunoprecipitation
DAISY	Diabetes Autoimmunity Study in the Young
DIPP	Diabetes Prediction and Prevention
DNMT	DNA methyltransferase
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
LFA	Lymphocyte function-associated antigen
MHC	Major histocompatibility complex
MS	Methionine synthase
NF-κB	Nuclear factor-κB
Seq	Sequencing
SNP	Single nucleotide polymorphism
TCR	T cell receptor
TEDDY	The Environmental Determinants of Diabetes in the Young
T_H	T helper
T1D	Type 1 diabetes
T_{reg}	T regulatory

19.1 Overview of Type 1 Diabetes

Type 1 diabetes is a complex autoimmune disease involving the interaction of numerous genes and environmental factors (Fig. 19.1). It results from the immune-mediated destruction of the insulin-secreting β-cells that reside in the pancreatic

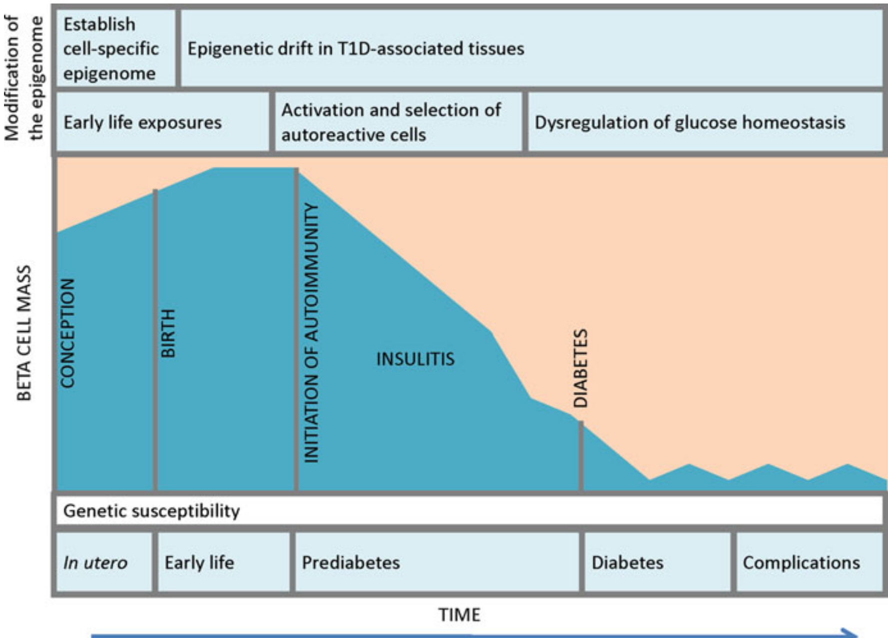


Fig. 19.1 Pathogenesis of Type 1 diabetes and possible role of epigenetic mechanisms. Type 1 diabetes (T1D) is a complex autoimmune disease involving the interplay of numerous genes and environmental factors, which may be regulated by epigenetic mechanisms. Genetically susceptible individuals are prone to the loss of self tolerance resulting in initiation, selection and progression of autoimmunity and insulitis, which culminates in the reduction of β cell mass, loss of glucose homeostasis and the requirement for exogenous insulin. Epigenetic modification of disease susceptibility may be dependent on timing as well as on the tissues involved. There are critical windows in development during which epigenetic marks are erased and re-established, making the epigenome of the developing embryo particularly vulnerable to changes in the intrauterine environment. Altered establishment of the epigenome may result in aberrant pancreas development and function making it more vulnerable to autoimmune attack. Early life experiences, such as the introduction of dietary components and exposure to infectious agents, mould the maturing immune system, a process regulated and perpetuated by epigenetic mechanisms. The epigenome of immune or β cells may also impact the timing of disease onset and disease severity. Finally, fluctuations in circulating glucose and insulin concentrations due to the diabetic state may modify homocysteine metabolism and cellular methylation potential in various tissues resulting in altered susceptibility to the development of T1D-associated complications (Modified from [10])

Islets of Langerhans (reviewed in [1]). Ultimately, the loss of β-cell mass becomes critical and glucose homeostasis is lost, resulting in lifetime dependence of the individual on exogenous insulin. Diabetes is associated with increased risk for a number of co-morbidities including heart disease, kidney failure, blindness, and limb amputation, which culminate in a decreased life expectancy.

A number of linkage and genome-wide association studies have identified the major risk loci for Type 1 diabetes. The DR3, DR4 and DQ2 susceptibility loci

are located within the major histocompatibility complex (MHC, also referred to as the human leukocyte antigen, HLA) class II region and can account for more than ~40% of increased risk [1, 2]. Overall, more than 40 risk loci have been associated with T1D, the majority of which are common polymorphisms (minor allele frequency of >10% in Western populations) in non-MHC genes that display odds ratios ≤1.3 [3, 4].

While underlying genetic susceptibility is necessary, it is not sufficient for the development of autoimmune diabetes leaving environmental factors as key contributors to disease risk. The incidence of T1D has increased more than 2–3 fold over the past half century, a pace that has not been paralleled by an increase in the frequency of the major risk alleles [3, 4]. In fact, the prevalence of the major MHC class II susceptibility loci appears to be decreasing among diabetic patients suggesting that the rise in incidence is due to the impact of environmental factors on individuals with lower-risk genotypes [5, 6]. Perhaps the most compelling indication that environment plays a significant role in diabetes development is the lack of 100% concordance among monozygotic (MZ) twins. The concordance rate among MZ twins is estimated to be between 25% and 65% [7–9]. A number of environmental factors have been implicated in the pathogenesis of autoimmune diabetes, including exposures to various dietary components and infectious agents [10, 11]. However, like the genetic side of the susceptibility coin, no single environmental agent has been found to explain all cases of the disease. As in many complex traits, autoimmune diabetes likely develops as a result of a variety of intricate interactions among many genetic loci and exposure to common environmental factors.

19.2 Epigenetic Regulation of Gene Expression and Its Potential Involvement in Autoimmune Diabetes

Epigenetics may explain, at least in part, non-genetic susceptibility to Type 1 diabetes (reviewed in [12]). Epigenetic mechanisms could act as the conduit by which some environmental factors induce variable phenotypes from identical genotypes. Of note, epigenetic modifications can occur at the level of the cell, tissue or whole organism and have the potential to be stable and mitotically heritable [13–15].

Epigenetic regulation of gene expression occurs by many mechanisms including DNA methylation, post-translational modifications of histones and the activation of microRNAs [16]. The cytosine in CpG dinucleotides in the genome can be methylated to form 5-methylcytosine. Multiple CpG dinucleotides, so called CpG islands, are often observed in the regulatory regions of genes and their methylation status influences gene expression. Heavily methylated CpG islands are often associated with gene silencing [17]. Second, post-translational covalent histone modifications, including acetylation, methylation, phosphorylation and ubiquitination also regulate gene silencing or expression within their associated genomic region(s) [18]. For example, lysine acetylation of histones H3 and H4 is commonly associated with an open

chromatin conformation that allows for active gene expression, such as the methylation of H3 lys4 (H3K4) and lys36 (H3K36). However, H3 and H4 hypoacetylation, di- and trimethylation of H3 lys9 (H3K9), trimethylation of H3 lys27 (H3K27) and methylation of H4 lys20 (H4K20) are associated with gene silencing [18]. A third mechanism involves the expression of short non-coding RNAs, the expression of which can lead to translational silencing through the specific binding and eventual degradation of transcribed RNA [19].

Despite general acceptance that environment plays a significant role in T1D [10, 11], with the exception of a few studies, the involvement of epigenetic mechanisms in T1D pathogenesis has gone essentially unstudied. Epigenetic mechanisms could play a role in the initiation or progression of autoimmunity, or alter the target tissue in such a way as to increase the likelihood that it will be targeted by an autoimmune attack. Here I propose potential epigenetic mechanisms that could be involved in T1D pathogenesis, identify a number of candidate epigenetically-regulated genes and suggest study designs that could be implemented to clarify the T1D-susceptibility epigenome.

19.2.1 Autoimmunity

T1D results from a T cell-mediated autoimmune attack on pancreatic β-cells. CD4+ T helper (T_H) cells, major protagonists involved in β-cell autoimmunity, originate from naïve $CD4^+$ T cells that differentiate into one of three main lineages, T_H1, T_H2 or T_H17, depending on extrinsic signals [20–22]. The transformation from naive to mature cells is a mitotically heritable phenotype that is passed on to all daughter cells and it is subject to complex epigenetic control (reviewed in [23–25]). A number of epigenetically-regulated genes associated with the immune response are presented in Table 19.1.

The differentiation of naïve T cells into T_H cell lineages is driven by the expression of specific cytokines. The treatment of thymoma cells with 5-azacytidine, which induces DNA hypomethylation, resulted in clones that constitutively produced interleukin (IL)-2 [26], suggesting that DNA methylation patterns were important for regulated gene expression. 5-Azacytidine treatment of stimulated naïve T_H cells was also associated with increased interferon (IFN)-γ and IL-4 expression [27], the main effector cytokines of T_H1 and T_H2 cells, respectively. The IFN-γ and IL-4 loci undergo extensive DNA and histone methylation changes that correlate with their respective gene expression during T_H1 and T_H2 cell differentiation [28–36]. Also, genes encoding T cell lineage specific transcription factors are targeted by particular epigenetic modifications [34]. Therefore, the specificity and balance of cytokine expression in differentiating naïve T_H cells is controlled at least in part at the level of the epigenome.

Interestingly, modification of methylation profiles in $CD4^+$ T cells can induce autoreactivity. Normally $CD4^+$ T cells do not react to self antigen; however, when

Table 19.1 Epigenetically-regulated genes involved in the immune response or β-cell development and function

Function/Tissue	Gene product	Epigenetic mechanism	Evidence	References
Immunity	IFN-γ	DNA methylation	IFN-gamma promoter undergoes differential methylation during in vitro T cell differentiation: hypermethylated in Th2 cells, hypomethylated in Th1 cells	[27, 35, 36]
	IFN-γ	Histone modification	Histone modifications characteristic of relaxed, accessible chromatin, observed at *Ifng* locus in T_H1 cells, and selective T-bet binding and activity, a key transcription factor associated with T_H1 pro-inflammatory responses	[34]
	IL-4	DNA methylation	Hypermethylated 5′ region of *Il4* locus becomes demethylated when naive $CD4^+$ T cells are stimulated to undergo T cell differentiation, demethylation associated with enhanced gene expression	[27, 28, 30]
	IL-4	Histone modification	H3K27me3 histone modification at *Il4* locus associated with gene suppression	[34]
	Foxp3	DNA methylation	Transcriptionally active region in *Foxp3* locus demethylated at CpG motifs in $Foxp3^+CD25^+CD4^+$ T_{reg} cells	[41, 44–46]
	Foxp3	Histone modification	H3 acetylation and trimethylation patterns indicative of open euchromatin observed at *Foxp3* locus region, associated with T_{reg} differentiation	[44]
	LFA-1	DNA methylation	Hypomethylation and upregulation of *Itgal* gene expression results in antigen-independent co-stimulation promoting T cell activation	[37–40]
	Il-17	Histone modification	Loss of the histone methyltransferase G9a during T helper cell differentiation results in loss of H3K9me2 repressive marks at the *Il17a* locus and increased expression	[116]
β-cell development and function	Insulin	Histone modification	Proximal promoter of *Ins1/2* gene displays H3 hyperacetylation and hypermethylation at H3K4, both of which are associated with euchromatin status and active gene transcription in pancreatic β-cells. Dimethylation status at H3K4 in the regulatory region of *Ins1/2* maintained by a transcriptional protein complex involving Pdx1, a key transcription factor involved in β-cell development, and the methyltransferase Set7/9, promoted continuous activation of *Ins1/2* and other genes involved in glucose-stimulated insulin secretion.	[54–58]
	PPARGC1A	DNA methylation	PPARGC1A is a transcriptional regulator associated with insulin secretion from islets. Its promoter region was hypemethylated in Type 2 diabetes patients and associated with reduced gene expression and insulin secretion	[117]
	miR-375	miRNA	Pancreatic islet specific expression inhibits insulin secretion in mouse β-cells	[59]
	miR-9	miRNA	Increased expression related to defective glucose-stimulated insulin secretion	[60]
	INK4a/Arf	Histone modification	Increased expression is associated with reduced β-cell regeneration, repression of expression in β-cells is regulated by Ezh2, a histone methyltransferase, and is dependent on H3K27me3 in the gene region	[118]

treated with 5-azacytidine, they lose the requirement for specific antigen stimulation and become activated in response to self MHC class II molecules [37]. The mechanism for this loss of self tolerance has been ascribed to the demethylation and activation of the *ITGAL* gene [38, 39]. The *ITGAL* gene product, lymphocyte function-associated antigen (LFA)-1, stabilizes T cell receptor-MHC interactions and provides co-stimulatory signals for the activation of T cell responses [40]. Its dysregulated overexpression is hypothesized to stabilize lower affinity TCR-MHC interactions and provide co-stimulation, which lead to autoimmunity similar to that observed in systemic lupus erythematosus. The data clearly indicate that changes to the methylation status of immune-activating genes can lead to the promotion of an autoimmune response by allowing indiscriminate interactions between T cells and antigen presenting cells.

