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Tung-chin Chiang *Editors*

Environmental Epigenetics

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Preface

Human population is simultaneously and continuously exposed to a wide variety of chemical substances, biological agents, physical agents, and other stressors in daily life. The exposures can modify health consequences or potentiate the response expected from the exposure. Over the past decades, it has been a raised awareness of the environmental pollution to the health of human. Despite the effort of remediation and better control of the pollution, many of nature disasters, such as oil spill in the Gulf of Mexico, Fukushima nuclear plant disaster in Japan, and levee failure in New Orleans after Hurricane Katrina, or manmade products, such as bisphenol-A in food containers and a widely used plasticizer, di(2-ethylhexyl) phthalate (DEHP), presence of fungicides, vinclozolin and prochloraz, in foods, inappropriate disposal of electronic boards that contain heavy metals, and pharmaceutical use of finasteride, continuously pose threat to the human health.

Epidemiological and experimental studies have linked epigenetic modulations with exposure to environmental toxicants. Many environmental toxicants affect epigenetic pathways mainly through DNA methylation and complex histone modifications. Evidence linking environmental factors with DNA hyper- or hypomethylation provides the most compelling support for such an association. Further, life style and dietary factors may modulate the toxicity of environmental pollutants, through either synergistic or antagonistic effects. Environmental toxicants may also cause aberrant modifications in the metabolic activity of nutritional factors. However, the relationship between environmental toxicants and epigenetic modulations is complex and an exact causal relationship has yet to be identified. In addition, the complexity of the relationship is compounded by the interaction of multiple toxicants and nutritional factors. Nonetheless, nutritional factors and environmental toxicants may affect similar biological and epigenetic pathways and thus should be examined simultaneously.

This book provides a compilation of the recent development and knowledge in the exciting field of environmental epigenetics through interdisciplinary approach. The book begins with the question “What is epigenetics?” in Chap. 1 to explore the history and fundamental knowledge of epigenetic research and followed by Chap. 2 to provide an overview of epidemiology of environmental health. Chapters 3, 4, 5,

6, 7, 8, and 9 utilize uterine leiomyoma, endocrine disease, breast cancer, obesity, cancers, aging, and cardiovascular diseases, respectively, as an examples to explore the potentially intricate relationship between environmental disrupting chemicals and these diseases. Some have suggested that epigenetics is a natural exhibition of gene and environmental interactions. Therefore, Chap. 10 provides a slightly different angle to summarize the endeavor of gene-environmental interaction on human health to date. The technical applications of epigenetic biomarkers to investigate epigenetic alterations from environmental exposures are reviewed in Chap. 11. Genome rearrangement during development that is driven by epigenetic markings has been a relatively new concept in epigenetics research, which is explored in Chap. 12. Finally, Chap. 13 catalogs existing laboratory technologies used to index epigenetic modifications and offers a concluding note to explore the concept of "modifiable trilogy."

Because this book started out from the introductory knowledge of the science of epigenetics and the identification of issues in environmental epidemiology, this book is intended to serve both as a reference compendium on environmental epigenetics for scientists in academia, industry, and laboratories and a textbook for graduate level environmental health courses. We are indebted to scientists worldwide to their contributions that made the publication of this book possible. Most importantly, we would like to acknowledge the chapter authors for their efforts in creating this book. Even though they all have many demands on their time, they generously contributed toward this effort to highlight the link between environmental exposure and epigenetics and the consequence to human health and diseases.

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L. Joseph Su is a Program Director at the Epidemiology and Genomic Research Program, Division of Cancer Control and Population Sciences, National Cancer Institute, where he manages a diverse grant portfolio on cancer epidemiologic studies that involves in nutrition, environmental exposure, genetics/epigenetics, and -omics application in population-based studies. He has collaborated with his colleagues at the National Institutes of Health leading the efforts to promote the advancement of epigenetics and metabolomics research. Before joining the National Cancer Institute, Dr. Su was an Associate Professor in Epidemiology and Pathology at the Louisiana State University Health Sciences Center (LSUHSC), New Orleans, where he directed research projects on cancer etiology, nutrition, environmental health, healthy aging, and healthcare access and utilizations funded by Federal, State, and not-for-profit agency and organizations. He pioneered epigenetics research works at the LUSHSC with Dr. Tung-Chin Chiang, coeditor for this book, at Tulane University Health Sciences Center, Center for Bio-environmental Research, and with Dr. Jovanny Zabaleta, a contributing author of this book, at the LSUHSC Stanley S. Scott Cancer Center. He continues epigenetics research on obesity and periconceptional environmental exposures to health outcomes later in life.

Chapter 1
What Is Epigenetics?

Tung-Chin Chiang and L. Joseph Su

Abstract Transgenerational health effects have been reported for many environmental chemical exposures. In order to explain many unanswered questions, researchers have made significant efforts looking for something beyond genetics, call "epigenetics". This chapter introduce the concept of epigenetics and many different forms of epigenetics modifications. Potential application of epigenetics in health research and complex disease treatment is explored. Highlights of chapters in this book are also summarized.

Keywords Epigenetics • Environmental exposure • Transgenerational • Application • DNA methylation • Histone deacetylation

1.1 Historical Aspects

1.1.1 Environmental Exposure and Transgenerational Health Effect

While investigating how environmental chemicals affect sex determinations in embryonic animals, Skinner and Cupp found that the first generation male offspring rats (F_1) from pregnant rats (F_0) exposed to insecticide (methoxyclor) and fungicide (vinclozolin) had decreased sperm counts and higher rate of infertility (Anway et al. 2005). The effect of the insecticide and fungicide was found to persist for at least

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four generations, even though the offspring since the first generation were not exposed to the chemicals (Schmidt 2013). This research suggests that the chemicals we exposed to in our lifetime could have consequence not just for our own and our children's health, but also for the health of generations to come. Because chemicals given to females (the F_0) interact not only with the fetal offspring (the F_1) but also with the germ cells developing within those offspring, which mature into the sperm and eggs that given rise to the F_2 generation, the F_3 animals are the first generation to be totally unexposed to the original agent. These types of transgenerational effects have been reported for many environmental chemical exposures, including permethrin, DEET, bisphenol A, phthalates, dioxin, jet fuel mixtures, nicotine, tributyltin, and etc. Most of the studies to date were on rodents (Manikkam et al. 2012; Rehan et al. 2012, 2013; Doyle et al. 2013). However, preliminary evidence that chemical effects can carry over generations in human is also starting to emerge. Some reports have linked nutritional deficiencies from famine and exposure to diethylstilbestrol (DES), a nonsteroidal estrogen used to protect against miscarriage from the 1940s to the 1970s, to persisted effect among grandchildren of exposed women (Heijmans et al. 2008; Titus-Ernstoff et al. 2010; Painter et al. 2008; Veenendaal et al. 2013; Pembrey et al. 2006; Veenendaal et al. 2012). Although there are strong evidence linking environmental exposures and transgenerational health effects, the underlying biological mechanism is still unclear.

1.1.2 The Needs to Know Beyond Genetics

Genome-environment interactions have been discussed extensively (Ghazarian et al. 2013; Steves et al. 2012; Franks 2011). They are important for understanding how health and disease are impacted by environmental exposures. A large number of environmental factors and lifestyle factors have been shown to influence a variety of diseases (Bauer et al. 2014). However, few have shown to promote DNA sequence mutation (Li 2002; Skinner et al. 2008). For the past decades, researchers have made significant efforts looking for something beyond genetics, call "epigenetics", in order to explain many unanswered questions (Esteller 2006). Questions such as, how one genotype can be translated into many different phenotypes? What is the main driving force for aging differently, even in monozygotic twins (Fraga et al. 2005a)? If environmental factor plays a role in aging and different response to diseases, how does it work? Furthermore, how does the impact of environmental stress, such as environmental estrogen or cigarettes smoking, pass down to the next generations? Can mutations on genomic DNA fully explain the differential responses between individuals and their offspring? Many studies have shown that differential gene expression from the same genetic code has played a role in differential responses in cancer, especially in the case of tumor suppressor genes (Esteller 2005, 2007). However, how are the gene expressions being differential regulated? How much do researches know and how can we use the accumulated knowledge to explore possible ways to cure disease or to adjust our lifestyle to accommodate the nature

force of modifications, either genetic or beyond? Emerging evidence suggests that epigenetics is involved in the ability of environmental exposures to regulate the expression of genome and to develop stable alternations in phenotypes within one to two generations (Jirtle and Skinner 2007). Environmental alternations in the epigenome appears to be a mechanism potentially involved in the abnormal transcriptomes associated with diseases (Egger et al. 2004). The question is "what is epigenetics?" This book offers a comprehensive understanding of epigenetics and its impact on health and diseases after the exposure to certain environmental and lifestyle factors.

1.1.3 Looking for Answers Beyond Genetic Codes: Epigenetic Signatures

In contrast to genetic modifications, which the DNA is altered, epigenetic, on the other hand, focuses on how much and when the gene expression occurred, without changing gene sequence (Esteller 2006). The "gene expression regulatory machinery" that epigenetic research has focused on is like a signature or a switch imprinted in the gene sequence. The epigenetic signatures, which is beyond the normal genetic coding A-T-G-C and is functioned like the 5th genetic code, can not only initiate gene expression but also be passed down to the next generation. What are the epigenetic signatures? There are many researches trying to find the "changes" when gene expression is altered without change in DNA sequence. Many of these "changes" have been observed and all are in the umbrella of epigenetic changes. Those changes include DNA methylation, histone modifications, RNA interference, chromosomal instability, transposons, and loss of imprinting (Su et al. 2011).

1.2 Forms of Epigenetic Modifications

Epigenetic modifications can be identified through one or more of the forms such as DNA methylation, histone modifications, RNA interference, chromosomal instability, transposons, and loss of imprinting.

1.2.1 DNA Methylation

The most studied epigenetic modifications include DNA methylation and histone modification. In general, DNA methylation is a major epigenetic phenomenon that predominantly involves the covalent addition of a methyl group (CH_3) to the 5′-position of cytosine that precedes a guanosine in the DNA sequence (the CpG dinucleotides), thereby regulating genetic expression and integrity in various

biological processes, such as differentiation, genomic imprinting, DNA mutation, and DNA repair (Fang et al. 2007). This is referred to as an epigenetic modification because it does not change the coding sequence of the DNA. DNA methylation occurs on CpG sites, which are stretches of CG dinucleotides found in promoter regions. Methyl-CpG binding proteins (MeCP1 and MeCP2) are actively recruited to the methylated CpG sites and serve as a base to recruit other proteins to modify transcription. These proteins include DNA methyl transferase 1 (DNMT); histone methyl transferase (HMTs); and histone deacetylase (HDAC). Histone modification, especially acetylation of specific lysine residues contained within the tails of nucleosomal core histones, is known to play a critical role in chromatin packaging and gene expression (Jenuwein and Allis 2001). In general, demethylation of CpG sites plus acetylation on lysine residues H3 and H4 result in a loosened chromatin structure that allows the gene expression machinery to access the promoter to turn on the gene. On the other hand, methylated DNA, plus deacetylated H3 and H4, would recruit DNMT1, HDAC, and HMT to form a closed chromatin state, which would result in epigenetic transcriptional silencing (Esteller 2005). The CpG dinucleotides are not distributed evenly in the whole genome, but tend to be clustered in small stretches of DNA termed "CpG islands" (Robertson and Wolffe 2000). These regions often are associated with the promoter regions of genes. Approximately one-half of the genes in the human genome have such CpG-rich promoter regions (Herman and Baylin 2003). A majority of the CpG dinucleotides in the human genome that are not associated with CpG islands are heavily methylated (Herman and Baylin 2003). On the contrary, the dinucleotides in CpG islands, in particular those associated with gene promoters, usually are unmethylated (Bird 2002). Patterns of DNA methylation are generated during development involving de novo methylation and demethylation activities. DNA methylation is an enzymatic process that is mediated by DNA methyltransferases (DNMTs). DNMT3 regulates de novo methylation during development, whereas *DNMT1* maintains DNA methylation patterns during cell replication. The genomic methylation pattern is precisely inherited during mitosis and is highly tissue specific. Cytosine methylation changes the structure of the major groove in the DNA molecule and disrupts the attachment of DNA-binding proteins and transcription factors. Genes methylated at specific sites, such as upstream of a promoter region, are either not transcribed into messenger RNA (mRNA) or are transcribed at a reduced rate, thus reducing translation of the gene into the encoded protein. As a result, epigenetic DNA methylation contributes to the control of gene and ultimately protein expression (Costello and Plass 2001; Duthie 2011). It can induce the gene to turn on or off, and to up- or down-regulate.

One of the most examined hypotheses regarding DNA methylation is its relationship with cancer. Aberrant DNA methylation patterns in cancer cells have been recognized for decades (Boehm and Drahovsky 1983). However, the interpretation of observed DNA methylation and cancer risk remains a challenge because virtually all types of cancer that have been examined have shown both global hypomethylation and gene-specific hypermethylation in gene promoter regions (Baylin et al. 1998). Hypermethylation of promoter regions, which is associated with transcription

silencing, similar to DNA mutation, has been proposed as a mechanism for tumor suppressor gene inactivation in human cancers (Jones and Baylin 2007). In addition, a number of candidate tumor suppressor genes, such as HIC1, INK4b (p15), TIMP3, and others that are not commonly inactivated by mutation, are transcriptionally silenced by methylation. The aberrant methylation of genes that suppress tumorigenesis appears to occur early in tumor development, accumulates progressively, and eventually leads to the malignant phenotype (Fearon and Vogelstein 1990; Davis and Uthus 2004). Hypermethylation has been found to be associated with the dysregulation of pathways involved in the cancer process, such as DNA repair (*hMLH1*, *BRCA1*, *MGMT*), cell cycle regulation (*p16*, *p14*, *p15*), carcinogen metabolism (*GSTP1*), apoptosis (*DAPK*, *APAF-1*), hormonal response (*RARβ2*), and cell adherence (*CDH1*, *CDH3*) (Costello and Plass 2001; Davis and Uthus 2004; Esteller 2008; Momparler 2003).

1.2.2 Histone Modifications and the Histone Code

Another commonly known epigenetic modification is histone modifications. A complex of DNA and globular proteins, known as "histones," package heritable genetic information in eukaryotic nuclei in the form of nucleosomes that constitute chromatin. Nucleosomes are composed of 147 bp of DNA wrapped around core histone proteins named H2A, H2B, H3, and H4. An additional histone, H1, is located at the outer surface of the nucleosome to anchor DNA to the nucleosome. H3 and H4 have long tails that extend out from the nucleosome and can be modified chemically by acetylation, methylation, ubiquitination, phosphorylation, sumoylation, citrullination, and ADP-ribosylation (Baccarelli and Bollati 2009). These chemical modifications can result in a closed chromatin conformation that suppresses transcription, or in an open conformation that activates transcription. The most common modifications are acetylation and methylation on histone lysine residues. Increased acetylation results in transcription activation, and methylation of histones can result in either repression or activation of transcription, as determined by the lysine residue position involved (Bollati and Baccarelli 2010).

Growing lists of histone modifications, histone-modifying complexes, and their interactions have been developed into what is commonly referred to as a "histone code." Together with methylation, the histone code ensures that chromatin exists in a transcriptionally inactive mode that undergoes activation of specific gene regions when needed. The timely on/off switching of genes requires relevant changes in the epigenetic profile. Given that chromatin transcriptional activity is related to germline development, X-inactivation, stem-cell identity, cell-cycle regulation, and mitosis/meiosis, the decoding of epigenetic mechanisms should hold insights for studying cancer (Delage and Dashwood 2008). When histone modifications and chromatin remodeling processes become deregulated, they could result in mutations in oncogenes, tumor suppressor genes, or DNA repair genes. Such changes then could result in genomic instability, oncogenic transformation, and the development

of cancer. Histone modifications are extremely dynamic and highly regulated. When histone modifying complexes, including histone acetyltransferases (HATs), deacetylases, or histone methyltransferases, display aberrant activity, cancer development by misregulation of chromatin structure and activity may occur, as happens frequently in human leukemia (Herceg 2007).

Histone acetylation typically results in an open chromatin configuration that facilitates transcription factor access to DNA and gene transcription. It also can silence tumor suppressor genes in cancer cells if deacetylated (Wade 2001; Nian et al. 2009). HATs are responsible for acetylation; whereas histone deacetylases (HDACs) are responsible for histone deacetylation. Overexpression and or increased activity of HDACs is found in many malignancies, and the repression of transcription can result in dysregulated cell cycle kinetics, apoptosis, and differentiation (Dokmanovic and Marks 2005; Mariadason 2008; McLaughlin and La Thangue 2004). Many dietary components, such as sulforaphane, a major component in cruciferous vegetables, have shown promising results in direct or indirect inhibition of HDAC activity as well as other histone modification activities in cancer prevention and therapy (Myzak et al. 2004; Meeran et al. 2010).

1.2.3 RNA Interference

RNA interference plays an important role in controlling the activity level of specific genes. The exact roles and mechanisms are still not fully explored but rapidly expanding. One type of small RNA molecule involved in RNA interference is microRNA (miRNA). miRNAs are single-stranded non-coding RNAs of approximately 21–23 nucleotides in length whose main function is to downregulate gene expression by interfering with messenger RNA processes. miRNA suppresses gene expression by affecting miRNA stability, targeting the miRNA for degradation, or both (Mathers et al. 2010). In limited cases, miRNA also may increase gene transcription (Barros and Offenbacher 2009). Close to 1,000 miRNAs have been identified in human cells with the potential to regulate the expression of about one-third of human mRNAs and influence almost all genetic pathways through their effects on transcription factors, receptors, and transporters (Esquela-Kerscher and Slack 2006). miRNAs have an established repressing role in cancer initiation, development, maintenance, and proliferation (Esquela-Kerscher and Slack 2006). Mutation or mis-expression of miRNA occurs in several human cancers, and miRNAs have been reported to repress the expression of cancer-related genes. Taken together, these two observations suggest the potential importance of miRNA in tumorigenesis (Esquela-Kerscher and Slack 2006). It has been demonstrated that chemopreventive agents, such as n-3 polyunsaturated fatty acid, pectin, folate, retinoids, and curcumin, favorably modulate carcinogen-directed non-coding miRNA signatures in various cancer (Shah et al. 2011; Davidson et al. 2009; Kutay et al. 2006; Sun et al. 2008; Wang et al. 2008). These data suggest a persistent effect of diet in miRNA-mediated oncogenic transformation.

1.2.4 Chromosome Instability

The stability of the genome in general and chromosomes in particular plays a large role in several diseases including cancer. Mechanisms by which stability is undermined include DNA metabolism and repair gene silencing by aberrant methylation near CpG promoter regions, and accelerated telomere shortening. Environmental and nutritional factors may control both mechanisms. For example, zinc and magnesium levels help determine the effectiveness of DNA repair and DNA metabolism, respectively, and telomere length may be associated with folate and nicotinic acid levels (Bull and Fenech 2008). Animal study has demonstrated that exposure to estrogen and ionizing radiation causes epigenetic dysregulation, activation of mitogen-activated protein kinase pathways, and genome instability in the mammary gland (Kutanzi and Kovalchuk 2013). Although evidence has started emerging linking environmental factors to the instability of genome and subsequent diseases, additional studies will offer better understanding of the exact epigenetic mechanisms involved.

1.2.5 Transposons

Transposable elements are sequences of DNA that move to new positions within the genome of a single cell. Transposons are among several types of transposable elements which include plasmids, bacteriophages, and certain introns (Wicker et al. 2007). Transposons can behave as mutagens that cause genomic damage by various pathways. For example a given section of DNA could be cut and pasted into another DNA region potentially resulting in disruption of the functional gene it was pasted into or leaving behind an irreparable gap in the gene it was cut from, both resulting in a mutagenic effect. In other cases multiple sequences of pasted copies result which disturb chromosomal pairing during mitosis and meiosis. Some human blood dyscrasias can be caused by transposons including hemophilia A & B and porphyria (Kazazian 2004). Eukaryotic cells use RNA interference to silence transposable elements immediately after they are transcribed thereby inhibiting transposon activity levels to protect against genomic damage.

1.2.6 Loss of Imprinting

Imprinting is a process in which genes are silenced, depending on whether they are maternal or paternal in origin. The epigenome cycles through a series of precisely timed methylation changes designed to ensure proper development, both in utero and throughout life, of the organism. In humans, the paternal genome is actively

demethylated, and the maternal genome is passively demethylated following fertilization (Bernal and Jirtle 2010). Genomic imprinting results in parent-of-origin-dependent monoallelic expression of a subset of critical autosomal genes (Murphy and Jirtle 2003). Loss of methylation can either shut down these critical genes or lead to overexpression of the gene product. Because of their monoallelic expression, imprinted genes are particularly susceptible to dysregulated expression that results from epigenetic aberrations. Environmental exposures that alter the imprinting of these genes will lead to enhanced susceptibility to disease (Das et al. 2009). For example, a study has demonstrated that loss of imprinting at the insulin-like growth factor 2 (*IGF2*) locus could be induced by synthetic methyl-donor-deficient post weaning diet in mice (Waterland et al. 2006). Loss of imprinting is associated specifically with hypomethylation in colon cancer (Cui et al. 2002).

1.3 Mechanisms of Epigenetics

Epigenetic signatures provide the best explanation for how one genotype can be translated into many different phenotypes. For example, a series of studies showed that alternation in DNA methylation, using a well-designed epigenetic model, led to changes in coat color in Agouti mice (Michaud et al. 1994; Dolinoy et al. 2007a, b). In this model, diet affects the methylation status of an inserted intracisternal A particle element that alters coat color. The same group successfully proved that the phytoestrogen genistein (a weak estrogen), Bisphenol A, and folic acid (a methyl donor) are all able to change the methylation status of pregnant mice and affect offspring coat color.

Numerous experiments have demonstrated the importance of epigenetics. Animal cloning studies have shown that insufficient epigenetic reprogramming is the key reason for aberrant growth and lethality (Humpherys et al. 2001). Studies on human assisted reproductive technology, which uses media with undisclosed concentrations of methyl-donors, indicate that this technology is associated with epigenetic errors, such as imprinting defects and cancers (Niemitz and Feinberg 2004). Studies involving monozygotic (MZ) twins show that each individual develops different disease susceptibilities with age and may be in part explained by epigenetic modifications since these diverge as MZ twin age (Fraga et al. 2005a). Global DNA methylation, global histone H3 and H4 acetylation and sequence-specific DNA methylation were examined followed by relative gene expression profiles and show little differences in young twins but significant alterations in aged twins. Epigenetic changes, which may result from external environmental exposure or internal metabolic differences, may lead to further understanding of biological outcomes beyond the sequence code.

1.4 Applications of Epigenetic Research

1.4.1 Cancer Research

Epigenetic changes play a significant role in cancer progression. Overall, global DNA hypomethylation and reductions of specific histone modifications, such as loss of monoacetylation at lysine 16 and trimethylation at lysine 20 of histone H4, are found in malignant cells (Fraga et al. 2005b; Seligson et al. 2005; Feinberg and Vogelstein 1983). These global epigenetic alterations are thought to induce harmful expression of inserted viral sequences, oncogene activation, loss of imprinting, X-chromosome inactivation and genomic instability via changes in structure elements, such as centromeres (Gaudet et al. 2003). On the other hand, DNA hypermethylation and deacetylation of CpG sites often result in silencing of tumor suppressor genes (Herman and Baylin 2003). In addition, studies have found DNA methylation and histone acetylation have synergistic effects in cancers (Esteller 2005; Jones and Laird 1999). This increases the complexity and makes interpretation of data on DNA methylation or histone acetylation alone and associated gene expression changes difficult.

Many genes have been found to be epigenetically silenced in cancer cells. In fact, it is suggested that there is a greater chance of genes silenced by epigenetic mechanism than by genetic aberrations (Herman and Baylin 2003). A profile of promoter hypermethylation for human cancers has been identified and includes genes involved in cell cycle regulation and apoptosis (p14 ARF, p16INK4a), DNA repair (hMLH1, BRCA1), as well as metastasis and invasion (CDH1,TSP-1). DNA methylation and histone modification profiles have become promising tools for cancer prognosis and assessments for therapy (Seligson et al. 2005; Sidransky 2002).

Since the early discovery by Wolff and colleagues (Wolff et al. 1998), a significant amount of efforts on epigenetic research has been devoted on cancer research. Recent cancer genome analysis have revealed that a significant fraction of cancers cannot be accounted for only mutations of known protein-coding genes and that epigenetic alternations of genes are unexpectedly frequent (Stratton et al. 2009; You and Jones 2012). Because epigenetic modifications play a central role during somatic reprogramming, elucidating the underlying mechanisms of the initiation and progression of human cancer can help identify potential targets of intervention for prevention, diagnosis, therapy, and prognosis of this disease. Aberrant DNA methylation and histone modifications to date are the best established epigenetic mechanisms of carcinogenesis. It has been observed that epigenetic abnormalities are maintained even after a somatic cell division (Feinberg 2004). These abnormalities can be causally involved in carcinogenesis (Ushijima and Takeshima 2013).

With the application of the knowledge gained from cell-cultures and animal models, population-based studies on epigenetics, such as epigenetic epidemiology to study of variation in epigenetic traits and the risk of diseases in population, have further provided insight into how lifestyle and environmental factors influence the epigenome and how epigenetic events may be involved in carcinogenesis (Wolff et al. 1998). The application of epigenetics research also has the potential to bring benefit to patients through identification of diagnostic markers that enable the early detection of disease and prognostic markers that can inform appropriate treatment strategies (Wolff et al. 1998). Because the occurrence of epigenetic changes prior to clinical diagnosis of cancer and their reversibility through pharmacologic/genetic approaches, epigenetic research offers a very promising avenue for basic and translational research of human cancer (Besaratinia and Tommasi 2014). For example, it has been suggested that radiotherapy for cancer treatment itself introduces epigenetic alterations (Smits et al. 2014). As epigenetic alterations can potentially be reversed, they could serve as radiotherapy sensitizers. The application of demethylating drugs or histone deacetylase inhibitors to sensitize patients for radiotherapy has been studied in vitro, in vivo as well as in clinical trials with promising results (Smits et al. 2014; Eramo et al. 2005; Zhang et al. 2011; Van Nifterik et al. 2012; Kawano et al. 2010; Kano et al. 2009; Berghuis et al. 2012; Sharma et al. 2013; Groselj et al. 2013).

1.4.2 Neuroscience Research

Study on neurological disorders is another field that epigenetic research has provided promising advancements. Study has shown that epigenetic modifications are more susceptible to alterations induced by environmental factors than are DNA sequences and that some drugs commonly used reverse mental-stress induced alterations to histone modifications in neural genes (Kubota et al. 2014). Therefore, application of diagnostic assays to detect epigenetic alterations may provide new insight into the characterization and treatment of neurodevelopmental disorders. Additionally, epigenetic regulation of gene expression, which includes DNA methylation and histone modification, play significant role in neutral stem cell proliferation and differentiation (Wakabayashi et al. 2014). Since miRNAs are small non-coding RNA molecules that post-transcriptionally regulate gene expression, the cross-talk between miRNA and epigenetic regulators appears to modulate neurogenesis in the adult mammalian brain (Szulwach et al. 2010; Jobe et al. 2012; Shi et al. 2010). Additionally, miRNA has been identified to govern many genes, including drug targets, are altered in disease development and drug administration.

It has recently been demonstrated that the dysregulation of the proopionmelanocortin (POMC) system function by fetal alcohol exposure (FAE) is induced by epigenetic mechanisms such as hypermethylation at POMC gene promoter and an alteration in histone marks in POMC neurons (Gangisetty et al. 2014; Bekdash et al. 2014). The developmental programming of the POMC system by FAE altered the

transcriptome in POMC neuron and induced a hyper-response to stress in adulthood. These long-lasting epigenetic changes influenced subsequent generations via the male germline. The study also demonstrated that the epigenetic programming of POMC system by FAE can be reversed in adulthood with the application f the inhibitors of DNA methylation or histone modification (Bekdash et al. 2014). Therefore, prenatal environment influence could epigenetically modulate POMC neuronal circuits and function to shape adult behavior patterns.

1.4.3 Autoimmune Diseases, Pain Management and Beyond

Additionally, epigenetic research has also been applied to the studies of autoimmune diseases and pain management. For example, epigenetic studies in systemic erythematosus (SLE) are expected to reveal novel therapeutic targets and disease biomarkers in autoimmunity (Ombrello et al. 2014). Pain management through the epigenetic modification of genes that regulate chronic inflammation has also been examined (Duenas-Gonzalez et al. 2014).

1.4.4 Pharmacology and Drug Development

In terms of drug development, due to the complexity of many diseases, such as cancer, neurological diseases, autoimmune diseases, and etc., the discovery pharmacoepigenetics provides a new way to solve the problem. Epigenetic modifications can influence drug efficacy and altering gene expression via changing chromatin structure. For example, studies have suggested that cancer cells possess the ability to reversibly change their properties between a drug sensitive state and a drug resistant state accompanied by epigenetic changes (Brown et al. 2014; Wilting and Dannenberg 2012; Sharma et al. 2010). Therefore, better understanding of cancer epigenetics is important for efficient cancer therapy. Very recently, Chaurasia and colleagues reported an impressive ex vivo expansion of human stem cells from cord blood using cytokines and altering epigenetic modifications (Broxmeyer 2014). This protocol provides potential application for clinical consideration because of the ability for chromatin modification of erythroid progenitor cells from cord blood. Researchers have also established a reprogramming technology that made it possible for researchers to artificially introduce epigenetic remodeling into somatic cells (Watanabe et al. 2013). This technology, accordingly, could be used to introduce the coordinated epigenetic reorganization. The potentials for the "real life" application of epigenetics research for treatment of complex diseases is near to fruition as several DNA demethylating drugs, such as 5-azacytidine (azacitidine) and 5-azadeoxycytidine (decitabine), and histone deacetylase inhibitors, such as Vorinostat and Romidepsin for cutaneous T cell lymphoma, are already approved and many next-generation epigenetic drug are under development. Right patients, right doses, and right combinations are key to further success.

There are many other applications of epigenetics research which are discussed more extensively in other chapters in this book. This book is intended to highlight some important accomplishments in the field of environmental epigenetics but not make an exhausted list of all applications to date.

1.5 Associations of the Chapters in this Book in Epigenetics

This book consists of 13 chapters. In addition to this Chapter which gives a broad overview of epigenetics. The following is the highlights of each chapter in this book.

Chapter 2: Epidemiology of environmental health

In order to better understand the reasons behind the study of environmental epigenetics, this chapter provide a general introduction on the importance of environmental health through the prospective of epidemiological studies.

Chapter 3: Epigenetic changes in hormonal related disease: Uterine Leiomyoma (fibroids)

Epigenetic modifications have been associated with etiology of hormonal related diseases. Environmental factors, such as environmental disrupting chemicals, are thought to play important roles in the development and progress of hormonal related diseases. This chapter utilizes uterine leiomyoma as an example to explore the potentially intricate relationship.

Chapter 4: Is Environmental Epigenetics Relevant to Endocrine Disease?

Endocrine disrupting chemicals have the potential to mimic endocrine endpoints at the receptor level dysregulated hormone mediated gene expression through epigenetic changes and changes in signal transduction. However, environmental compounds may exert endocrine effects through other alternative pathways. The concepts for endocrinology related to environmental epigenetics are reviewed in this chapter.

Chapter 5: Pregnancy Exposures Determine Risk of Breast Cancer in Multiple Generations of Offspring

The maternal exposure to various environmental factors during pregnancy may change various carcinogenesis-related hormone levels and alter the epigenome, which predispose offsprings to breast cancer risk in their later life. This chapter systematically summarizes findings from both experimental and epidemiological studies examining the relationship between maternal pregnancy exposures and offspring's breast cancer risk.

Chapter 6: Environmental epigenetics and obesity – evidences from animal to epidemiologic studies

Agouti mouse model was one of the first direct evidence that epigenetic alternations early in life lead to obesity later in adult life. Natural human experiment, such as Dutch famine study, also suggested that peri-conceptional and peri-natal nutrient deficiency resulted in overweight or obesity later in life. Evidences from animal experiments to epidemiologic studies are summarized and etiologic factors are explored.

Chapter 7: Environmental toxicants, epigenetics, and cancer

The vast majority of cancers are sporadic, in which there is no genetic predisposition or family history of the disease. For these cancers, the etiology must lie, at least in great part, in environmental factors including environmental toxicants. This chapter examines sources of environmental toxicants that are encountered by most people in their everyday lives for their known or potential roles in cancer development through epigenetic mechanisms.

Chapter 8: The epigenome and aging

The epigenetics of aging is a relatively new field. Global DNA methylation has been examined for some time; however, only recently have age-related differentially methylated regions been elaborated. Studies have examined expression of microRNA during aging in various organisms, including human, point to the possibility that these gene regulatory molecules may also be involved in aging. The interface between the genome and the environment that epigenetic mechanisms provide is explored.

Chapter 9: Environmental contributors to epigenetics and cardiovascular disease risk

Cardiovascular diseases are the leading causes of death and disability worldwide. The advanced knowledge in epigenetics allows for a newer perspective of how a cell's DNA sequence dynamically responds to environmental stimuli and insults. This chapter highlights emerging epigenetic concepts in cardiovascular health in relation to a range of environmental stressors from starvation to microfluidic hemodynamics in vasculature.

Chapter 10: Gene-environment interactions in human health

Risk of most complex diseases is determined by a combination of environmental and genetic factors. By studying gene-environment interactions, it may be possible to describe disease mechanisms, discover novel genetic variants associated with disease, better understand heterogeneity between populations, identify populations with higher risk from exposure, and target preventive and therapeutic interventions. This chapter explores opportunities to better understand the complex interplay between genes and environment may result in improved insights in disease and treatment outcomes.

Chapter 11: Environmental epigenomics: applications of epigenetic biomarkers to investigate epigenetic alterations from environmental exposures

Given that almost 25 % of all human diseases are estimated to be caused by environmental exposures, this chapter reviews current studies of xenobiotic exposures for which there is growing mechanistic evidence for indirect DNA alteration through epigenetic mechanisms. Epidemiological methods used to incorporate epigenetic alterations into studies of disease etiology as well as current methods used to apply and critically evaluate study results reporting associations between environmental exposure and disease are also discussed.

Chapter 12: Interplay of epigenetics, genome rearrangement, and environment during development

Genome rearrangement, characterized by insertion, deletion, amplification, inversion, and/or transposition of DNA segments, during development are driven by epigenetic markings, which are modifiable by external and internal environments.

It has been suggested that genome rearrangement, which introduces genome diversity, is a driving force of mammalian development. This chapter summarizes the current state of evidence and explores the interplay among factors contributing to the genome rearrangement.

Chapter 13: Environment, epigenetic, and disease: the modifiable trilogy

Contrary to genetic approaches, epigenetic changes, including those caused by environmental exposures, are reversible with specific treatments. Lifestyle modifications may help ameliorate damage, or reduce disease risk, by changing the epigenetic "landscape." Burgeoning technologies have helped to identify specific gene profiles differentially expressed in disease conditions. This chapter summarized the existing laboratory technologies used to catalog epigenetic modifications and explore the "modifiable trilogy".

1.6 Conclusions

Over the past 5 years, massively parallel, high-throughput, next-generation sequencing techniques have revolutionized genetic and genomic, identifying the causes of many Mendelian diseases. The application of whole-genome sequencing and whole exome sequencing to large populations has produced several publicly available sequence datasets that have revealed the scope of human genetic variation and have contributed to important methodological advances in the study of both common and rare genetic variants in genetically complex diseases (Ombrello et al. 2014).

However, despite of the advancement in epigenetics knowledge, there are still many questions to be answered in epigenetics research. For example, suitability of blood as a surrogate tissue to assess epigenetic changes and interpretation of genome-wide DNA methylation information are questions to be answered.

Epigenetic therapy is highly anticipated as a next player to molecular target drugs. "Omics" technologies have been proposed and applied to drug toxicology research. However, we cannot fully conceptualize the application of epigenetics to the field of drug toxicology, as there are still many challenges to overcome before DNA methylation-based biomarkers can be effectively used in drug development. Comprehensive whole-genome DNA methylation methods for an unbiased analysis based on either microarray or next-generation sequencing need to be evaluated in an intensive and systematic manner. Additionally, robust analysis systems need to be developed to decode the large amounts of data generated by whole-genome methylation analyses as well as protocol standardization for reproducibility to develop meaningful database that can be applied to drug toxicology (Duenas-Gonzalez et al. 2014).

The more we understand the complicate biologic system, genetically and epigenetically, the more challenge we will face. What we saw to date is a tip of iceberg. The good news is we have started the first step. This book is to serve as an introduction for beginner to understand the importance of epigenetics and hope to bring more idea in understanding the concert between genetic and epigenetic signatures.

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Chapter 2
Epidemiology of Environmental Health

Chen-Yu Liu

Abstract There has been a growing awareness of environmental effects among human. More epidemiologic studies have been triggered toward investigating exposure effects of chemical, social or physical factors in relation to the common or complex diseases, such as heart disease, cancer, and diabetes afterward. Following recent advances in genomics and risk assessment, environmental epidemiologic studies are incorporating gene-environment interactions and epigenetic changes to explore the multidisciplinary nature of individual. The challenges for human population work in this field include the complexity of exposure biology and the small effects that are easily disturbed. Several strategies have been developed with an attempt to resolve these challenges, such as the development of exposome, the two-stage designs and Mendelian randomization. In this chapter, the conventional study design features, including particular strengths and limitations, have been summarized.

Keywords Case-control • Cohort • Confounding • Bias • Biomarkers • Critical developmental windows • Epigenetics

2.1 Introduction of Epidemiology

2.1.1 Definition

The word "epidemiology" literally meaning "the study of what is upon the people" is made up by three Greek word roots: epi, meaning upon or among, demos, meaning people, and logos, meaning study. Epidemiology is the study of the distribution of health-related events or conditions occur in specified populations and the

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investigation of why. The application of the study is to prevent and effectively control health problems (Last 2001). It aims to characterize person, time, and space and relate to the states of human health. When the health states are not distributed uniformly across people, times, and spaces, the epidemiologist is able to generate hypotheses of the underlying causes.

While early studies in epidemiology were largely concerned about communicable diseases, more attention has been paid toward the exposure effects of chemical, social or physical factors in relation to the common or complex diseases, such as heart disease, cancer, and diabetes afterward. Both environmental and genetic factors play an important role in most complex human diseases. One of the major challenges of exploring mechanisms and treatment of complex diseases is that neither purely environmental factors, nor purely genetic factors can fully explain the observed estimates of disease incidence and progression. Epidemiologic studies of environment and health incorporation with gene-environment interactions and epigenetic changes investigations help exploring the multidisciplinary nature of individual. Discussions of epidemiologic study designs and analysis issues in the area of genes and environment interaction effects can be found in Hunter (2005), Thomas (2010), and Liu et al. (2012).

The role of epigenetics has been increasingly recognized as a mechanism of gene-environment interaction. Epigenetics refers to changes in gene function without altering DNA sequence. This field remains particularly compelling because a number of epigenetic events have been recognized as tissue-specific and reversible, this may help explain why exposures affect specific organs and the complexity of individual susceptibility among the exposed population. Because many epigenetic changes can be affected by both internal and external factors and have the ability to change gene expressions, epigenetics is a potential major mechanism of how the genomes interact with the environmental exposures. Thus, epigenetics provide a new strategy in the search for etiological factors in many environment associated diseases.

Bringing epigenetics into the studies of environmental epidemiology helps investigators recognize mechanisms linking exposure and disease outcome, and provides new opportunities to explore disease and exposure biomarkers and new strategies for disease prevention and treatment (Michels 2010). However, some challenges remain in the areas of study design and epigenetic data interpretation among human populations. In the interpretation of an epidemiologic study, it is essential to examine if there are factors other than that under investigation might explain the result. In this chapter, we focus on design issues and recent developments in studying epidemiology of environment and health. For more extensive discussion about research principles in epidemiology, the interested readers are encourage to consult textbook in the area (Koepsell and Weiss 2003; Rothman and Greenland 1998a).

2.1.2 Epidemiologic Study Designs

Epidemiologic study designs comprise experimental studies and observational studies. Experimental studies use randomization to assign exposures to individuals, such as clinical trials or field trials, or to communities (community trials). Experimental studies are conducted to investigate preventions and treatments for diseases. A well-conducted experimental study can provide more scientifically rigorous data, however, several difficulties such as high cost, noncompliance, and ethical issues make the conduction of experimental study really challenging.

The majority of environmental health studies are conducted by using observational designs. The choice of study design is almost always determined by the research question of interest and the feasibility. For example, the commonly used epidemiologic study designs in the search for biomarkers of genetic and environmental effects, primary including case-control, cohort, and cross-sectional studies.

2.1.2.1 Data Collection

The data collection for observational studies may be based on existing records such as health examination records or the information collected specially for the investigators. Many lifestyle related information, such as cigarette smoking, alcohol consumption, or diet, are usually not able to obtain from the existing records. Interviews and questionnaires are especially useful in collecting this information. Exposure measurements by incorporating questionnaires, environmental sampling, personal monitoring, and biomonitoring, may also be collected in the environmental health study. Direct exposure monitoring includes personal monitoring by measuring toxics on or near the body, such as measuring air pollutants exposure levels at the breathing zone, or by sampling biological properties, such as the measurement of urinary 1-hydroxypyrene (1-OHP) as a biomarker of short-term polycyclic aromatic hydrocarbon (PAH) exposure (Jacob and Seidel 2002). *Biomarkers* of exposure are biological indicators of exogenous agents within the biological system, or other event in the biological system related to the exposure. Examples of biological samples collected for biomarker investigations include urine, blood, or other tissues of subjects.

2.1.2.2 Types of Epidemiologic Studies

Cross-Sectional Study

In a cross-sectional study the exposure information is usually collected simultaneously with the disease status. A cross-sectional study can be useful in estimating the health status or the disease pattern of a population at a specific time. When investigating a sudden disease outbreak, a cross-sectional study can measure several exposures and be

the first step in exploring the cause. For example, in the late 1960s, cross-sectional studies were used during the outbreak of blackfoot disease and reported associations of arsenic in drinking water and blackfoot disease (Tseng 1977; Tseng et al. 1968). It is relatively quick, inexpensively, and less time-consuming to conduct cross-sectional study. The association of multiple exposures and disease outcomes can be examined at the same time and therefore can be used as hypothesis generation. More rigorous study design such as cohort study or randomized control trial should be followed to test the hypothesis.

Case-Control Study

In a case-control study, subjects are enrolled by disease status (or other outcome variable) and historical exposure statuses of cases and controls are *retrospectively* assessed for the possible cause. Cases are individuals selected on the basis of disease. Controls are individuals without the disease when they enroll the study. Controls are selected to represent the source population where the cases come from. Its purpose is to provide information of exposure distribution in the source population as a reference so the occurrence of possible cause can be compared between cases and controls.

Cases can be either incident or prevalent cases. Incident cases are the cases enrolled in the study with disease newly diagnosed. Prevalent cases are cases already have existing disease before the enrollment of the study. The advantage of using incident cases in case-control study is to avoid the exposure-disease relationship be affected by the disease prognosis and duration. However, during rare disease research, prevalent cases may still have to be included. Investigators need to understand which kind of misleading conclusion could be introduced.

Case-Crossover Study

The case-crossover design was developed by Maclure (1991) to study effects of transient short-term exposures on acute events. In a case-crossover study, cases compare their own exposure during a risk period related to the causation of the outcome event to one or more than one exposure during control periods. Therefore, each case serves as his or her own control.

Cohort Study

A cohort study is a group of individuals who are free of disease of interest at the baseline (the start of follow-up) and are followed to determine the health outcomes. The exposure status is assessed among every subject at the baseline and possibly during the follow-up. The unexposed (or low-level exposure) group is used as

reference. Investigators compare the disease rates by exposure status. If the disease development is substantially different by exposure status, the investigator may be able to conclude the association between exposure and disease.

Cohort studies are useful in studying rare exposure since investigators can base on exposure status to select study subjects and make sure sufficient exposed subjects are enrolled.

A cohort study can be *prospective* or *retrospective* depending on if the data collection is before or after the development of health outcomes. In a prospective study, information of environmental exposures and other covariates of interest and biological samples, such as blood, are collected at the start of the study before the onset of disease or other health outcome. The study subjects are then followed over time to measure disease occurrence. Exposure status and questionnaire data as well as biological samples may be also updated during follow-up.

In a retrospective study, information is collected after the health outcome has already occurred. Exposure status and health outcome are assessed by using existing data or by subjects' recall. Investigators therefore have limited control over data collection. The existing data may not be appropriate for the study purpose and the measurements may be inaccurate, incomplete, or inconsistent between subjects.

Birth Cohort

The concept of fetal origins of adult diseases demonstrates the critical nature of exposure timing in producing later health effects (e.g., the association of maternal smoking during pregnancy and reduced fetal growth (Agrawal et al. 2010), obesity (Kristensen 1992), decreased lung function (Wiencke et al. 1991) and diabetes (Montgomery and Ekbom 2002) in the offspring). An increasing number of animal studies provide evidence of the role of environmental epigenetics both in disease susceptibility and in heritable environmentally induced transgenerational alterations in phenotype (Jirtle and Skinner 2007).

Genome-wide epigenetic reprogramming occurs at the stages of preimplantation and germ cell lineage development. Epigenome may therefore be more vulnerable to the environmental insults during these critical life periods (Sasaki and Matsui 2008; Feng et al. 2010). Epigenetic mechanisms in somatic cells also provide a potential explanation of how early life environmental exposures can program long-term effects in chronic disease susceptibility (Gluckman and Hanson 2004; Waterland and Michels 2007). In a birth cohort, pre-conceptual and prenatal exposures can be assessed. Follow-up a birth cohort allows tracking of development and variations in exposures and health outcomes over time. Birth cohort studies can measure DNA methylation profiles and chromatin states of mothers and their offspring at birth in tissues that can be easily obtained such as saliva, cord blood, and placenta. Additional serial sampling at multiple time points across life course enable the assessment of early effects, temporal variations in biomarkers, or susceptibility of exposures over time.

Nested Case-Control Design and Case-Cohort Studies

Cohort studies are less feasible in studying rare disease with long latency because of the number of subjects required and the follow-up period. A pseudo case-control design can be used to reduce the number of subjects for whom covariate data are required. A so-called 'case-control nested within a cohort' design (Liu et al. 2012) by matching each subject developing disease to one or more subjects without disease at the same time using incidence-density sample (Liu et al. 2012). Only cases and their matched controls require the covariate measurements (see for example, (Breslow et al. 1983; Lubin and Gail 1984; Whittemore 1981; Whittemore and McMillan 1982)).

A case-cohort design proposed by Prentice (Prentice and Pyke 1979) involves the random sample of a subcohort from the entire cohort. The covariate data are collected only among these random subcohort and all the cases. In nested case-control designs, controls are selected from individuals at risk at the times cases are identified, while in case-cohort studies, controls are a subcohort selected from the entire cohort at baseline. The subcohort in a given stratum provides a comparison set of cases occurring at a range of failure times and a basis for covariate monitoring during the cohort follow-up. Very similar designs have also been proposed by Kupper et al. (1975) and Miettinen (1982). These more efficient designs have started being used to study gene-environment interactions in cohort studies (Bureau et al. 2008).

Two-Stage Designs

One of the challenges environmental epidemiologists facing is the study of small effect that are easily affected by the confounding factors. In the situations when the exposure of interest and the disease are both rare, very large number of subjects are required and can be very expensive. One solution originally proposed by White is a two-stage design (White 1982). The first stage is the screening stage, exposure and disease information is ascertained on a large sample, but complete covariate information and maybe more refined exposure measurement is then collected on only a subsample in the second stage. The sampling fractions for stage 2 can depend jointly on disease and exposure status.

Twin and Family-Based Study

Twin- and family-based studies, such as triads of mother, father, and child, have been used to estimate the genetics basis of traits and the heritability of epigenetic profiles, including chromatin states (Rothman and Greenland 1998a) and DNA methylation (Koepsell and Weiss 2003; Hunt and White 1998; Ludwig and Weinstein 2005). Increasing gene-environment interaction studies have been conducted in the environmental health investigations. Individual variation in their sen-

sitivity to exposure is one of the aims of environmental epidemiology. Family-based studies of gene–environment interaction sometimes may be more powerful than population-based studies (Gauderman 2002). In family-based study, investigators are studying if the familial aggregation presence in the disease and if such aggregation can be explained by environmental or genetic factor. In twin study, the heritability of traits may be estimated based on comparisons between monozygotic and dizygotic twins since monozygotic twins have 100 % of their genes in common while dizygotic twins have 50 % of their genes in common. An increased disease concordance among monozygotic twins may represent a greater extent to which genetic effects contribute.

Collect DNA samples from family members can be more difficult than from unrelated cases and controls, especially for long latency or late-onset diseases. The use of relatives as controls may lead to overmatching on a range of genetic and environmental factors (Wacholder et al. 1992). Besides, the over sampling of intact families would also not be expected to represent social environments in the general population.

2.1.3 Study Design Issues

2.1.3.1 Confounding and Bias

When designing epidemiologic studies, there are a few issues required for consideration: feasibility, efficiency, expense, and potential sources of bias. In contrast to controlled clinical trials, epidemiological studies often suffer from confounding bias due to measured and unmeasured confounders (van Rein et al. 2014).

Confounding occurs when a disease outcome appears to be associated with an exposure merely due to its correlation with some other risk factors of the disease. For example, age is a potential confounder in epigenetic epidemiology, since DNA methylation levels change with age and age is a risk factor for lots of diseases. At the stage of study design, confounding effects can be minimized by randomization, matching, and restriction. Confounding effects can also be addressed during data analysis by performing stratification, standardization, regression modeling. Careful examination of the criteria for a possible confounder should be done before attempting to control for confounding effects to avoid overadjustment and the introduction of new bias (Jager et al. 2008).

While the occurrence of confounding is mostly due to the nature of epidemiologic study that various characteristics unevenly distributed among humans, the occurrence of bias is an error introduced by investigator during study design or conduction. In the retrospective study design such as case-control studies, the collection of data on environmental exposures and biological samples is retrospectively after the disease diagnosis. The assessment of exposure retrospectively is fraught with potential *recall bias*. While biomarkers of exposure can reduce such bias, these measures rarely can reconstruct past exposure and may be affected by the

current disease status, which may be one of the great challenges of retrospective studies. Another potential concern is *selection bias*. A fundamental requirement of a case-control study design is that cases and controls should be selected from the same population (Rothman and Greenland 1998b). Population-based incidence cases allow investigators to maximize the generalizability of the findings. Selection bias occurs when controls do not represent the population from which the cases arose (Last et al. 2001). Case-control studies are subject to both selection bias and recall bias. For rapidly fatal diseases, since only some of the incidence cases may be available for interviewing, *survivor bias* can occur if exposure status differs by survival time.

In prospective cohort studies, because this approach has the advantage of prospective collection of environmental information and biomarkers, which both precede the disease and will be unaffected by recall bias (Albert et al. 2001). A cohort study can be affected by selection bias if the participation or follow-up is related to exposure and health outcome. Analysis of data from cohort studies can be subject to bias due to loss of follow-up. Effective follow-up should minimize selection bias by attrition, and yet, at the same time, more time and expense is required. The expense of cohort studies often limits feasibility, especially as incidence rates of most diseases are low. Even with many years of follow-up a cohort study often requires collection of an *extremely* large number of individuals before the onset of disease and a sufficient follow-up time. Hence, prospective studies are considerable challenges for diseases with low incidence rate. *Risk-based sampling* is being used to increase the power of prospective studies by enrolling first-degree relatives of probands, such as the Sister Study for breast cancer risk (Weinberg et al. 2007; Medlin 2001) or the on-going Early Autism Risk Longitudinal Investigation (EARLI) study for autism risk. For common pediatric diseases such as asthma, obesity, and some adverse birth outcomes, a prospective cohort study will be extremely valuable to identify environmental risk factors (Manolio 2009; Manolio et al. 2006). Prospective cohort studies on a national scale (Collins and Manolio 2007), by pooling data from existing prospective cohorts (Willett et al. 2007), or by collaborations of cohorts across large regions such as Birth Cohort Consortium of Asia (BiCCA) and Environmental Health Risks in European Birth Cohorts (ENRIECO) should be conducted to ensure sufficient power. The U.S. Congress, through the Children's Health Act of 2000, authorized the National Institute of Child Health and Human Development (NICHD) "to conduct a national longitudinal study of environmental influences (including physical, chemical, biological, and psychosocial) on children's health and development" (CHA 2000). The National Children's Study is a 21-year prospective cohort study of 100,000 US-born children. Environmental exposures, including chemical, physical, biological, and psychosocial exposure, will be assessed repeatedly during pregnancy and childhood in children's homes, schools, and communities. The National Children's Study will provide great opportunities to gene-environment interactions for common pediatric diseases.

In a cross-sectional study since exposure and disease status are assessed at the same time, the temporal sequence of exposure and disease status usually cannot be identified. Unless in the situation that exposure is a characteristic such as gender and

the disease developed through time, such a temporal sequence of exposure and disease development is more possible. Otherwise, for the exposure factors different by time, the causality is hard to define. In addition, like many retrospectively studies, assessment of environment exposure retrospectively is fraught with potential recall bias. Further, because cases in a cross-sectional study are existing cases at the time, cases have to survive long enough to be included and may mislead the conclusion.

2.1.3.2 Measurements of Exposure

Measurements of environmental exposures have been a great challenge in epidemiologic studies due to the complex pattern of long-term exposures and the need to collect accurate and repeated individual exposure data in large populations (Morgenstern and Thomas 1993). Measurement errors, such as misclassification of exposure status, can exist regardless of study design. Misclassification of exposure generally leads to attenuation of the main effects when the error is non-differential (Carroll et al. 2006). Non-differential misclassification occurs when the misclassification of exposure does not depend on disease status or when the misclassification of disease does not depend on exposure status. Non-differential misclassification can also bias away from the null in some circumstances, including (1) if the exposure is multilevel (>2 levels), the intermediate levels of exposure could be biased away from null (Dosemeci et al. 1990; Weinberg et al. 1994); (2) if the misclassifications are correlated with other errors (Kristensen 1992; Chavance et al. 1992); (3) if the measured exposure do not change monotonically with the true exposure (Weinberg et al. 1994; ES 1991).

The use of questionnaires for exposure assessment relies on personal memory and has the potential for recall bias. Several technologies have been developed to improve measurements of environmental exposures. To incorporate qualitative and quantitative changes of environmental exposures, such as atmospheric conditions and topography, over time and space, as well as individuals' diverse demographic characteristics, lifestyles, activity patterns, geographic information systems (GIS)/global positioning system (GPS), personal monitoring, and biomonitoring are now being used in environmental epidemiology. Combined geospatial tools with statistical models allow investigators to model the transport of the pollutants from source to residence, e.g., using wind speed, temperature, and traffic density in addition to measurements from the central site, to estimate an individual-level exposure as well. Monitoring data, such as personal monitoring and measurements of biomarkers, can hold great promise for improving exposure assessment by providing objective individual-level measurements. Biomarkers take into account individual differences in absorption, distribution, and metabolism of the compound within the body and therefore can be used to reflect the effects of earlier exposures and the association between exposure and disease at the molecular level (NRC 1987; Perera and Weinstein 1982; Rothman et al. 1995). The usefulness of a biomarker is strongly depending on the specificity, sensitivity, assay reliability, accessibility, and cost (Hemstreet et al. 2001). Examples of biomarkers for exposure or effect range from

patterns of gene expressions, proteins, or metabolic profiles in cells and tissues change. Glycosylated hemoglobin for instance, a measure of chronic serum glucose, can be used to study diabetic risk factors with more power than a study focused on clinical diabetes. In spite of these potential advantages, the results of biomarker measurements sometimes can confuse the investigators a lot. Different conclusions may arise due to the differences of specimen kinds, collection and processing methods, laboratory error, and individual variation in the biomarker levels over time (Little and Sacks 2009). For instance, the correlation between epigenetic profiles in different tissues is complex and locus dependent. The tissue specificity and may be the cell type specificity in epigenetic patterns is challenging in epidemiologic studies. Population-based studies may inevitably rely on the use of easily accessible tissue, such as peripheral blood, saliva, or buccal cells. These sources may be able to reflect the environmental induced epigenetic changes or an overall indicator of the health status of a group of individuals; however, they may not accurately represent epigenetic variations of the diseased target tissue of interest (Talens et al. 2010).

Exposure Effects Across Life Stages

Measuring environment has added complexity beyond issues of measurement error or selection bias. Even measuring cumulative exposure prospectively may be insufficient. During prenatal life and childhood, critical biological events occur that establish the number, connections and proper function of cells within given tissues. As an example, environmental exposure, particularly in early development, may induce changes in gene expression modulated through DNA promoter methylation or chromatin remodeling (Fleming et al. 2008). Toxicological studies show that the central nervous system is especially vulnerable to toxic injury (Rodier 2004) and epidemiological studies clearly show an association between adverse neurodevelopment and in utero exposure to chemicals such as methyl mercury (Amin-Zaki et al. 1974; Marsh et al. 1980), PCBs (Tilson et al. 1990), while exposure later in life demonstrates less toxicity. The concept of fetal origins of adult diseases demonstrates the critical nature of exposure timing in producing later health effects (e.g., the association of maternal smoking during pregnancy and reduced fetal growth (Agrawal et al. 2010), obesity (Kristensen 1992), decreased lung function (Wiencke et al. 1991) and diabetes (Montgomery and Ekbom 2002) in the offspring). All individuals are exposed to a variety of hazardous agents and chemicals in the environment. The concept of exposome represents the totality of exposures received by a person during life and defines exposures as levels of biologically active compounds (Rappaport 2011). Because sources and levels of exposure vary by time, exposomes can be constructed by analyzing toxicants in blood specimens obtained during critical stages of life.

Because genotypes do not vary over time and can always be presumed to precede phenotype, genotype related measurements and prognostic markers are still identified in case-control studies. Otherwise, early detection or risk predication markers are preferred in nested case-control studies with biological samples collected

pre-diagnostically. A prospective study can address timing of exposure in an unbiased manner, however, it is still challenging to assess the details of exposure timing and risk as the critical window likely differs for different phenotypes and for different exposures. These dynamic features make their interpretation in human studies challenging. Single measurement may not be reliable especially in those investigating long-term chronic effects. Incorporating different exposure assessment techniques with long-term monitoring data especially during critical periods is needed to provide an integrated view of exposure in complex exposure–disease relationships (Lioy 1995; Weis et al. 2005). Unfortunately, for most adult diseases, an unbiased reconstruction of childhood exposure is difficult or even impossible. Thus, a major limitation of adult epidemiologic research will continue to be the inability to reconstruct childhood factors that predict disease. A measure of cumulative exposure, while preferable to cross-sectional measures, cannot capture exposure during the critical developmental life stage predisposing to disease. Therefore, rather than modeling a chemical in a single dose response curve for toxicity, chemicals appear to have different dose response curves depending on the life stage at which exposure occurs. For example, in utero diethylstilbesterol exposure is associated with vaginal cancer in offspring, while mothers who took the drug do not appear to be at risk.

Another difficulty is that it is not possible to know with certainty what the critical exposure window is a priori (i.e. in utero vs. childhood vs. puberty). The difficulties in assessing the exposure effects by timing present in carefully designed observational studies and even trial results. An example is the initial report from Women's Health Initiative (WHI) randomized trial and epidemiologic data on the risk of coronary heart disease (CHD) and the menopausal hormone therapy. Large observational studies include Nurses' Health Study (NHS) suggested a reduced risk of CHD among postmenopausal hormone therapy (Hemstreet et al. 2001; Angerer et al. 2007) while WHI randomized trial found increased risk of CHD among women assigned to the menopausal hormone therapy compared to the placebo group (Chatterjee et al. 1969). Hernán et al. re-analysis of the Nurses' Health Study and concluded that most of the difference could be attributed to the age distribution at the time of initiation of hormone therapy and length of follow-up (Hernan et al. 2008).

Mendelian Randomization

Mendelian randomization is an approach, instead of studying unknown effects, using the established associations between genetic variations and exposure intermediate phenotypes to maximize causal inference. These genetic variations can mimic the modifiable exposure effects and serve as a surrogate to test the association between exposure and disease. Mendelian randomization provides an approach for making causal inferences about the exposure by using the nature of randomly assigned genotypes from parents to offspring before conception (Gray and Wheatley 1991; Davey Smith and Ebrahim 2003). However, as well with all genetic association studies, potential confounding effects by population stratifications and other limitations can still occur (Davey Smith and Ebrahim 2003; Cui et al. 2001).

Population stratification refers to the different genotype frequencies between cases and controls due to differences in ancestry rather than association between genes and disease. Careful study conduction and thorough verification remains essential before considering the causality.