The maturation and function of T regulatory (T_{reg}) cells, which are required for the maintenance of self tolerance [41], is also regulated by epigenetic mechanisms [42]. The transcription factor Foxp3 drives T_{reg} cell differentiation and its loss in mice results in the failure to generate $CD25^{+}CD4^{+}$ T_{reg} cells and the development of severe autoimmunity [43]. A conserved and transcriptionally active region within the *Foxp3* locus demonstrated epigenetic patterns associated with open and active euchromatin. Specifically, CpG motifs were demethylated and histone H3 was acetylated at the *Foxp3* locus, which correlated with gene expression in naïve $Foxp3^{+}CD25^{+}CD4^{+}$ T_{reg} cells, but not $CD25^{-}CD4^{+}$ T cells [41, 44–46]. Normally T_{reg} cells promote immune tolerance but this function is lost during autoimmunity. Therefore the dysregulation of T_{reg} cells has been suggested to play a role in T1D pathogenesis [42].

Other genes involved in the inflammatory and/or autoimmune process are also epigenetically regulated. These include the major diabetes susceptibility gene CTLA4, TGF-β, p38 mitogen-activated protein kinase, Toll-like receptors, IL-8, macrophage chemoattractant protein-1, and IL-6 [47–49]. One could speculate that the dysregulation of any one or combination of these epigenetically-regulated genes could predispose an individual to mount an autoimmune response or promote the uncontrolled progression of an autoimmune attack. However, this has not been empirically tested.

Only a few studies have examined epigenetically-regulated gene expression in the context of Type 1 diabetes. Abnormal global methylation of $CD4^{+}$ T cells has been observed in patients with latent autoimmune diabetes in adults [119] compared with healthy control subjects. Global methylation was increased compared to control subjects. Concomitantly, Foxp3 expression was decreased due to hypermethylation of its promoter region. Miao et al. [50] observed that lymphocytes from T1D patients demonstrated distinct histone modification patterns in comparison to control subjects. However, an important caveat to these studies is the earlier findings that human THP-1 monocytes cultured in high glucose conditions also revealed alterations in histone lysine methylation of various T1D-related loci [51]. Because blood glucose levels vary considerably in patients with T1D, it is difficult to differentiate whether such epigenetic changes are related to diabetes susceptibility or result from variations in blood glucose in overt diabetes [50].

In fact, cellular methylation potential is significantly influenced by glucose and insulin concentrations, which will be discussed in Sect. 19.3, highlighting the importance of obtaining tissue samples in the pre-diabetic period. Nonetheless, the data suggest that altered epigenetic regulation of gene expression could potentially influence the autoimmune process or impact diabetes-associated complications.

19.2.2 β-Cell

Type 1 diabetes is associated with progressive dysfunction of insulin-secreting β-cells, resulting in part from the dysregulation of epigenetically-regulated gene expression that is required for appropriate β-cell development and function [52, 53] (Table 19.1). For example, insulin is a major autoantigen in T1D and its gene expression is dependent on specific patterns of DNA methylation, histone acetylation and methylation. The expression of insulin mRNA is negatively correlated with DNA methylation of specific CpG islands in its promoter regions in patients with Type 2 diabetes [120]. Specifically, the proximal promoter of the *Ins1/2* gene in pancreatic β-cells displays H3 hyperacetylation and hypermethylation at H3K4, which are associated with euchromatin status and active gene transcription [54, 55]. Dimethylation status at H3K4 in the regulatory region of *Ins1/2* was shown to be maintained by a transcriptional protein complex involving Pdx1, a key transcription factor involved in β-cell development [56], and the methyltransferase Set7/9, which promoted the continuous activation of *Ins1/2* and other genes involved in glucose-stimulated insulin secretion [57, 58]. Also, two microRNAs, miR-375 and -9, have been reported to regulate glucose-stimulated insulin secretion [59, 60]. Aberrant insulin expression due to altered epigenetic regulation of *Ins1/2* could promote or antagonize an autoimmune response.

Specific genomic regions undergo a sequence of histone modifications during pancreatic organogenesis, with specific events occurring over the course of β-cell development. In zebrafish, the loss of DNMT1, which is required for the maintenance of DNA methylation, was associated with increased beta-cell regeneration, implicating DNA methylation as an important regulator during pancreatic development [121]. Also, in the absence of HDAC5 or HDAC9, two class IIa histone deacetylases, is associated with increased numbers of insulin-producing beta-cells [122]. In β-cells from mice, H3K27 trimethylation was increased among regulators of inappropriate developmental fate, allowing for the specific repression of genes that would be detrimental to the development, function and survival of β-cells [61]. This marker of gene repression was also associated with decreased mRNA expression of these factors. Interestingly the majority of the H3K27me3 marks were acquired *de novo* throughout developmental stages, from the multipotent progenitor cell stage through to terminal differentiation. Therefore, changes to histone modifications associated with the insulin gene and other developmental regulators required for β-cell development could modify the maturation or function

of β-cells resulting in predisposition for autoimmune diabetes. Additionally, aberrant gene expression could also result in the targeting of β-cells by the autoimmune response.

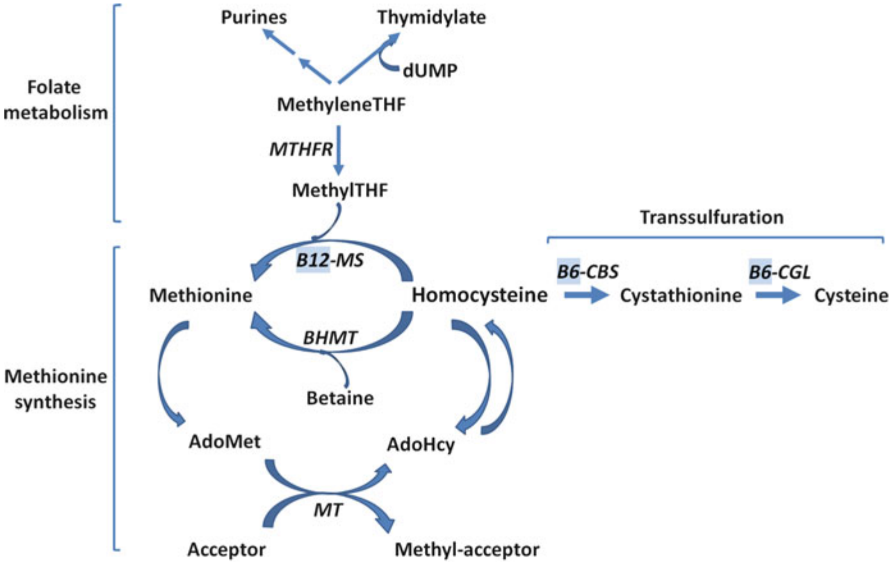
19.3 The Diabetic State, Homocysteine Metabolism and Cellular Methylation Potential

The establishment and maintenance of methylation patterns of CpG dinucleotides in DNA and histones depend on the cellular methylation potential. Homocysteine and methionine metabolism are interdependent and regulate the availability of methyl groups for cellular methylation reactions (Fig. 19.2) [62]. Homocysteine can be remethylated to form methionine, which in turn can be converted to S-adenosylmethionine (AdoMet). AdoMet is the major methyl group donor in cellular transmethylation reactions including the methylation of DNA, histones and other molecules [62]. AdoMet-dependent transmethylation reactions, mediated by methyltransferases, result in the production of S-adenosylhomocysteine (AdoHcy), which can then be converted to homocysteine, completing the methionine cycle. Alternatively, homocysteine can be catabolised by the transsulfuration pathway through its irreversible conversion to cystathionine.

Methyl group and homocysteine metabolism depend on the availability of a number of factors including folate, vitamin B6, vitamin B12, choline, betaine and methionine. Folate, the biologically active form of folic acid, mediates one-carbon metabolism (Fig. 19.2) [62]. Folate carries and activates one-carbon units for their use in the *de novo* synthesis of purines, thymidylate and methionine [63]. Methionine synthase (MS), a vitamin B12-dependent enzyme, transfers the methyl group of 5-methyltetrahydrofolate to homocysteine in its remethylation to form methionine [63]. Folate-mediated one-carbon metabolism is also dependent on vitamin B6, niacin, riboflavin, zinc, cobalt and vitamin A [62]. Folate-independent remethylation of homocysteine utilizes methyl groups donated by betaine, which can be taken up from the diet or derived from choline (Fig. 19.2) [64, 65]. The irreversible transsulfuration of homocysteine by cystathionine β-synthase (CBS) to cystathionine is vitamin B6-dependent (Fig. 19.2). Therefore, nutrient deficiencies or single nucleotide polymorphisms (SNPs) in enzymes involved in various aspects of methionine and homocysteine metabolism can influence cellular methylation potential.

19.3.1 Glucose and Insulin Regulate Homocysteine Metabolism and Cellular Methylation Potential

In the context of studying the epigenetic epidemiology of T1D, it is important to note that the diabetic physiological state can modify cellular methylation potential by altering methyl group metabolism. Specifically, it has been clearly demonstrated



↑ Glucose ∝ ↓ Homocysteine	Insulin treatment
-↑ flux through transsulfuration -↑ flux through remethylation -Potential altered methylation capacity	-normalizes homocysteine -normalizes transsulfuration

Fig. 19.2 Homocysteine and methionine metabolism. Cellular methylation potential is dependent on the production of S-adenosylmethionine (*AdoMet*), the major methyl donor in the cell. Homocysteine can be remethylated to form methionine. A methyl group can be transferred by the B12-dependent enzyme methionine synthase (*MS*) from 5-methyltetrahydrofolate (*THF*), the product of methyleneTHF reductase and the folate metabolic pathway. In the liver and kidney, homocysteine can also be remethylated by betaine:homocysteine methyltransferase (*BHMT*) using betaine as the methyl donor. Betaine can be derived directly from the diet or from choline. Methionine is converted to AdoMet, which is used by methyltransferases (*MT*) to transfer methyl groups to a variety of acceptor molecules, including DNA and histones. The reaction produces S-adenosylhomocysteine (*AdoHcy*) that can be converted to homocysteine. Alternatively, homocysteine can be irreversibly metabolized to cysteine by the B6-dependent enzymes cystathionine-β-synthase (*CBS*) and cystathionine-γ-lyase (*CGL*) in the transsulfuration pathway. Vitamin co-factors B6 and B12 are highlighted. Circulating homocysteine in T1D patients is lower than in non-diabetic control subjects as a result of increased flux through the transsulfuration pathway, and also potentially by increased flux through remethylation. Insulin treatment normalizes homocysteine by decreasing its metabolism by transsulfuration

that fluctuations in glucose and insulin concentrations, as observed in diabetes, perturbs homocysteine and methionine homeostasis.

Glucose regulates homocysteine metabolism. The concentration of circulating homocysteine is inversely correlated with blood glucose concentrations in diabetic

patients without renal dysfunction [66–68] and rodent models of diabetes [69]. The activities of enzymes involved in homocysteine transsulfuration, including CBS and cystathionine γ-lyase, are also increased in the liver of rats with uncontrolled diabetes [69] and CBS activity positively correlates with glucose concentration in cultured hepatocytes [70]. Chiang et al. suggested that homocysteine clearance under high glucose conditions in cultured hepatocytes could also be attributed to increased homocysteine remethylation flux in a methionine-dependent fashion [71]. High glucose levels were associated with increased cellular methylation potential, as indicated by the AdoMet to S-adenosylhomocysteine (AdoHcy) ratio, increased AdoMet synthase activity and DNA methyltransferase activity and global methylation potential in cultured hepatocytes [71]. The cumulative data suggest that the observed diabetes-related reduction in circulating homocysteine is due to its catabolism by the transsulfuration pathway and potentially by increased rates of its remethylation. The overall result is that the diabetic state impacts cellular methylation potential.

Conversely, insulin normalizes circulating homocysteine in T1D. Insulin treatment normalized the rates of homocysteine remethylation, methionine synthesis and homocysteine transsulfuration in T1D patients [71–73]. Similarly, insulin treatment of rats with streptozotocin-induced diabetes resulted in a normalization of circulating homocysteine [69, 74]. Insulin treatment was associated with decreased activities of transsulfuration enzymes which resulted in a dose-dependent increase in plasma homocysteine [75]. In addition, the flux through the transsulfuration pathway was reduced in hepatocytes cultured with insulin [71]. Insulin also induced folate-dependent homocysteine remethylation under methionine-restricted and normoglycemic conditions in hepatic cells [71]. Together the data suggest that insulin substitution normalizes homocysteine concentrations by reducing the capacity to eliminate it by transsulfuration.