2.2 Discussion

Following recent advances in genomics and risk assessment, current research has shown that both internal and external factors of human bodies are critical to disease risk and progression. Future improvements in disease prevention and treatment are anticipated. More research efforts need to be directed towards investigating the exposure biology such as identifying new biomarkers for better measuring exposures, and the roles of genome and epigenome to various environmental agents. The integration of specimen bank among stored biological materials from cohort studies and the increasing collaboration between basic bench work and epidemiology is becoming more and more important. Study design selection is strongly depending on the feasibility and cost and should be guided by the appropriateness of the research question of interest. As summarized above, each epidemiologic study design has various strengths and limitations that make it more or less suitable for particular exposure effect investigation. In the interpretation of an epidemiologic study, it is essential for the researcher to examine if there are factors other than that under investigation might explain the result.

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Chapter 3
Epigenetic Changes in Hormonal Related Disease: Uterine Leiomyoma (Fibroids)

Tung-Chin Chiang

Abstract Epigenetic modifications have been associated with etiology of hormonal related diseases such as breast cancer and prostate cancer. In this chapter, the epigenetic status of a less studied hormonal related disease, uterine leiomyoma, was investigated and its application in potential therapeutic was further discussed. Differential methylation status, the most studied epigenetic signatures, is associated with differential gene expression. Here we investigate whether there are differential epigenetic signatures and their involvement in differential gene expression, apoptosis between two uterine stable cell lines, uterine leiomyoma UtLM-hT (LM) and normal uterine smooth muscle cell lines UtSM-hT (SMC). We first analyzed DNA methylation status using a cancer methylation panel bead array and our data showed that there are differential methylation patterns between the two cell lines. We then selected nine genes with significant differential methylation patterns from the bead array and verified that eight of the nine selected genes show significant differential gene expression between the two cell lines. Additionally, four of the eight genes that demonstrate differential gene expression respond to treatment with a demethylation agent, 5-Aza-2′-deoxycytidine (DAC). Cellular retinol binding protein 1 (CRBP-1) and Tumor necrosis factor super family 10 (TNFSF10) are genes that shows significant differential methylation patterns, gene expression, and responses to DAC between LM and SMC. Altered extracellular matrix (ECM) genes and reduced apoptosis are found in LM and are associated with epigenetic silenced CRBP-1 and TNFSF10 genes.

This chapter showed a systemically investigation from a methylation bead array to explore the possible role of epigenetic changes and their associated biological impacts and potential epigenetic therapy in uterine leiomyoma.

Keywords Epigenetics • Uterine leiomyoma • Methylation • DAC: 5-Aza-2′ deoxycytidine • RBP-1: retinol binding protein 1 • TNFSF10 (tumor necrosis factor superfamily)

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3.1 Epigenetics Changes in Hormone Related Diseases

Many studies have suggested that epigenetic changes play a role in etiology of hormonal related diseases, such as endometrial cancer, breast cancer and prostate cancer (Hervouet et al. 2013; Aschelter et al. 2012; Takai et al. 2005). Deregulated expression of estrogen receptors (ER) is a common findings associated with breast cancer. Restored ER expression via chemical epigenetic modulators increased response to estrogen treatment in breast cancer. Similarly finding in prostate cancer has shown that epigenetic alternations in androgen receptor (AR) play a role in prostate cancer carcinogenesis and disease progression. In addition, a profile of miRNA, another type of epigenetic marker, has shown to down regulate tumor suppressors in breast cancer and some deregulated miRNA is associated with breast cancer progression (O'Day and Lal 2010).

In this chapter, an understudied hormonal related disease, uterine leiomyoma, will be discussed. The purpose is to establish the role of epigenetic changes in uterine leiomyoma and explore possible treatment options. This chapter will include the following topics: (1) Background of uterine leiomyoma (UL) and its role as a hormonal related disease; (2) Molecular characterizations of UL and the potential role of epigenetic changes in UL; (3) Strategies used to investigate epigenetic changes: from the in vitriol model, methylation array, to verification with gene expressions; (4) Further modification of methylation status: reverse methylation changes and analysis of relative gene expression (5) Biological role of selected epigenetic modified genes, CRBP-1 and TNFSF10; a further investigation of the role of apoptosis in UL (6) Conclusions and potential role of epigenetic therapy for UL.

3.2 What Is Uterine Leiomyoma (Fibroids): A Hormonal Related Disease?

Uterine leiomyomas, also known as fibroids, are benign smooth muscle tumors found in approximately 33 % of pre-menopausal women (Lurie et al. 2005). Most affected women have an average of six tumors in their uterus with varying sizes and locations. Patients with leiomyomas have a broad spectrum of clinical symptoms, such as menorrhagia and pelvic pain, infertility and pregnancy complications. Treatments for fibroids include Gonadotropin-releasing hormone (GnRH) agonist therapy; myomectomy; and hysterectomy. GnRH treatment, which reduces ovarian hormone production, is the most well-established therapy, but is tempered by significant side effects such as hypoestrogenism with accompanying hot flashes and bone demineralization. Thus, GnRH is often used prior to myomectomy to reduce the tumor size. Myomectomy is the surgical removal of fibroids while preserving the uterus. While initially effective, the recurrence rate of fibroids is approximately 30 % for women following myomectomy. Hysterectomy is the surgical removal of the uterus, which effectively relieves symptoms but results in infertility. In the

United States, approximately 600,000 hysterectomies are performed at the cost of 1.7 billion dollars each year, with fibroids accounting for 40 % of procedures performed (Wilcox et al. 1994). Despite the high frequency of fibroids and their socioeconomic impact, little research has been done in this area (Barker et al. 2006).

Although, the nature of the initial event in uterine leiomyoma formation is unclear, it is known to be a steroid hormone dependent disease. Although estrogen and progesterone both likely play roles, estrogen has been found to be very important for the growth of fibroids. Uterine leiomyomas occur only after puberty and regress with falling hormone production in menopause. Also, treatment with Gonadotropin-releasing hormone (GnRH) agonist, which reduces the circulating levels of ovarian hormones to create a hypoestrogenic state, reduces leiomyoma growth; re-enlargement of tumors occurs when GnRH treatment is discontinued (West et al. 1987). Several risk factors for development of fibroids, such as obesity, early use of oral contraceptives and ethnicity, are also associated with high estrogen levels (Marshall et al. 1998; Evans and Brunsell 2007). Studies on prenatal diethylstilbestrol (DES) exposure have shown increased uterine fibroid occurrence in mice (McLachlan et al. 1980) and rats (Cook et al. 2005). Another study found that women prenatally exposed to DES had a greater risk of developing fibroids and of having a larger tumor size (Baird and Newbold 2005). From these studies, it is clear that estrogen plays an important role in leiomyoma tumor growth.

3.3 Molecular Characterizations of Uterine Leiomyoma

Molecular characterizations of uterine leiomyoma have focused on genes that affect the balance between proliferation and apoptosis and chromosomal aberrations. Studies have shown a consistent trend of higher proliferation in leiomyomas compared to the myometrium in various stages of the menstrual cycle (Matsuo et al. 1997; Shimomura et al. 1998). Consistent with this finding, the proliferating cell nuclear antigen (PCNA) is expressed at a higher level in leiomyoma tissue than in myometrium in all phases of the menstrual cycle.

Studies on apoptosis in leiomyoma, however, are less conclusive. An anti-apoptotic protein, bcl-2, has been found to be increased in fibroids with differences between phases of the menstrual cycle. Another study using Eker rats, which carry a germ-line defect in one allele of the homolog to human tuberous sclerosis complex (Tsc2) tumor-suppressor gene and have a high (65 %) prevalence of fibroids, showed that estrogen treatment increases cell proliferation but does not affect the rate of apoptosis as compared to untreated leiomyoma cells (Burroughs et al. 1997). Nierth-Simpson et al. demonstrated that human uterine smooth muscle cells differ from leiomyoma cells in their response to estrogen via rapid estrogen signaling pathways and may affect the proliferation rate (Nierth-Simpson et al. 2009). However, how estrogen may regulate the balance between cell proliferation and apoptosis in leiomyoma is still not clear.

A variety of chromosomal abnormalities have been found in approximately 40 % of uterine leiomyomas. The most commonly observed chromosomal translocation is on t(12;14), although this accounts for only 20 % of karyotypically abnormal leiomyoma (Meloni et al. 1992). Other types of abnormalities include deletion of chromosome 7 bands q22–q32, which accounts for 17 % of karyotypically abnormal fibroids. Rearrangements of chromosome 6 p21 include: t(1:6) (q23:21); t(6;14) (p21;24); and t(6;10) (p21;q22), which accounts for 5 % of karyotypically abnormal fibroids (Dal Cin and Van den Berghe 1997). With the fact that 50–60 % of fibroids are cytogenetically normal, it is suggested that cytogenetic abnormalities occur as secondary events and facilitate growth of existing tumors (Ligon and Morton 2001).

3.4 The Potential Role of Epigenetic Changes in Fibroids

Several groups have demonstrated that epigenetic changes alter tumor suppressor genes in various tumors, resulting in down-regulated gene expression (Takai et al. 2005; Campan et al. 2006; Hellebrekers et al. 2007; Rhee et al. 2002). Research projects on endocrine disrupting chemicals (EDC), (compounds that mimic or block endogenous hormones) have proposed that environmental changes in early developmental stages may influence genetic factors without change in the primary DNA sequences, and these epigenetic changes result in mitotically heritable alteration in gene expression (Crews and McLachlan 2006).

Uterine leiomyoma is an estrogen-dependent disease, and differential gene expression in estrogen-regulated genes, such as ER, PR, IGF-1 and IGF-1R, between uterine leiomyoma and myometrium has been found in clinical tissues from many studies (Maruo et al. 2004; Li and McLachlan 2001). With the development of DNA microarrays, many studies also show a broad spectrum of differential gene expression between leiomyomas and normal tissues (Catherino et al. 2003; Tsibris et al. 2002; Zaitseva et al. 2006; Arslan et al. 2005; Ahn et al. 2003; Chegini et al. 2003; Skubitz and Skubitz 2003; Wang et al. 2003; Weston et al. 2003; Hoffman et al. 2004; Quade et al. 2004). Several genes and pathways consistently show altered expression in leiomyoma from different microarray studies and include genes involved in the retinoic acid pathway, IGF2 metabolism, TGF-beta signaling and extracellular matrix formation.

In addition to differential gene expression, altered DNA methylation of the lactoferrin (LTF) gene has been found in neonatal DES treated mice, which have increased the occurrence of uterine fibroids (McLachlan et al. 1980; Li et al. 1997). Also, Li et al. found differential DNA methyltransferases 1, 3a and 3b (DNMTs) gene expression from 23 pairs of human tissues of fibroids and their adjacent myometrium (Li et al. 2003). DNMT1 is an enzyme responsible for maintaining normal methylation patterns and is upregulated in leiomyoma. On the other hand, DNMT3a and 3b, the enzymes responsible for de novo methylation, are expressed at lower levels in leiomyomas. Moreover, global DNA hypomethylation was detected in leiomyoma tissues from the same study. Differential gene expression, altered DNMTs,

and global hypomethylation found in leiomyomas indicate the significance of epigenetic changes in etiology of uterine leiomyoma.

In order to further understand the role of epigenetic changes in uterine leiomyoma, this chapter discuss in detail the strategies used to investigate methylation modifications and the potential applications in epigenetic therapy.

3.5 Strategies Used to Investigate Epigenetic Changes in Fibroids

3.5.1 Models for Studying Fibroids

Mechanistic studies of uterine fibroids have been limited by the lack of suitable in vitro or in vivo models. Fibroids are rare in nonhuman species, leading to difficulties in developing animal models. The best-characterized animal model is the Eker Rat, which exhibits fibroid-like growths in 65 % of female rats. However, the pathology of this model is not similar to human fibroids and often transforms to malignant tumors (Hunter et al. 2002). Primary cells lines are hard to maintain, suffer early senescence in ten passages, and lose their estrogen and progesterone responses in cultures, all of which result in difficulty in hormone response analysis (Severino et al. 1996; Carney et al. 2002). Immortalized human uterine leiomyoma (UtLM-hT; as known as LM) and normal uterine smooth muscle cell lines (UtSM-hT; also known as SMC) were generated via retroviral transfection of human telomerase, which allow cells to bypass their normal programmed senescence (Carney et al. 2002). These immortalized cells contain similar levels of the estrogen receptor alpha (ER alpha) and the progesterone receptor (PR) protein as their parental cells and show no anchorage-independent growth. Furthermore, UtLM-hT cells are karyotypically more stable than the parental line (Varella-Garcia et al. 2006). The development of these immortalized cell lines provides an important tool to understand the molecular pathways involved in the growth of uterine leiomyoma while maintaining a response to estrogen. Studies have not only characterized the cell lines, but discovered a mechanism involved in LM sensitivity to the mitogenic effects of estrogen (Nierth-Simpson et al. 2009; Martin et al. 2006).

3.5.2 Methylation Analysis on an Array: Analyzing Thousands of Methylation Sites at a Time

One of the best-studied epigenetic alterations is the methylation status. Studies have shown a strong association between epigenetic changes, especially DNA hypermethylation of CpG sites on promoter regions, and the silencing of tumor suppressor genes in many cancers (Esteller 2005; Herman and Baylin 2003). Typical methylation analysis includes sodium bisulfite conversion of DNA, which converts Cytosine into Uracil. Since 5-meC, methylated cytosine at C5 position, cannot be converted,

it is thus possible to distinguish between methylated cytosines and unmethylated cytosines (Frommer et al. 1992).

DNA promoter region methylation patterns of SMC and LM were analyzed using Golden Gate methylation cancer panel I. A ß value is generated, which is the relative abundance of methylated to unmethylated signals from an average of 30 probes for a specific locus. Methylation ß values close to one indicate almost 100 % prevalence of methylated CpG loci and are considered fully methylated. Methylation ß values close to zero indicate almost 0 % prevalence of methylated CpG loci and are considered unmethylated at that specific locus. Figure 3.1 is a heatmap based on methylation ß values of four normal SMC and tumor LM cell samples. Differential methylation patterns are found between the two cells. Two independent biological replica of each cell line showed a strong correlation ($r = 0.98$ and 0.81 of LM and SMC respectively). One hundred and thirty-one CpG loci are hypermethylated and 44 CpG loci are hypomethylated in LM cells versus SMC cells.

3.5.3 *mRNA Expression of Selected Genes with Differential Methylation Profiles Between SMC and LM*

In general, DNA methylation correlates with decreased gene expression and demethylation correlates with increased gene expression. To investigate whether methylation differences correlate with relative gene expression, gene expression of nine selected genes with differential methylation profiles (Table 3.1) were further examined. Eight of the nine selected genes with differential methylation patterns showed significant differential gene expression between SMC and LM ($p < 0.001$) (Fig. 3.2). Four genes, CRBP-1, TNFSF10, THBS1 and PR, showed decreased gene

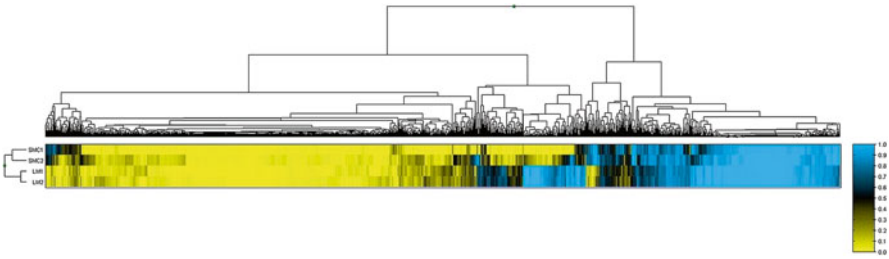

Fig. 3.1 A heat map of a methylation pattern of uterine smooth muscle cell (SMC) and leiomyoma cell lines (LM). SMC1/SMC2 and LM1/LM2 are independent biological repeats of uterine smooth muscle cells and leiomyoma cells, respectively. DNA samples were isolated, bisulfate treated and loaded on a methylation bead array. All samples were clustered based on the methylation beta values of 1,505 cancer-specific markers. The *green* area indicates the mid-point of the root branch of clusters. The *dark blue* area indicates a high methylation status with a methylation value close to 1, and the *yellow* area indicates a low methylation status with a methylation beta value close to zero. LM1 and LM2 have similar methylation patterns ($r^2 = 0.98$) and show a trend of differentiation from the pattern of SMC1 and SMC2 ($r^2 = 0.81$).

Table 3.1 Methylation patterns of selected genes for PCR verification (Chiang 2009)

		Mean averaged beta value[a]		
Gene ID	Target ID	SMC	LM	Gene full name
CRBP1	RBP1_P426_R	0.15	0.96	Retinol binding protein 1, cellular
TNFSF10	TNFSF10_E53_F	0.09	0.97	Tumor necrosis factor superfamily
THBS1	THBS1_E207_R	0.05	0.97	Thrombospondin 1
PR	PGR_E183_R	0.47	0.95	Progesterone receptor
ACVR1	ACVR1_P983_F	0.07	0.92	Activin A receptor, type I
ABCG2	ABCG2_P310_R	0.05	0.83	ATP-binding cassette, sub-family G
IGFBP3	IGFBP3_P423_R	0.04	0.84	Insulin-like growth factor
IGFBP3	IGFBP3_P1035_F	0.04	0.81	Binding protein 3
FRK	FRK_P258_F	0.06	0.76	Fyn-related kinase
PITX2	PITX2_P183_R	0.86	0.03	Paired-like homeodomain 2

[a]Mean average beta value of each gene was derived from averaged beta value of two biological repeats

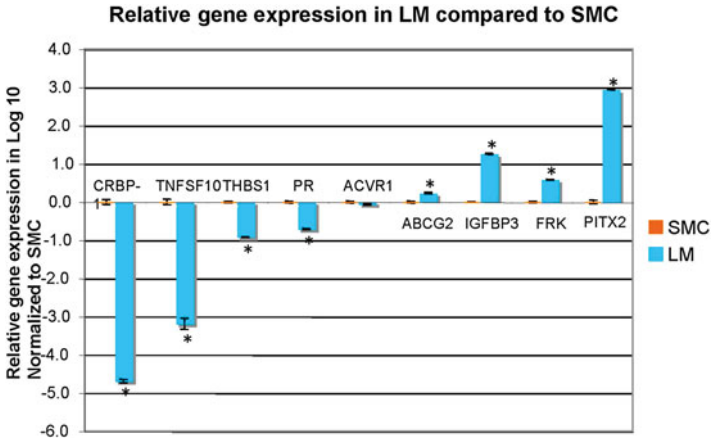

Fig. 3.2 Methylation and gene expression. Cells were cultured and harvested. RNA was isolated, and real time PCR was conducted on our nine selected genes with clear differential methylation patterns between LM and SMC. The bars represent relative gene expression normalized to SMC cells of each gene in Log 10 values. *Orange bars* represent SMC, and *blue bars* represent LM. All data were from triplicate biological repeats with three technical repeats from each biological repeat. Each bar represents the mean +/− SE (p value < 0.001). Eight of the nine selected genes showed significantly differential gene expression between SMC and LM. Abbreviation: *CRBP-1* cellular retinol binding protein 1, cellular (CRBP-1), *TNFSF10* tumor necrosis factor (ligand) superfamily, member 10, *THBS1* thrombospondin 1, *PGR* progesterone receptor, *ACVR1* activin A receptor, type I, *ABCG2* ATP-binding cassette, sub-family G (WHITE), member 2, *IGFBP3* insulin-like growth factor binding protein 3, *FRK* fyn-related kinase, *PITX2* paired-like homeodomain 2

expression that corresponded with selective CpG loci being hypermethylated in LM compared to that of SMC. Likewise, the gene expression of PITX2 is increased, which correlates with the selective CpG locus being hypomethylated in LM compared to that of SMC.

3.5.4 Modification of Methylation Status by 5-Aza-2′-Deoxycytidine (DAC)

In order to associate altered gene expression with epigenetic changes, specifically DNA methylation, methylation altering chemicals were used to alter the methylation status to compare changes of relative gene expression associated with methylation changes. One of the chemicals is 5-Aza-2′-deoxycytidine (DAC), a modified deoxycytidine, which prevents DNA methyltransferase 1 (DNMT1) from adding methyl groups on the DNA. DNMT1 is one of four DNMTs (DNMT2, 3a, 3b) and is known as the maintenance enzyme that keeps methylation patterns in daughter strands identical to parent strands (Bestor 2000; Okano et al. 1999). DAC treatments are associated with increased DNA demethylation and gene expression in general. Many studies have used DAC to re-express genes silenced by methylation, many of which are tumor suppressor genes (Takai et al. 2005; Hellebrekers et al. 2007). In fact, DAC has been successfully applied in clinical as a chemo-reagent to treat leukemia by systematically inducing a hypomethylated status (Kaminskas et al. 2005; Silverman et al. 1993; Saunthararajah 2013). In addition, DAC is also at phase II clinical trial for Refractory advanced Solid Tumors and ovarian cancer (Fan et al. 2014; Glasspool et al. 2014).

The LM and SMC cells were treated with DAC and the gene expression level of selected genes, from the methylation arrays, was expected to be altered. Hypermethylated genes with low expression levels were expected to have increased relative gene expression upon treatment with DAC, thus strengthening the correlation between the specific hypermethylated CpG locus and gene silencing. The five genes, CRBP-1, TNFSF10, THBS1, PR and PITX2, showed differential methylation and expected gene expression patterns (methylated gene was decreased and demethylated gene was increased) between cell lines, which were further examined for their relative gene expression with DAC treatments. Four of the five selected genes' expression increased with DAC treatments in either one or both cell lines (Fig. 3.3). These four genes are CRBP-1, TNFSF10, THBS1 and PITX2. DAC responses, the ratio of relative gene expression after DAC treatment vs. before DAC treatments, were further compared between SMC and LM. CRBP-1 and TNFSF10 showed significantly differential response to DAC in LM compared to that of SMC. CRBP-1 and TNFSF10, which are hypermethylated at selected CpG loci in LM, gene expression increased with DAC treatment (328 times and 1,029 times, respectively) in LM. However, gene expression of these two genes, which are hypomethylated at their respective CpG loci in SMC, only increased less than three times in SMC.

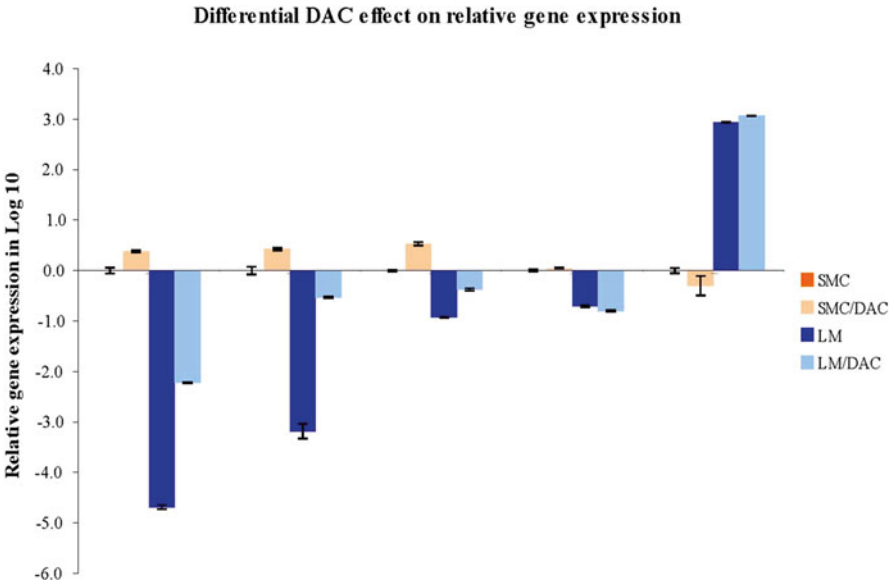

Fig. 3.3 DAC effects on gene expression. Cells were cultured and treated with DAC. RNA was isolated, and real time PCR was conducted on five selected genes, CRBP-1, TNFSF10, THBS1, PR and PITX2, with clear differential methylation patterns and basic gene expressions, from Fig. 3.2, between LM and SMC. The bars represent relative gene expression normalized SMC control of each gene in log 10 values. *Orange bars* represent SMC control; *tan bars* represent SMC treated with DAC; *sky blue bars* represent LM control; and *pale blue bars* represent LM treated with DAC. All data were from triplicate biological repeats with three technical repeats from each biological repeat. Each bar represents the mean +/− SE ('*' p value < 0.005). Four of the five selected genes' expressions increased with DAC treatments in either one or both cell lines. DAC responses, the ratio of relative gene expression after DAC treatment vs. before DAC treatments, were further compared between SMC and LM. CRBP-1 and TNFSF10, showed a significantly differential response to DAC in LM compared to that of SMC ('**'p value < 0.005). Abbreviation: *CRBP-1* retinol binding protein 1, cellular, *TNFSF10* tumor necrosis factor (ligand) superfamily, member 10, *THBS1* thrombospondin 1, *PGR* progesterone receptor, and *PITX2* paired-like homeodomain 2

The impacts induced by epigenetic modifications are the results of interplays between many epigenetic events, such as methylation changes or histone modifications, miRNA, and chromatin insulator etc. (Choudhry and Catto 2011; Sandoval and Esteller 2012; Singh et al. 2012; Kozaki et al. 2008; Pavicic et al. 2011). Those epigenetic events may work alone or concurrently happened with other epigenetic events. Therefore, the strategy applied here is to focus on selecting genes highly associated with one epigenetic event, methylation changes. The selected genes are sifted from hundreds of genes applied to methylation array, followed with real time PCR verification and tested by the methylation modification reagent, DAC. This strategy expedites the selection process and brings the research to the next step, investigating the biological role and its application.

3.6 Biological Significance of Genes with Epigenetic Differences in Uterine Leiomyoma

Gene expressions of CRBP-1 and TNFSF10 were found to be significantly reduced in LM as compared to SMC, and their methylation statuses were coordinately methylated compared to that of SMC. The biological roles of CRBPP-1 and TNFSF10 and their association in etiology in uterine leiomyoma were further explored.

3.6.1 Biological Role of CRBP-1 and Its Association with Cancers

CRBP-1 is essential for regulation of vitamin A esterification and storage in the liver (Ghyselinck et al. 1999) and breast epithelia cells (Yuvarani et al. 2001). In addition to the roles in retinol metabolism, CRBP-1 also plays a role as a tumor suppressor and is associated with sensitivity to cell detachment-induced anoikis (Yuvarani et al. 2001). Reduced CRBP-1 gene expression is found in many cancers, such as breast cancer and prostate cancer (Kuppumbatti et al. 2000; Jeronimo et al. 2004).

3.6.2 Biological Role of CRBP-1 and Its Association of Deregulated Extra Cellar Matrix (ECM) in LM

Microarray studies showed altered expression of genes involved in the retinoic acid pathway and extracellular matrix (ECM) formation from leiomyoma tissues (Tsibris et al. 2002; Leppert et al. 2004). ECM offers structural support for cells. The network of ECM proteins, such as collagens and Fibronetin (FN) support stromal microenvironment, which consists of smooth muscle cells and other cells. Alternating the compositions of ECM proteins is associated with cell death, proliferation and tumor progression (Pietras and Ostman 2010). In fact, changes in the extracellular matrix is one of the hallmarks of uterine leiomyoma in addition to the observations of deregulated retinoic acid signaling found in uterine leiomyoma tissues (Catherino and Malik 2007). Furthermore, the use of All-Trans-Retinoic Acid (ATRA), which results in increased CRBP-1 and other retinoic acid signaling genes, changes the composition of ECM in leiomyoma to the level similar to that in normal smooth muscle cells. Clearly, CRBP-1 is associated with ECM and plays a role in etiology of uterine leiomyoma (Malik et al. 2008).

3.6.3 Biological Role of TNFSF10 (TRAIL) in Apoptosis

Study has showed that ECM is associated with apoptotic response of cells and one of the ECM proteins, EMILIN2, functions like TRAIL, which is one of the epigenetically silenced genes found in LM (Mongiat et al. 2007). TRAIL, also known as TNFSF10, is a member of the TNF superfamily of death inducing ligand and exerts its biological function through a sub type of apoptosis, a p53 independent extrinsic pathway. TRAIL is of special interest for cancer therapy, since it has been shown to predominantly kill cancer cells, while sparing normal cells (Ashkenazi 2002).

3.6.4 Imbalanced Apoptosis and Modified ECM Associated Genes in Uterine Leiomyoma

It is clear that the epigenetic silenced genes, CRBP-1 and TNFSF10, found in LM are associated with modification of ECM, which plays a role in apoptosis. The level of apoptosis between LM and SMC cells were further investigated. There is a clear differential apoptosis level between LM and SMC as shown in Figs. 3.4 and 3.5.

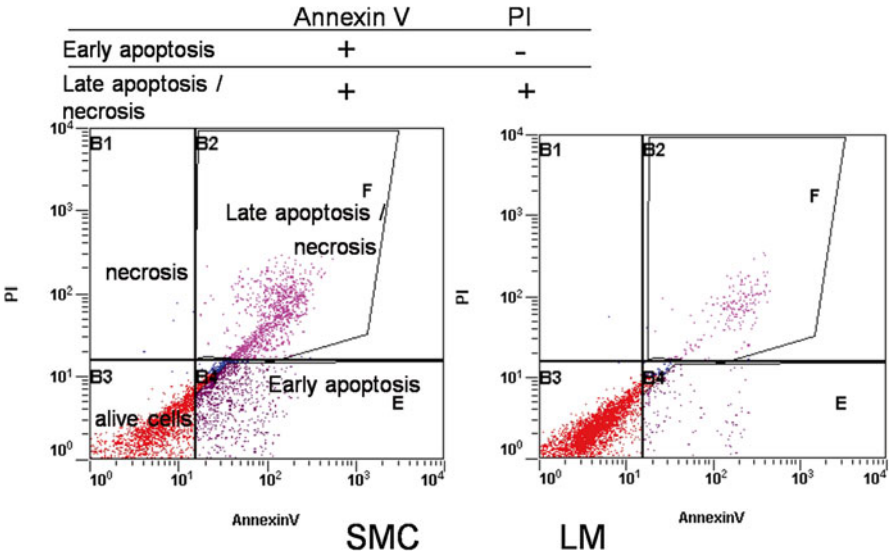

Fig. 3.4 Tracing different phases of apoptosis: flow cytometry analysis of LM and SMC. This is a representative figure of triplicate samples. Cells were seeded overnight in three 10 cm plates and harvested. Cells were reacted with the Annexin V and PI kit as suggested by the manufacture protocol and analyzed within 1 h. The fluorescent signals that detected early apoptosis are represented at the *lower right* of the figure, and cells in necrosis are represented at *upper left*. Cells detected in late apoptosis/necrosis phases are represented at *upper right*. Cells at the *bottom left* corner represent living cells. It is clear that SMC have more cells located at early apoptosis than that of LM

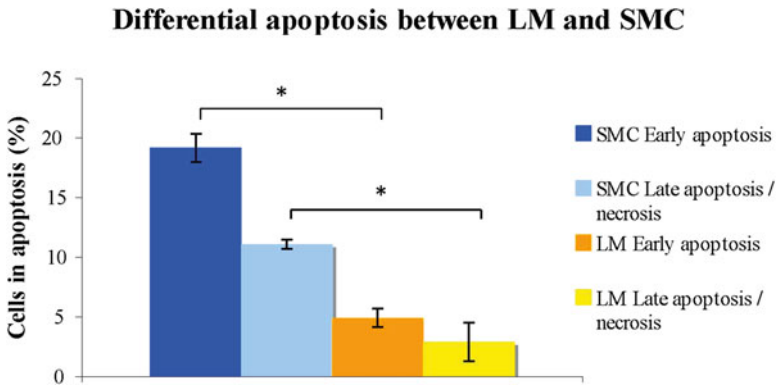

Fig. 3.5 Differential apoptosis levels between SMC and LM. Cells were seeded in 10 cm plates overnight and stained with annexin V/PI reactions as suggested by the manufacture. The *blue bar* represents SMC at early apoptosis, the *pale blue bar* represents SMC cells in late apoptosis, the *dark golden bar* represents LM cells in early apoptosis while the *light golden bar* represents LM cells in late apoptosis phases. The cells in the early apoptosis phase are significantly less in LM compared to that in SMC at 24 h in both early and late apoptosis. Data were from triplicate biological repeats. Each *bar* represents the mean +/− SE ('*' p value < 0.05)

Decreased apoptotic cells are found in LM compared to that of SMC. In addition, the selected ECM associated genes, such as Collagen 7A1 (COL7A1); Thrombospondin-1(THBS1); Fibronectin (FN1); and Versican (VCAN1), were also investigated. There is a clear differential expression in all four genes between LM and SMC cells as in Fig. 3.6. The gene expression of pro-apoptotic ECM gene, THB1 gene, is reduced in LM compared to that in SMC. Furthermore, pro-survival ECM gene, FN1, is increased in LM compared to that in SMC. The specific differential ECM genes profiles supported the findings that LM cells have reduced apoptotic cells found in the same study with flow cytometer staining. Further study found the demethylation reagent, DAC, reduced the differential apoptotic related ECM genes expression between LM and SMC cells (Fig. 3.7). These results suggested that differential methylation of the two selected genes, CRBP-1 and TNFSF10, are associated with apoptosis and their related ECM proteins in LM.

3.7 Conclusions

3.7.1 From Epigenetic Finding to Biological Impact: Apoptosis in LM

The biological roles of epigenetic silent genes, CRBP-1 and TNFSF10, are found to be associated with apoptosis. Their relative gene expression patterns are in concert with the differential apoptosis status between LM and SMC. In addition to the reduced apoptosis, we found altered ECM gene expressions and their responses to

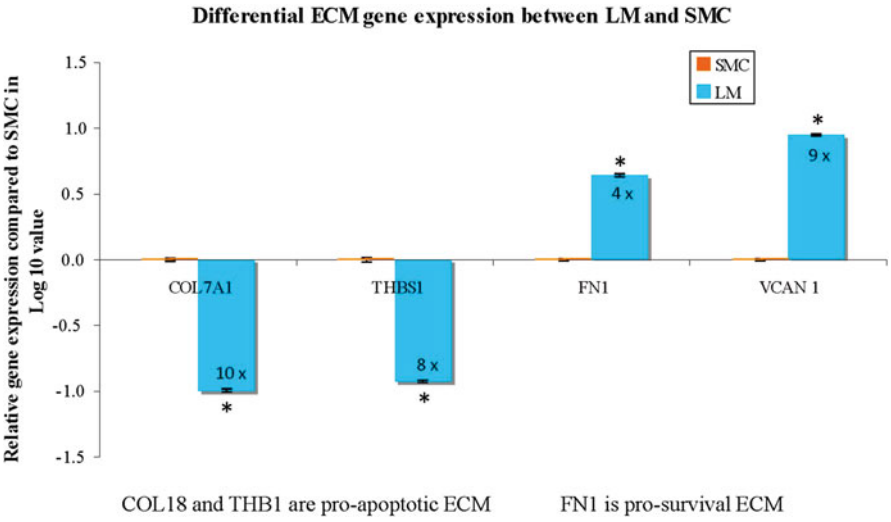

Fig. 3.6 Basel level of ECM gene expression in LM and SMC. The *bars* represent relative gene expression to SMC control for each gene in Log 10 value. *Orange bars* represent SMC control and *sky blue bars* represent LM control. All data were from triplicate biological repeats with three technical repeats from each biological repeat. Each *bar* represents the mean +/− SE. All four ECM genes are differentially expressed between LM and SMC ('*' p value < 0.0001). Abbreviations: *CRBP-1* cellular retinol binding protein, and *COL7A1* collagen, type VII, alpha 1

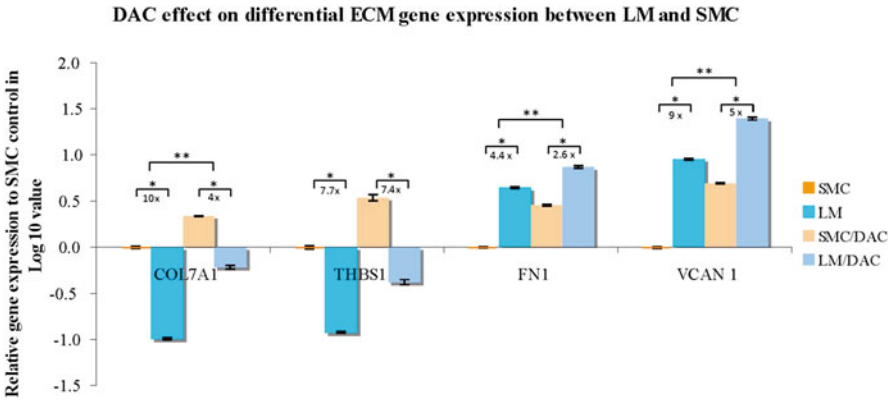

Fig. 3.7 DAC reduced differential Extra Cellular Matrix (*ECM*) associated genes expression between SMC and LM. The *bars* represent relative gene expression to SMC control for each gene in Log 10 value. *Orange bars* represent SMC control; *sky blue bars* represent LM control; *tan bars* represent SMC treated with DAC; and *pale blue bars* represent LM treated with DAC. All data were from triplicate biological repeats with three technical repeats from each biological repeat. Each *bar* represents the mean +/− SE. All four genes showed significant differences before and after DAC treatment in both cell lines ('*' p value < 0.0001). Also, the differences between LM and SMC are significantly reduced after DAC treatment compared to that of non-DAC treatment in all three genes, except for THB1 gene ('**' p value < 0.0001). Abbreviations: *CRBP-1* cellular retinol binding protein, and *COL7A1* collagen, type VII, alpha 1

demethylation agents in LM compared to that in SMC. Together, these findings indicate the association of epigenetic silenced genes and their biological impact, which provide information on the possible mechanism of leiomyoma, and its potential clinical applications, as an alternative treatment or as a bio-marker.

3.7.2 Epigenetic Therapy and Markers

Methylation profiles provide markers for potential epigenetic therapy and risk assessment. Epigenetic therapy, either used singly or in combination with other chemotherapy or radiotherapy, is a novel focus of cancer treatments. For example, DAC, the drug which reduces methylation used in this chapter, is currently being used for treating myelodysplastic syndrome (MDS) and other leukemia (Egger et al. 2004; Yoo and Jones 2006; Griffiths and Gore 2008). The DAC response between LM and SMC cells was compared to further characterize the epigenetic role of selected sites, and to establish a possible therapeutic role of DAC for uterine leiomyoma. Half of selected genes, with differential methylation patterns between LM and SMC, and increased gene expression with DAC treatments, which indicates a strong epigenetic role of selected genes from the methylation array, further demonstrating the usefulness of this technique.

Cancer-specific methylation markers have been proposed as potential biomarkers due to the fact that hypermethylation of CpG islands is a common event in carcinogenesis, and DNA is a stable source for deriving molecular information (Laird 2003). Studies have shown that cancer patients have increased levels of free DNA in their serum relative to a non-cancerous person (Leon et al. 1977). Apoptotic cells may be the source of this increased free DNA (Jahr et al. 2001), which provides a good target for measuring biomarkers, such as DNA methylation. Many methylation markers from serum samples have shown high clinical sensitivity and specificity, including CDKN2A for bladder cancer and liver cancer, as well as GSTP1 for prostate cancer (Goessl et al. 2000; Wong et al. 1999; Valenzuela et al. 2002). Hess et al. found that the methylation profiles of 25 tumor suppressor genes allowed risk stratification of acute myeloid leukemia (AML), especially on patients with a methylated ESR1 gene (Hess et al. 2008). Another large population-based cohort study showed that methylation of the BRCA1 gene is associated with increased mortality in breast cancer patients (Xu et al. 2009). The combination of methylation levels and patterns of promoter regions may provide more specific characteristics of a disease than would level of gene expression.

Retinoid therapy has been used both as a chemopreventive and for treatment in various cancers, such as cervical cancer, leukemia and breast cancer (Abu et al. 2005; Lengfelder et al. 2005; Decensi et al. 2003; Fukuno et al. 2003). All trans retinoid acid (ATRA) treatment leads to a reduction in ECM related gene expression, and changes between uterine leiomyoma and smooth muscle primary cells (Malik et al. 2008). DAC also leads to reduced changes in ECM associated genes between LM and SMC cell lines and is similar to the treatment effects induced by ATRA. The

similarity between ATRA and DAC on ECM protein expression in LM indicating that epigenetic therapy may provide an alternative treatment for leiomyoma.

3.7.3 Future Studies

These studies strongly suggest that epigenetic differences exist between LM and SMC and may be useful in further investigating the etiology of this disease. The high prevalence of differential gene expression from genes with differential methylation patterns suggests the strong association between epigenetic differences and differential gene expression in uterine leiomyoma.

The finding leads in many directions for future studies, such as over expression or knock out target genes, to further understand the role of selected epigenetically modified genes in the etiology of fibroids at the molecular level. As mentioned before, methylation change is one of many epigenetic events. Those epigenetic events could work together or alone in different sequences and result in different impacts. The study model the first step in investigating a complex biological issue. Together, we wish to bring an alternative treatment for uterine fibroids in the future.

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Chapter 4
Is Environmental Epigenetics Relevant to Endocrine Disease?

Abby F. Fleisch, Robert O. Wright, and Andrea A. Baccarelli

Abstract Endocrine disrupting chemicals that are structurally similar to steroid or amine hormones have the potential to mimic endocrine endpoints at the receptor level. Endocrine disrupting chemicals may dysregulate hormone-mediated gene expression through changes in signal transduction and through epigenetic changes. However, epigenetic-induced alteration in gene expression may occur outside of receptor-mediated effects and has emerged as an alternative way in which environmental compounds may exert endocrine effects. We discuss general implications of DNA methylation, histone modification, micro RNAs, and other more recently recognized epigenetic modifications for endocrinology, and we discuss potential for transgenerational inheritance of epigenetic marks. We also review concepts related to environmental epigenetics and relevance for endocrinology through three broad examples, (1) effect of prenatal and early-life under- and overnutrition on future metabolic disease, (2) effect of lifetime environmental exposures such as ionizing radiation on endocrine cancer risk, and (3) potential for compounds previously classified as endocrine disrupting to additionally or alternatively exert effects through epigenetic mechanisms. The field of environmental epigenetics is still nascent, and additional studies are needed to confirm and reinforce data derived from animal models and preliminary human studies. Current evidence suggests that environmental exposures may significantly impact expression of endocrine-related genes and thereby affect clinical endocrine outcomes.

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Keywords Diethylstilbestrol (DES) • Toxic Substances Control Act (TSCA) • DNA methyltransferase (DNMT) • Methyl binding proteins (MBPs) • Cytosine-phosphate-guanine (CpG) • Histone deacetylases (HDACs) • High fat diet (HFD) • Bisphenol A (BPA)

4.1 Introduction

In the 1970s, diethylstilbestrol (DES), an estrogen agonist used to prevent miscarriages, was associated with vaginal clear cell adenocarcinoma in adult female offspring exposed in utero (Robboy et al. 1977). Mothers who took DES, however, were not at increased risk for this rare adenocarcinoma, suggesting that in utero programming was the mechanism for this observation. Since then, evidence that the endocrine effects of multiple exogenous agents play a role in epigenetic programming, has increased dramatically.

Exogenous compounds that alter "hormonal and homeostatic systems" (i.e.,—endocrine endpoints) have been termed "endocrine disrupting chemicals" and were officially recognized in an Endocrine Society statement in 2009 (Diamanti-Kandarakis et al. 2009). The majority of currently identified endocrine disruptors are synthetic industrial chemicals, rather than drugs. In the US, although the Toxic Substances Control Act (TSCA) of 1976 provides a legislative framework to limit the spread of toxic chemicals, companies are not required to perform monitoring for adverse health effects of product chemicals prior to their incorporation in commercial products. Therefore, toxicity is typically discovered only after the product is widely used. For this reason, many chemicals with suspected or even confirmed endocrine disruption properties are already in current daily use.

Many endocrine disrupting chemicals have structural similarity to steroid or amine hormones. Acting as ligands, they can either activate or antagonize the hormone's receptor, leading to altered endocrine endpoints (Diamanti-Kandarakis et al. 2009). However, more recently, the classification of endocrine disrupting chemicals has expanded. Agents such as heavy metals that do not act directly on steroid receptors have been shown to alter hormone metabolism via epigenetic alterations. Also, compounds such as DES that are structurally similar to hormones (Fig. 4.1) may induce epigenetic changes through interaction with hormone receptors.

4.2 What Is Epigenetics?

Epigenetics is defined as changes in gene expression that occur without changes in DNA sequence (Wolffe and Guschin 2000) and can be transmitted through mitosis and/or meiosis. Given that all cells in the human body contain the same DNA sequence, epigenetics can be thought of as those processes that regulate gene

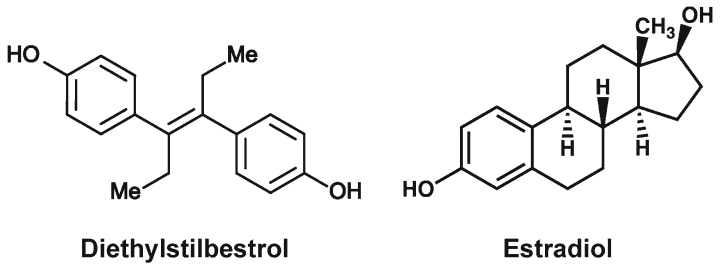

Fig. 4.1 Many endocrine disrupting chemicals such as DES have structural similarity to steroid hormones such as estrogen

expression in a given cell leading to its cellular phenotype, a definition first proposed by CH Waddington (1942). One of the more intriguing facets of epigenetics is the responsiveness of many epigenetic marks to environmental factors. In recent years, as the hereditary component of complex, multifactorial processes such as obesity (Lindgren et al. 2009), diabetes (Voight et al. 2010; Schafer et al. 2011), osteoporosis (Duncan et al. 2011), and pubertal timing (He et al. 2009; Perry et al. 2009) was not explained by DNA sequence changes, the potential role of heritable epigenetic marks as alternative mechanisms of disease grew. Epigenetics offers a complementary mechanism for heredity that is independent of DNA sequence variation and may account for much of the "missing" heritability noted in genomic studies of complex disease.

Epigenetics involves several categories of biological 'marks' added to DNA or histone binding proteins leading to transient or persistent changes in gene transcription. Epigenetic marks can also refer to small noncoding RNA molecules that may regulate expression at the level of translation. Each organism has a unique epigenetic signature that is partially inherited and partially created in utero and continues to be modified throughout adult life. Changes in epigenetic marks may arise from environmental exposures that in turn interact with genotype to produce changes in gene expression, or epigenetics may explain health outcomes independent of genotype as these marks alone can either turn off or turn on gene expression, functioning as an "epimutation".

The most widely studied epigenetic mark is DNA methylation, although histone modifications and microRNAs (miRNAs) are other established epigenetic modifications. Recently, 5-hydroxymethylcytosine and 5-formylmethylcytosine (intermediates in the DNA degredation pathway) have been recognized as unique epigenetic marks (Cortellino et al. 2011; Ito et al. 2011). In addition, mitochondrial DNA expression has been found to be independently regulated by methylation (Dawid 1974). Since mitochondria have an independent genome from the nucleus, epigenetic regulation of gene expression should be considered a cellular, rather than purely nuclear process.

4.2.1 DNA Methylation

DNA methylation refers to the addition of a methyl group to a cytosine (C) nucleotide at position 5 [(5-methylcytosine (5-mc)] and most commonly occurs when a cytosine is positioned next to a guanine (G). Because phosphates (P) link nucleosides in DNA, this particular arrangement is termed a "CpG dinucleotide" or "CpG repeat" in which multiple CpG occur in sequence. Regions of the genome dense in CpG dinucleotides are termed CpG islands. Although CpG islands have the potential for methylation and are frequently overrepresented at gene promoters, the majority of the time, they are not methylated (Jirtle and Skinner 2007). Regions with lower CpG density bordering the CpG islands are termed CpG shores and have been proposed to occur with increased frequency in regulatory sites involved in tissue differentiation (Doi et al. 2009). Keeping the "island" terminology in mind, CpG shelves refer to regulatory sites farther still from the gene that is regulated.

Tissue-specific methylation of CpG dinucleotides by DNA methyltransferases (DNMTs) can lead to gene silencing (Wolffe and Matzke 1999; Orphanides and Reinberg 2002). The simplest way to understand DNA methylation is to think of DNA not as strand, but as a coil wrapped tightly around a histone. Methylation leads to gene silencing because it alters DNA's three-dimensional structure such that the coil becomes tighter at the locus corresponding to the promoter region. Within this tightened coil, transcription factors can no longer be recruited to their regulatory binding sites. This structure also facilitates interactions with methyl binding proteins (MBPs) that can interact with methylated CpGs and actively repress gene transcription (Fig. 4.2) (Bird and Wolffe 1999).

The endocrine system is a key player in gene expression in all tissues, and there is evidence that physiologic DNMT activity is under hormonal control. For example, DNMT1 and MBP levels vary with menstrual cycle phase and with estrogen and progesterone secretion in endometrial explant tissues (van Kaam et al. 2011). Thus, based on their analogy to steroid hormones, some endocrine disrupting compounds have the potential to affect DNMT activity and consequently affect epigenetic marks.

4.2.2 Histone Modifications

Histones are globular proteins around which DNA is packaged. The combination of DNA bound to histones forms chromatin. Like DNA methylation, enzyme modifications of histones such as acetylation and methylation of lysine residues in the amino terminus lead to a chromatin conformational change which can be open (increased transcription) or closed (decreased transcription). Acetylation leads to an open three-dimensional conformation and increased DNA accessibility to transcription factors and RNA polymerase, and methylation can either increase or decrease DNA accessibility depending upon the specific site of histone methylation and the type of histone protein being methylated (Yan and Boyd 2006). DNA methylation and histone modification often work in tandem in regulating the conformation of

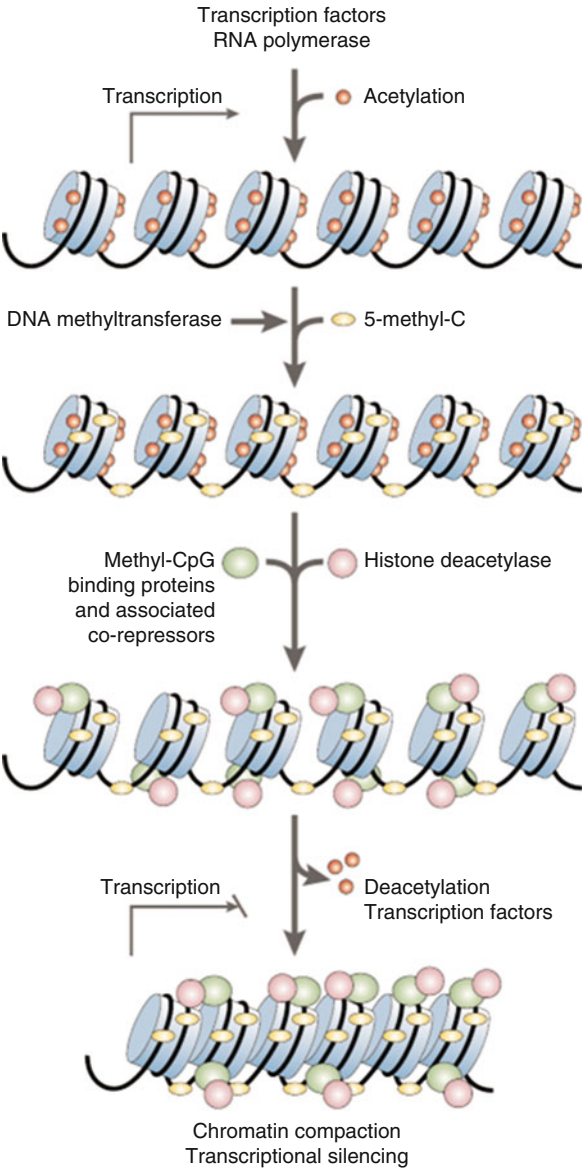

Fig. 4.2 DNA methylation and histone modification work in tandem to alter DNA's three-dimensional structure. Methyl binding proteins recruited by DNA methylation may exert their effects by recruiting histone deacetylases (HDACs), leading to chromatin condensation (i.e., closed conformation) and transcriptional inactivation (Reprinted by permission from Macmillan Publishers Ltd: Robertson and Wolffe (2000), copyright 2000)

local DNA regions, as MBPs recruited by DNA methylation may exert their effects through recruitment of histone deacetylases (HDACs) resulting in chromatin condensation (i.e., closed conformation) and transcriptional inactivation (Fig. 4.2) (Jones et al. 1998; Nan et al. 1998).

4.2.3 MicroRNAs

miRNAs are small (21–23 nucleotide), non-coding RNA molecules that induce gene silencing by base pairing with a sequence complementary to the 3′ untranslated region of the target mRNA molecule. Once expressed, miRNAs are transported to the cytoplasm for processing forming "hairpins" in which their 3′ and 5′ strands form a partial imperfect complement. This hairpin structure is further processed to a ribonuclear protein complex called a "RISC" (RNA induced silencing complex), which is the active form. The remaining strand of RNA in the RISC complex forms imperfect complementary bonds with one or more mRNA molecules. The complex either directly degrades mRNA or accelerates its degradation through deadenylation (Fig. 4.3). The resulting mRNA degradation and interference with mRNA translation disrupts gene expression (Chen and Rajewsky 2007; Bartel 2009). miRNA activity has been implicated in the pathogenesis of obesity via regulation of adipogenesis [reviewed in (Romao et al. 2011)]. Also, mice exposed to miRNAs from the let-7 family developed high-fat diet induced obesity and insulin resistance, and this was reversed by anti-let-7 miRNAs (Zhu et al. 2011).

4.2.4 Recently Recognized Modifications

Demethylation occurs through a pathway that includes oxidative degradation of 5-mc to 5-hydroxymethylcytosine (5-hmc) and 5-formylcytosine (5-fmc) followed by base excision by DNA repair machinery (Cortellino et al. 2011; Ito et al. 2011). Methodology has recently been developed to quantitatively sequence these intermediate methylation marks (5-hmc and 5-fmc) (Booth et al. 2012; Raiber et al. 2012). 5-hmc is enriched in actively transcribing genes and enhancer elements (Song et al. 2011), and levels were recently demonstrated to change in response to a non-genotoxic carcinogen (phenobarbital) in mouse liver DNA (Thomson et al. 2013). Whether other environmental exposures impact the formation of these epigenetic marks and/or the demethylation pathway remains to be elucidated and is poised to become an active topic of future research.

Mitochondrial DNA (small circular genome independent from nuclear DNA) can also undergo methylation (Dawid 1974) under the control of a mitochondrial-specific DNMT1 (Shock et al. 2011). Individuals occupationally exposed to high levels of fine particulate matter air pollution had altered mitochondrial DNA methylation as compared to those with lower-level exposures (Byun et al. 2013),

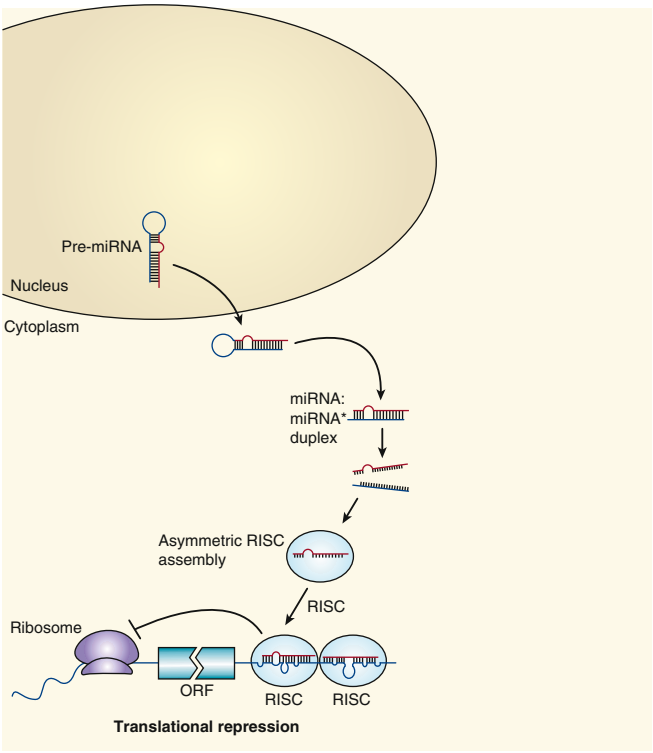

Fig. 4.3 miRNAs enter the cytoplasm where they form hairpin structures in which their 3′ and 5′ strands form a partial imperfect complement. This structure is further processed to a ribonuclear protein complex called a RISC which either directly degrades mRNA or accelerates its degradation through deadenylation (Reprinted by permission from Macmillan Publishers Ltd: He and Hannon (2004), copyright 2004)

suggesting that environmental exposures can impact epigenetic mechanisms in mitochondrial DNA. Impaired mitochondrial function has been associated with insulin resistance (Fleischman et al. 2009) and decreased adult height (McCormack et al. 2011), so methylation-induced impairment in mitochondrial function is another epigenetic pathway through which environmental exposures may impact endocrine outcomes.

4.3 Epigenetic Reprogramming and Inheritance

Epigenetic patterns undergo erasure and reprogramming in two phases during embryonic development. One phase occurs in somatic cells (to drive cell differentiation), and the other phase occurs in gametes (to establish gender-specific imprinting patterns).

Humans are diploid at all loci except the sex chromosomes, but the expression of each gene's two alleles is not equal. Imprinting refers to the monoallelic expression of a gene that correlates to the allele's parent of origin. In some imprinted genes only the allele from the father is expressed, in others, only the maternal allele is expressed. One important result of epigenetic reprogramming is the correct establishment of imprinting at sites with allele-specific methylation.

In somatic cells, during pre-implantation, all genes (with the exception of imprinted genes) become demethylated. After implantation, DNA methylation is restored de novo and rapidly acquires cell lineage-specific patterns to drive cell differentiation. This is the basis for the tissue-specific gene methylation pattern seen after birth and through adulthood (Reik et al. 2001; Shi and Wu 2009; Perera and Herbstman 2011). Because the methylation pattern of imprinted genes is unchanged in somatic cells, independent of gender, human somatic cells contain one haploid set of chromosomes inherited from the mother that carry female-specific imprints, and a second haploid set of chromosomes inherited from the father that carry male-specific imprints.

In gametes, DNA methylation – including methylation at imprinted loci – is erased and later *re-established* to match the gender of the fetus. At imprinted loci, DNA methylation is reprogrammed so that it will take female-specific imprints in oocytes and male-specific imprints in spermatozoa. This must occur in order for the correct imprinting marks to be passed on to subsequent generations, as gender can alternate across generation.

The prenatal erasure and reprogramming of DNA methylation patterns makes the in utero time period a window of potential vulnerability for epigenetic dysregulation from environmental exposures. This is particularly relevant in endocrinology where there is burgeoning evidence that the fetal environment may program adult outcomes such as obesity and type 2 diabetes mellitus (Law et al. 1992; Stocker et al. 2005). Other potential vulnerable windows for epigenetic dysregulation that might affect endocrine systems include puberty, during which time there is an overall rapid increase in DNA turnover and cell growth, as well as old age, which has been associated with progressive age-related changes in DNA methylation (Bjornsson et al. 2008) and hydroxymethylation (Song et al. 2011; Thomson et al. 2013) (Fig. 4.4).