19.3.2 Evidence That the Diabetic State Modifies Gene Expression

Fluctuations in insulin and glucose regulate homocsyteine metabolism and impact cellular methylation potential. Therefore, the diabetic physiological state can potentially impact cellular methylation reactions and methylation-dependent epigenetically-regulated gene expression, a concept for which evidence is accumulating. High glucose concentrations have been associated with dysregulation of methyltransferase activity and expression, global DNA methylation and gene expression [71]. Uncontrolled diabetes in streptozotocin-treated rats was associated with altered homocysteine metabolism, increased expression of hepatic DNA methyltransferase (DNMT) 1 and global hypomethylation of genomic DNA [76]. El-Osta et al. found that transient exposure of aortic endothelial cells to high glucose resulted in stable activating epigenetic changes in the promoter of the pro-inflammatory

nuclear factor κB (NF-κB) subunit p65 and long-lasting activation of its expression [77, 78]. Specifically, transient high glucose conditions resulted in persistent mobilization of the histone methyltransferase Set7 and monomethylation of H3K4 at the proximal promoter of p65 [77]. Also, transient glucose exposure was associated with recruitment of the histone demethylase LSD1 and the loss of suppressive H3K9m2 and H3K9m3 methylation marks at the p65 promoter [78]. Furthermore, cardiomyocytes cultured in high glucose demonstrate decreased IGF-1 receptor expression and were more susceptible to apoptosis. The IGF-1 receptor expression was associated with decreased association of acetylated H4 with the IGF-1 receptor promoter region and consequent reduced promoter activity [123]. In addition to diabetes-dependent effects on cellular methylation potential, new evidence has linked glucose derived acetyl-CoA, the substrate of histone acetylases, to modifications of chromatin structure and global gene expression [79, 80]. Many of these studies focused on the consequences of the diabetic state in tissues related to complications of diabetes rather than those directly involved in disease pathogenesis, leaving the role played by epigenetic mechanisms on diabetes initiation or progression unclear.

19.4 Strategies to Study the Epigenetic Epidemiology of Type 1 Diabetes

A number of strategies at the population level have been employed to successfully identify genetic risk factors associated with Type 1 diabetes (reviewed in [81]). Linkage studies using pairs of affected relatives, usually siblings, allow for the identification of genomic regions associated with risk, but the results tend to be limited to loci that have a large effect. Genome-wide association studies using case–control or family-based designs are able to identify loci with a small or moderate impact on susceptibility. The addition of high-throughput sequencing and SNP genotyping technologies have aided significantly in the identification of candidate risk genes.

Similar population sampling strategies in combination with genome-wide high-throughput screening methods can be implemented in the search for epigenetically regulated susceptibility loci. The "methylome", or genome-wide methylation status of an individual can be queried using array-based or genome-wide methods (reviewed in [82, 83]). Array-based methods, including SNP arrays and high density promoter and CpG island arrays, have been used extensively but are limited in terms of genome coverage and their restriction to specific gene promoters and CpG islands. Alternatively, high-throughput sequencing methods allow for genome-wide analysis of the methylome but are currently limited by their high cost. Genomic DNA associated with specific histone modifications can also be determined using chromatin immunoprecipitation (ChIP) assays followed by microarray (ChIP-on-chip) or direct sequencing of bound DNA (ChIP-Seq). Use of these methods, or a combination

thereof, will allow for the determination of the diabetes-risk epigenome. The key to any study will be the population from which the samples are drawn, the tissues that are sampled and how the data are interpreted.

19.4.1 Prospective Studies of Genetically At-Risk Children

The diabetic state is characterized by fluctuations in insulin and glucose, which can influence cellular methylation potential, global methylation profiles and gene expression. As such, epigenetic changes observed in overt diabetic patients could be related to the diabetic state rather than diabetes risk, making the interpretation of differential epigenetic modifications difficult. Therefore, it will be important to study genetically at-risk, normoglycemic individuals before the initiation of the autoimmune process to elucidate the pre-existing risk epigenome.

A number of prospective cohort studies of genetically at-risk children have already been implemented to determine environmental risk factors for Type 1 diabetes. These include the BABYDIAB study in Germany [84], the Diabetes Autoimmunity Study in the Young (DAISY) in the USA [85], the Finnish Diabetes Prediction and Prevention (DIPP) study [86] and The Environmental Determinants of Diabetes in the Young (TEDDY) study in the USA and Europe [87]. These studies have examined the relation among genetics, *in utero* exposures, diet and infectious agents, to name but a few, in the development of autoimmunity, as indicated by the presence of autoantibodies and progression to overt diabetes. It is easy to conceive that these studies or ones modeled after them could also identify diabetes-associated epigenetic modifications and their relation with various environmental factors. Ideally, sampling would occur in the prediabetic state, as well as during the progression to disease. This would allow for the epigenetic features of diabetes risk to be differentiated from those of the diabetic state, as well as the determination of key epigenetic events that occur during disease pathogenesis. Also, the identification of epigenetic changes due to active disease will be important as they could play a causal role in the development of diabetes complications, including cardiovascular disease. Furthermore, it will be imperative to collect detailed information on exposures to various environmental factors to begin to understand the association of specific factors with changes to the epigenome.

19.4.2 Monozygotic Twin Studies

As previously mentioned, the strongest evidence that environmental factors modify T1D is the lack of 100% concordance in twin pairs. Studies in MZ twins suggest that while genetics play a significant role in the development of autoimmune T1D, it is not sufficient to explain 35–75% of cases [7–9]. Some differences in disease concordance may be explained by environment-dependent differences in the epigenome

between twin pairs. Indeed, DNA methylation content and histone modifications can vary significantly in genetically identical twins. Significant differences in 5-methylcytosine content and H3 and H4 acetylation patterns of genomic DNA were observed in up to 35% of monozygotic twin pairs ranging in age from 3 to 74 years [88] .The epigenetic differences also correlated with age and time spent together, with younger twin pairs being more similar than older twin pairs, and those who had spent more time together being more similar than those who had been separated. Of note, the epigenetic patterns correlated with changes in gene expression [88]. Interestingly, even newborn MZ twins demonstrated variable methylation profiles at differentially methylated regions, such as the *IGF2/H19* locus, indicating the importance of the intrauterine environment in the establishment of the epigenome [89]. Another study demonstrated that the epigenetic profile of an individual can change over time, the concept of epigenetic drift, as demonstrated by changes in global DNA methylation. In two samples taken 11–16 years apart, more than 25% of individuals showed a 10% or greater difference in global DNA methylation [90]. Furthermore, Waterland et al. have identified candidate metastable epigenetic loci, the methylation profile of which significantly correlate among tissues within an individual but demonstrated increased inter-individual variation [91]. Of note, MZ twin pairs demonstrated discordant methylation patterns at some of the identified metastable loci, indicating the stochastic nature of the establishment of methylation patterns at these loci [91]. While these early studies relied on small samples sizes and, in some cases, less sophisticated analyses of epigenetic variation, such as global DNA methylation, together they support the concept that the epigenotype of genetically identical individuals can diverge in a time- and environment-dependent manner. In terms of diabetes discordance in monozygotic twins, this epigenetic drift could explain differences in time to disease onset, disease severity and predisposition to secondary complications.

Twin studies will provide a unique opportunity to elucidate the role of epigenetics in autoimmune diabetes, as they have for other autoimmune diseases such as systemic lupus erythematosus [92]. The pairwise examination of discordant twins will eliminate the confounding effect of genetic polymorphisms on the epigenome, which can influence methylation patterns. For example, a SNP in the SUV39H2 histone methyltransferase was shown to be associated with risk for diabetic nephropathy [124]. However, there exist some limitations. Twin studies could be confounded by significant shared environment and experiences, including those *in utero*. The tissues selected for study will also be important. As indicated previously, many genes involved in the autoimmune process are regulated by epigenetic mechanisms, which could be independent of disease susceptibility. By choosing tissues that are not involved in the immune process and by comparing multiple tissues within and between twin pairs it should be possible to minimize the impact of the autoimmune process. However, the diabetic state can also impact on cellular methylation potential and methylation-dependent epigenetic mechanisms, which could be misinterpreted as being associated with disease risk. It will be necessary to include MZ twins in a prospective cohort of genetically at-risk individuals to allow for their assessment in a prediabetic, normoglycemic state.

19.4.3 Comparative Studies Examining the Effect of In Utero and Neonatal Exposures

The epigenome is likely the most plastic and sensitive to environmental-mediated changes during embryonic development. Upon fertilization, gamete-related epigenetic marks are erased and replaced with embryonic marks [93]. Later in fetal development, developing gametes undergo epigenetic reprogramming in which parental marks are replaced by stem cell marks [93]. It is during these periods of flux that the epigenome will be the most susceptible to modifications by nutritional and other environmental factors. In addition, since epigenetic mechanisms regulate many of the cytokines involved in immune responses, including tolerance to dietary antigens and immunity to infections, exposures to environmental factors in the neonatal period can impact the establishment and maturation of the immune system, which will have long term consequences for future immune responses.

19.4.3.1 Gestational Diabetes

Gestational diabetes is diagnosed when an abnormal oral glucose tolerance is observed for the first time during pregnancy (reviewed in [94]). The incidence of gestational diabetes is increasing in parallel with the obesity epidemic, however, 10% of cases are of autoimmune origin. Consequences for offspring affected by gestational diabetes include increased risk for obesity, metabolic syndrome and Type 2 diabetes. However, evidence is mounting that Type 1 diabetes could also be associated with this *in utero* experience [95, 96]. Increased maternal body mass index (BMI) before and excessive weight gain during pregnancy, risk factors for the development of gestational diabetes, are associated with increased risk of islet autoimmunity in genetically at-risk children [97]. Also, children born to mothers with gestational diabetes are more likely to have increased birth weight, faster weight gain and higher BMI, which have been associated with increased risk for islet autoimmunity, progression to T1D and earlier age at disease onset in genetically at-risk children [98–101].

Of note, diabetic pregnancies have a 2–5-fold increased risk of being complicated by a congenital anomaly [102, 103]. Animal studies show that folic acid treatment can prevent gestational diabetes-associated anomalies [104], indicating that diabetes-related altered folate metabolism impacts the developing embryo. As such, altered folate metabolism during pregnancy as a result of gestational diabetes could also impact the cellular methylation potential and epigenome of the offspring. Ideally, prospective samples will be taken from children exposed to gestational diabetes *in utero* beginning at birth to determine the effect of the intrauterine environment on the epigenetic underpinnings of disease risk, as well as the relation with timing and severity of disease development. Information such as maternal BMI, diet, supplement use and other lifestyle factors from the periconceptional period and pregnancy should be collected to allow for associations to be made between *in utero* experiences and epigenetic-dependent risk in the offspring.

19.4.3.2 Maternal and Neonatal Diet

Early diet exposures, including exposures *in utero*, have been associated with the development of Type 1 diabetes in both humans and animals [11]. Whether or not the various dietary manipulations affect epigenetic-dependent disease susceptibility remains to be determined. However, the Agouti mouse model has been exploited to demonstrate an elegant proof of principle that maternal diet during gestation modifies the epigenome [105]. The Agouti mouse has an IAP retrotransposon insertion that contains a cryptic promoter upstream of the *agouti* gene. Hypomethylation of the cryptic promoter results in ectopic expression of the gene and yellow coat color. Conversely, hypermethylation inhibits promoter activity and results in brown coat color. Therefore, coat color acts as a phenotypic proxy for the methylation status of the promoter. Maternal nutrition, specifically the intake of methyl donors during pregnancy, was shown to modify coat color as a result of altered methylation of the IAP transposable element in the offspring [105]. In addition to these early animal studies, the methylation of candidate metastable epialleles in humans was associated with maternal nutritional status during early pregnancy [91].

In relation to T1D, maternal diet during gestation has been associated with the progression of autoimmunity and the development of disease [106]. One example is maternal intake of vitamin D during pregnancy, which has been associated with decreased risk of and increased time to autoantibody development in the offspring [107, 108]. Also, the introduction of diet components and the timing of their introduction in the neonatal period are important in determining the risk for diabetes in genetically at-risk infants. For instance, the timing of exposure to specific cereals has been associated with increased risk for autoantibody development in at-risk children [109, 110]. Other early diet exposures linked to diabetes risk have included duration of breast-feeding and introduction and timing of introduction of cow's milk formula [111].

In addition, heavier babies at birth, a potential indication of over-nutrition during gestation, have a small but significant increased risk for developing T1D [101]. Conversely, animal models demonstrate that maternal under-nutrition during pregnancy is associated with reduced risk for diabetes and abnormal development of the pancreas in offspring. Under-nutrition due to caloric restriction *in utero* reduces the incidence of diabetes in NOD mice [112]. A low protein diet fed during pregnancy and lactation was associated with decreased islet mass and decreased autoimmunity in NOD mice, and decreased islet mass and islet vascularity in rats [113, 114]. The smaller islet size and impaired insulin release observed in the offspring of low protein fed dams could be attributed to changes in the balance of beta cell replication and apoptosis in fetal and neonatal life [115]. It is possible that the changes observed in offspring are due to nutrition-dependent epigenetic modifications in the developing embryo but this relationship requires empirical determination. This can be achieved with the careful selection of study subjects, such as mother's that are themselves at genetic-risk for T1D, as well as detailed information about food intake, nutritional status and supplement use during pregnancy.

19.4.4 Complications of Type 1 Diabetes

Chronic diabetes is associated with a number of complications including diabetic nephropathy, neuropathy, and cardiovascular disease, which diminish quality of life and life expectancy. As previously noted, the diabetic state defined by high glucose and low insulin can modify the cellular methylation potential by altering the flux of homocysteine remethylation and transsulfuration, thereby regulating the availability of AdoMet for cellular methylation reactions (Sect. 19.3, Fig. 19.2). The careful choice of control subjects will be critical for defining the impact of the diabetic state on epigenetic drivers of diabetes complications. It will be necessary to use control subjects with T1D but without the specific co-morbidity in addition to healthy control subjects to differentiate diabetes/dysregulated glucose homeostasis-dependent from diabetes complication-dependent epigenetic signatures. Other critical factors to consider will be age at diagnosis, length of disease and disease severity. A recent study performed a genome-wide CpG island methylation analysis to identify differentially methylated sites associated with diabetic nephropathy. The methylation status of 19 CpG sites were shown to correlate with time to nephropathy, including one CpG site in close proximity to a gene that had previously been associated with nephropathy [125]. The cases and controls were selcted to control for T1D duration, age at diagnosis, age and sex.

19.5 Conclusion

Based on our understanding of disease mechanisms and the growing body of evidence for the involvement of epigenetics in autoimmune and other diseases, it is plausible that epigenetic mechanisms underlie at least a proportion of the risk for Type 1 diabetes. However, very few studies have empirically tested the relationship. A number of epigenetically-regulated genes associated with T1D have been identified, representing a starting point for a candidate gene approach to determine their role in disease pathogenesis. Altered epigenetic regulation could affect T1D susceptibility in a number of ways and could be dependent on timing as well as on the tissues involved. The intrauterine environment, especially during periods of epigenetic flux such as post-fertilization, may be the most important environment to affect the T1D-susceptibility epigenome. Epigenetic mechanisms could act as T1D-susceptibility modifiers during other periods such as pancreas development, immune system maturation, the initiation and progression of the autoimmune process and even once overt diabetes has been established, through the impact of epigenetically regulated genes on the development of T1D-related co-morbidities. As such, studies must be thoughtfully designed to address numerous caveats, including the timing of recruitment and sampling, the collection of data relevant to environmental exposures including diet and infections, and the tissues sampled. Ideally, data will be derived from prospective cohort studies of genetically at-risk individuals using

powerful genome-wide high-throughput technologies. Only then will it be possible to begin to unravel the T1D risk epigenome and the environmental factors that influence it.

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Chapter 20
Epigenetic Epidemiology of Obesity, Type 2 Diabetes, and Metabolic Disorders

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Abstract Knowledge and inclusion of epigenetic variants as potential risk factors for obesity and type 2 diabetes (T2D) will improve diagnosis and mechanistic understanding of these complex metabolic disorders. A number of practical and conceptual issues still have to be worked out before epidemiological studies can fully integrate epigenomic approaches. Altered patterns of histone modifications,

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DNA methylation, and microRNA levels are implicated in obesity and T2D. Recent studies are discussed, thereby illustrating strengths and challenges to accurately identify and measure epigenetic differences that covary with metabolic disorders. Unlike classical, DNA sequence-based approaches, the choice of cell or tissue type as source material may confound the results of such epigenomic studies. It is becoming clear that various types of epigenetic signatures exist; some are very stable and do not change over many years within an individual, whereas others are liable to change and perhaps more influenced by age and environmental and physiological conditions. A standardized classification system to accurately describe and characterize the properties of newly identified epigenetic signatures will enable the generation of reproducible epigenetic data.

Abbreviations

AS	Angelman syndrome
BMI	Body mass index
CNV	Copy number variation
DMR	Differentially methylated region
GWAS	Genome-wide association study
LOI	Loss of imprinting
PBL	Peripheral blood lymphocyte
PWS	Prader-Willi syndrome
SNP	Single nucleotide polymorphism
T2D	Type 2 diabetes
VMR	Variably methylated region

20.1 Introduction

This chapter discusses challenges and issues related to the budding field of epigenetic epidemiology of T2D and non-syndromic obesity. Recurrent themes are the choice of tissues and cells, the difficulty to efficiently measure epigenetic marks with accuracy in many samples, and the necessity to collect additional information such as environmental variables, age, and gender – all of which can influence the nature and stability of epigenetic marks. Since almost any change in cellular physiology is likely to result in some change in epigenetic marks, there will always be an association with metabolic disease. Distinguishing between association and causality of epigenetic marks will become a main issue for this research field. In this context, the small number of available studies are examined that highlight strengths and problems in determining the role of epigenetic variation in T2D, obesity, and related metabolic disorders.

20.2 Complementing Genetic Approaches to Understand Metabolic Syndrome: Challenges of a Young Discipline

Epigenetic research into T2D and obesity is still a very young field. The role of epigenetic mechanisms in the etiology of these disorders and related metabolic abnormalities such as dyslipidemia, hypertension, and hyperglycemia is not well understood. Nevertheless, interest is growing rapidly and epigenetic concepts are now being integrated in models to understand complex disease phenotypes [1].

This comes at a time when we realize that variations in DNA sequence reveal much less about disease predisposition than was widely expected. The genetic contribution to T2D and obesity – long presumed to be significant with heritability estimates reaching 70% in twin studies [2–4] – may be lower in reality. Genome-wide association studies (GWAS) have extracted a number of single nucleotide polymorphisms (SNPs) that are consistently associated with disease risk within the general population. Their contributions are small and currently can explain less than 10% of the presumed heritability of metabolic conditions [5]. As SNPs fail to sufficiently enlighten the heritable basis of non-syndromic T2D and obesity, attention has also turned to larger structural differences among human genomes. Quantitative variation of genomic regions, including insertions and deletions (indels) and common copy number variations (CNVs), were thought to add to disease risk. Yet, recent studies indicate that these types of genomic differences also fail to explain susceptibility to metabolic syndrome-related diseases [5, 6].

Studies on the role of epigenetics in T2D and obesity are lacking. At present, there is a disparity between the large number of hypothesis-based review articles and the few reports documenting solid, experimental evidence of an epigenetic role in T2D and obesity in human populations. Although for the remaining part of this chapter epigenetics is discussed as likely contributing factor, it is possible that epigenetics plays no causal role in the etiology of complex metabolic disorders.

Several factors account for the paucity of epigenetic data. Because it is a young research field, resources are only starting to be committed to this new line of investigation. Genomic loci carrying epigenetic signatures must be identified, which are unmistakably associated with phenotypes of metabolic syndrome. A number of candidate genes for such studies now exist, but further characterization is necessary before they can be confirmed as suitable biomarkers. It has been argued that large networks of non-imprinted and imprinted genes may acquire epigenetic modifications during early stages of life, resulting in a disease predisposition [7, 8]. Microarray-expression studies suggest that transcription levels of hundreds or thousands of genes are changed in animals and humans with symptoms of T2D and obesity [9, 10]. Whether epigenetic modifications underlie and maintain these altered gene expression states over time has not been established. If, as we expect, many genomic loci become modified and acquire epigenetic marks associated with T2D or obesity as a result of adverse lifestyle factors such as poor diet, lack of exercise [11], we must further determine whether they are causative and disease-promoting.

Existing methods enable epigenetic profiling of the entire human genome (Chap. 4). Many of these techniques were developed by probing DNA, histones, or chromatin from cell lines – homogenous populations of cells – and the resulting datasets have moderate levels of “background noise.” The complexity and heterogeneous mix of cell types typical for human tissues is a complicating factor that adds to the challenges of epigenetic epidemiology. What is the currently available evidence in humans on the role of epigenetic variation in metabolic disorders and what can we learn?

20.3 Muscle: Genetic and Epigenetic Analysis of COX7A1

A small cohort study explored the relation between genetic and non-genetic factors and their potential roles in contributing to altered gene expression in insulin resistance and type 2 diabetes [12]. Insulin resistance in skeletal muscles is an early characteristic in diabetics and insulin-insensitivity may be linked with impaired mitochondrial function, which is another symptomatic abnormality. Nuclear-encoded genes contributing to mitochondrial function are often down-regulated in insulin-resistant muscle [13–15]. One of these genes is *COX7A1*, which encodes a component of the mitochondrial respiratory chain. A compelling feature of the work by Ling and colleagues is that they examined *COX7A1* in twins by sampling the *vastus lateralis* muscle [12]. This muscle is prone to become insulin insensitive and is pathologically affected in obese and diabetic individuals. Variables contributing to a reduction of *COX7A1* expression levels in muscle were considered and included (a) SNPs, representing variations in genotype; (b) age, representing a non-genetic factor; and (c) DNA methylation, representing epigenetic variation.

Genotype: Although one SNP (rs753420) appeared to influence basal *COX7A1* expression, the effect of this particular genotype was only detectable in muscle of young twins ($n=110$/age 28.0 ± 1.9), which were a subset of the study participants [12]. Overall, *COX7A1* genotypes did not reveal any associations with T2D-risk in the sampled group ($n=4{,}296$).

Age: One of the key findings was that *COX7A1* expression levels decrease significantly with age. This non-genetic variable must be considered when conducting and interpreting gene-expression data related to metabolic traits – age appears to have a strong influence on certain genomic loci (see also Chap. 10)

DNA methylation: To measure DNA methylation levels of the *COX7A1* promoter, samples from a small subset of individuals were analyzed, which included ten young twins (age 28.0 ± 1.9) and ten older twins (age 62.4 ± 2.0). PCR products derived from sodium bisulfite converted DNA were cloned and subsequently sequenced. The promoter region contains 22 CpG dinucleotides; these are potential methylation sites. The sequencing data from each individual was pooled and used to determine the proportion of *COX7A1* methylation by dividing the number of methylated CpGs

by the total number of CpGs in the data set compared to the young twins, DNA methylation levels were higher in older twins. While the *COX7A1* DNA methylation levels were higher, they were also found to be more diverse among individuals of the elderly twin-group. Thus, DNA methylation patterns diverge with age and as a result of environmental exposures, a phenomenon that has to be considered in the design of population-based, epigenetic studies [16] (see also Chap. 10).

DNA methylation levels surrounding a promoter are generally assumed to provide an adequate proxy for gene activity, yet Ling and colleagues did not observe a significant correlation between DNA methylation levels and *COX7A1* expression [12]. Measurements of this epigenetic modification were performed on DNA samples derived from skeletal muscle – relevant to disease – and the same tissue samples were used to determine *COX7A1* mRNA levels. Does the lack of correlation between DNA methylation and gene expression indicate a lack of functionality? Is it worth to assess DNA methylation levels at individual loci, or will such studies consume disproportionate amounts of resources without providing meaningful information, as so often is the case for SNP-studies? Is there a possible explanation for the mismatch between the *COX7A1* methylation profile and expression levels?

For each individual, methylation patterns of ten DNA molecules (epialleles) from ten cells were analyzed. While this approach – subcloning and sequencing of PCR products derived from bisulfite-treated genomic DNA – yields the most accurate information of cytosine methylation patterns of individual DNA molecules, it also bears several disadvantages, particularly if only a limited number of sequences are being used to measure DNA methylation levels. Biased PCR amplification of a subset of template DNA molecules, or biases during the subcloning procedure, can often lead to a quantitative misrepresentation. Failing to reflect the true distribution of the many possible types of epialleles, even subtle biases in sampling regularly contribute to the distortion of data sets. Redundant sampling of the same DNA molecule – and thus the same DNA methylation pattern – is particularly frequent when only limited amounts of template DNA are available. The muscle biopsies studied by Rönn and colleagues were likely composed of different cell types making the comparison, on the basis of about ten cells per individual, more challenging. The above-mentioned technique to analyze DNA methylation patterns is unsurpassed at the level of individual DNA molecules – every potential cytosine methylation event within the targeted region can be detected. This is a suitable method if the DNA methylation state of a genomic locus is examined for the first time and is considered as a potential epigenetic biomarker for T2D and obesity.

To establish robust methylation profiles for loci that have a broad range of DNA methylation densities, a large number of epialleles must be examined. Exceptions would be genes and promoters that normally remain methylation-free in all human tissues and cell types; here, any trace of cytosine methylation could indicate a disease predisposition. Such epigenetic lesions exist in tumor samples (see Chap. 12). Identification of this type of clear-cut, unambiguous epigenetic alteration associated with metabolic disease risk would greatly advance population-based screens.

Given an unbiased sampling, working with large numbers of epialleles also reduces the problem of cell type diversity in tissue specimen. The more epialleles we analyze per tissue sample, the better the representation will be of the various cell types present in a specimen. Thus, even if the epialleles are derived from a tissue composed of heterogeneous cell types, an overall representative methylation profile will emerge from a locus that carries diagnostic value. That is, the epigenetic profile will likely differ between healthy and diabetic/obese individuals, but the differences might be very subtle. Unlike genetic testing, the results will have to be interpreted in "shades of grey" rather than "black and white." Technically, small research groups

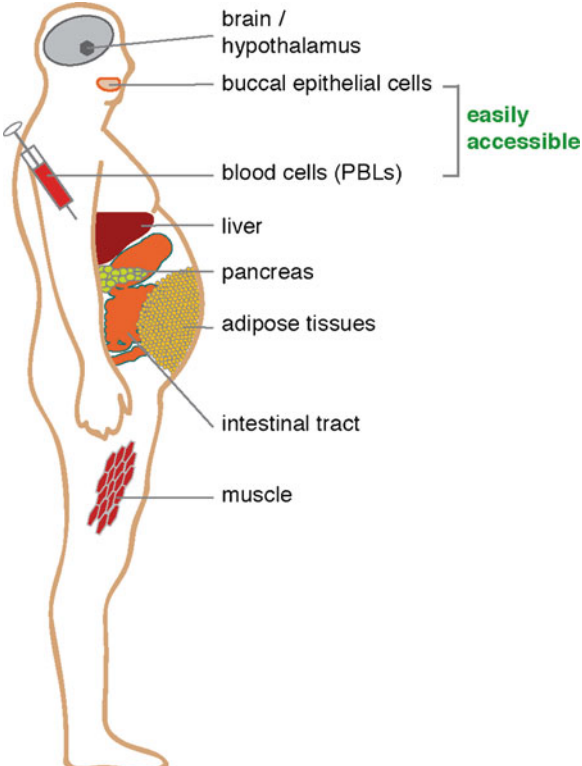

Fig. 20.1 Energy homeostasis is a fundamental physiological process that requires interaction and regulation of multiple organ systems, tissues, and cell types of the body. The central nervous system – the hypothalamus in particular – integrates signals from peripheral tissues and controls food intake, satiety, and energy expenditure. Most of the tissues relevant for our understanding of T2D and obesity (muscle, intestinal tract, adipose tissues, pancreas, liver, and brain) cannot be easily sampled and are not suitable for large, epigenetic population studies. In contrast, peripheral blood cells and buccal epithelial cells can be collected without difficulty and are frequently used as a DNA source for genetic screens; whether these cell types carry epigenetic biomarkers that identify disease risk for either T2D or obesity is insufficiently studied

can carry out this technique. Nevertheless, it is labor and time intensive, and as a result costly and not well suited for epidemiological studies.