4.4 Potential for Transgenerational Inheritance of Epigenetic Modifications

The phenomena of imprinting suggests that erasure of DNA methylation during embryogenesis may not be complete, creating the potential for transgenerational inheritance of epigenetic modifications. In addition, there are epigenetic marks other than DNA methylation which might be the mode of inheritance. Experimental evidence for transgenerational inheritance can come only from studying the generation with no direct environmental exposure. For example, if an F0 generation

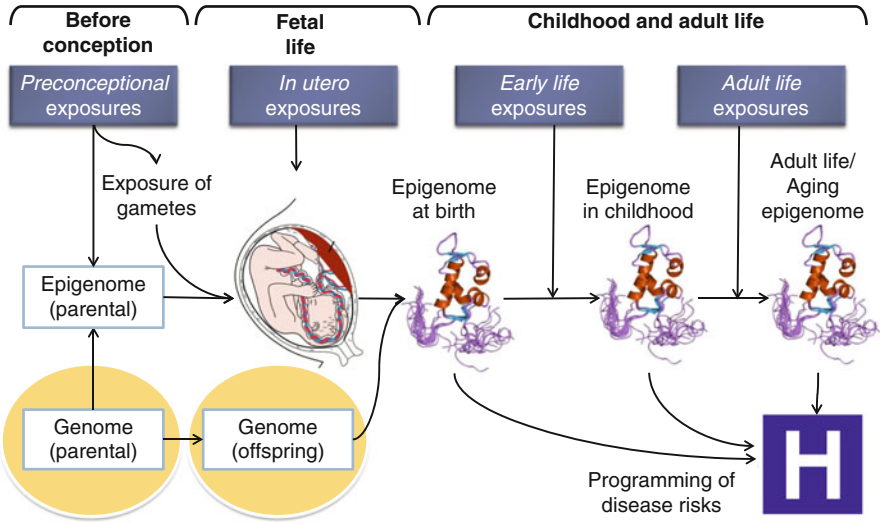

Fig. 4.4 Exposures that occur preconception, *in utero*, in early life, and in adult life may result in epigenetic dysregulation

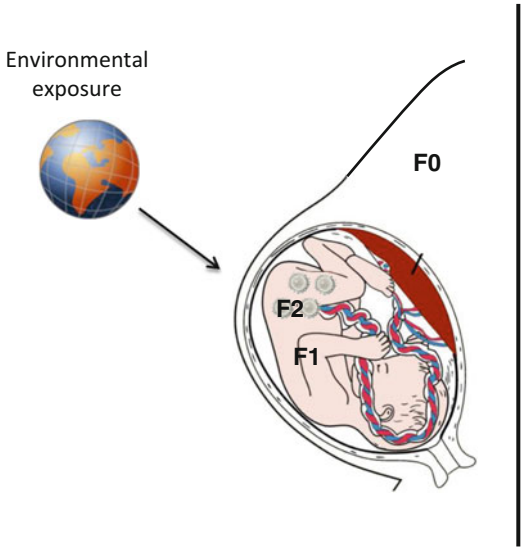

Fig. 4.5 An environmental exposure during a woman's pregnancy has the potential to impact her epigenome (F0), the epigenome of her child (F1), and the epigenome of her child's gametes that will ultimately produce her grandchild (F2). Thus, only the epigenome of her great grandchildren [F3 (not shown)] can indicate whether the epigenetic changes induced by a given environmental exposure can be inherited

pregnant female has an exposure, the F1 generation offspring will have had germline and somatic cell exposure while in utero. Because the F1 generation's germline was directly exposed, the F2 generation grandoffspring will still have cells that reflect the original exposure. Only the F3 generation great grandoffspring will be able to be tested for true transgenerational effects (Skinner 2008) (Fig. 4.5). A few rodent

models have examined transgenerational transmission of health outcomes following F0 generation exposure (Pentinat et al. 2010; Bruner-Tran and Osteen 2011; Kujjo et al. 2011). In humans, global methylation pattern in maternal blood was significantly correlated with that in offspring umbilical cord blood (Kile et al. 2010). There is a need for additional studies to follow tissue-specific epigenetic modifications through the F3 generation in rodents and humans.

4.5 Epigene-Environment Interaction

Epigenetic dysregulation can result from environmental exposures including dietary factors, physical activity, social stressors, and environmental toxicants (Mathers et al. 2010; Alegria-Torres et al. 2011). However, there is a paucity of human studies that document the causal pathway from environmental exposure to epigenetic modification to clinical outcome (Fig. 4.6).

We have chosen to demonstrate these themes by reviewing three broad examples where environmental epigenetics has impacted endocrinology. These include

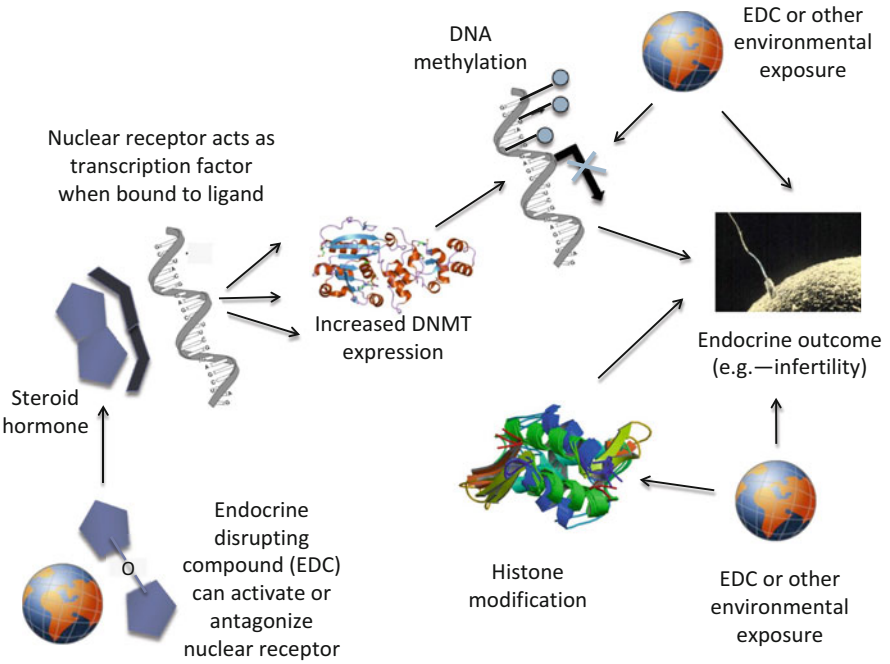

Fig. 4.6 There are multiple potential causal pathways through which an environmental exposure may lead to epigenetic modifications which may, in turn, impact clinical outcomes. Additional human studies that extend over the entire causal pathway are needed to establish cause-and-effect relationships between exposures and outcomes

(1) effect of prenatal and early-life nutritional exposures on future obesity and insulin resistance, (2) effect of lifetime environmental exposures such as ionizing radiation on endocrine cancer risk, and (3) potential for endocrine disrupting compounds to affect endocrine endpoints through epigenetic modifications. We review available data and suggest avenues for future research.

4.6 Nutritional Status and Epigenetic Changes

Both under- and overnutrition during pregnancy and early life have been associated with offspring metabolic disease later in life. Epigenetic programming has been implicated as one possible pathway through which the *in utero* environment in general, and prenatal nutrition in particular, may influence future offspring health [reviewed in (Langley-Evans 2009; Lillycrop 2011)].

4.6.1 Undernutrition

In rodent and human studies, poor maternal nutrition during pregnancy has been associated with altered DNA methylation and later life metabolic disease in offspring. Offspring of rats fed a protein-restricted diet during pregnancy had hypomethylation and increased mRNA expression of hepatic genes *PPARα* and *GR* early in life (Lillycrop et al. 2005; Burdge et al. 2007). While increased *GR* activity promotes gluconeogenesis, *PPARα* physiologically opposes *GR* activity by increasing insulin sensitivity. However, as offspring aged, *PPARα* expression declined, possibly as a result of epigenetic drift, and the rats developed insulin resistance and hepatic steatosis (Erhuma et al. 2007). In a related study in humans, lower maternal carbohydrate intake during early pregnancy was associated with hypermethylation of the *RXRA* gene in umbilical cord tissue and later childhood adiposity. RXRA may be involved in insulin sensitivity, adipogenesis, and fat metabolism based on its interaction with *PPAR* when serving as a transcription factor (Godfrey et al. 2011). Finally, adult offspring of women exposed to the Dutch Hunger Winter famine of 1944 in early gestation had hypomethylation of the imprinted *IGF2* gene (Heijmans et al. 2008) and increased obesity and cardiovascular disease (Painter et al. 2005) as compared to unexposed siblings. These rodent and human studies suggest that maternal nutritional status in pregnancy affects the DNA methylation profile and subsequent adult onset metabolic disease in offspring.

Poor prenatal nutrition may affect methylation status in part as a result of a decrease in dietary sources of methyl group donors such as folate, betaine, methionine, and choline in conjunction with decreased availability of B vitamins (B_2, B_6, and B_{12}) (Mathers et al. 2010). Agouti viable yellow mice offspring of mothers with prenatal folate, betaine, choline, and vitamin B12 supplementation had hypermethylation of the agouti gene promoter as well as altered coat color and decreased

obesity and cancer (Wolff et al. 1998). Offspring of sheep with deficiencies in vitamin B12, folate and methionine supplementation during pregnancy had hypomethylated fetal liver DNA as well as obesity, elevated blood pressure, and insulin resistance (Sinclair et al. 2007). In humans, among 24 infants, cord blood homocysteine level, an inverse marker of maternal folate supplementation, was associated with LINE-1 hypomethylation (Fryer et al. 2009). While an association between maternal methyl donor intake and offspring methylation was found in two additional small human cohorts (Park et al. 2005; Steegers-Theunissen et al. 2009), no association was seen in a larger, folate replete cohort residing in the United States (Boeke et al. 2012). Future studies are needed to explore the pathways through which poor nutrition may alter epigenetic programming and to understand the impact on metabolic disease in offspring.

4.6.2 Overnutrition

There is also evidence in animal and human models that overly rich nutrition prenatally or in early life may alter the offspring epigenome and later life metabolic disease risk. While some studies have linked prenatal overnutrition with methylation of genes directly involved in insulin resistance and/or obesity pathways, others have identified genes that control more ubiquitous functions. For example, in a rat model of neonatal overfeeding created by culling liter size per nursing mother, overfed rats developed rapid early weight gain and a metabolic syndrome phenotype, as compared to their normally-fed counterparts. The obese, overfed rats had high levels of leptin and insulin and thus should have upregulated the appetite suppressing gene *POMC*; however, the *POMC* gene was hypermethylated, and there was no increase in expression despite high levels of leptin and insulin (Plagemann et al. 2009). In the same model, the insulin receptor gene *IR* was also hypermethylated, and the extent of methylation was correlated with the degree of glucose intolerance (Plagemann et al. 2010). In a different rodent model, female offspring of high fat diet (HFD) fathers had lower birth weight and developed glucose intolerance, impaired insulin secretion, and decreased pancreatic islet and β–cell mass as compared to control offspring. Within pancreatic tissue, *Il13ra2*, part of the Jak-Stat signaling pathway, was hypomethylated and upregulated in HFD offspring, suggesting that a HFD may have affected the epigenetic profile of the paternal germ cells (Ng et al. 2010). In human studies, although epigenetic analysis was not performed, paternal insulin resistance was similarly associated with low infant birth weight and increased risk of diabetes in offspring (Lindsay et al. 2000; Hypponen et al. 2003).

Other studies considering the effect of prenatal overnutrition have evaluated offspring epigenetic profile more generally and found genes with broad ontologies affected. In an agouti viable yellow mouse model of natural onset obesity, when offspring were fed a HFD, insulin resistance and hepatic steatosis was more pronounced in those with obese as compared to lean mothers. When the hepatic epigenome of non-obese, non-HFD offspring of obese versus normal weight mothers

was compared, 36 loci with a >1.5-fold change in methylation involved primarily developmental rather than metabolic ontologies (Li et al. 2013). Similarly, in a human study of 50 adult siblings born to mothers pre- and post- bariatric surgery, methylation and expression differences primarily involved genes responsible for general biological functions that could affect metabolic disease including diabetes, inflammatory processes, and cardiovascular disease (Guenard et al. 2013).

Finally, there is potential for transgenerational inheritance of nutritionally induced phenotypes. Mice overfed in infancy developed obesity, insulin resistance, and glucose intolerance, and the male offspring and grand-offspring of these mice also developed obesity, insulin resistance, and glucose intolerance as compared to controls (Pentinat et al. 2010). Future studies are needed to determine whether transgenerational inheritance may be a result of stable epigenetic modifications and transmit phenotypes acquired in previous generations.

4.7 Epigenetics and Endocrine Cancers

Abundant literature exists linking environmentally-induced epigenetic modifications to tumor formation and progression in non-endocrine cancers through altered expression of tumor-suppressor genes and proto-oncogenes. For example, benzene exposure has been associated with increased risk of acute myelogenous leukemia (Bird et al. 2005). Gasoline-station attendants and traffic-police officers with higher than average benzene exposure had a significant reduction in methylation of selected genomic repetitive elements (i.e., viral-like retrotransposable sequences repeated thousands of times in one haploid genome) in conjunction with hypermethylation of tumor-suppressor gene *p15* and hypomethylation of the *MAGE-1* gene, suggesting epigenetic modification as a possible pathogenic mechanism (Bollati et al. 2007).

With regard to endocrine-related cancers, epigenetic modifications have also been implicated in thyroid carcinoma via hypermethylation and inactivation of tumor suppressor genes including cyclin-dependent kinase inhibitor *p16INK4A*, microtubule stabilizer *RASSF1A*, GTPase-activating protein *Rap1GAP*, and PI3K/akt pathway modulator *PTEN* (Russo et al. 2011). The *PTEN* promoter is hypermethylated in 50 % of papillary carcinomas and almost 100 % of follicular carcinomas, which is notable because a deleterious mutation in this gene causes PTEN hamartoma tumor syndrome leading to increased risk of thyroid carcinomas (Hobert and Eng 2009). As far as a potential environmental trigger for this epigenetic profile, ionizing radiation exposure has traditionally been thought to increase risk of thyroid cancer as a result of DNA mutagenesis, for example, through RET/PTC rearrangements (Caudill et al. 2005; Christodouleas et al. 2011). However, ionizing radiation has also been associated with global DNA hypomethylation that may be genotype-specific (Giotopoulos et al. 2006) and that even occurs in cells not directly irradiated (Tamminga et al. 2008). Prior ionizing radiation has also been associated with gene-specific hypermethylation in cancer patients (Figueroa et al. 2009; Bennett et al. 2010; Mathers et al. 2010). Additional research is needed to establish

whether ionizing radiation leads to specific epigenetic changes seen in thyroid carcinoma and subsequent thyroid carcinoma development.

Pituitary adenomas have also been associated with hypermethylation-related silencing of tumor-suppressor genes such as *RB1* and cyclin-dependent kinase inhibitors *p15* and *p16* (Vandeva et al. 2010). In some studies, growth hormone-secreting adenomas have resulted in tissue-specific loss of imprinting at the paternal stimulatory G-protein allele, which leads to increased expression of *GNAS* and constitutive activation of adenylyl cyclase (Hayward et al. 2001; Mantovani et al. 2010). As imprinting is an early embryonic process, *in utero* exposure may be responsible for dysregulated imprinting and subsequent development of growth hormone-secreting adenomas; however, this has not yet been evaluated.

4.8 Endocrine Disruptors and Epigenetics

A growing number of animal models have linked prenatal endocrine disruptor exposure to offspring epigenetic modifications. Bisphenol A (BPA), a synthetic chemical and weak estrogen agonist found in food and beverage containers (Le et al. 2008), baby bottles (Nam et al. 2010), and dental materials (Fleisch et al. 2010), is associated with hypomethylation in a regulatory region of a mutated allele in which the insertion of an intracisternal A particle (IAP) retrotransposon into the 5′ end of the wildtype *A* allele created a novel promoter in the proximal end of the transposed sequence. Methylation at this insertion particle correlates highly with expression of the Agouti gene and both fur color and obesity. Increased expression of the Agouti gene in prenatally-exposed mice leads to yellow rather than brown fur, as well as obesity, diabetes, and tumorigenesis (Dolinoy 2008). Furthermore, rodent mothers with the agouti phenotype were more likely to have offspring with that phenotype in the second generation. This important study serves as proof of principle that prenatal exposure to synthetic estrogen agonists such as BPA can affect the epigenome and thereby lead to endocrinologic sequelae.

As previously noted, diethylstilbestrol (DES), an estrogen agonist found to cause vaginal clear-cell adenocarcinoma in the female offspring of women exposed in pregnancy, has been associated with epigenetic modifications in offspring uterine tissues. Specifically, in mouse models of prenatal DES exposure and resultant genital tract neoplasia, hypomethylation occurred along with increased uterine expression of estrogen-sensitive *LTF* and proto-oncogenes including *EGF* and *SRF* (Nelson et al. 1994; Falck and Forsberg 1996; Li et al. 1997).

Exposure to vinclozolin, a fungicide and an antiandrogen, during embryogenesis decreased adult sperm motility and concentration and increased the rate of kidney and prostate disease, immune system abnormalities, hypercholesterolemia, and tumorigenesis in rat first-generation male offspring. This phenotype was transferred through four generations (F1–F4) of male offspring born to an F0 treated mother and was associated with alterations in the sperm methylation profile through the F3 generation (Anway et al. 2005). This is one of the first studies to suggest the potential

for transgenerational inheritance of epigenetic marks presumably resulting, in this case, from incomplete erasure of epigenetic marks during gametogenesis. However, similar studies attempting to replicate the above findings in vinclozoilin-exposed mice demonstrated changes in sperm methylation profile in the F1 and F2, but not the F3 generation (Schneider et al. 2008; Inawaka et al. 2009; Stouder and Paoloni-Giacobino 2010) suggesting that the phenotype of the F2 generation may be due to exposure of the F1 germline to the maternal environment rather than true transgenerational inheritance.

Persistent organic pollutants such as PCBs, used as coolants and insulating fluids for transformers and as flame retardants, and organochlorine pesticides (e.g., DDT and DDE), are endocrine disruptors with structural similarity to steroid hormones thyroxine and estrogen. POP exposure has been associated with spontaneous abortions and impaired male fertility. Lifetime POP exposure was associated with DNA global hypomethylation in a Greenlandic Inuit population (Rusiecki et al. 2008), suggesting a need for additional studies to examine whether global hypomethylation might be on the causal pathway linking POP exposure with endocrine endpoints.

4.9 Future Directions

Environmental epigenetics offers several clear research opportunities, particularly as it relates to endocrinology, including the following:

- Human studies of fetal epigenetic vulnerability to environmental exposures at physiologically relevant doses will confirm existing animal models. Human observational studies able to show the relationship from exposure to epigenetic modification to outcome over a lifespan will be required to differentiate causality from mere association.
- Additional endocrine tissue- and gene-specific studies of epigenetic modifications will further delineate causality.
- Finally, integrated teamwork including clinical endocrinologists, environmental toxicologists and epidemiologists will strengthen study design and thereby lead to heightened understanding of the impact of environmental epigenetics on endocrinology.

4.10 Conclusions

Although the field of environmental epigenetics is just moving out of its infancy, it has already begun to demonstrate the breadth of potential impact of environmental exposures on the expression of endocrine-related genes. These results not only suggest the need for increased biomonitoring of synthetic compounds, but also point out specific windows of human susceptibility as well as potential mechanisms that could represent the substrate for future preventive interventions.

Declaration of Interest The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Chapter 5
Pregnancy Exposures Determine Risk of Breast Cancer in Multiple Generations of Offspring

Zhenzhen Zhang, Shaowei Chen, Zhuang Feng, and L. Joseph Su

Abstract Breast cancer is the most common non-skin cancer in women in the United States. The incidence rate of breast cancer among migrant women from Asian countries, where the breast cancer incidence is low, could achieve the rate of the United States within two generations. Maternal exposures to various environmental factors during pregnancy have been hypothesized to be associated with offsprings' breast cancer risk. These exposures may change various carcinogenesis-related hormone levels and alter the epigenome among offsprings, which increase their breast cancer risk in later life. The effect of maternal pregnancy exposures on offsprings' breast cancer via epigenetic modifications could be carried out through multiple generations of offspring. In this chapter, we aim to summarize findings from both experimental and epidemiological studies investigating associations between maternal pregnancy exposures and offspring's breast cancer risk.

Keywords Pregnancy • Offspring • Breast cancer

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5.1 Overview of Pregnancy Exposures and Offspring's Breast Cancer Risk

In the U.S., breast cancer is the most common non-skin cancer and the second leading cause of cancer-related death among women (Siegel et al. 2012). A migration study showed the breast cancer incidence among Asian women who migrated to the U.S. increased over two generations to near the rate of the U.S. women (Ziegler et al. 1993), suggesting substantial effects of environmental factors on breast cancer through generations. Women who were exposed in utero to the Dutch famine during the end of World War II had 1.6 times increased risk for breast cancer compared to those without the exposure (Painter et al. 2006). In rat model, administering a high fat diet or ethinyl-oestradiol (EE2)-supplemented diet to pregnant mother rats increased mammary tumor incidence in two to three generations of daughter rats (de Assis et al. 2012). The link between pregnancy exposures and offspring breast cancer risk has been proposed to be mediated by epigenetic modifications through generations (de Assis et al. 2012), since high penetrance gene (such as BRCA-1 and BRCA-2) mutations account for only 20–25 % of familial breast cancer occurrence (Oldenburg et al. 2007). Human and animal species have similar mechanisms of mammary gland development and carcinogenesis (Rudel et al. 2011), therefore, studies from animal models could facilitate better understanding of human breast cancer etiopathogenesis. In this chapter, we (1) introduce the important role of intrauterine period for breast development; (2) review the influence of several prenatal risk factors (parental age, maternal weight gain, fetal growth, preeclampsia) on offspring's breast cancer; (3) evaluate the association between maternal dietary intake/environmental-disrupting chemicals (EDC) exposure during pregnancy and offspring's breast cancer risk; (4) summarize the potential association between several pregnancy biomarker levels and offspring's breast cancer risk; (5) and more importantly, discuss the epigenetic implications, especially DNA methylation, on the relationship between pregnancy exposures and offspring's breast cancer risk.

5.2 Importance of In Utero Period in Breast Development

Normal human breast develops in three major stages: in utero, puberty and pregnancy (Barbieri 2009). As the start of breast development, female human breast develops from budding and branching at gestational age 6–20 weeks (Rudel et al. 2011). Then the fetus in utero undergoes tremendous mammary morphogenesis, including the growth of epithelial elements into the underlying mesenchyme and the development of rudimentary ductal system (Barbieri 2009; Howard and Gusterson 2000). Breast development in utero is tightly regulated by multiple hormones (estrogens, progesterone, prolactin, growth hormone, etc.) and stromal factors (Falcone and Hurd 2007; Medina 2005). In utero exposures to various environmental agents

have been shown to lead to permanent changes in mammary gland development in both humans and rodents (Rudel et al. 2011), which suggests that in utero period is an etiologically relevant window for offspring's breast cancer development.

5.3 Intrauterine Risk Factors and Offspring's Breast Cancer Risk

Human epidemiological studies have investigated many intrauterine risk factors that are potentially associated with daughter's breast cancer development, such as older parental age, higher maternal weight gain during pregnancy, higher birth weight and length, and maternal preeclampsia (Xue and Michels 2007).

5.3.1 Mother or Father Age

One study from the Offspring Cohort of the Framingham Heart Study indicated father's age at birth was associated with daughters' increased susceptibility to develop breast cancer, possibly mediated through altering the leukocyte telomere length (LTL) (Arbeev et al. 2011). Mother's age was also reported to be positively associated with breast cancer risk in daughters from several epidemiological studies (Potischman and Troisi 1999). Three other studies reported both older maternal and paternal age increased breast cancer risk in daughters (Choi et al. 2005; Hodgson et al. 2004; Xue et al. 2007). Potential biological mechanisms underlying this association may implicate various levels of breast cancer-related biomarkers at different ages. For example, androgen and estrogen levels in the first trimester were found to be higher in younger age mothers (Troisi et al. 2008); whereas lower androgen and estradiol levels, and higher progesterone levels were observed in first-half pregnancy among older age women (Toriola et al. 2011).

5.3.2 Maternal Weight Gain During Pregnancy

Higher weight gain in pregnancy has been hypothesized to increase prenatal exposure to maternal estrogen (Hilakivi-Clarke et al. 2005; Kinnunen et al. 2004), which may in turn increase breast cancer risk. However, results from the Nurses' Mothers study did not identify an association between maternal pregnancy weight gain and daughter's breast cancer risk (Wilson et al. 2011). Possible explanation for the discrepancy may be due to the different pattern of pregnancy weight gain among this nurse population born between 1921 and 1964, when rigid restriction of weight gain was encouraged (Abrams et al. 2000).

5.3.3 *Fetal Growth*

Most epidemiological studies have suggested a positive relationship between birth weight/length and breast cancer risk, and an inverse relationship between gestational age and breast cancer risk (Ruder et al. 2008). Birth weight and length are positively associated, and are influenced by gestational age and fetal growth rate. A meta-analysis revealed women with higher birth weight had 23 % higher risk for breast cancer compared to women with lower birth weight (Michels and Xue 2006). The effect of birth weight on offspring's breast cancer was found to be independent of maternal weight gain (Wilson et al. 2011). A meta-analysis demonstrated women with longer birth length had 28 % increased risk for breast cancer compared to women with shorter birth length (Xue and Michels 2007). These associations are biologically plausible because higher rates of fetal growth is associated with greater maternal estrogen and insulin-like growth factor (IGF)-I levels (Nechuta et al. 2010). Studies on rat models also showed higher birth weight was related to shorter latency for mammary tumorigenesis, possibly by activating mitogen-activated protein kinases and protein kinase B in the tumors (de Assis et al. 2006).

5.3.4 *Preeclampsia*

Preeclampsia is a complication occurring in pregnancy, characterized by hypertension and proteinuria. Many studies have identified an inverse association between in utero exposure to maternal preeclampsia and breast cancer in later life (Troisi et al. 2007a). A study following a cohort of daughters of preeclamptic pregnancies and normotensive pregnancies found a lower estrogen level and 20 % (OR = 0.80, 95 % CI = 0.70–1.00) lower risk of breast cancer development among daughters of preeclamptic pregnancies (Forman 2011). Preeclampsia during pregnancy was shown to be associated with lower IGF and higher insulin-like growth factor binding protein (IGFBP) levels in maternal and fetal blood (Giudice et al. 1997), which may account for the inverse association between breast cancer risk and in utero exposure to preeclampsia. Sex hormones, and other hormones involved in energy balance and glucose homeostasis may also contribute to the mechanisms of preeclampsia-associated reduction in breast cancer risk (Troisi et al. 2007a).

5.4 Maternal Pregnancy Dietary Intake and Offspring's Breast Cancer

Maternal dietary intake can interact with gene polymorphisms, modify epigenetic variation, and affect later health outcomes (Burdge et al. 2012). Recent studies found dietary factors including fat, folic acid, protein and total energy intake may

alter specific genes through epigenetic regulation in offspring (Burdge et al. 2012). Table 5.1 shows the summary of effects of several maternal dietary intakes during pregnancy on offspring's mammary gland in animal models.

5.4.1 Fat

Total dietary fat has been established to be modestly associated with increased risk of breast cancer in many epidemiological studies (Smith-Warner and Stampfer 2007). Data from animal studies suggested different types of fat might exert different effects on mammary tumorigenesis (De Assis and Hilakivi-Clarke 2006). Experiments conducted on rats in 1994 demonstrated maternal high fat diet increased the incidence of induced mammary tumors in the first generation of female offspring (Hilakivi-Clarke et al. 1994). Later, the same research group showed maternal intake of corn oil with high n−6 polyunsaturated fatty acids (PUFAs) was associated with increased offspring's breast cancer risk (Hilakivi-Clarke et al. 1997), while maternal intake of n−3 PUFAs was associated with decreased offspring's breast cancer risk (Hilakivi-Clarke et al. 2002). Another study, comparing canola oil (20 % n−6 PUFA and 10 % n−3 PUFA) with corn oil (50 % n−6 PUFA), showed the canola oil group had a significantly lower mammary tumor incidence, fewer tumors per mouse and lower tumor weight (Ion et al. 2010). Moreover, maternal high fat diet with 40 % of the energy source as safflower oil could increase mammary tumor incidence in offspring (Lo et al. 2009). The timing of fat exposure, in utero rather than during lactation, appeared more critical in the etiopathogenesis of mammary tumor development (Su et al. 2010).

The above results are biologically plausible because high fat diet during pregnancy increased estradiol level in the mother (Hilakivi-Clarke et al. 1994). Biological mechanisms of altered fetal epigenome, and modification in the fetal metabolite profile have also been suggested (Cox et al. 2009).

5.4.2 Soy Food

Childhood/adolescence soy intake has been consistently found to be associated with a decreased breast cancer risk (Korde et al. 2009; Shu et al. 2001; Wu et al. 2002; Lee et al. 2009). No study was conducted on in utero exposure to soy food and offspring's breast cancer in humans. However, it has been hypothesized that different timing of exposure to soy food products may have different effects on breast cancer risk (De Assis and Hilakivi-Clarke 2006). Genistein is a weak estrogenic compound mainly derived from soy. Studies using mice and rats models showed in utero exposure to genistein increased mammary cancer risk in female offspring (Hilakivi-Clarke et al. 1998, 1999). However, another study found maternal genistein intake had protective effect on offspring's mammary tumor development in a dose-response relationship (Fritz et al. 1998).