In order to advance the research field it will be important to establish epigenetic profiles for genomic loci in tissues, such as muscle, which play a pathophysiological role. Because of the obvious difficulties of obtaining these tissues, other research groups cannot easily validate such data. Future studies will benefit from comparative epigenetic testing, by matching data from, for example, muscle, with data derived from peripheral blood cells from one and the same person. In this way, it will be possible to build databases that allow us to determine whether the epigenetic state of a given genomic locus is similar in disease-affected tissues and simple-to-collect tissues. In an ideal setting, the epigenotype in the affected tissue will be mirrored by the epigenotype in peripheral blood lymphocytes (see also Fig. 20.1).

20.4 Pancreatic Tissue

Suboptimal insulin secretion and changes in the number of beta-cells – one of the endocrine cell types in pancreatic islets – contribute to the etiology of T2D [17]. Altered gene expression programs in pancreatic islets could be an underlying factor for disease risk. Several research groups have recently started to investigate the role of chromatin, epigenetic modifications, and genetic variants in pancreatic islets.

20.4.1 Specific Epigenetic Signatures and Genetic Variation

Gaulton and colleagues established a map of open chromatin sites in human pancreatic islets and compared the results with chromatin maps of non-islet cell lines [18]. Their chromatin-profiling experiments demonstrated that pancreatic islets have a distinct, tissue-specific distribution of open chromatin sites. Thus, a particular epigenetic information – relevant for our understanding of T2D and obesity – can be confined to difficult-to-collect tissues.

The authors discovered that one of the islet-specific open chromatin sites overlaps with the genomic position of a T2D-associated SNP (rs7903146) [18]. This SNP maps to intron 3 of the gene encoding transcription factor 7-like 2 (*TCF7L2*). The T risk-allele was found to be in a more “open chromatin” structure, indicating reduced nucleosome occupancy at this locus in comparison with the non-risk C-allele. Such a “relaxed” chromatin state correlates well with increased *TCF7L2* expression of the T risk-allele, which had been observed earlier [19, 20]. Carriers of the *TCF7L2* risk-allele are found to have impaired insulin secretion [21, 22].

Establishment of cell type specific chromatin profiles and their correlation with disease-associated SNPs is a promising approach to unravel the molecular and epigenetic mechanisms that lead to the phenotypes associated with the metabolic syndrome [23].

20.4.2 Epigenetic Nomenclature and the Characterization of Epialleles

The correlation between the T risk-allele and an open chromatin state suggests that epigenetic variation at the *TCF7L2* locus may either be "obligate" or "facilitated." These terms have been proposed by Richards and allow classification of epigenetic variants [24, 25]. An obligate epigenetic variant directly corresponds with a given genotype; in this case, the T-risk allele would always be associated with open chromatin. The facilitated epigenetic variant is somewhat dependent on the genotype, but would not necessarily have to be linked with the DNA sequence variation; in this case, the T risk-allele would often be associated with open chromatin. It is likely that many associations between epigenotype and T2D/obesity-risk phenotype exist that are completely independent of the genotype [7]. Using the nomenclature proposed by Richards such genotype-independent epigenetic variants would be labeled as "pure" [24].

20.4.3 The Histone Methylation Profile of Pancreatic Islets

Human pancreatic islets were also subject of a recent epigenetic analysis by Kaestner's group [26]. A genome-wide map of histone modifications was established by chromatin immuno-precipitation and massively parallel sequencing technology (ChIP-seq). The relative distribution of two marks was recorded: methylation of histone 3 lysine 4 (H3K4me) is usually associated with active genomic loci; in contrast, methylation of histone 3 lysine 27 (H3K27me) usually correlates with genomic repression. Unlike many other epigenomic studies that sample cell lines – and therefore obtain fairly clear-cut results – this study looked at the mixture of cells present in the pancreatic islets. Assuming that these pancreatic cells were obtained from individuals with no apparent metabolic dysfunction, the results provide a glimpse of the normal histone-modification profile present in cells enriched from this endocrine tissue. For example, pancreatic and duodenal homeobox 1 (*PDX1*) and *MAFA* are genes involved in differentiation and regulation of beta-cell function and despite relative low expression levels, their promoters are highly occupied with H3K4 trimethylated nucleosomes. Unexpectedly, only low levels of H3K4 trimethylation were detected around the insulin (*INS*) and glucagon (*GCG*) promoters, leading the authors to speculate that alternative epigenetic modifications and regulatory mechanisms promote high-level transcription of both genes in vivo [26].

The genome-wide map of H3K4me3 marks in pancreatic islets created by Kaestner and colleagues corroborate the tissue-specific distribution of this histone modification [26]. The pancreas dataset was compared with a high-resolution histone modification profile derived from CD14+ T-cells described by Barski et al. [27]. Islet cells have comparatively high H3K4me3 levels in a small subset of genes (< 100) and the associated gene promoter sequences have a low content of CpG dinucleotides. This small list of genes with a pancreatic-specific histone modification profile could prove very useful for comparative epigenetic studies and might indicate where differences in chromatin structure and gene expression will be detected

in the diseased state. As pancreatic islets play a significant role in the etiology of diabetes and carry their own distinct epigenetic profile, it becomes clear that this tissue could provide important insights, but unfortunately it is an impractical choice for large, population-based studies (Fig. 20.1)

20.4.4 Epigenetic Signatures of the Insulin Locus

The epigenetic makeup of the insulin (*INS*) locus has been characterized in more detail by Mutskov and Felsenfeld, who also used freshly isolated human pancreatic islets as study material [28]. *INS* maps to a region of human chromosome 11p15.5, within an ~ 80 kb gene-rich cluster including tyrosine hydroxylase (*TH*), insulin-like growth factor 2 antisense (*IGF2AS*), and insulin-like growth factor 2 (*IGF2*). High levels of histone acetylation and H3K4 dimethylation – both epigenetic marks associated with gene activity – were present throughout the genomic region, indicating that this gene cluster lies within an unexpectedly large and open chromatin domain. Overall the epigenetic profile was characteristic for all tested pancreatic islet cells. However, the authors noted some variation among the samples without further exploring the cause of these presumably subtle, epigenetic differences [28]. Variations in the purity and quality of the isolated pancreatic islets may explain such differences, exemplifying one of the practical difficulties that must be overcome in order to establish "normal" base-line data. It will be of interest to create similar epigenetic profiles in pancreatic islets from obese or diabetic individuals and to determine whether or not the large, open *INS* chromatin domain at 11p15.5 is significantly altered in the diseased state.

Tissue-specific, intergenic transcription of non-coding RNA was observed within the 80 kb *INS* gene cluster in pancreatic islets, but was absent in HeLa cells and human brain tissue [28]. Islet-specific intergenic transcription correlates in a linear fashion with *INS* expression levels. In addition, two sites within the *INS* locus are devoid of DNA methylation, perhaps demarcating transcriptional start sites for non-coding, intergenic islet transcripts. At this stage we do not know if and how DNA methylation levels change at 11p15.5 in individuals with metabolic syndrome, nor is it clear whether alterations of intergenic RNAs around *INS* locus correlate with – or even contribute to – diminished metabolic health. A role of non-coding RNAs must be considered, as microRNAs are known to modulate insulin promoter activity and insulin mRNA levels in the adult mouse pancreatic islets [29].

20.5 Brain Tissue

The brain, and the hypothalamus in particular, plays a key role in modulating appetite and energy expenditure (Fig. 20.1). This region of the brain integrates information from peripheral organs, including the intestinal tract and adipose tissues and connects

via neuronal pathways with different brain regions and the endocrine system [30–32]. Within the hypothalamus the suprachiasmatic nucleus (SCN) orchestrates peripheral, circadian body clocks, which in turn regulate crucial metabolic processes. Recent studies provide evidence for a "circadian epigenome," linking daily metabolic rhythms with oscillating chromatin changes and histone modifications (reviewed in [33]). Because this tissue cannot be easily sampled, animal studies may have to suffice in the identification of T2D or obese-specific epigenetic patterns in the hypothalamus.

20.6 Peripheral Blood Lymphocytes

Peripheral blood lymphocytes (PBLs) are routinely collected and are an economic and feasible source for molecular epidemiology studies. A pertinent question then is whether this source of DNA can be used to probe epigenetic differences that accurately reflect a given complex phenotype.

20.6.1 Example and Concept of PBL DNA for Epigenetic Studies

For certain conditions, such as Fragile-X syndrome, DNA methylation patterns derived from PBLs are indeed good diagnostic indicators. The degree of cytosine methylation mosaicism at a single locus – the *FMR1* promoter – in PBL DNA was shown to correlate remarkably well with the degree of cognitive impairment of a fragile X individual [34, 35]. The symptoms of fragile X syndrome can range from mild learning impairments to severe intellectual disabilities [36] and although blood cells do not contribute to the fragile-X phenotype they are suitable for epigenetic profiling of this predominantly neurological disorder because aberrant DNA methylation at the *FMR1* locus is likely established in the germline or during early stages of embryonic development [37, 38]. As cells divide and differentiate, *FMR1* methylation is maintained in neurons but also in the multiple distinct cell types present in peripheral blood. Proportions of the different cell types in a population of PBLs can vary dramatically even between samples taken from a single individual at different time points; an infection, for example, significantly alters the cell type ratio in PBLs. Thus, if an epigenetic marker is not derived from the germline, or undifferentiated embryonic cells, but is particular to one blood cell type, proportional changes in the PBL pool of cells can result in apparent – but misleading – changes of the epigenotype.

20.6.2 Establishment of Epigenetic Patterns Reflecting T2D and Obesity Risk

Epidemiologic studies suggest that at least some epigenetic patterns, indicative of a predisposition for metabolic disorders, could be established during early stages in

life, propagated and maintained in cells of an adult person [39–43]. That is, a population of embryonic and fetal cells may acquire epigenetic marks that are passed on to developing tissues that contribute to metabolic disease. These include the pancreas, muscle, adipose, and brain, but also to cell types not directly associated with T2D and obesity, such as hematopoietic stem cells, the precursors of mature blood cells. Questions then arise about the causes, timing and stability of varying epigenetic states. Early life events indeed seem to have lasting effects, influencing metabolic disease risk in adulthood; this topic is covered in detail in Chaps. 6 and 7. Briefly, there is clear evidence that environmental factors – nutrition in particular – experienced during pregnancy and perinatal development can predispose to the metabolic syndrome. Studies of the "Dutch hunger winter" cohort are a classic example. A transportation blockade imposed by the Nazis caused a severe famine during the winter of 1944/1945 in the western region of the Netherlands. Epidemiological studies revealed that individuals who were exposed in utero to this severe food shortage are significantly more likely to be diabetic and overweight [44, 45].

20.6.3 Results from the Dutch Hunger Winter Cohort

Heijmans and colleagues recently reported their first results of epigenetic profiling of individuals of the Dutch hunger winter cohort, using DNA derived from PBLs [46, 47]. Genes chosen for their methylation measurements have been implicated in the etiology of obesity, T2D or related metabolic dysfunctions. More than 60 years after the Dutch famine, DNA methylation levels were detected that appear to differ between individuals who were exposed to the famine during pregnancy and their same-sex siblings who were either born before, or conceived after the hunger winter [46, 47]. In exposed individuals, seven loci were found to have different DNA methylation levels; they include the imprinted genes *IGF2, GNASAS, MEG3*, and *INSIGF* and non-imprinted genes *IL10, ABCA1, LEP*. The results are remarkable and imply that hematopoietic stems cells of adults retain epigenetic memories of environmental events that occurred in early life. The results further suggest that it may be possible to use PBLs for epigenetic epidemiology studies of the intrauterine origin of T2D and obesity although these cell types are not directly implicated in contributing to the complex, metabolic disease phenotypes (although it could be argued that infiltration of marcophages and pro-inflammatory T-lymphocytes in adipose tissue is an important feature in obese and insulin-resistant patients [48–52]).

DNA methylation levels in the Dutch hunger winter cohort were measured by a method based on mass spectrometry (Epityper, Sequenom) [46, 47]. While the quantitative nature and reproducibility of this approach is generally considered to be accurate, the results provide only average cytosine methylation estimates for all CpGs combined of a given genomic region, and can be influenced by genetic polymorphism. Importantly, this approach does not reveal the nature of methylation patterns on individual DNA strands; this is relevant for our understanding of the processes that shape the DNA methylation profile at a given locus.

Leptin (*LEP*), encoding an adipocyte hormone regulating energy homeostasis, was one of the genes found to have higher methylation levels in exposed individuals of the Dutch hunger winter cohort study [47]. Cytosine methylation patterns vary considerably at the human *LEP* locus in adipose tissues, where *LEP* is highly expressed [52, 53]. Pronounced epigenetic variation – mosaicism of cytosine methylation – of the *LEP* promoter has also been detected in DNA isolated from PBLs [52]. Examples of such methylation mosaicism among *LEP* epialleles from PBLs are shown in Fig. 20.2; they illustrate the complex nature of epigenetic patterns that may be present in many autosomal, non-imprinted genomic loci. Based on the broad range of methylation densities in healthy individuals, it may be a challenging task to differentiate between normal methylation levels and those that predispose to disease.

We can assume that *LEP* methylation densities reported for the individuals by Heijmans et al. reflect the heterogeneous mixture of epialleles present within each of the sampled PBL-DNAs. As Fig. 20.2 depicts, some individuals have a larger proportion of more densely methylated epialleles and will therefore register to have higher *LEP* methylation in PBL-DNA. If *LEP* methylation densities also reflect an early-life event – as the results by Heijmans and colleagues imply – it must be assumed that DNA methylation patterns do not change significantly at the *LEP* locus as cells divide and differentiate from a pool of hematopoietic stem cells into the short-lived PBLs. This would suggest that epigenetic marks established at the *LEP* promoter are very stable in cells of the hematopoietic system and the locus would have the potential to be used as an epigenetic biomarker for metabolic disease risk set in utero. Detailed studies and longitudinal methylation profiles need to be established that trace the *LEP* DNA methylation in order to determine the epigenetic stability of this genomic locus.

There is evidence that DNA methylation levels are influenced by gender. PBL-derived DNA of males has methylation levels that are reportedly somewhat elevated in *Alu* and *Line-1* elements, as well as in differentially methylated regions (DMRs) of imprinted loci, including *PEG3, NESP55*, and *H19* [54]. Sex-dependent variations were also observed in DNA samples of the Dutch hunger winter study. In particular, exposure to famine around the time of conception influences the methylation levels of *LEP, INSIGF*, and *GNASAS* in males [47]. Such findings add another layer of complexity and any epigenetic biomarker considered must first be scrutinized for gender-specific differences if epidemiologic studies are to succeed.

20.6.4 Results from the AGES Cohort

A recent study by Feinberg and colleagues also suggests the existence of epigenetic marks in lymphocyte DNA that correlate with the body mass index (BMI) of an individual [55]. An approach termed "CHARM" was used to measure DNA methylation levels across the genome. CHARM is a comprehensive high-throughput

Hematopoietic stem cells

- Are *Leptin* methylation patterns established in early life?
- Do individual stem cells maintain a particular pattern throughout their life span?
- Do these methylation patterns change significantly during differentiation?
- Are they influenced by environment, age, gender, circadian rhythms, etc ?
- Do these patterns reflect DNA methylation levels in other tissues of a person?
- Do these methylation patterns modulate *Leptin* expression levels?

Leptin methylation in mature, peripheral blood lymphocytes

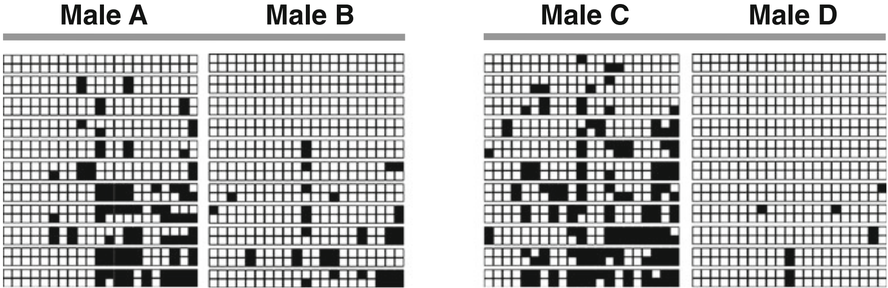

Fig. 20.2 An example of DNA methylation mosaicism. Marked variation in methylation densities is characteristic for the Leptin (*LEP*) promoter in cells of an individual, as well as between individuals. DNA samples from peripheral blood lymphocytes (PBLs) were analyzed in two age-matched pairs of adult males: males A & B, and males C & D, respectively. The DNA samples were received anonymized and it was not possible to determine whether the *LEP* methylation status of an individual correlates with body mass index, health status, or environmental variables. Further epigenetic characterization of the *LEP* promoter will be necessary before we know if this genomic locus can be used as a reliable biomarker for epigenetic epidemiological studies. It is not clear if and how different methylation densities of the promoter influence transcription levels of *LEP* mRNA. The data were obtained using hairpin-bisulfite PCR, a method that provides information on the methylation status of CpG sites on both strands of individual DNA molecules. For each male, a column is depicted with methylation patterns from 11 double-stranded DNA molecules. The two rows of boxes within each thin rectangle stand for CpG sites on the two strands of individual DNA molecules; unmethylated CpG sites are shown in *white*, while methylated CpG sites are shown in *black*

array-based relative methylation analysis platform, combining tiling arrays and statistical procedures [56]. With a hypothesis-neutral analysis of around four million CpG sites (approximately one seventh of all CpGs of the human genome), this study currently represents the largest screen to identify genomic loci whose epigenetic make-up relate to BMI [55].

The authors had access to samples of lymphocyte DNA from participants of the Age, gene/environment, susceptibility (AGES) cohort. The AGES cohort consists of Icelandic individuals partaking in an ongoing longitudinal study, which originally began in 1967 [57]. The phenotypes of these individuals have been documented in detail, including body composition and metabolism. Two DNA samples from each individual, collected about 11 years apart, were included in the methylation analysis. At the time of the first of these two DNA sample collections, individuals were between 58 and 85 years of age. Overall, Feinberg and colleagues analyzed data from 74 individuals [55].

Methylation levels of more than 200 genomic loci were found to vary considerably among the tested individuals. These variably methylated regions (VMRs) could be further categorized into three groups. Site-specific "dynamic" VMRs (41 out of 227) seem to undergo large changes in methylation levels over time within an individual (intra-individual variation). About a third of the VMRs (67 out of 227) are "ambiguous" with respect to intra-individual methylation change over time. The third group of VMRs (119 out of 227) was categorized as "stable"; the methylation levels of these particular genomic regions did not change in an 11-year time span. Indeed, in analogy to a person's SNP profile, it was possible to identify individuals on the basis of their unique methylation profiles of stable VMRs [55]. It is not clear why the methylation levels of these VMRs are stable but vary among individuals. Using Richards' aforementioned classification system [24], future studies will likely clarify whether these stable VMRs are "obligate" (entirely related to an individual's genotype), "facilitated" (somewhat influenced by genotype), or "pure" (entirely uninfluenced by genotype) epigenetic variants.

Detection of stable VMRs allowed Feinberg and colleagues to explore the relationship between epigenotype and BMI [55]. Following stringent statistical procedures four stable VMRs located near genes were found to correlate over time with an individual's BMI. The four genes are *PM20D1, MMP9, PRKG1*, and *RFC5*, respectively. For example, the level of methylation at *PM20D1* was elevated by about 20% in obese participants (≥30 BMI), in comparison with normal-weight, individuals (BMI ≤25). *PM20D1* encodes an unstudied putative carboxypeptidase and its role in energy metabolism is not known. The authors point out that the remaining three genes with stable VMRs may be implicated in metabolic syndrome [55]. That is, the matrix metalloproteinase 9 (*MMP9*) is upregulated in preadipocytes of obese individuals [58]. Overactivity of *MMP9* has also been observed in cultured adipocytes exposed to macrophage-conditioned medium [59] and in atherosclerotic plaques from subjects with T2D [60]. The cyclic GMP-dependent protein kinase-1 gene (*PKRG1*) is linked with food-related behaviors in genetic model organisms [61]; in humans, however, genetic variations associated with obesity have not been found in *PKRG1* [62]. Deregulation of *RFC5,* encoding a

component of the DNA replication and repair clamp loader machinery, may be involved in diabetes-associated DNA damage as the authors suggest [55]. These four genomic regions were not detected previously by large, genome-wide association studies as potential risk loci for common obesity. This may indicate that SNPs do not significantly influence DNA methylation levels of the associated genes. In other words, genetic variation does not influence the variance of methylation of the four BMI-related VMRs; these loci appear to harbor "pure" epigenetic variants.

CHARM is clearly an effective approach to establish an individual's gene-specific DNA methylation profile on a genome scale. In principle, it should be possible to scale-up the procedure permitting large, population-based epigenomic screens of complex phenotypes such as obesity. It remains to be shown if other research groups will adopt the CHARM approach, or if rapid advancement of technologies will supersede this procedure.

The methylation data were obtained from blood DNA. As discussed earlier, we currently do not know if epigenomic signatures from lymphocyte DNA are a suitable proxy for other tissues of an individual. It is promising that four loci were identified whose DNA methylation levels showed covariation with BMI. Will these VMRs turn out to be accurate epigenetic biomarkers for diagnosis of obesity? The study by Feinberg et al. – as all work referred to in this chapter – illustrates that it is still early days for the research field of epigenetic epidemiology of obesity. Confidence will grow in epigenetic biomarkers if independent measurements lead to similar results.

20.7 Imprinted Genes: A Contributing Factor in the General Population?

Imprinted genes carry parental-specific epigenetic information. Epigenetic variation and instability in this class of genes was observed soon after the first imprinted genes had been discovered [63]. Perturbations of genomic imprints have mainly been analyzed in the context of tumor biology, where loss of imprinting (LOI) is a common event in a variety of cancers [64]. Whether or not degeneration of parental imprints commonly occurs in metabolic diseases remains to be determined [8, 65, 66]. A possible role for imprinted genes in obesity and T2D can be inferred from to kinship theory of genomic imprinting. This hypothesis predicts that imprinted genes are generally involved in managing resources, ranging from regulating cell growth to feeding behaviors [67–69].

A recent case in point is the maternally expressed, imprinted gene *KLF14*, which encodes a Krüppel-like transcription factor [70]. *KLF14* operates as a master trans regulator, modulating the expression of a gene network in adipose tissue [70]. *KLF14* and many of its target genes have been associated with T2D and related metabolic phenotypes in large population-based studies [71].

20.7.1 Rare Imprinting Disorders Associated with a Metabolic Phenotype

Certainly some rare human imprinting syndromes—caused by epigenetic or genetic abnormalities—feature metabolic imbalances as part of the phenotypic spectrum. For example, perturbations of parent-of-origin specific gene expression on human chromosome 15q11-13 can cause Prader-Willi syndrome (PWS) or Angelman syndrome (AS), respectively. PWS individuals become obese during adulthood as they have hypothalamic abnormalities and a defective satiety-sensing system promoting uncontrollable hyperphagia [72].

A cluster of imprinted genes map to 15q11-13, and although all the genes and transcripts have been identified, it is still not clear how many of them contribute to the clinical manifestations of PWS. Some genes such as *UBE3A and ATP10A* show tissue-specific imprinting; in neuronal tissues the paternally inherited allele is mostly inactive, while bi-allelic expression is observed in all other tissue types [73, 74]. Hypothalamic tissue would be the choice for epigenetic studies of the imprinted gene cluster on 15q11-13, but use of this tissue is obviously not feasible in any epidemiologic approach to metabolic disorders.

The imprinted *GNAS* locus on 20q13.2-13.3 has been linked with the development of an early-onset obesity phenotype in a subgroup of individuals with Albright hereditary osteodystrophy (AHO). Mainly patients with mutations in their active, maternally inherited G-protein alpha-subunit gene ($G_s a$) allele become obese. Metabolic phenotypes observed in a range of different *Gnas* knock-out mouse models support the finding that perturbation of imprinting at this locus affects glucose and energy metabolism [75, 76].

20.7.2 The Imprinting Cluster on Chromosome 11p15.5 and *IGF2*

Another large genomic region harboring at least 12 imprinted genes lies on human chromosome 11p15.5 and includes the aforementioned *INS* chromatin domain (Sect. 20.4.4). Prominent imprinted genes in this cluster are *IGF2, CDKN1C, KCNQ1*, and the non-coding transcripts *H19* and *KCNQ1OT1*. Both genetic and epigenetic defects in this locus are associated with a number of human diseases such as the Beckwith-Wiedemann syndrome and transient neonatal diabetes mellitus. Reduced expression in the pancreas of *CDKN1C*, a cyclin-dependent kinase inhibitor, has been observed in hyperinsulinism of infancy and is associated with an increased proliferation of pancreatic beta-cells [77]. SNPs associated with T2D susceptibility were recently identified in the imprinted region on 11p15 [5, 78]; some of them confer disease risk when inherited from the father. Interestingly, alleles carrying the risk variant of one of these SNPs (rs2334499) have decreased methylation levels in

PBL-DNA at five CpG sites located within a DMR of the 11p15 imprinting cluster [79]. If confirmed, this SNP would provide another example of an "obligate" or "facilitated" epigenotype, where a genetic variation modulates the epigenotype of a genomic region associated with T2D risk in the general population.