Table 5.1 Pregnancy exposure to dietary factors and offspring's breast cancer risk in female animal models

Study	Maternal pregnancy dietary factors	Animal species	Mammary gland assessment	Observations in offspring following in utero exposure	Comments
Rahal et al. (2013)	Blueberry (3 %) Control: casein diet	Wnt1-transgenic mice	Oncogene Wnt1-induced mammary tumorigenesis	Offspring with blueberry exposure demonstrated higher tumor suppressor (PTEN and Cdh1) and lower proproliferative (Ccnd1), anti-apoptotic (Bcl2) and proangiogenic (Figf, Flt1 and Ephb4) transcript levels in tumor tissue; higher gene expression of PTEN and Cdh1 in mammary tissue; and higher chromatin-modifying enzyme Dnmt1 and Ezh2 transcript levels in mammary and tumor tissues	Blueberry-exposed offspring can exhibit lower body weight, serum insulin and serum leptin/adiponectin ratio
de Assis et al. (2012)	AIN93G based high fat (HF) diet containing 43 % energy from corn oil Control: AIN93G diet	Sprague-Dawley rats	Carcinogen-induced mammary tumorigenesis	Offspring exposed to HF showed a multigenerational increase in mammary tumor incidence in two generations	In utero exposure to HF diet can increase breast cancer risk in multiple generations of offspring
Cho et al. (2012)	High-dose lipotropes (8.3 g L-methionine, 5.0 g choline, 10.0 mg folic acid and 100 μg vitamin B_{12}/kg diet) Control: AIN-93G semi-purified diet with basal level lipotropes	Sprague-Dawley rats	N-nitroso-N-methylurea (NMU) induced mammary tumorigenesis	Offspring exposed to high-dose lipotropes exhibited decreased mammary tumor incidence, multiplicity and volume, and increased tumor latency and survival rate, as well as significantly decreased histone deacetylase 1 (HDAC1) messenger RNA expression in tumor tissue	Maternal high-dose lipotropes exposure may decrease offspring's mammary tumor risk

Ly et al. (2011)	Folic acid supplementation (5 mg/kg) Control: folic acid (2 mg/kg)	Sprague-Dawley rats	DMBA (7,12-dimenthylbenz[α] anthracene) induced mammary tumorigenesis	Offspring exposed to folic acid demonstrated a significantly higher risk and faster rate of developing mammary adenocarcinoma as well as an increased risk for tumor multiplicity	Global DNA methylation is significantly reduced by maternal but not postweaning folic acid supplementation
Ion et al. (2010)	Canola oil (20 % n−6 PUFA, 10 % n−3 PUFA) Control: corn oil (50 % n−6 PUFA)	Transgenic SV 129 mice	Tumor growth rates, incidence, multiplicity (tumors per mouse) and weight	Offspring exposed to canola oil exhibited significantly less multiplicity, lower incidence, less total weight, and slower development of mammary tumor	Maternal canola oil diet may decrease offspring's breast cancer risk possibly through increasing CEBPβ expression in the mammary gland
Su et al. (2010)	High n−6 fat diet containing 20 % sunflower oil with fish oil supplementation Control: high n−6 fat diet with no fish oil supplementation or fish oil supplementation later in life	Sprague-Dawley rats	DMBA induced mammary tumorigenesis	Maternal exposure to high n−6 diet with fish oil significantly decreased the incidence of carcinogen-induced mammary tumor in offspring compared with control. In utero exposure to high n−6 fat diet increased the risk of mammary tumor in female offspring than later exposure during lactation	Fish oil supplementation negates the adverse effect of high n−6 fat diet on the risk of carcinogen-induced mammary tumor in offspring
Lo et al. (2009)	High-fat diet (40 % of the energy from safflower oil) Control: chow diet-fed rats	Sprague-Dawley rats	DMBA induced mammary tumorigenesis	Offspring exposed to high-fat demonstrated increased mammary tumor incidence	The timing of high-fat exposure is more critical in determining the risk of carcinogen-induced mammary tumor in offspring. In utero high-fat exposure poses a greater risk of mammary tumor than exposure in later life

(continued)

Table 5.1 (continued)

Study	Maternal pregnancy dietary factors	Animal species	Mammary gland assessment	Observations in offspring following in utero exposure	Comments
Wu et al. (2009)	Blueberry (2.5 %, 5 % and 10 %)	Sprague-Dawley rats	Mammary epithelial differentiation	The 5 % blueberry group showed greatest mammary branch density and terminal end bud size. The 5 % blueberry group had lower nuclear phosphorylated histone 3 and higher nuclear tumor suppressor PTEN levels in mammary ductal epithelial cells than control. Sera from the 5 % blueberry group induced higher nuclear PTEN level in human MCF-10A mammary epithelial cells. There was no difference in terminal end bud numbers among all diets	Blueberry components exert a substantial regulatory effect on the developing mammary glands in early life, possibly through modulation of the PTEN signalling pathway
	Control: casein diet group	Human MCF-10A mammary epithelial cells			
Sie et al. (2009)	Folic acid supplementation (5 mg/kg)	Sprague-Dawley rats	The number of terminal end buds, alveolar buds, terminal ducts and lobules	Offspring exposed to folic acid had a significantly lower number of terminal end buds and higher number of alveolar buds than control	Maternal folic acid supplementation has no statistically significant effect on the mean number of terminal ducts and lobules
	Control: folic acid (2 mg/kg)				

Kovacheva et al. (2009)	Choline intake 36 (mmol/kg) Control: (8 mmol/kg)	Sprague-Dawley rats	DMBA induced mammary tumorigenesis	Maternal dietary choline content showed an inverse association with tumor growth rate/tumor size, along with distinct expression patterns of 70 genes in tumors. DNA methylation within the tumor suppressor gene stratifin is positively correlated with maternal dietary choline content and inversely correlated with its mRNA and protein expression levels in tumor tissue	In utero choline exposure may affect breast cancer risk in offspring possibly through epigenetic mechanisms
Dolinoy et al. (2007)	Methyl donors like folic acid or phytoestrogen genistein Bisphenol A (BPA)	Viable yellow agouti (Avy) mice	–	In utero BPA exposure decreased DNA methylation at CpG sites in early development, which was negated by maternal nutrient supplementation, with either methyl donors like folic acid or phytoestrogen genistein	Maternal nutrient supplementation can reverse BPA-induced DNA hypomethylation in offspring through epigenetic modification
Khan et al. (2007)	Flaxseed diet (5 % and 10 %)	Sprague-Dawley rats	DMBA induced mammary tumorigenesis	In utero exposure to flaxseed at 5 % and 10 % decreased mammary tumor latency and lowered ER-beta protein levels in the terminal end buds, lobules and ducts. Flaxseed at 10 % increased tumor multiplicity and lobular ER-alpha protein levels. The number of terminal end buds and epithelial cell proliferation were not affected by flaxseed exposure	Whether the effect of flaxseed is caused by cadmium present in flaxseed is unclear

(continued)

Table 5.1 (continued)

Study	Maternal pregnancy dietary factors	Animal species	Mammary gland assessment	Observations in offspring following in utero exposure	Comments
Fernandez-Twinn et al. (2007)	Isocaloric low-protein diet (8 % casein) Control: 20 % casein	Wistar rats	Mammary gland development assessed by morphometry	Offspring exposed to low-protein exhibited reduced ductal branching and epithelial invasion, and twofold increase in early mammary tumor incidence, followed by compensatory mammary growth along with accelerated protein expression of insulin, IGF-1 and estrogen receptors	Early nutrition has a significant impact on mammary gland development, potentially through compensatory upregulation of growth factor activities and hormone signalling
Yu et al. (2006)	Fiber (6 %) from either cellulose (control), oat, whole wheat or defatted flax flour	Sprague-Dawley rats	DMBA induced mammary tumorigenesis	Offspring exposed to high-fiber diet demonstrated decreased number of terminal end buds and increased apoptotic epithelial cells in the mammary glands, and significantly increased BRCA1 and p53 mRNA and/or protein expression	Maternal consumption of high-fiber diet may decrease offspring's breast cancer risk, possibly by improving DNA damage repair mechanisms
Hilakivi-Clarke et al. (2004)	Alcohol (16 g, 25 g/kg) Control: no alcohol	Sprague-Dawley rats	DMBA induced mammary tumorigenesis	Offspring exposed to alcohol displayed denser mammary epithelia tree more susceptible to breast cancer, more carcinogen-induced mammary tumors, and higher ER-alpha levels	In utero alcohol exposure may increase offspring's risk of mammary carcinogenesis, possibly through remodelling the fetal mammary gland in morphology and gene expression

Hilakivi-Clarke et al. (2002)	AIN-93-based diets containing 16 % or 39 % energy from fat with components of n−3 PUFA and n−6 PUFA AIN-93-based diets (15 mg, 150 mg or 300 mg/kg genistein/ diet)	Sprague–Dawley rats	DMBA induced mammary tumorigenesis	DMBA induced mammary tumorigenesis	The effect of maternal n−3 PUFA intake on offspring's mammary tumor risk may be mediated through modulation of pregnancy estrogen levels
Hilakivi-Clarke et al. (1999)	Genistein (20, 100 or 300 μg), zearalenone (20 μg)	Sprague-Dawley rats	DMBA induced mammary tumorigenesis	Offspring exposed to genistein demonstrated increased incidence of DMBA-induced mammary tumors, reduced mammary protein kinase C (PKC) activities, and increased ER protein levels. Zearalenone had no significant effect on mammary tumor incidence	In utero genistein exposure may increase mammary tumor risk in offspring, possibly through upregulation of ER protein levels and downregulation of PKC activities in the mammary gland
Fritz et al. (1998)	Genistein (0, 25 and 250 mg genistein/kg AIN-76A diet)	Sprague-Dawley rats	DMBA induced mammary tumorigenesis	Perinatal genistein exposure provided dose-dependent protective effects against DMBA-induced mammary tumors. Genistein-exposed offspring showed fewer multiplicity, fewer terminal end buds, and smaller proliferative compartment for terminal end buds	Perinatal dietary genistein enhances epithelial cell differentiation, and renders mammary gland less susceptible to cancer

(continued)

Table 5.1 (continued)

Study	Maternal pregnancy dietary factors	Animal species	Mammary gland assessment	Observations in offspring following in utero exposure	Comments
Hilakivi-Clarke et al. (1997)	Isocaloric high n−6 PUFA diet (43 % or 46 % of energy from corn oil)	Sprague-Dawley rats	DMBA induced mammary tumorigenesis	Offspring exposed to high n−6 PUFA had a significantly higher mammary tumor incidence, shorter tumor latency, earlier puberty onset and higher number of epithelial structures	High n−6 PUFA exposure may increase maternal pregnancy blood 17β-estradiol levels

5.4.3 Methyl Nutrients

Methyl nutrients include vitamins (folate, riboflavin, vitamin B12, vitamin B6, and choline) and amino acids (methionine, cysteine, serine, and glycine) (Glier et al. 2014). They can generate adenosylmethionine, the primary methyl donor for DNA, RNA and protein (Finkelstein 2003). The imbalance of methyl nutrient intake and metabolism process may be implicated in epigenetic changes involving DNA methylation and lead to various diseases. Several animal studies have been conducted to assess dietary methyl nutrients intake and breast cancer development.

An animal study on pregnant rats revealed maternal diet high in methyl nutrients lipotropes (methionine, choline, folate and vitamin B12) could diminish the induced mammary tumor development, possibly by significantly decreasing histone deacetylase 1 (HDAC1) messenger RNA levels (Cho et al. 2012). In a study assessing maternal folic acid supplementation on the development of carcinogen-induced mammary tumor, folic acid was found to significantly increase mammary adenocarcinomas risk (Ly et al. 2011). On the contrary, another study showed folic acid could reduce the number of terminal end buds, alveolar buds, terminal ducts and lobules, which further decreased mammary tumor development (Sie et al. 2009). The reasons for the conflicting results are unclear, but may in part due to the use of different animal models (carcinogen induced vs. spontaneous). In addition, maternal choline intake, another methyl nutrient, was inversely correlated with carcinogen-induced mammary tumor growth rate as well as mRNA and protein expression of the tumor suppressor gene stratifin (Kovacheva et al. 2009).

5.4.4 Others

Protein Restriction A study using rat model found maternal protein restriction could inhibit p21 gene expression in the mammary gland of offspring, thereby predisposing the offspring to breast cancer later in life (Zheng et al. 2012). Moreover, offspring born with maternal low protein intake doubled mammary tumor incidence (Fernandez-Twinn et al. 2007). *Blueberry*: Two studies have shown an anti-tumor effect of maternal pregnancy blueberry diet on offspring's breast cancer outcome (Rahal et al. 2013; Wu et al. 2009). *Alcohol*: In a study assessing the role of maternal alcohol intake on offspring's breast cancer risk, alcohol could make the mammary glands more susceptible to carcinogenesis (Hilakivi-Clarke et al. 2004). *Flaxseed*: The maternal diet of flaxseed was found to shorten mammary tumor latency in offspring, and higher dose of flaxseed exposure could increase tumor multiplicity. However, the effect of cadmium present in flaxseed could not be excluded (Khan et al. 2007). *Fiber*: higher fiber intake was shown to be associated with offspring's decreased mammary tumorigenesis, possibly by improving DNA damage repair mechanism (Yu et al. 2006).

5.5 Maternal Exposures to Environmental-Disrupting Chemicals (EDC) and Offspring's Breast Cancer

Maternal pregnancy exposure to EDCs may alter mammary gland developmental processes in offspring and modify the epigenome, which may predispose the offspring to later breast cancer development as a result of interruption of normal hormonal development (McKinney and Waller 1994). These EDCs include a variety of synthetic chemicals such as dioxins polychlorinated biphenyls (PCBs), bisphenol A (BPA), Diethylstilboestrol (DES), 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and organochlorines dichlorodiphenyltrichloroethane (DDT) (Calle et al. 2002). PCBs and DDT have been banned in the U.S., but their environmental effects may still persist. In a study from the Michigan fish-eater cohort, maternal exposure to oestrogenic dichlorodiphenyl-dichloroethylene (DDE), a chemical similar to DDT, was associated with obesity among daughters (Karmaus et al. 2009), a risk factor for postmenopausal breast cancer. A study combining three cohort studies found women exposed to in utero DES had increased risks for multiple health outcomes including infertility, spontaneous abortion, preterm delivery, loss of second trimester pregnancy, ectopic pregnancy, preeclampsia, still birth, early menopause, cervical intraepithelial neoplasia and breast cancer (Hoover et al. 2011).

Table 5.2 shows the summarization of prenatal exposure to EDCs and offspring's breast cancer in female animal studies. In animal models, administration of ethinyl oestradiol to gestational rats altered morphological structures of mammary glands in offspring rats (Mandrup et al. 2012). In utero exposure to BPA, a chemical used mainly in the manufacture of plastics, increased breast cancer risk via DNA hypomethylation, which can be counteracted by maternal nutritional supplementation with methyl donors (Dolinoy et al. 2007). In addition, maternal BPA exposure could increase the occurrence of ductal hyperplastic and neoplastic lesions (Durando et al. 2007). TCDD is a typical persistent organic compounds used in herbicide production. In mice, maternal TCDD exposure increased the incidence of mammary tumors in offspring, and high fat diet could sensitize the mammary tissue to subsequent exposure of TCDD (La Merrill et al. 2010). In utero exposure to TCDD could lead to more terminal end buds and fewer lobules in offspring rats (Brown et al. 1998). DES, a strong synthetic non-steroid estrogen analogue, was used as estrogen-replacement therapy and preventive medicine for miscarriages and other pregnancy complications from the 1940s to the 1970s. Results from The DES Combined Cohort Follow-up Study showed daughters exposed to in utero DES had an about 83 % increase in breast cancer risk at age 40 years and older (Troisi et al. 2007b; Palmer et al. 2006). Earlier animal studies on DES consistently found a positive relationship between in utero DES exposure and offspring's mammary tumor incidence (Walker 1990; Boylan and Calhoon 1983). Another synthetic non-steroid compound with similar structure to DES is tamoxifen, which is widely used as a selective estrogen receptor modulator to treat breast cancer. Tamoxifen was found to be associated with increased incidence of offspring's mammary tumors and more aggressive phenotype among rats (Halakivi-Clarke et al. 2000). Fetal exposure to

Table 5.2 Pregnancy exposure to environmental endocrine disruptors and offspring's breast cancer risk in female animal models

Study	Environmental compound	Animal species	Mammary gland assessment	Observations in offspring following in utero exposure	Comments
Mandrup et al. (2012)	Ethinyl oestradiol (0, 5, 15 or 50 µg/kg/day)	Wistar rats	Whole mount of mammary glands	Perinatal estrogen exposure increased mammary gland development in pre-pubertal rats	The most sensitive parameters were the distance to the fifth gland, the relative growth towards the lymph node and the overall density
de Assis et al. (2012)	Ethinyl-oestradiol (EE2) (0.1 p.p.m) supplemented to AIN93G diet Control: AIN93G diet	Sprague-Dawley rats	Carcinogen-induced mammary tumorigenesis	EE2-exposed offspring exhibited a transgenerational increase in mammary tumor incidence in three generations	In utero exposure to EE2 diet can increase breast cancer risk in multiple generations of offspring
La Merrill et al. (2010)	1 mg/kg TCDD or vehicle	TCDD-responsive mouse strain FVB/NJ	DMBA induced mammary tumorigenesis	Offspring with in utero exposure to TCDD demonstrated increased mammary tumor incidence, higher cytochrome P450 1B1 expression and lower catechol-O-methyltransferase expression	High fat diet increases the sensitivity of mammary tissue to in utero TCDD exposure
Kawaguchi et al. (2010)	4-n-octylphenol (OP) (10, 100, 1,000 ppm)	Sprague-Dawley female adult rats	DMBA induced mammary tumorigenesis	In utero exposure to 100 and 1,000 ppm OP increased the incidence and number of mammary tumors but not those of benign proliferative lesions	Proliferative lesions included solid masses and gross cysts

(continued)

Table 5.2 (continued)

Study	Environmental compound	Animal species	Mammary gland assessment	Observations in offspring following in utero exposure	Comments
Durando et al. (2007)	Bisphenol A (BPA) (25 µg/kg/day)	Wistar rats	Carcinogen N-nitroso-Nmethylurea (NMU) induced mammary tumorigenesis	Prenatal exposure to BPA increased the occurrence of mammary ductal hyperplasias and neoplastic lesions in offspring	In utero BPA-exposed offspring has increased stromal mast cells in the mammary gland
Jenkins et al. (2007)	2,3,7,8-tetrachlo-rodibenzo-p-dioxin (TCDD) (1 µg/kg on post-conception day 15)	Sprague-Dawley CD rats	DMBA induced mammary tumorigenesis	Offspring with in utero exposure to TCDD displayed a downregulation in superoxide dismutase 1 (SOD1)	Maternal TCDD exposure can program mammary gland with enhanced susceptibility for carcinogenesis in offspring by altering the mammary proteome
Murray et al. (2007)	BPA (2.5, 25, 250 and 1,000 ug/kg/day)	Wistar-Furth rats	DMBA induced mammary tumorigenesis	Increased incidence of Ductal hyperplasia and carcinoma in situ at postnatal day 50 and 95	No effects on body weight
Halakivi-Clarke et al. (2000)	Tamoxifen (20 mg gestation day 15 and 20)	Sprague-Dawley rats	DMBA induced mammary tumorigenesis	Offspring exposed to in utero tamoxifen exhibited increased incidence of mammary tumors and more aggressive phenotype	Tamoxifen may increase the estrogen level during pregnancy

Hilakivi-Clarke et al. (1998)	Estradiol (20 ng), Genistein (20 μg), Zearalenone (2 μg),	Outbred CD-1 mice	Mammary gland morphology, puberty onset, physical maturation	Estradiol increased terminal end bud density, and accelerated body weight gain, physical maturation and puberty onset	Maternal genistein exposure may increase mammary tumor risk in offspring
	Tamoxifen (2 μg)			Genistein decreased epithelia differentiation	
				Zearalenone increased epithelial differentiation and density	
Brown et al. (1998)	TCDD 1 μg/kg on post-conception day 15	Sprague-Dawley rats	DMBA induced mammary tumorigenesis	Offspring exposed to in utero TCDD had more terminal end buds and fewer lobules II	Maternal TCDD exposure may predispose offspring to mammary cancer risk as a result of altered gland differentiation
	Control: vehicle (sesame oil)				
Walker (1990)	Diethylstilboestrol (DES) (1 μg/g body weight)	CD-1 mice	Analyses of several tumors including mammary tumors	Mammary tumors were more frequent with prenatal exposure to high dietary fat in vehicle-injection mice but not in DES-lineage mice	Early exposure to DES may predispose offspring to mammary tumors
Boylan and Calhoon (1983)	DES (total dose: 1.2 mg)	Sprague-Dawley rats	DMBA induced mammary tumorigenesis	Offspring exposed to in utero DES had higher incidence of mammary tumors and shorter latency for tumor development	The dose of the carcinogen exerts significant effects on the association between prenatal DES exposure and later tumor development

3-n-octylphenol (OP), a weak estrogenic disruptor, was also found to be associated with an increased mammary tumor incidence in a dose-response relationship (Kawaguchi et al. 2010).

All these evidences contribute to the effects of maternal exposures to EDCs on the increased risk for breast cancer in offsprings. Whether the effects of EDCs on female offspring's breast cancer risk are mediated by transplacental or lactational pathway is still unclear (Snedeker and Diaugustine 1996).

5.6 Biomarker Level During Pregnancy and Offspring's Breast Cancer Risk

As the above animal and human studies suggested, exposures to multiple risk factors during pregnancy could affect offspring's breast cancer development in later life. Biological mechanisms underlying the association between pregnancy exposures and offspring's breast cancer risk are still unclear. Hormonal milieu involving sex hormones, insulin and insulin-like growth factor (IGF), human chorionic gonadotropin (HCG) and alpha-feto protein (AFP) have been implicated.

5.6.1 Sex Hormone System

It is hypothesized that high estrogen concentrations in pregnancy increased the breast cancer risk in daughters, who get the greatest exposure to endogenous estrogen in utero (Trichopoulos 1990). In addition, maternal pregnancy exposure to food components with estrogenic effects may also affect the fate of mammary epithelial cells differentiation in offsprings (Hilakivi-Clarke et al. 1998).

Estradiol, Estrone, Estriol The family of endogenous estrogens includes estriol, 17β-estradiol, estrone and their conjugates (Kuhl 2005). Estriol is produced in high amount by the placenta during pregnancy, while it is very low in concentration among non-pregnant women (Kuhl 2005; Mucci et al. 2003). Estriol is a short-acting estrogen and the "weakest" estrogen (Files et al. 2011). Estradiol is a predominant sex hormone in women and the major estrogen in humans (Files et al. 2011). Estrone obtains its peak concentration after menopause (Files et al. 2011). The human ovaries produce both estradiol and estrone. In addition, estradiol can be converted from estrone, not from estriol; and estrone can be converted from estradiol in adipose tissue (Files et al. 2011). Maternal serum unconjugated Estriol is routinely measured in prenatal screening during the second trimester of pregnancy. Many studies have examined the associations between well-known risk factors for breast cancer and blood estrogen levels. However, to our knowledge, there is no epidemiological study directly investigating maternal serum estradiol, estrone or estriol levels during pregnancy and offspring's breast cancer risk.

Progesterone and Sex Hormone-Binding Globulin (SHBG) Pregnancy levels of estrogen and progesterone are a double-edged sword (Ferretti et al. 2007). Progesterone is a steroid hormone involved in menstrual cycle, pregnancy and embryogenesis. It has been implicated in mammary gland carcinogenesis through animal studies and epidemiological studies (Brisken 2013). SHBG regulates the bioavailability of the circulating estradiol, therefore, SHBG has an important role in breast cancer development (Fortunati et al. 2010). Evidence on both the pregnancy progesterone and SHBG levels and offspring breast cancer is lacking in human epidemiological studies. As reviewed above, many studies demonstrated women exposed to pre-eclampsia in utero had a significantly lower breast cancer risk (Ekbom et al. 1992, 1997). Serum progesterone was found to be elevated in pre-eclamptic mothers (RR = 2.65) at the 27th gestational week (Tamimi et al. 2003), whereas serum SHBG (RR = 0.61) was reduced in pre-eclamptic mothers (Lagiou et al. 2006). Proper diet during pregnancy could affect progesterone and SHBG levels which may decrease the offspring's breast cancer risk.

5.6.2 IGF System

IGF and insulin systems are interrelated systems that regulate human body's normal physiological functions (Pollak 2008). Insulin increases the physiological activity of IGF-1, a protein with mitogenic and anti-apoptotic effect, by increasing its synthesis and decreasing its binding proteins levels (Kaaks 2004). A pooled statistics from 17 prospective studies found circulating IGF-I was positively associated with women's breast cancer risk (Key et al. 2010). However, a nested case-control study demonstrated no association between pregnancy IGF-I/IGF-II and maternal breast cancer risk (Jeffreys et al. 2011). There is no study investigating pregnancy IGF levels and offspring's breast cancer. IGF-I is the major IGF that regulates postnatal growth (Liu and LeRoith 1999), while IGF-II is the major growth factor for fetal growth (Juul 2003). Both maternal and fetal serum IGF-I and IGF-II levels increase dramatically during pregnancy (Luo et al. 2012), in accordance with the increasing placental growth hormone production as gestation advances (Chellakooty et al. 2004). A singleton pregnancy cohort study revealed maternal IGF-I rather than IGF-II was positively associated with placental and fetal growth (Luo et al. 2012), which may shed light on the potential role of IGF-I in the etiopathogenesis of offspring's breast cancer since fetal overgrowth is associated with later development of breast cancer.

5.6.3 Human Chorionic Gonadotropin (HCG) and Alpha-Fetoprotein (AFP)

Serum total hCG and AFP levels in the second trimester of pregnancy are routinely measured as part of the traditional triple screening test and the current quadruple screening test. AFP is a major fetal protein that can suppress estrogen-dependent

breast cancer cell growth (Bennett et al. 1997). HCG is another important protein produced during pregnancy, which has proapoptotic, antiproliferative and tumor suppressor functions (Rao et al. 2004). The intact heterodimeric hCG in pregnancy, rather than free beta subunit of hCG produced by tumor cells, may initiate tissue differentiation and therefore reduce the population of stem-like cells that are susceptible to factors promoting breast cancer formation (Iles et al. 2010). Four studies examined hCG and/or AFP in pregnancy in relation to maternal breast cancer (Toniolo et al. 2010; Lukanova et al. 2008; Richardson et al. 1998; Melbye et al. 2000). Among them, two studies of maternal pregnancy AFP (Richardson et al. 1998; Melbye et al. 2000) and one study of hCG (Toniolo et al. 2010) determined a statistically significant inverse association between serum hCG/AFP levels and maternal breast cancer risk, which might imply a potential inverse association between these two biomarkers and offspring's breast cancer. However, no studies have been conducted on the association between pregnancy hCG/AFP level and offspring's breast cancer.

5.7 Epigenetic Implications for the Relationship Between Pregnancy Exposures and Offspring's Breast Cancer Risk

Given all the findings discussed above, an essential question yet to be answered is: How transient maternal pregnancy exposures can lead to lasting effects on breast cancer risk among female offspring decades later or even in multiple generations? Especially, in most cases (if not all cases), no known genetic mutations are identified.

In recent years, research has brought to light a series of mechanisms involved in heritable and potentially reversible alterations in gene expression that occur without a change in DNA sequence itself (Berger et al. 2009). These mechanisms are described with the term "epigenetic modifications", which include DNA methylation and histone modifications. The best example of epigenetic modifications is the process of cellular differentiation during embryonic development. All types of cells in the body are derived from a single fertilized egg cell and contain identical DNA sequences. However, the cells carry unique epigenetic information, i.e. distinct patterns of epigenetic modifications, which distinguish them from each other. Thus, from the epigenetic point of view, embryonic development, which includes mammary development, is basically a process of epigenetic reprogramming of the genome. Importantly, epigenetic modifications can dynamically respond to the environment, such as nutrients, toxicants, stress, and other factors. Growing evidence indicates that changes in the intrauterine milieu during "sensitive" periods of embryonic development can affect the accuracy of epigenetic reprogramming, resulting in birth defects and/or predisposing the offspring to diseases in later life. It is now increasingly realized that epigenetic modifications play important roles in

various types of cancers, including breast cancer. Therefore, the need for studies examining the association between maternal pregnancy exposures and stable epigenetic imprinting has become apparent. These studies will provide new insights into the pathological mechanisms of breast cancer, and open new avenues for future preventive and therapeutic strategies, through pharmaceutical agents that target epigenetic modifications. Such epigenetic drugs [e.g. HDAC inhibitors and DNA methyltransferase enzymes (DNMT) inhibitors] are already in clinical use or under investigation for the treatment of cancers, including breast cancer.

Among epigenetic modifications, DNA methylation is one of the most widespread and best characterized markers and is well recognized as a critical epigenetic biomarker in breast cancer carcinogenesis. Latest studies indicate that increased breast cancer susceptibility resulting from maternal pregnancy exposures involves changes in the DNA methylation machinery and methylation patterns in mammary tissue in female offspring (de Assis et al. 2012). While histone modifications are relatively unstable in the epigenetic change detection, few studies have used histone modifications as an epigenetic biomarker. Therefore, we will discuss DNA methylation and breast cancer in more details next.

5.8 DNA Methylation and Breast Cancer

DNA methylation refers to covalent addition of a methyl group by DNA DNMTs to the number 5 carbon of the cytosine pyrimidine ring or the number 6 nitrogen of the adenine purine ring. DNA methylation is normally stable and can be passed onto daughter DNA strands through cell division in a semi-conserved manner. Copying the methylation patterns from an existing DNA strand to its new synthesized partner during DNA replication is catalyzed by the maintenance DNMT (DNMT1). Typically, genome-wide DNA demethylation occurs twice during development: first in the male pronucleus of the zygote right after fertilization; and then in the primordial germ cells when they reach genital ridge (Shearstone et al. 2011). Thereafter, de novo DNA methylation is re-established through successive cell divisions. The enzymes that control the acquisition of new DNA methylation are de novo DNMTs (DNMT3a and DNMT3b) (Bestor 2000; Okano et al. 1999). Two recent studies revealed that hydroxylation of methyl groups occurs rather than complete removal of methyl groups in zygote (Iqbal et al. 2011; Wossidlo et al. 2011). Therefore, some methylation modifications can be inheritable in regulating gene expressions, resulting in partial genomic imprinting in multiple generations.

In mammalian genome, DNA methylation predominantly occurs in the sequence context of CpG dinucleotides that overall 60–90 % of CpGs are methylated, with the exception of CpG islands, where a high frequency of unmethylated CpG sites is found, typically, at the promoter regions of genes. In human, about 70 % of all gene promoters contain CpG islands (Tucker 2001). DNA methylation causes inactivation of the targeted genomic loci by two major mechanisms. First, the methylation of DNA itself may cause a physical block of the binding of chromatin remodelling

proteins to the DNA strand. Second, the methylated DNA may serve as a platform to recruit multiple proteins known as Methyl-CpG-binding domain proteins (MBDs). Then, MBDs in turn recruit other chromatin remodelling proteins, such as histone methyltransferases and histone deacetylases, thereby forming tightly packed, inactive chromatin (Robertson 2005; Scarano et al. 2005; Ushijima et al. 2006).

Intriguingly, DNA methylation patterns differ largely between normal and cancer cells. A common paradoxical epigenetic feature observed in cancer cells is the coexistence of global genomic DNA hypomethylation and gene-specific promoter hypermethylation, though the underlying mechanisms remain unclear. In particular, ~50 % cases of breast cancer show global DNA hypomethylation when compared with normal tissue counterparts (Bernardino et al. 1997; Soares et al. 1999). DNA hypomethylation in breast cancer mainly occurs in repetitive DNA sequences and pericentromeric satellite regions, which are normally heavily methylated in non-malignant cells. Loss of DNA methylation in these regions leads to genomic instability and early breast cancer development (Veeck and Esteller 2010). By contrast, promoters of more than 100 genes have been found to be hypermethylated in breast cancer cells. Many of these genes are tumor suppressors or other cancer-related genes that play important roles in cell-cycle regulation (e.g. CCND2, BRCA1, hTERT, p16 and RAR-β,), apoptosis (e.g. BCL2, DAPK, HOXA5, GPC3 and p53), tissue invasion and metastasis (e.g. APC, BCSG1, E-Cad, H-Cad, Nm23-H1, and PROSTASIN, SYK), angiogenesis (MASPIN, THBS1, and TIMP3) and hormone signalling (e.g. ER-α and ER-β,) (Jovanovic et al. 2010). Consequently, gene-specific DNA hypermethylation and thereby inactivation of tumor suppressors and cancer-related genes provides another important mechanism contributing to breast cancer tumorigenesis and progression. It's worthy to point out that the patterns of DNA methylation vary in different breast cancer subtypes, and are considered as promising epigenetic biomarkers for breast cancer diagnosis, prognosis and prediction of treatment outcome in breast cancer (Jovanovic et al. 2010; Jones et al. 2010). For example, genome-wide analysis of DNA methylation has identified distinct DNA methylome subgroups in breast cancer with diverse metastatic behavior. Coordinate hypermethylation at numerous genes that make up the metastasis transcriptome accounts for much of the transcriptomal diversity, and is associated with high metastatic risk and death (Fang et al. 2011).