IGF2 is an imprinted gene of interest within the 11p15 imprinting cluster. Epigenetic lesions are thought to cause bi-alleleic expression of *IGF2*, a gene that is normally transcribed only from the paternal allele. Within the general population bi-allelic *IGF2* expression has been observed at a frequency of about 10% [80, 81 #45]. Loss of imprinting and other, as yet unknown, events causing elevated *IGF2* levels can promote cell growth and division. In culture, growth-arrested pancreatic beta-cells resume cell proliferation upon treatment with IGF2 at physiological levels [82, 83]. Similarly, transgenic mice with beta-cell specific *IGF2* overexpression have pancreatic islet hyperplasia and subsequently develop insulin resistance as a consequence of prolonged hyperinsulinemia [84]. Is there a relationship between the *IGF2* imprinting status and pancreatic beta-cell mass? The number of beta-cells is thought to vary considerably among individuals [85]. Again, the difficulty to collect these endocrine micro-organs in humans makes it impractical to explore if epigenetic dysegulation of *IGF2* is associated with changes in beta-cell mass in metabolic disorders.

20.8 Conclusions

Integration of epigenetics as a component of population-based T2D and obesity studies will undoubtedly accelerate our understanding and shape our views of these public health problems in the coming years. Much expectation was placed in the power of genetics. We must now look to epigenetics to fulfill the promises of mechanistic insight, accurate diagnosis, prognosis, and potential therapies. As PBLs will be a preferred source for epidemiological studies, genomic loci from these cells will have to be rigorously characterized. In addition to classic epigenetic systems, non-coding RNAs have been identified as regulators of adipose tissue and insulin sensitivity [86]. MicroRNAs are likely to become a relevant class of biomarkers alongside DNA and histone modifications. The dynamic nature of epigenetic marks, and the many variables that can influence them, pose considerable challenges for this research field. The emerging data may not provide easily identifiable markers, and will probably require the adjustment or development of new statistical tools. Collection and use of additional data will bring the results into focus. Cumulative environmental exposures, age, gender, and even circadian rhythms are factors that must be included when embarking on epigenetic epidemiology studies. The task is complex – but so is the metabolic syndrome.

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Chapter 21
Epigenetic Epidemiology of Atherosclerosis

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Abstract The multitude of atherosclerotic risk factors can be divided into three main categories – genetical, environmental, and habitual issues. This classification can be simplified even further to genetical and environmental issues only as human habits manifest as environmental factors at cellular level. The link between large artery cellular responses to changed environment and epigenetic modifications of the chromosomal DNA has been established only recently. This review discusses the general epigenetic mechanisms operational in atherosclerotic tissue.

Abbreviations

ApoE	Apolipoprotein E
CHD	Coronary heart disease
CpG	Cytosine-guanine dinucleotide
CTCF	CCCTC-binding factor
ECM	Extracellular matrix
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
FGF2	Fibroblast growth factor 2
HDAC	Histone deacetylase
HAT	Histone acetyl transferase
Hcy	Homocysteine
HIF1α	Hypoxia inducible factor 1α
IGF2	Insulin-like growth factor 2
KAT2B	Lysine acetyltransferase 2B
LDL	Low density lipoprotein
LDLR	LDL receptor
MBD	Methyl-cytosine binding protein
NOS	Nitric oxide synthase
POL2	RNA polymerase II
PBL	Peripheral blood lymphocytes
RISC	RNA induced silencing
SAH	S-Adenosylhomocysteine
SAM	S-adenosylmethionine
SiRNA	small interfering RNA
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism
SRF	Serum response factor
TGS	Transcriptional gene silencing

21.1 Introduction

Atherosclerosis is a complex disease, and most of the complexity lies in the interplay of factors contributing to the disease etiology. Male gender, genetic variability, and some environmental and habitual issues have been recognized as the most important risk factors for atherosclerosis. The impact of genetic variability on atherosclerosis, with only a few exceptions, has been very difficult to assess as the contribution of the identified single nucleotide polymorphism (SNP) is usually only marginally above normal variability [1]. The chromosomal region 9p21 appears the strongest polymorphic site that has been related to atherosclerosis, but the statistical significance of the detected variability still means only a small increase in the risk for disease development [2]. On the other hand, research done on experimental animals has shown that there are strong non-genetic factors that can be linked to the increased risk for atherosclerosis in the progeny [3]. These hereditary events not explainable by genetics have attracted attention long before any possible mechanisms for that had been proposed and the widely used term epigenetics was invented by Conrad Waddington already in 1942. Waddington defined epigenetics as something that is needed in addition to genes "to bring the phenotype into being" [4].

Epigenetic mechanisms include several levels of regulation which contribute to the activity of gene expression. The principal mechanisms of epigenetic regulation in mammals are DNA methylation and modifications of histone tails which alter chromatin structure [5]. Importantly, these changes in chromosomal structure are not permanent and they leave the primary genetic code (= nucleotide sequence) unchanged. Thus, epigenetic regulation necessitates thinking that a particular phenotype is not entirely defined by the DNA primary sequence. Moreover, the most striking piece of information from epigenetic studies is supporting the assumption that some acquired traits can be passed to progeny, both in humans [6] and in experimental animals although direct evidence in humans is still lacking [3].

Genetic variability and epigenetic mechanisms of gene regulation are intricately combined in the phenomenon of sexual dimorphism [7]. The impact of sexually dimorphic genes on complex diseases can only be analyzed after sequencing of possibly thousands of genomes with the ability to collect data separately from maternal and paternal genomes. Human haploid genome lengths can vary by up to 3%, when all insertions and deletions are included. Recent advancements in DNA sequencing technology have increased the speed of sequence data collection by eight orders of magnitude during the last 25 years, but the biggest challenges are still in the computational efficiency of simultaneous comparisons of thousands of genomes and relating findings to phenotypic information [8].

Most studies relating epigenetics to human pathologies have focused on cancer. Studies about the involvement of epigenetic mechanisms in cardiovascular diseases have been disappointingly sparse. The first study demonstrating a link between hypercholesterolemia and chromatin structure was published already in 1980 [9]. Acridine orange intercalated to unwound DNA regions devoid of histones and made it possible to distinguish between actively transcribed and/or replicating euchromatin

and densely packed heterochromatin. Lehmann and coworkers could detect more acridine orange binding nuclei in aortic wall of hypercholesterolemic rabbit [9]. Already decades ago researchers noticed that commonly recognized risk factors (genetic background, diet, and lifestyle) do not explain all cases of cardiovascular morbidity [10]. Studies in the area of epigenetics may significantly contribute to our understanding of the pathogenesis of cardiovascular diseases and suggest new therapeutic possibilities for atherosclerosis-related diseases.

21.2 Etiology of Atherosclerosis

Atherosclerosis is a chronic disease of large and medium sized arteries which is characterized by accumulation of cholesterol in the arterial wall together with proliferation of arterial smooth muscle cells (SMC) and accumulation of extracellular matrix components which lead to occlusion of blood vessels, myocardial infarction, stroke, aneurysms, and peripheral vascular disease [11–14]. Chronic inflammatory response with infiltration of monocyte–macrophages and T-cells and endothelial dysfunction are also prominent features of atherogenesis [15, 16].

Atherosclerosis develops over several decades of life. Its incidence reached epidemics in the Western world in the 1960s and 1970s whereafter the incidence declined, thanks to effective blood lipid lowering, treatment of hypertension, dietary changes, and the decline of smoking in many countries. However, in other parts of the world, such as in the Far East and in Third World countries, its incidence is rapidly increasing. It is obvious that unfavorable changes in dietary habits and smoking contribute to the changes via increases in blood lipid levels and uptake of cholesterol in the arterial wall. However, it has been speculated that epigenetic changes might also contribute to atherogenesis since it involves polyclonal proliferation of SMCs and some dietary components, recognized as risk factors of atherosclerosis, may affect methylation machinery in the arterial cells [17, 18] (Fig. 21.1).

21.3 Hypercholesterolemia and Potential Dietary Effects: An Epigenetic View

Hypercholesterolemia is central to the initial stages of atherosclerosis in large arteries [19]. Genetic studies have provided support for the existence of inherited patterns of cardiovascular diseases and maternal hypercholesterolemia appears to be associated with a higher incidence and a faster progression of atherosclerosis in humans [6], rabbits [20], and mice [21, 22]. However, there is at least one report where the link between maternal hypercholesterolemia and enhanced atherosclerosis in mouse progeny was not demonstrated [23].

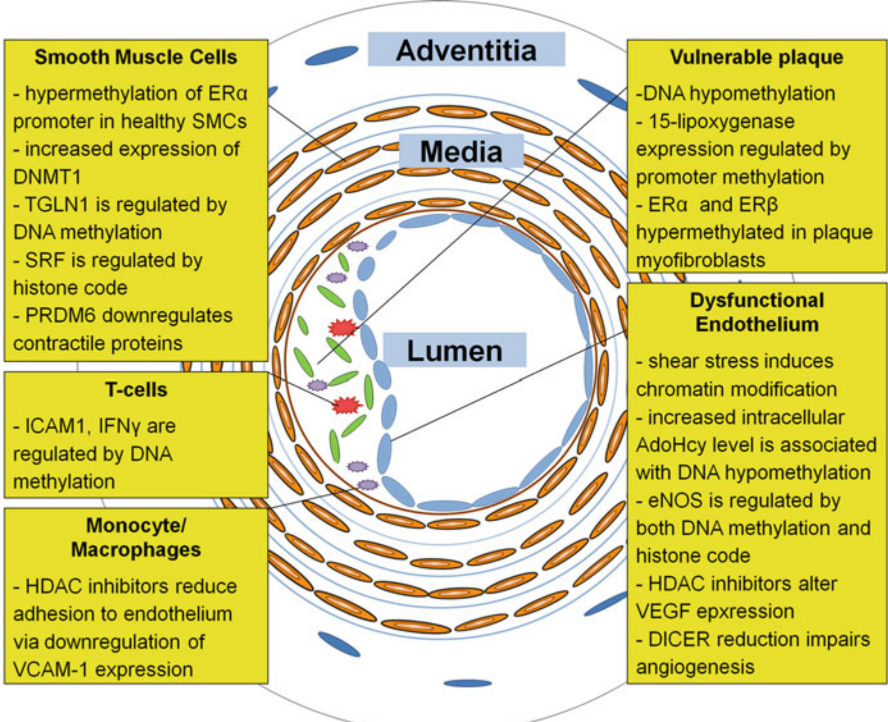

Fig. 21.1 Summary of epigenetic effects in atherosclerotic vascular wall

Dietary modification can have a marked effect on epigenetic modifications of the genome. A diet deficient for methionine and folate impairs the biosynthesis of necessary methyl donor S-adenosylmethionine (SAM), and this, in turn, can lead to altered imprinting of IGF2 [24]. While the risk of colorectal cancer is linked to dietary folate deficiency and some variants of methylenetetrahydrofolate reductase that direct folate to remethylation of homocysteine (Hcy) to methionine [25], high folic acid supplementation has also been linked to a promotion of underlying colorectal cancer [26–28]. Epigenetic mechanisms may connect dietary and other environmental exposures with the risk of cardiovascular disease.

21.4 The Role of Homocysteinemia in Atherosclerosis

About 10% of human population may be affected by hyperhomocysteinemia and elevated plasma total Hcy is associated with increased risk for ischemic heart disease and stroke [29, 30]. Hcy is metabolized to methionine after activation to SAM,

which is known to act as a methyl donor. Underlying pathogenic mechanisms that lead to endothelial dysfunction and atherosclerosis have been thoroughly studied but remain poorly understood. Recent evidence suggests that the pathologic role of hyperhomocysteinemia in vascular diseases might be mediated via S-adenosylhomocysteine (SAH) accumulation and DNA methylation [31].

Castro et al. have observed that patients with vascular disease have increased plasma Hcy and SAH concentrations and disturbed global DNA methylation status [31]. High blood Hcy levels (> 75 μmol/l) correlate with DNA hypomethylation and atherosclerosis [32] and can lead to a 35% reduction in DNA methylation status of peripheral blood lymphocytes [31]. High Hcy concentration competes with SAM for binding sites on DNA methylases and may lead to passive loss of methylation in replicating DNA [33]. Aberrant DNA methylation secondary to environmental and nutritional factors has been documented in atherogenesis, both in humans and in animal models [34, 35].

Sharma et al. [36] reviewed literature of 135 genes which either modulate the blood level of Hcy or are regulated by the elevated level of Hcy. Mapping of these genes to their respective pathways revealed that an elevated level of Hcy may lead to atherosclerosis either by affecting lipid metabolism and transport or via oxidative stress and endoplasmic reticulum stress. Elevated level of Hcy also decreases the bioavailability of nitric oxide [36].

21.5 DNA Methylation and Atherosclerosis

DNA methylation is an epigenetic mechanism maintaining chromosomal DNA structure and regulating gene expression (e.g., X chromosome inactivation, imprinting, controlling transposable genetic element mobility and activity of developmentally regulated genes). DNA methylation pattern is replicated in embryogenesis and in tissue repair at each mitotic cycle [37]. Methyl-cytosine binding (MBD) proteins effectively prevent gene transcription from methylated promoters [5] and induce stable blockade of transcription by recruitment of transcriptional co-repressors with histone methylase and deacetylase activities [38, 39].

In healthy human somatic cells about 1% of all cytosines appear to be methylated [40], but there is some variation between tissues and even 3–4% of all cytosines have been reported to be methylated in some tissues [41]. This corresponds to 70–90% of all cytosines in CpG dinucleotides [42]. CpG dinucleotides present in the 5′ regulatory areas are mostly unmethylated, whereas CpG islands in intronic and repetitive DNA elements are usually methylated [42]. Regulatory DNA regions rich in CpG dinucleotides are known as CpG islands with lengths varying from a few hundred to several thousand base pairs [37]. Some components of the DNA methylation machinery are shared with the enzyme complex involved in histone methylation (see below; [39, 43]).