Recently, it was reported as the first evidence that dysregulation of DNA methylation is associated with increased breast cancer susceptibility caused by maternal diet during pregnancy in multiple generation (de Assis et al. 2012). The researchers fed pregnant rats (F0 generation) with a high-fat (HF) or ethinyl-oestradiol (EE2)-supplemented diet, and then examined carcinogen-induced breast cancer risk in multiple generations of female offspring – daughters (F1 generation), granddaughters (F2 generation) and great-granddaughters (F3 generation). Incidence of breast cancer was increased by 55–60 % in the daughters and granddaughters of rats administered a high fat diet during pregnancy compared to the offspring of control rats on a normal diet during pregnancy. The increased risk of breast cancer may be transmitted to granddaughters through either female or male germ lines. However, the effects of the high fat diet were not passed on to the great-granddaughters.

Meanwhile, the daughters of mice exposed to EE2 had a 50 % higher incidence of breast cancer than the daughters of the controls. Interestingly, unlike HF, EE2 exposure had an opposite effect on male and female offspring (F1 generation) regarding their ability to transmit susceptibility of breast cancer to their offspring. Offspring resulting from Control (female) × F1 EE2 (male) breeding exhibited an increased breast cancer risk (62 %), whereas offspring resulting from F1 EE2 (female) × Control (male) breeding exhibited a decreased breast cancer risk (33 %). Nevertheless, the breast cancer risk-lowering effect on F1 EE2 male offspring was transient and not transgenerational, as evidenced by the fact that F3 generation females, produced by F2 EE2 (female) × F2 EE2 (male), had increased incidence of breast cancer. This finding is in agreement with previous reports that DNA methylation is transmitted prominently through maternal gametes (Schulz et al. 2010; Borgel et al. 2010). Quantitative real-time PCR (qPCR) analysis showed that both HF and EE2 in utero exposures increased the levels of DNMT1 mRNA in mammary glands in F1 generation, and this increase is extended to F2 and F3 generations in the EE2 offspring but not in the HF offspring. In addition, mammary mRNA levels of DNMT3a and DNMT3b were increased in the F2 and F3 generations in the EE2 offspring, respectively, but were not altered in the HF offspring. More importantly, genome-wide analysis of DNA methylation status identified 375 common alterations (214 hypermethylated and 161 hypomethylated gene promoter regions) in mammary glands across F1, F2 and F3 EE2 offspring. The observation that the hypermethylated promoter regions involve a number of polycomb group target (PCGT) genes (Pax6, Runx3, Foxe3, Gata4 and Vgf) is in line with recent studies showing that hypermethylation-mediated silencing of the PCGT genes is a hallmark of breast cancer as well as other cancers (Moelans et al. 2011; Dallol et al. 2012; Teschendorff et al. 2010; Fourkala et al. 2010). Taken together, these results implicate an epigenetic transgenerational effect of EE2 exposure during pregnancy, and its contribution to the increased breast cancer susceptibility in multiple generations of offspring. However, it remains to be determined how DNA methylation is effected by EE2, and how these changes are transmitted through multiple generations. In addition, whether the changes in DNA methylation indeed account for the increased breast cancer risk in the EE2 offspring, or they are just simply associated needs further investigation.

5.9 Future Directions

Breast cancer is a complex disease with multiple biological mechanisms intertwined. The research on the association between maternal dietary intake, environmental exposures or biomarker levels during pregnancy and offspring's breast cancer development is scarce in humans. Future well-designed prospective cohort epidemiological studies involving multiple generations will provide informing evidence regarding to the association between maternal pregnancy exposures and offspring's breast cancer risk. Laboratory studies examining the association between maternal

pregnancy exposures and stable epigenetic imprinting are also needed to shed light on the etiopathogenesis of breast cancer and provide new preventive and therapeutic strategies. In addition, since the quantification of environmental exposures in general is extremely difficult and the gene-environmental interaction is important in breast cancer development, genome wide assessments of epigenetic modification may help to understand the nature of genetic modification and genome-scale sequences (Hardy and Singleton 2009).

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Chapter 6
Environmental Epigenetics and Obesity: Evidences from Animal to Epidemiologic Studies

L. Joseph Su

Abstract Obesity is a major health problem today that grows into a global epidemic. Obesity has been associated with increased risk of many non-communicable diseases, such as type-2 diabetes mellitus, hypertension, cardiovascular diseases and cancer. There are multiple factors that cause obesity. Accelerated lifestyle, fast food, unhealthy eating habits and sedentary lifestyle have been considered as the major risk factors of overweight and obesity development. It has been suggested that genetic factors may play an important part of the onset of obesity. However, the observed rapid rise of obesity worldwide has occurred too rapidly to be solely explained by fixed genomic variation in combination with adult lifestyle factors. Epigenetic modifications can be programmed in the intrauterine environment and can be modulated by environmental influences including diet besides genetic influences. It has been widely accepted that genes together with adult lifestyle factors determine the risk of developing non-communicable diseases. Animal study models have demonstrated that epigenetic alternations early in life lead to obesity later in adult life. Natural human experiment, such as Dutch famine study, also suggested that peri-conceptional and peri-natal nutrient deficiency resulted in overweight or obesity later in life. The results from epidemiologic studies are less consistent. This chapter reviewed the evidence from animal studies, ecologic evaluation, to epidemiologic studies to assess whether there is a causal relationship between epigenetics modification from diet and environmental exposure and obesity. Challenges and opportunities to improve the future study design in human are explored.

Keywords Diet • Obesity • BMI • Epigenetics • DNA methylation

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6.1 Overview: The Problems of Obesity

Obesity has drawn a lot of attention in the recent decades because of its significantly negative health consequences. The prevalence of obesity worldwide has almost doubled between 1998 and 2008. An estimated 205 million men and 297 million women were obese with body mass index (BMI) equal or greater than 30 kg/m^2 (WHO 2014). The matter may be even more significant in some developed countries. For example, more than one third (35.7 %) of the U.S. adult population is considered as obese as of 2009–2011 (Ogden et al. 2009).

The incidence of non-communicable diseases, such as type-2 diabetes mellitus, hypertension, cardiovascular diseases and cancer, has risen sharply over the past two decades. Together these non-communicable diseases account for 60 % of all deaths worldwide. The rise of non-communicable diseases has occurred too rapidly to be solely explained by fixed genomic variation in combination with adult lifestyle factors (Lillycrop and Burdge 2012). Obesity has been associated with increased risk of many of the non-communicable diseases mentioned. It is a metabolic condition that has paradoxically been associated with poverty, low quality of life, malnutrition, and an imbalanced intake of nutrients (Soubry et al. 2013; Dyer and Rosenfeld 2011; Tanumihardjo et al. 2007).

Despite of significant efforts to promote weight loss and a healthier lifestyle, the prevalence of the metabolic syndrome is still rising. Early developmental environment that is known to exert long-lasting influences through metabolic programming may be an important factor contributing to the etiology of obesity, metabolic syndrome and related diseases. Growing evidence support the concept that epigenetic mechanisms play a role in fetal programming which may well be a common mechanism in the development of different organs (Heerwagen et al. 2010; Jimenez-Chillaron et al. 2012). A large number of epidemiological studies have shown the association between impaired fetal nutrition and development of obesity and diabetes (Barker and Osmond 1986; Ravelli et al. 1998; Nohr et al. 2008; Fraser et al. 2010; Laitinen et al. 2012). Although both animal and human studies have demonstrated the association between early developmental events and adult obesity and metabolic diseases, the underlying biological mechanisms behind these programming effects have remained largely unclear. The importance of fetal and early postnatal life is currently extensively studied to clarify the physiological and molecular links between events during this developmental period and long term health. Epigenetic modification has been hypothesized to have contribution to certain extent (Barnes and Ozanne 2011).

Epigenetic processes are integral in determining when and where specific genes are expressed. Alterations in epigenetic regulation of genes can lead therefore to profound changes in phenotype (Cox et al. 2002; DeBaun et al. 2003). Epigenetic marks are tissue specific. The major epigenetic processes include but not limited to DNA methylation, histone modification and non-coding RNAs. Epigenetic modifications can be programmed in the intrauterine environment and can be modulated by environmental influences including diet besides genetic influences.

It has been widely accepted that genes together with adult lifestyle factors determine the risk of developing non-communicable diseases (Lillycrop and Burdge 2012). However, substantial evidence has suggested that the mechanism by which the environment can alter long term disease risk may involve epigenetic process. Epigenetic processes play an essential role regulating tissue specific gene expression and hence alternations in these processes can induce long-term changes in gene expression and metabolism which persist throughout life. Epigenetic changes can activate or silence genes by such mechanisms as recruiting methyl-CpG binding proteins which then block transcription factor from binding to the promoter sites or change chromatin structure enhancing heterochromatin formation (Jones and Takai 2001).

To what extent epigenetic modifications contribute to obesity in human and to the total heritability of common form of obesity is presently unknown. However, DNA methylation status has been extensively demonstrated using the Agouti mouse model (Wolff et al. 1998). The coat color, Fig. 6.1, is linked to the methylation status of the Agouti gene, which is highly dependent on maternal diet. Altered epigenetic change is associated with the increased risk of diabetes-like condition, obesity, and tumorigenesis (Yen et al. 1994; Dolinoy 2008; Duthie 2011). There are some evidences that specific nutrients in human diet that are strongly associated with the risk of cancer can modulate DNA methylation (Kim et al. 2009). For example, it is found that identical twins possess the same genotype and no distinguishable

Fig. 6.1 Fat *yellow* mice and skinny *brown* mice are genetically identical. The coat color of Agouti mice is linked to the methylation status of the Agouti gene, which is highly dependent on maternal diet. When a mouse's agouti gene is completely unmethylated, its coat is *yellow* and it is obese and prone diabetes and cancer. When the agouti gene is methylated (as it is in normal mice), the coat color is *brown* and the mouse has a low disease risk (Image created by Randy Jirtle and Dana Dolinoy, in 2007 and used in compliance with creative commons attribution 3.0 unported license)

epigenetic differences in their early life but showed remarkable differences in genomic methylation and histone acetylation patterns in their later life (Fraga et al. 2005). These epigenetic differences may result in different gene expression and disease susceptibility.

Obesity is a major public health problem in the U.S. From translational standpoint, DNA methylation has provided useful biomarkers for the correct diagnosis, prognosis, and prediction of chemosensitivity of malignancies (Heyn and Esteller 2012). Global DNA methylation from white blood cells estimated by the methylation of repetitive sequences in the genome has been suggestive of an indicator of susceptibility to chronic diseases. Since epigenetic modifications have been shown to be reversible, at least in experiment animals, there is great potential in obesity prevention if we can identify epigenetic changes prior to the onset of obesity and other metabolic syndrome phenotypes and implement proper preventive strategies at the right period in life to reverse the continue increase in the prevalence of obesity in the world. A somewhat comprehensive summary of evidence in epigenetics and obesity is explored in this Chapter.

6.2 Environmental Contaminants and Epigenetics

Environmental contaminants released in the past few decades have posted significant threats to ecosystem health. These chemicals, also called endocrine disruptors, mimic natural hormones, elicit opposing hormonal actions and altered either the synthesis and/or the excretion of hormones. These chemicals, which include synthetic organic compounds such as pesticides (e.g., organochlorine), pharmaceutical drugs (e.g., diethylstilbestrol (DES)), chemical used for the polymerization of plastics and resins (e.g., bisphenol A (BPA)), flame-retardant (e.g., polybrominated diphenyl ether (PBDE)), and the list goes on and ever increasing, are ubiquitous in the environment (Karoutsou and Polymeris 2012). They have been associated with changes in epigenetic marks following in utero and adult exposure, and these changes may be transmitted to subsequent generations through the germline (Li et al. 2003). At the same time, the prevalence of obesity and its known complications is rising and these conditions are now manifesting at younger ages. Although the exact causes for this surge in worldwide overweight and obesity is still not clear, epigenetics modifications from exposures to environmental contaminants may contribute the epidemic of obesity.

Among many known endocrine disrupters, DES has been studied more extensively. DES is a synthetic non-steroidal estrogen that is a known human carcinogen. It was prescribed to pregnant women between 1947 and 1971 to reduce the risk of miscarriage (NTP (National Toxicology Program) 2005). In a finding that usually has been observed among older women, female offspring of mothers exposed to DES experienced a higher risk of clear-cell adenocarcinoma of the vagina or cervix at younger ages (Herbst et al. 1977). DES has been shown to increase expression of the *c-fos* proto-oncogene and significantly reduce methylation in mice exposed in

utero (Li et al. 2003). Recent studies have shown that in utero exposure to DES caused hypermethylation of *HOXA10*, which is expressed in uterine epithelial and stromal cells (Bromer et al. 2010) and directs embryonic uterine development; *DNMT1* and *DNMT3b* also were overexpressed (Bromer et al. 2009). DES increased histone H3 trimethylation in human cells and in mice exposed in utero (Doherty et al. 2010). Most recently, Jensen and Longnecker used data from a multicenter prospective study of pregnant women and their offspring found that DES use was associated with offspring overweight or obesity (Jensen and Longnecker 2014).

Food is a major intermediating source of exposure to endocrine-disrupting chemicals such as phthalates and BPA. BPA is a known endocrine disruptor. BPA is used in the production of polycarbonate plastics and epoxy resins. Because of its pervasive use in plastics, it is ubiquitous in the environment. Although the available evidence does not suggest that BPA is carcinogenic on its own, evidence indicates that it increases cancer susceptibility through developmental reprogramming as a result of epigenetic changes (Keri et al. 2007). Similar to DES, BPA has been associated with epigenetic alterations following exposure in utero (Schoen et al. 2006). For example, exposing pregnant mice to BPA produced alterations in *HOXA10* expression associated with significantly reduced methylation in offspring, but this reduced methylation was not due to repression of DNA methyltransferases (Bromer et al. 2010). BPA exposure in utero also has been shown to alter expression of *EZH2* in mammary tissue. Overexpression of *EZH2* is associated with human breast cancer and reduced expression of DNA repair mediators (Doherty et al. 2010). Similar to DES, BPA increased H3 trimethylation in human MCF-7 cells and in mice exposed *in utero*. These epigenetic changes persisted into adulthood (Doherty et al. 2010). In the male rat, hypomethylation of *PDE4D4* in the prostate tumors of male rats was observed after neonatal BPA exposure; these epigenetic changes appeared to be permanent (Schoen et al. 2006). BPA is present at low but detectable levels among pre-adolescent and adolescent males and females (Wolff et al. 2006; Calafat et al. 2007). Cross-sectional evaluation using the NHANES surveys has suggested that urinary BPA level is positively associated with higher chance of obesity and abnormal waist circumference-to-height ratio among children (Bhandari et al. 2013; Eng et al. 2013; Trasande et al. 2012; Shankar et al. 2012; Carwile and Michels 2011). Although meta-analyses of existing epidemiological studies do not support significant association between phthalates or BPA and obesity (Lakind et al. 2014; Goodman et al. 2014), existing data, mostly from animal or cross-sectional human studies, provide compelling evidence to suggest that exposure to BPA early in life may predispose the epigenetic impact that may affect health later in adult life.

Additionally, diet (Cong et al. 2012; Sohi et al. 2011) or environmental exposure to a number of chemical agents like heavy metals (e.g., cadmium, arsenic, nickel, chromium, and methylmercury) (Cheng et al. 2012; Martinez-Zamudio and Ha 2011; Reichard and Puga 2010; Sadli et al. 2012; Wang et al. 2012a), and air pollutants (e.g., particulate matter, black carbon and benzene) are able to induce epigenetic changes (mainly through DNA methylation, histone acetylation/descetylation (Ferrari et al. 2012), and non-coding microRNAs (Chuang and Jones 2007; Tang and Ho 2007)), which are involved in a wide range of metabolic diseases, including

obesity (Martinez et al. 2012; Milagro et al. 2013), abnormal hepatic triglyceride accumulation (Sookoian and Pirola 2012), and the metabolic syndrome (Sookoian and Pirola 2012; Wang et al. 2012b), and type-2 diabetes (Berends and Ozanne 2012; Gilbert and Liu 2012). Epidemiological and experimental studies have linked epigenetic modulations with both nutritional factors and environmental toxicants (Baccarelli and Bollati 2009; Feil 2006). Nutrition and environmental toxicants affect epigenetic pathways described so far mainly through DNA methylation and complex histone modifications. Evidence linking nutritional and environmental factors with DNA hyper- or hypomethylation provides the most compelling support for such an association (Mathers et al. 2010). Further, nutrition may modulate the toxicity of environmental pollutants, through either synergistic or antagonistic effects (Hennig et al. 2007). Environmental toxicants also may cause aberrant modifications in the metabolic activity of nutritional factors (Hennig et al. 2004). The relationship between nutrition and environmental toxicants and epigenetic modulations is complex, however, and an exact causal relationship has yet to be identified. In addition, the complexity of the relationship is compounded by the interaction of multiple toxicants and dietary factors. Nonetheless, nutritional factors and environmental toxicants may affect similar biological and epigenetic pathways and thus should be examined simultaneously.

6.3 Natural Experiments of Epigenetics and Obesity

Epidemiologic studies have not been focused on the environmental challenges in early life on future risk of a range of non-communicable diseases, including obesity, type-2 diabetes, hypertension, and cardiovascular diseases, until a recent effort from the National Institutes of Health to support this type of studies. However, through the observation of a "natural experiment" on the Dutch Famine Cohort, evidence has emerged for a potentially causal association between nutrition during development and future risk of disease. Increased body mass index (BMI) and waist circumference were found among adults exposed to famine in utero compared to those born before or conceived after the famine (Ravelli et al. 1999). Genomic DNA methylation level on the parentally imprinted insulin-like growth factor (*IGF*) – *2*/*H19* locus of individuals experienced Dutch Famine Winter in utero was found to be 3 % higher than the level among non-exposed siblings (Heijmans et al. 2008). The study also found differences with similar magnitude in the methylation status of non-imprinted genes associated with inflammatory response, regulation of appetite and energy balance (Tobi et al. 2009). Additionally, inversed association between birth weight and abdominal fat, a risk factor for cardiovascular diseases and a better indicator for obesity than BMI alone, was found in several studies (Barker et al. 1997; Byberg et al. 2000; Law et al. 1992; Okosun et al. 2000; Rasmussen et al. 2005). These findings demonstrated that nutritional challenges in early life can have impact on obesity in adulthood possibly through the epigenetics mechanisms. However, there is little compelling epigenetic studies to explain the

role of epigenetics in obesity, especially in developmental prospective. The differences found in DNA methylation analyses from the Dutch Famine Cohort were too small to interpret in terms of gene function.

Polymorphisms in the methylenetetrahydrofolate reductase (*MTHFR*) is a ubiquitous key enzyme required for methyl group transfer and cell division (Hall and Solehdin 1998; Goyette et al. 1994). Folate intake provides dietary methyl groups. The 677 C → T allele encodes a small change in protein structure with potentially large biological effects (Hall and Solehdin 1998; Goyette et al. 1995). In vitro and animal studies have provide evidence that both *MTHFR* polymorphism and folate intake affect DNA methylation, a key epigenetic contributor for the maintenance of gene silencing (Lucock and Yates 2005; Sinclair et al. 2007). There have been reports on the association between *MTHFR* polymorphism on low birth weight (Yila et al. 2012; Glanville et al. 2006; Relton et al. 2005; Chen et al. 2004). Although low birth weight has been linked to obesity and metabolic syndromes later in life through the epigenetics hypotheses, the direct relationship between *MTHFR* polymorphism modifiable by peri-conceptional exposure to folic acid and obesity has not been observed. A recent pilot study by the author of this Chapter was able to examine the DNA methylation level in adolescents whose mother participated in a double-blind peri-conceptional folic acid/placebo trial in China. Because the Chinese population under study had a wide-variety of exposures to low and adequate folate and other nutrients involved in methylation during gestation (Berry et al. 1999). It is hypothesized that there would be observable differences in methylation patterns in those exposed to folic acid supplementation peri-conceptionally compared to those who received no supplementation. There may also be differences in the Southern and Northern regions, which are the surrogate indicator for the locations of nutrient sufficiency and insufficiency, respectively, during the original study and to a lesser extent currently (Hao et al. 2007). Preliminary results suggested that peri-conceptional folic acid supplementation at 400 mcg level do not have significant epigenetic impact on adolescent offspring. A larger scale of follow-up on the original cohort may offer an opportunity to examine the epigenetic effects stratified by genetic polymorphism, diet, and other potential contributing factors.

6.4 Animal Studies Linking Epigenetics and Obesity

Much of the current knowledge about the role of epigenetic changes in the etiology of obesity associated diseases is based on studies in animal models. Established epigenetic patterns during the fetal period can be changed in adult life by environmental factors including nutrition. Diet can profoundly alter epigenetic patterns in animals. The Agouti mouse has been used extensively to demonstrate the impact of maternal nutrition on the fetal epigenome and the phenotype of the offspring (Wolff et al. 1998). The murine mouse Agouti gene encodes a signaling molecule that regulates a switch from producing black (eumelanin) to yellow (phomelanin) coat color (pigment in the hair follicles). The coat color is linked to the methylation

status of the Agouti gene, which is highly dependent on maternal diet. Wolf et al. were the first to demonstrate that feeding pregnant black *a*/*a* dams a methyl-supplemented diet shifts the coat color distribution of their *Avy* offspring (Wolff et al. 1998). Since evident suggested that *Avy*/*a* coat color correlates with *Avy* methylation status (Morgan 1999), it was argued that maternal methyl-donor diets change coat color of the offspring via *Avy* methylation (Cooney et al. 2002). Waterland and Jirtle were the first to demonstrate in 2003 that dietary supplementation of viable yellow Agouti (*Avy*) mice during pregnancy with methyl donors (folic acid, vitamin B12, choline, and betaine) alter the coat color of their offspring because of increased DNA methylation of a transposable element upstream of the Agouti gene rather than mutation of the gene (Waterland and Jirtle 2003). These observations provide clues that epigenetic modifications resulting from dietary effects may have long-term health consequences. Most importantly, altered epigenetic change is associated with the increased risk of diabetes-like condition, obesity, and tumorigenesis (Yen et al. 1994; Dolinoy 2008; Duthie 2011).

Instead of using mutant Agouti mouse, Lillycrop, Burge and colleagues found that feeding pregnant rats with moderate reduction in protein content would induce hypomethylation of the *PPARα* and glucocorticoid receptor (*GR*) promoter as well higher mRNA expression of these gene and of their respective targets in the liver of offspring (Lillycrop et al. 2005; Burdge et al. 2007a). These offspring was subsequently found to have higher plasma β-hydroxybutyrate and glucose concentration after fasting (Burdge et al. 2008). This finding suggested that energy homeostasis can be induced in the offspring by moderate changes to maternal macronutrient intake in non-mutant animal model. To better understand the underlying mechanism of the finding, the effect of methylation on *PPARα* due to maternal diet modification was later confirmed through a sequencing analysis showing hypomethylation of specific CpG loci on putative transcription factor response element among offspring of dam fed protein-restricted diet when compared to the controls (Lillycrop et al. 2008). Other studies of rats also identified epigenetic changes of GR and *PPARα* regulations in the heart (Slater-Jefferies et al. 2011), whole umbilical cord (Burdge et al. 2007b), and in the angiotensin receptor 1β promoter in adrenal gland (Bogdarina et al. 2007) in offspring of maternal rats fed with protein-restricted diet.

Increased histone modifications, which including acetylation of H3 and H4 and methylation of histone H3 and lysine K4, were also observed in these rats as the results of maternal protein-restricted diet (Lillycrop et al. 2007). The maternal protein-restricted diet was also found to induce epigenetic silencing by histone modification in pancreatic β-cell in the offspring, which weakened the interaction between *HNF4α* promoter and its downstream enhancer and subsequently through decreased mRNA expression (Sandovici et al. 2011). Furthermore, age-related decrease in *HNF4α* activity was found to be greater in the offspring of dam fed with protein-restricted diet than controls. *HNF4α* is involved in glucose sensing and insulin secretion (Odom et al. 2004) and has been implicated in type 2 diabetes mellitus (Silander et al. 2004). Therefore, epigenetic silencing of *HNF4α* transcription could explain the relationship between prenatal nutrition constraints for risk of diabetes.

In addition to animal studies of mouse and rats, it is found that maternal obesity was associated with altered expression of non-coding RNA species in the liver of the offspring in sheep (Nicholas et al. 2013). At the same time, maternal weight loss altered the mRNA expression of genes involved in insulin signaling in the liver of the offspring. It is unclear, however, whether exposure to a high level of maternal nutrition before pregnancy and exposure to a high transplacental nutrient supply in later pregnancy would have the same impact of the finding of maternal obesity on offspring. Zhang et al. using pregnant sheep demonstrated that maternal overnutrition in late pregnancy resulted in an upregulation of *PPARγ* activated genes in fetal visceral fat and a subsequent increase in the mass of subcutaneous fat in the postnatal lambs (Zhang et al. 2011). However, they found that exposure to maternal overnutrition during the peri-conceptional period alone resulted in an increase in total body fat mass in female lambs only on visceral fat depots. Therefore, the early programming of later obesity hypothesis can only be supported if both maternal overnutrition during the peri-conceptional period and fetal overnutrition during late pregnancy. They also found that a short period of dietary restriction during peri-conceptional period reverse the impact of peri-conceptional overnutrition on the obesity programming, which is evident by the increased lamb adrenal weight and cortisol response that changed the epigenetic state of the insulin like growth factor 2 (*IGF2*) gene in the adrenal.

As demonstrated earlier, glucocorticoids have been shown to play an important role in many epigenetic processes. A study examining the effect of maternal nutrient reduction on methylation of fetal baboon liver phosphoenolpyruvate carboxykinase I (*PEPCK1*), the rate-limiting enzyme in hepatic gluconeogenesis, demonstrated that fetal liver *PEPCK1* promoter methylation was reduced at six of nine CpG-dinucleotides in fetuses of nutrient-reduced mothers compared with fetus of control mothers (Cox et al. 2013). They also found overall phenotype would pre-dispose to later-life type-2 diabetes among offspring of nutrient-reduced mothers (Cox et al. 2013).

Variants in the FTO gene are associated with increased body mass index in humans. The FTO locus, encoding a DNA-demethylase enzyme, was found to code for an enzyme involved in the control of epigenetic modification in mice (Gerken et al. 2007). At the same time, genetic disruption of the histone H3k9-specific demethylase (*Jhdma2a*), one of the enzyme involved in the pathway, has been shown to result in obesity and hyperlipidemia in mice (Tateishi et al. 2009). There also have been a few other genes that were found to be critical to obesity are regulated by epigenetic mechanisms. For example, *MC4R* gene has reduced methylation in the brain following long-term high-fat diet exposure in mice (Widiker et al. 2010). Promoter methylation in the hypothalamus at the *POMC* gene is sensitive to overfeeding and administration of leptin in early life (Plagemann et al. 2009; Palou et al. 2011). High-fat diet was found to modify leptin gene promoter methylation in adipose tissue (Milagro et al. 2009).

As described earlier, reduction of food intake of rats during pregnancy induced hypermethylation and liver expression of PPARα and GR in the liver of offspring, which was associated with obesity and impaired glucose homeostasis (Vickers et al.

2000). However, these effects were found to be reversible by neonatal treatment of leptin (Vickers et al. 2005). It was also found that treatment of leptin during the neonatal period was found to induce activation of PPARα isoform in adipose tissue through reaction in the methylation status of specific CpG loci in its promoter (Garratt et al. 2013). Such induction of PPARα in adipocyte by leptin has been shown to switch the fat metabolism from storage to catabolism (Lee et al. 2002). Therefore, the knowledge on leptin exposure in early life that modulates the epigenetic regulation of genes in peripheral tissues could be an important determinant of energy balance throughout the life course (Burdge and Lillycrop 2014). It is also found that folic acid supplementation for rats during their juvenile-pubertal period induced impaired lipid homeostasis, including down-regulation of hepatic fatty acid β-oxidation, hepatosteatosis and increased weight gain, which was associated with hypermethylation of PPARα in the liver and hypomethylation of insulin receptor in adipose tissue (Burdge et al. 2009).

It has been shown that animals exposed to several endocrine disruptors, including phytoestrogens, synthetic estrogens, and environmental endocrine disruptors cause changes in body weight and development of obesity. For example, Takai et al. demonstrated that blastocysts exposed to BPA at weaning resulted in heavier adult mice when compared to control despite having similar weight at birth (Takai et al. 2001). Mice fed high amount of dietary phytoestrogens can lead to sex-specific changes in DNA methylation patterns of the *Acta1* promoter in the liver of offspring (Guerrero-Bosagna et al. 2008). *Acta1* is developmentally regulated and associated with morphometric features in mouse (Crawford et al. 2002). The proto-oncogene *cfos* has an estrogen response element that binds the estrogen receptor, which may be a key factor in relating estrogenic stimuli to methylation changes (Guerrero-Bosagna et al. 2005). Additionally, neonatal exposure to soybean products enriched in phytoestrogens was found to alter body weight, adiposity and adipokines in adult female mice (Newbold et al. 2009). This finding support an earlier study finding that that brief exposure to phytoestrogens early in life affects mouse white adipose mass and serum adenosines (Naaz et al. 2003).