It is now generally accepted that DNA methylation patterns are replicated during embryonal development and that changes introduced into DNA methylation pattern

in early life can be passed to the progeny at least in rodents [3]. There is also some evidence that the epigenetic programming in early life can be reversed by pharmacological therapy later in adulthood [6, 44–46].

Age-related changes in global methylation status of DNA appear to be genetically determined. Bjornsson et al. have shown that some individuals gain and others loose cytosine methylation in their DNA over time and the paired data on intra-individual changes could be clustered family-wise [47]. This result may explain why the averaged levels of DNA methylation in age groups did not change [48].

SMCs make up most of the cellular mass in arterial wall, and consequently epigenetic changes detected in vascular wall are mainly a characteristic of SMCs. Transgelin is the earliest marker of SMC differentiation and is regulated also by DNA methylation [49]. Another example of epigenetic regulation is the estrogen receptor (ER) α promoter, which is hypermethylated in proliferating arterial SMCs, but not in endothelial cells [50]. Endothelial nitric oxide synthase (eNOS) is hypomethylated in endothelium, but heavily methylated in other cell types [51], including SMC lines [52]. Inducible NOS (iNOS) is expressed in atherosclerotic plaque neointima [53, 54], but repressed by methylation in most other tissues [51].

21.5.1 Hypermethylation and Atherosclerosis

Hypermethylation of promoter CpG islands is characteristic to transcriptional silencing. Gene repression by DNA methylation is achieved either by prevention of the binding of transcription factors to their cognate sites by cytosine methylation or by occupying the promoter region by one of the five methyl-cytosine recognizing proteins MBD1, MBD2, MBD4, MeCP2, or Kaiso [5]. In atherosclerosis the binding of certain transcription factors, such as HIF1α [55], myc [56], and CTCF [57] have been shown to be affected by DNA methylation. CTCF target sites on paternal chromosomal DNA are methylated and CTCF regulates maternal genes like IGF2 [58]. YY1 is another eukaryotic insulator regulating paternal genes (e.g., XIST, GNAS, and PEG3, which are all methylated on maternal chromosome). Kaiso recognizes also unmethylated TNGCAGGA and interacts with CTCF [59].

Transcriptional silencing by hypermethylation is poorly studied in other diseases than cancer. There is some existing controversy about the existence of DNA hypermethylation in peripheral blood lymphocytes (PBL) in coronary heart disease (CHD) patients. Two recent articles [60, 61] have reported an increase in PBL methylation status of CHD patients, whereas some earlier reports have documented opposite findings [32, 62].

The complex nature of atherosclerosis can be seen in the dualistic effects of epigenetic regulation of specific genes (Table 21.1). For example, while hyperhomocysteinemia has the potential to reduce DNA methylation in atherosclerotic patient PBL [31], some promoters like iNOS [63] in cultured human monocytes and FGF2 [64] in cultured human coronary artery endothelial cells are still hypermethylated. Increased methylation of ERα promoter has been detected in atheromatous

Table 21.1 Genes related to atherosclerotic diseases which are at least partly regulated by epigenetic mechanisms

Gene	Function	Target	Mechanism	Reference
eNOS	Blood pressure control	Endothelium	DNA methylation and histone code	[52, 104]
iNOS	Blood pressure control	Inflammation, macrophages	DNA methylation and histone code	[51]
Fads2	Fatty acid desaturase	Contributes to pathology of Hcy	DNA methylation	[105]
c-*fos*	Transcription factor	Shear stress	Histone code	[106]
Estrogen receptor α	Transcription factor	Coronary artery	DNA methylation	[50]
Estrogen receptor β	Transcription factor	Coronary artery Mammary artery Carotid artery Femoral artery Saphenous vein	DNA methylation	[66]
P66Shc	Docking protein in cell signaling	End-stage renal disease	DNA methylation	[107]
15-LO (ALOX15)	Lipid peroxidation	Pathogenesis of atherosclerosis	DNA methylation	[108]
EC-SOD	Oxidative stress	Pathogenesis of atherosclerosis	DNA methylation	[17]
H19/Igf2	H19 – tumor suppressor IGF2 – fetal growth factor	Regulated by hyperhomo-cysteinemia	Imprinting, normally maternally expressed	[105, 109]
MMP-2	Matrix degradation	Extracellular matrix	DNA methylation	[110]
MMP-7	Matrix degradation	Extracellular matrix	DNA methylation	[110]
MMP-9	Matrix degradation	Extracellular matrix	DNA methylation	[110]
TIMP-3	Inhibitor of ECM degradation	Extracellular matrix	DNA methylation	[111]
IFN-γ	Cytokine	Inflammatory response	DNA methylation	[112]
PDGF-A	Growth factor	Cell proliferation	DNA methylation	[113]
ICAM-1	Adhesion	Inflammatory reactions	DNA methylation	[114]
p53	Tumor suppressor	Apoptosis	DNA methylation	[115]

proliferating SMCs [50, 65]. Also, ERβ promoter shows a high level of methylation in cells from the plaque area compared to non-plaque regions [66]. Although it is apparent that ERα and ERβ play a role in actively proliferating atheromatous SMCs, the interpretation of the exact role of ER in vivo remains unclear. There is a steady age-dependent increase in ER promoter methylation, which may reach 99% methylation level in the arterial SMC isolated from elderly cadaveric patients [65].

Age-related hypermethylation of promoters is not limited to ERα [67, 68] and hypermethylated promoters of immunomodulatory genes and tumor suppressors [68] may have profound effects also on the pathogenesis of atherosclerosis.

Inflammation promotes global hypermethylation of peripheral blood lymphocyte DNA, and inflammation in dialysis patients correlates with increased cardiovascular mortality [69].

21.5.2 Hypomethylation and Atherosclerosis?

Loss of DNA methylation can be either passive (DNA replication-dependent) or active (DNA-replication independent) [42]. It is not known which of these processes predominates in atherosclerosis, although active replication of SMCs in neointima seems to favor the idea of passive hypomethylation.

Ageing and atherosclerosis occur simultaneously, and it is somewhat difficult to distinguish between these two processes as causes for global hypomethylation. Global hypomethylation of genomic DNA has been considered as a characteristic of aging [70], but recent data show that hypomethylation is characteristic to only 20–30% of the population [47]. Global hypomethylation is common in some other pathological conditions like tumor growth [71], hyperhomocysteinemia [32], and schizophrenia [72]. Global hypomethylation is also characteristic of areas of SMC proliferation in advanced human atherosclerotic plaques where a 9% decrease in cytosine methylation has been detected compared to normal artery [41]. In mice atherosclerosis and aging can be studied separately, since in ApoE knock-out mice DNA hypomethylation appears to precede any histological signs of atherosclerosis [73]. Similar hypomethylation is present in atheromas of ApoE knockout mice [73] and in neointimal thickenings of New Zealand White rabbit aortas [17, 41]. A significant decrease in DNA methylation status has also been detected in ApoE knockout mouse aortas already as early as 4 weeks of age, and considerable advancement in demethylation was detectable at 6 months [73]. Whereas hypomethylation of eNOS and iNOS promoter areas are characteristic of healthy human vascular endothelial cells, reduction in the methylation status of extracellular superoxide dismutase [17] and 15-lipoxygenase [41] promoters are characteristic of human atherosclerotic lesions.

Transcriptional regulator specifically recognizing unmethylated DNA is CXXC1 [74] which acts in concert with histone 3 lysine 4 methylases MLL, MLL2, MLL3, and hSET1 [75]. Hypomethylation of DNA repeated sequences can lead to activation of transposable elements which, in turn, may cause insertional mutagenesis and chromosomal instability by DNA recombination events as shown in human myeloid leukemia [76]. However, hypomethylation in atherosclerotic lesions is a somewhat paradoxical finding since DNMT1 mRNA has been shown to be upregulated in atheroma tissue [41]. Atherosclerosis is also associated with humoral and adaptive immunological responses, including autoantibody formation against oxidized LDL [77]. In this context it is interesting to note that hypomethylating agents can promote autoimmunity in mice [78].

21.6 Histone Code and Atherosclerosis

Inflammatory processes in atherosclerosis are largely mediated by transcription factor NFkB activation which includes histone code modulation. Histone acetylation of KAT2B has a costimulatory effect on NFkB-dependent transcription of inflammatory genes in PBL from diabetic humans [79, 80], whereas histone deacetylase activation suppresses inflammation [81]. In addition to histone modifications, both KAT2B and p300 can acetylate NFkB proteins directly and thereby increase its binding affinity to DNA and thus its transactivating activity [82, 83]. Trichostatin A, a specific inhibitor of histone deacetylase, significantly increased the formation of fatty streak lesions and macrophage infiltration in LDLR knock-out mice [84]. Dysregulation of epigenetic histone modifications may be a major underlying mechanism for metabolic memory and sustained proinflammatory phenotype in SMCs in diabetic vascular disease [43, 85].

Histone lysine demethylase KDM1 (LSD1), a component of the CoREST2CtBP co-repressor complex, was discovered in 2004 and since then a number of histone demethylases have been identified and shown to play important roles in the regulation of gene expression, as well as in cellular differentiation and development [86]. Recently, Reddy et al. showed that SMCs derived from a mouse model of type 2 diabetes displayed enhanced inflammatory gene expression and proatherogenic responses [85]. These results demonstrated functional roles for LSD1 and H3K4 methylation in SMCs and inflammation. Dysregulation of their actions may be a major mechanism for chronic vascular inflammation, and metabolic memory associated with diabetic vascular complications.

21.7 Small RNAs and Atherosclerosis

Small RNA molecules have been shown to regulate gene transcription by interacting with the promoter region and modifying the histone code [87–90]. A process known as transcriptional gene silencing (TGS) involves promoter-targeted small interfering RNA (siRNA) and leads to silent state epigenetic profile containing dimethylated histone H3 lysine 9 (H3K9me2) and trimethylated histone H3 lysine 27 (H3K27me3) [91]. This process was first recognized in plants but has since been identified also in mammals [89, 92]. Interestingly, besides transcriptional gene silencing, the small RNAs have been reported to induce gene activation [88, 90]. This is associated with a loss of H3K9me2 at the targeted promoter sequences and seems to require a member of a RISC complex, Argonaute-2 protein. TGS has earlier been noticed to require Argonaute-1 and -2 [93].

The chromatin modifications induced by siRNAs have been shown to require RNA polymerase II (POL2) and the transcription of the homologous target sequence in *S. pombe* [94]. Truncation of the C-terminal domain of POL2 did not cause any deficiencies in transcription or in production of siRNAs but it impaired

the RNAi-directed chromatin modifications. Thus, POL2 via its C-terminal domain might stabilize the interactions between the components of RNAi machinery and the transcript (the RNA/RNA model). It has been also proposed that during transcription the passage of POL2 might act by opening the chromatin and providing access for siRNAs to the complementary DNA sequence, allowing the siRNA-DNA interactions to take place (the RNA/DNA model). One suggested mechanism of action for promoter targeted small RNA mediated gene activation and silencing is that the antisense strand of the small RNA binds to a complementary non-coding promoter-associated antisense RNA [95–97].

21.8 Conclusions and Future Perspectives

Clonal proliferation of lesion SMCs has been documented in human atherosclerotic lesions [98]. It is likely that some SMCs acquire properties that enable them to proliferate more rapidly and thus increase their progeny in the lesion area. No data is available about possible association of epigenetic modifications with this clonal proliferation process. However, SMC-specific gene expression is dependent on regulatory elements controlled by epigenetic mechanisms altering chromatin structure. The binding of serum response factor (SRF) to SMC-specific promoters is associated with modification of adjacent histones by methylation and acetylation [99, 100]. Likewise, myocardin-induced SMC-specific transcription is modulated by the acetylation of histones flanking SRF-binding sites [101]. A recently discovered histone methylase PRDM6 specifically downregulates contractile protein expression in SMCs and aids switching to the rapidly proliferating synthetic phenotype [102]. Because of the rapid fluctuation in the incidence of cardiovascular diseases within various countries, epigenetic effects are likely involved since human genetic code does not change that rapidly through classical mutations. Similarly, epigenetic mechanisms could explain, at least partly, marked increases with age of several common diseases, including cardiovascular diseases.

Potential links of direct dietary effects on vascular cells and their gene expression via epigenetic mechanisms cannot be excluded. This is an area that clearly requires further study. Several drugs are currently in development that target epigenetic changes in malignant cells and processes like autoimmunity. Testing these compounds for potential effects on atherosclerosis might prove fruitful and provide direct evidence whether these processes are involved in the pathogenesis of cardiovascular diseases.

Double stranded RNAs have recently been shown to influence epigenetic properties of several cell types [89, 91, 92, 103]. Indeed, microRNAs expressed from genes and intergenic areas constitute a large pool of potential effectors and modifiers of gene expression, some effects of which are likely to involve epigenetic changes at DNA methylation and histone level [90]. The role of microRNAs in fine tuning gene expression and cellular genetic memory are areas where further studies are clearly warranted.

In summary, epigenetic changes have been detected in atherosclerotic lesions. These changes may contribute to the pathogenesis of vascular lesions and modify properties and gene expression in lesion cells. Treatments directed toward modification of epigenetic changes in vascular cells may provide new possibilities for the treatment of cardiovascular diseases.

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