Nutrient intakes play an important role in regulating one-carbon metabolism (Davis and Uthus 2004; Lee et al. 2005). Excessive or deficient nutrient status may have an effect on DNA methylation. This is based on the observation that the primary methyl donor, S-adenosylmethionine (SAM) may be regenerated from S-adenosylhomocysteine (SAH) through a series of several chemical reactions that rely on the presence of several nutrients, such as folate, betaine, choline, zinc, vitamin B_6, and vitamin B_{12}, as cofactors or intermediates (Fig. 6.2) (Delage and Dashwood 2008). Deficiency on any of these nutrients can result in a depletion of cellular pools of SAM, which may cause aberrant DNA methylation (Gilbert and Liu 2010). Folate, vitamin B_6, vitamin B_{12}, methionine, and choline are involved in the one-carbon metabolism provide the most compelling data for the interaction between nutrients and DNA methylation because they influence the supply of methyl groups and hence the biochemical pathways of methylation process (Mas et al. 2007). Other dietary factors, such as alcohol, coumestrol, equol, genistein, nickel, selenium, tea polyphenol, vitamin A and zinc are also know to influence

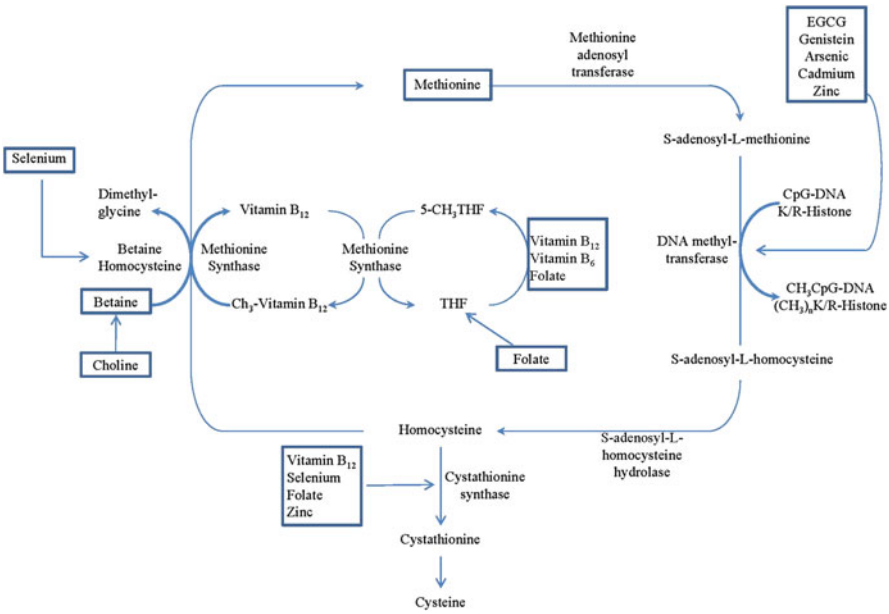

Fig. 6.2 Overview of the methionine-homocysteine-folate-B12 cycle, which provides methyl donors for methyltransferase. Nutritional regulations of the cycle are identified by *rectangle* frames

DNA methylation and cancer susceptibility (Davis and Uthus 2004). Certain bioactive food components, such as sulforaphanes in broccoli, diallyl disuphides in garlic, and resveratrol in wine, have been shown to alter epigenetic processes in cell functions, such as control of proliferation, upregulated apoptosis, and reduction in inflammation (Ross et al. 2008). However, it is still unclear whether there is a causal link between diet and epigenetics in the development of obesity and whether diet could be differentially effective at various stages in the lifespan of human despite observations on abnormal epigenetic control of gene expression increases obesity risk.

6.5 Epidemiologic Evidence on Epigenetics and Obesity

Rodents have been used extensively for obesity research. A few other animal models had also been used to demonstrate the potential pathways involved in epigenetics leading to the onset of obesity. The translation of these findings to the human condition has achieved mixed results. Human obesity is thought to be interplay among genetic variations at several susceptible loci with variable contribution from environmental factors such as diet and physical activities. As illustrated in Fig. 6.3, the timing and magnitude of each, directly or indirectly, contribute to this mechanistic pathways makes it even more complex to have a good handle on the effort of obesity prevention.

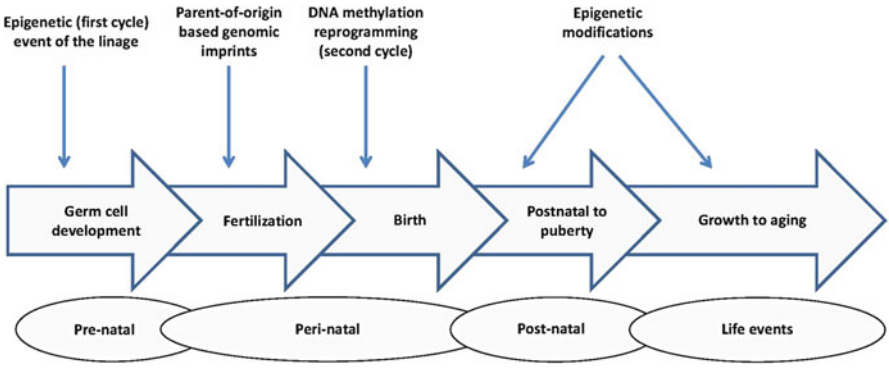

Fig. 6.3 Environmental and dietary influences on epigenetics modifications that result in obesity can occur throughout the life cycle: from germ cell development to the aging process

The field of obesity and genetics was dominated by candidate gene approaches examining the association of polymorphisms in one or a few candidate genes with obesity or obesity-related phenotypes. The series of Human Obesity Gene Maps incorporated published results up to the end of October 2005 summarizing evidence from single-gene mutation obesity cases, Mendelian disorders exhibiting obesity as a clinical feature, transgenic and knockout murine models relevant to obesity, quantitative trait loci (QTL) from animal cross-breeding experiments, association studies with candidate genes, and linkages from genome scans (Rankinen et al. 2006). The review identified numerous genes related to the central regulation of energy balance, adipogenesis, lipid turnover, adaptive thermogenesis, and signaling by insulin and other extracellular signals imping on energy metabolism. However, due to inadequate statistical power of the studies and/or insufficient biological and genomic knowledge, none of these gene or combination was able to be firmly validated (Palou and Bonet 2013). The candidate gene approach was later overwhelmed by powerful genome-wide association studies (GWAS) to scan the entire genome for common disease-associating single nucleotide polymorphism in a hypothesis-free spirit in large cohort of non-familial related individuals with small effect size. The fat mass and obesity associated gene (*FTO*) stood out from about 40 loci replicated to human obesity as the most replicated gene with the highest statistical significance (Sandholt et al. 2012).

Unfortunately, despite the advances in genetics of obesity, the combined effect of all loci identified to date account for only about 2–45 of the total heritability of common form of obesity, which is estimated to be about 40–70 % among all obesity (Palou and Bonet 2013). The vast majority of obesity susceptibility variants identified in GWAS lies in non-coding regions including intronic or intergenic regions, which could either be linkage disequilibrium markers of the causal variants or true causal variants influencing gene function or transcript regulation. The functional assessment remains a major challenge. Besides, the contribution of a given SNP to obesity may be modulated by the presence of other SNPs. The influence of haplotypes and gene-gene interaction needs to be considered.

Maternal gestational BMI and high-fat overfeeding have been found to increase the methylation level at the promoter region of *PGC1* gene in human umbilical cord and muscle, respectively, independent of birth-weight (Gemma et al. 2009; Brons et al. 2010). Additionally, methylation at candidate genes, retinoids X receptor-α (*RXRA*) and endothelial nitric oxide synthase (*eNOS*), promoters at birth has been shown to be independently associated with child's later adiposity (Godfrey et al. 2011). A mother's diet is passed on to her fetus and affects fetal growth and development. The fetal origins of disease hypothesis, also known as Barker's hypothesis, essentially proposes that under-nutrition during gestation increases the risk of disease later in life (Barker 2007). In 1989, Barker and colleagues published data showing that men with the lowest weights at birth and at 1 year had the highest death rates from ischemic heart disease, and increasing weight was associated with a graded decrease in the risk (Barker et al. 1989). The authors proposed that an environment that induces poor fetal and infant growth will be followed by high risk of ischemic heart disease in adulthood. This idea has been validated in several prospective cohort studies. For example, in Sweden (Leon et al. 1998), Finland (Forsen et al. 1997), Denmark (Baker et al. 2008), Norway (Risnes et al. 2009), England (Syddall et al. 2005), and Scotland (Lawlor et al. 2005), mortality from ischemic heart disease was inversely associated with birth weights. In the United States (Rich-Edwards et al. 1997, 2005) and India (Stein et al. 1996), a strong inverse association between birth weight and non-fatal coronary heart disease were reported. A recent systematic review of 17 published studies concluded that the findings are consistent with low birth weights increasing risk for ischemic heart disease (Huxley et al. 2007). If indeed, early life exposures affect cardiovascular disease (CVD) risk, by logical extension it may also affect obesity risk because some of the biological risk factors for CVD, such as abnormalities in lipid metabolism and non-insulin-dependent diabetes mellitus, intimately related to obesity.

There are also some evidences that specific nutrients in the human diet that are strongly associated with the risk of obesity can modulate DNA methylation (Kim et al. 2009). For example, it is found that identical twins possess the same genotype and no distinguishable epigenetic differences in their early life but showed remarkable differences in genomic methylation and histone acetylation patterns in their later life (Fraga et al. 2005). These epigenetic differences may result in different gene expression and disease susceptibility.

DNA methylation is a well-defined epigenetic mechanism involving in the regulation of a wide variety of biological processes, including gene expression, genomic stability and parental imprinting. Methylation involves addition of methyl groups to cytosine to form 5-methyl-cytosine (5mC) mostly occurring in a 5′-CpG-3′ sequence context. CpG dinucleotides are often aberrantly methylated in human cancers to give an overall reduction of 5mC despite regional hypermethyaltion at some CpG islands (Heijmans et al. 2008). Both systematic genome-wide (global) reductions of methylated cytosines and region-specific increases of methylated cytosines in CpG islands are common epigenetic events thought to play a role in obesity development. While hypermethylation occurs chiefly in gene promoter regions affecting gene control via impairing transcription, genomic hypomethylation

occurs not only in transcription control regions such as promoters but also in repetitive DNA sequences, such as satellite and LINE repeats, retrotransposons, and endogenous retroviral elements, causing altered chromatin structure, chromosomal instability, aneuploidy, and higher mutation rates. Global DNA methylation, presented as percentage of methylated cytosine (%mC) has been used in epidemiologic studies to examine its relationship with risk of diseases (Burdge et al. 2008; Smolarek et al. 2010; Huang et al. 2012). It is estimated that more than one-third of DNA methylation occurs in repetitive elements (Byberg et al. 2000; Law et al. 1992; Okosun et al. 2000), which represent a large portion of human genome. Among these repetitive elements, LINE-1 is one of the most plentiful families in the human genome (Rasmussen et al. 2005). Therefore, LINE-1 methylation has later been used as surrogate markers for estimating global DNA methylation levels due to its high representation (Okosun et al. 2000).

A prospective study recently reported that LINE-1 DNA methylation from peripheral leukocytes of 533 children aged 5–12 at baseline does not appear to be associated with the indicators of adiposity, such as Z-scores for BMI-for-age, skinfold thickness ratio, or waist circumference at baseline (Slater-Jefferies et al. 2011). There was no obvious association between the changes in adiposity and LINE-1 DNA methylation either after 30-month follow-up. Although there was no direct statistical assessment for the relationship between BMI and LINE-1 methylation, a nested case-control study of microsatellite stable colorectal cancer demonstrated a clear inverse association between LINE-1 DNA methylation and BMI (Ogino et al. 2009). Another study demonstrated that increased folic acid status during pregnancy, in particular among low maternal cobalamin status, was associated with increased risk of insulin resistance in 6 year old children (Yajnik et al. 2008). However, increased intake of folic acid during pregnancy in another study has not exhibited altered body composition after follow-up the children up to 9 years old (Lewis et al. 2009).

Most recently, an analysis of the data from the Prostate, Lung, Colon, and Lung Cancer trial by the author of this Chapter (not published) examined the relationship between lymphocyte LINE-1 and overall global DNA methylations and obesity among 493 control subjects only in a nested case-control study of colon cancer. The results of this study suggested that lymphocyte DNA methylation, as measured as level of methylation at LINE-1 element, was reflective of the obesity status in a linear fashion at the time (PLCO baseline) of the biological sample was collected. Obesity status in the recent past (age 50s – approximately 10 years ago) appeared to be predictive of the level of DNA methylation at LINE-1 as well (Table 6.1). However, obesity status at distant past (age 20s) was not associated with level of LINE-1 methylation. DNA methylation status measured as percent methylated cytosine was not associated with obesity status, regardless the time frame of the measurement, in this examination of non-cancer study subjects. Percentage of methylated cytosine may not be an appropriate indicator linking obesity status and DNA methylation. However, the association observed was more or less simply indicative of marker for obesity due to the cross-sectional nature of the sample collection and offered minimal information regarding the causal relationship. A

Table 6.1 Association[a] between leukocyte DNA methylation status and obesity at PLCO baseline (BMI ≥ 30 kg/m^2) among controls

	Obesity	
	N (BMI 30+ vs. <30)	OR (95 %CI)
BMI at baseline		
% methylation cytosine		
1st tertile	54/151	1.10 (0.66, 1.83)
2nd tertile	40/130	0.98 (0.57, 1.68)
3rd tertile	34/105	1.00
Test for trend		0.68
LINE-1 methylation		
1st tertile	66/150	2.21 (1.27, 3.87)
2nd tertile	40/123	1.51 (0.82, 2.77)
3rd tertile	22/113	1.00
Test for trend		0.004
BMI at age 50s		
% methylation cytosine		
1st tertile	35/170	1.40 (0.74, 2.64)
2nd tertile	18/139	0.93 (0.45, 1.92)
3rd tertile	18/133	1.00
Test for trend		0.25
LINE-1 methylation		
1st tertile	33/172	2.41 (1.14, 5.10)
2nd tertile	27/138	2.63 (1.21, 5.73)
3rd tertile	11/132	1.00
Test for trend		0.04
BMI at age 20s		
% methylation cytosine		
1st tertile	35/170	1.05 (0.59, 1.85)
2nd tertile	18/139	0.59 (0.30, 1.15)
3rd tertile	18/133	1.00
Test for trend		0.74
LINE-1 methylation		
1st tertile	33/172	1.06 (0.55, 2.06)
2nd tertile	27/138	1.62 (0.83, 3.13)
3rd tertile	11/132	1.00
Test for trend		0.93

[a]Model adjusted for age, study year, gender, race, randomization year, smoking status, family history of colon cancer, and NSID use

follow-up interview was conducted in 2011 for all study subjects with questions on their current body weight. The analysis with the inclusion of this additional information offered little evidence that DNA methylation, measured on LINE-1 element or as global methylation, at baseline is predictive of obesity approximately 10 years later.

6.6 Future Directions

Although the primary factors for obesity to be excess dietary intake combined with limited physical activity given certain genetic characteristics of individuals, the increase in the incidence of obesity worldwide has been accompanied by a parallel increase in academic research aimed to provide reasonable mechanistic explanations for the excess fat deposition and identify potential new therapeutic targets to reduce the burden of obesity epidemic (Symonds et al. 2013). While the genome-wide association studies have offered some explanations to elucidate the pathways to obesity, the proportion of variance in obesity explained by genetic variants remains low. As we have summarized throughout this chapter, existing evidence, in particular animal models and ecologic studies, offers a very convincing argument linking contributions from epigenetics to obesity etiology. Despite the finding from studies on human population is yet to prove to be consistent, the knowledge gained so far warrant the further exploration to confirm the causal pathways between epigenetics modification and obesity.

Unlike animal studies where researchers can control the exact exposures to experimental animals at exact time period and observe them through their life, similar to all epidemiological study design for chronic diseases, the inherited limitations on all human subjects research inhibit the ability for researchers to conduct epigenetics studies on human with similar type of controls over study subjects as animal experiments. Epidemiologic researchers face the challenges to cope with various variations, such as genetic, behavior, lifestyle, and environment, experienced among free-living human individuals. Nevertheless, a few challenges have been identified that may be able to significantly improve the quality of future studies if these issues are appropriately addressed. First of all, studies have suggested that markers for epigenetics alternations may be developmental-stage specific, cell-type specific, and/or tissue specific (Anwar and Lehmann 2014; Herzog et al. 2013). The question is whether we are measuring the right target at the right time. A study examined 17 somatic tissues from four autopsied humans with Illumina Infinium Human Methylation 450 BeadChip covering 486,428 CpG sites (Lokk et al. 2014). The investigators found that only 2 % of the CpGs analyzed were hypermethylated in all 17 different tissue specimens. These permanently methylated CpG sites are located predominantly in gene-body regions. Contrary, 15 % of the CpGs are hypomethylated in all specimens and they are primarily located in regions proximal to transcription start sites. It is obviously important to identify the right tissue at the right loci for DNA methylation study on obesity. Additionally, it has been postulated that circulating leukocyte DNA methylation would represent the overall epigenetic patterns throughout the human body because of the fact it reaches all tissues and organs. However, leukocytes are produced and derived from a multipotent cell in the bone marrow and they live for about 3–4 days in the average human body. The number of leukocyte in the blood is often sensitively affected by health status, such as inflammation, oxidative stress, chronic diseases, cancers, and etc. Besides, epigenetic changes, of which DNA methylation is the most commonly characterized, can occur throughout

the course of life (Janssen et al. 2014). Therefore, the timing of the measurement is very important. Given DNA methylation level may be a marker for obesity as demonstrated earlier, sample collection for the evaluation of leukocyte DNA methylation for the etiologic study of obesity should be taken prior to the onset of overweight and obesity.

Given potential variation in epigenetics measurement current available, stability and variability should be put into significant consideration when design human study on epigenetics and obesity. The exponentially increase in DNA methylation studies in human is dependent on the quality and stability of available biological archives. Previous studies have shown that DNA samples stored for several years at −80 °C are amenable to use for further applications, such as DNA methylation (Janssen et al. 2014; Ferrer et al. 2008; Vilahur et al. 2013). In terms of temporal stability of DNA methylation, the information available is limited. Dynamic changes in DNA methylation are found during developmental stages (Laurent et al. 2010). It has been suggested that DNA methylation is relatively stable in adult population (Dolinoy et al. 2007). However, a study on 63 healthy individuals measuring leukocyte DNA methylation collected 4 days apart found that temporal behavior ranged from high (IL-6, ICC = 0.89) to low stability (APC, ICC = 0.08) (Byun et al. 2012). The study suggested that density of CpG dinucleotides nearby the sequence analyzed (measured as CpG(o/e) or G+C content within ±2,000 bp) was positively associated with DNA methylation stability. Another study evaluated methylation of CpG sites in 8 candidate loci (*NR3C1*, *IGF2R*, *GRB10*, *LEP*, *ABCA1*, *KCNQ10T1*, *MEG3*, *GNAS A/B*) among 34 individuals with archived blood and buccal cells stored 11–20 years and 2–8 years, respectively. This study found that five out of eight loci were stable over time ($p > 0.75$) for both tissues, indicating that prospective epigenetics studies may be possible. On the other hand, prospective multiple repeated measurements will help to identify methylation on which loci are more stable for the evaluation of methylation over time to examine the association with obesity.

The advancement of high throughput DNA methylation profiling using microarray and sequencing-based DNA methylation technologies has made larger scaled human study on epigenetics and obesity much faster and more affordable than a decade ago. However, the advancement also brings new challenges for scientists studying epigenetics alternations on obesity in human. For example, the white paper by Illumina regarding their Infinium Human Methylation 450K BeadChip states demonstrated strong correlation between replicates ($r > 0.98$) (Infinium Human Methylation 450 BeadChip 2012). However, it also means that approximately 2 % of the 485,000+ sites may not correlate well. Using the categorical analysis for methylation status (hypo-, hyper-, and normal-methylated), our preliminary study (unpublished) suggested that the number of targets that are discordantly categorized into one of the methylation status are in the range of hundreds to a few thousands. As the finding in the evaluation of the global methylation patterns by Lokk et al. methylation patterns are consistent among certain tissues but not the others (Lokk et al. 2014). The traditional approach examining percentage of CpG site is methylated versus unmethylated may bias the finding toward the null. Attentions should be made to identify CpG methylation sites in certain tissues that are appropriate for

obesity research. Additionally, with the tremendous amount of data generated from the high throughput technologies, appropriate statistical analysis is another challenge. There is an immerse need for innovative approaches to examine the epigenetics results associated with obesity. All published analyses presume appropriately adjusted for multiple comparisons. However, multiple comparisons assume all methylation sites are independent and not functional correlated. We know that, for example Illumina Infinium 450K BeadChip, multiple CpG sites per gene and many functional related genes were included in the array. The multiple adjustment approach taken currently may artificial bias the finding toward the null. Besides, analyses often put gene and environment into consideration. These factors are known correlated with each other before the introduction of CpG methylation sites. Advanced pathway approach focusing on specific loci and capturing the intercorrelation among methylation sites measured may need to be further developed.

Animal studies have offered the opportunities to examine the effects of epigenetic programming on obesity with a mechanistic approach and have provided significant support hypothesis. The evidence to date in human studies, although is less solid, also offered some indication regarding the association between epigenetics modification and obesity outcome. With the newly developed technologies and better refined study design, the field in epigenetics and obesity research is very promising in identifying the mechanistic pathways between environmental exposure and epigenetics alternation leading to the onset of obesity.

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Chapter 7
Environmental Toxicants, Epigenetics, and Cancer

Hannah Lui Park

Abstract Environmental toxicants, including tobacco smoke, air pollution, and water and food pollution have been shown to either contribute to or be associated with development of some human cancers. Exposure to these toxicants are also associated with epigenetic changes detected in human specimens, including blood, saliva, and sputum. In healthy individuals, some of these epigenetic changes are consistent with those seen in cancer patients such as global hypomethylation and tumor suppressor gene hypermethylation. For example, benzene exposure was found to be associated with global hypomethylation and *p15* hypermethylation. In cancer patients, exposure to environmental toxicants have been found to be associated with different cancer subtypes with distinct epigenetic profiles. For example, smoking was found to be associated with increased colorectal cancer risk for CIMP-positive tumors. This chapter will review evidence supporting the potential roles of environmental toxicants in human cancer development by way of epigenetic changes.

Keywords Environmental toxicant • Tobacco smoke • Air pollution • Water pollution • Food pollution • Pesticides • Metal • Arsenic • Cadmium • Nickel • Chromium

7.1 Significance of Environmental Factors in Cancer Development

Approximately 5–10 % of cancer is inherited, in which individuals inherited a highly penetrant germline genetic mutation from one or both parents which gave them a much higher chance of developing cancer than if they did not have the genetic mutation (Nagy et al. 2004). For example, up to 85 % of women with a mutation in BRCA1 or BRCA2 will develop breast and/or ovarian cancer in her lifetime

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(Nagy et al. 2004; Antoniou et al. 2005). Virtually 100 % of males with HNPCC, in which they have a mutation in one of five DNA mismatch repair genes (MLH1, MSH2, MSH6, PMS1, or PMS2), will develop colorectal cancer by age 70 (Aarnio et al. 1999). Women with HNPCC have a 42–60 % lifetime risk of endometrial cancer and 54 % chance of getting colorectal cancer (54 %) (Aarnio et al. 1999; Vasen et al. 1996; Millar et al. 1999). A higher percentage of cancer, about 10–15 %, is considered "familial," in which there are more cases of cancer in a family than predicted by chance alone, but the features of hereditary cancer are not present (Nagy et al. 2004). Familial cancers may be caused by the interaction of low-penetrance genes, gene–environment interactions, or both. Thus, the vast majority of cancers are sporadic, in which there is no genetic predisposition or family history of the disease. For these cancers, the etiology must lie, at least in great part, in environmental factors including environmental toxicants.

7.2 Effect of Environmental Toxicants on Epigenetics in Cancer

Environmental toxicants include chemical, biological and physical agents, whether naturally occurring in nature or a consequence of human manufacture, that are harmful to living organisms (Wright and Welbourn 2002). The *Fourth National Report on Human Exposure to Environmental Chemicals*, *Updated Tables* (2013) presents data on levels of 261 chemicals found in blood and urine collected from participants in the CDC's National Health and Nutrition Examination Survey (NHANES), which obtains and releases health-related data from a nationally representative sample every 2 years (Centers for Disease Control and Prevention 2013). The list of chemicals were selected based on factors included suggested exposure in the U.S. population, seriousness of health effects, and availability of accurate and sensitive analytical methods. Most of the chemicals described in this chapter, which were based on the availability of published data on their known or potential role in increasing cancer risk or morbidity through epigenetic mechanisms, can be found on this list.

This chapter will describe environmental toxicants primarily within the contexts of human exposure. Most evidence will be drawn from epidemiologic studies examining potential associations between exposure and cancer-related epigenetic aberrations and some evidence will be drawn from experimental research. The reasoning for the primarily contextual approach is due to the complex makeup of most environmental factors contributing to cancer. For example, tobacco smoke contains more than 7,000 chemical components. While we all know smoking is a major risk factor for lung cancer, who is to say which of the 7,000 compounds in tobacco smoke is the culprit? Most epidemiologic studies examining addressing individual environmental toxicants that were encountered in the writing of this chapter did not adjust for the presence of toxicants other than the one in question.

This chapter will examine the following sources of environmental toxicants that are encountered by most people in their everyday lives, for their known or potential roles in cancer development through epigenetic mechanisms.

- Tobacco smoke
- Air pollution
- Water and food pollution (pesticides, metals)

In some controlled cases, such as experimental studies, findings on specific chemicals will be discussed. An overview of epigenetic changes (mainly in DNA methylation, histone modifications, miRNA) that occur in cancer development are described in Chap. 1. This chapter will focus on environmental toxicants that either cause or are associated with epigenetic changes that could potentially lead to cancer. Because there is so much information available about the effects and associations with tobacco smoke, and because tobacco smoke is still such a pervasive problem in many societies, not only through direct smoking but in maternal fetal transmission and second-hand smoke, tobacco smoke will be the first substance discussed.

7.2.1 Tobacco Smoke

According to the CDC, tobacco smoke contains more than 7,000 chemical components, and at least 69 of these chemicals can cause cancer, including formaldehyde, benzene, arsenic, lead, cadmium, chromium, carbon monoxide, ammonia, polycyclic aromatic hydrocarbons (IARC 2004a). While it is well established that active cigarette smoking is a major risk factor for >15 different types of human cancer (IARC 2004a; Secretan et al. 2009), environmental exposure, or exposure to second-hand smoke, also has adverse effects. Second-hand smoke has also been classified as a human carcinogen which causes lung cancer, with an average excess risk of lung cancer among never-smokers regularly exposed to second-hand smoke in the order of 20–30 % (IARC 2004a; U.S. Department of Health and Human Services 2006). Ten to fifteen percent of all lung cancer deaths occur in subjects with no history of smoking (IARC 2004a; U.S. Department of Health and Human Services 2006), with an estimated 10,000–15,000 US lung cancer deaths occurring annually occur among people who have never smoked (Thun et al. 2006). In addition, there is now limited evidence for an association between second-hand smoke and cancers of the larynx and pharynx (Secretan et al. 2009) and suggestion that it may also be linked to breast cancer (Betts 2007). This section describes some studies on direct smoking and some on exposure to second-hand smoke.

7.2.1.1 Associations Between Tobacco Smoke and DNA Methylation in Subjects with Cancer

The first epidemiology studies investigating the role of DNA methylation in cancer were conducted using tumor tissues from biopsies or resections as the main source of DNA. In most epidemiology studies, smoking status is routinely captured. Given the associations between aberrant DNA methylation and cancer, and between

smoking and cancer, it is not surprising that smoking is also associated with differences in DNA methylation in subjects with cancer. While epigenetic changes in cancer have been discussed in a previous chapter in this book, the following sections discusses observations that have been made on smoking status and DNA methylation in subjects with different types of cancer and in subjects without cancer.

Smoking is a strong risk factor many cancers, but it has the strongest association with lung cancer. While most lung cancers are associated with cigarette smoking, it is statistically estimated that 25 % of lung cancers are not attributable to cigarette smoking (Jemal et al. 2009). Due to observed differences in patient demographics, subtype, responsiveness to therapy, and survival between lung cancer in smokers and non-smokers, lung cancer in smokers are often regarded as a separate cancer category than lung cancer in non-smokers (Jemal et al. 2009). Differences in DNA methylation among smoking and non-smoking patients with lung cancers have been observed in a number of studies. For example, differential methylation of CDKN2A was found in NSCLC tissue from heavy smokers (OR, 3.95; 95 % CI, 1.21–12.90) compared to non-smokers analyzed by methylation-specific PCR (Tan et al. 2011). Another study on DNA methylation of a panel of seven genes, MYF6, SIX6, SOX1, RARB, BCL2, PHOX2A and FOLX2, using tumor tissue from 101 stage I NSCLC patients, found that co-methylation of BCL2 and RARB was associated with smoking (OR, 9.52; 95 % CI, 1.20–75.49). MYF6, SIX6 and FOLX2 (OR, 2.60; 95 % CI, 1.11–6.66) or the co-methylation of SIX6, BCL2 and RARB (OR, 2.37; 95 % CI, 1.27–4.44) were also associated with smoking (Zhao et al. 2013). Another study revealed that starting smoking under age 18 was significantly related to RASSF1A methylation [prevalence ratio (PR) = 1.6, 95 % confidence interval [CI] = 1.1–2.3] in patients with NSCLC (Marsit et al. 2006). Methylation in the MTHFR promoter of lung tumor DNA was also significantly higher in current smokers than in never smokers (Vaissiere et al. 2009).

A meta-analysis on the association between cigarette smoking and CDKN2A methylation in surgically resected tumor tissues from non-small cell lung carcinoma (NSCLC) patients was done using data reported on 2,037 smoker and 765 nonsmoker patients in 19 cross-sectional studies. The frequency of CDKN2A hypermethylation was higher in NSCLC patients with current or previous smoking status than that in non-smoking patients (OR = 2.25, 95 % CI = 1.81–2.80) (Zhang et al. 2011). The association was stronger in Asian patients and in the studies with larger sample sizes.

In an analysis on blood samples from 95 NSCLC patients, CDKN2A and ESR1 methylation were found to be strongly associated with smoking history as measured by real-time PCR. 16.2 % of non-smokers v. 27.5 % of smokers had CDKN2A methylation, and 36.5 % of non-smokers v. 51.0 % of smokers had ER methylation. 40.5 % of non-smokers v. 65.0 % of smokers had either CDKN2A or ER methylation ($p < 0.01$) (Suga et al. 2008).

On the other hand, there have also been many reports wherein there are no differences between smoking and non-smoking patients. For example, the sensitivity and specificity of methylation of a four-gene panel (CDKN2A, TERT, WT1 and RASSF1) analyzed in bronchial washings were similar in smoker and non-smoker lung cancer patients (Nikolaidis et al. 2012).

While there are ample examples of associations between DNA methylation and smoking, there are further differences associated with lung cancer status even among smokers. For example, methylation status of CDKN2A, MGMT, and CDH1 were found to be significantly higher in sputum of smokers with lung cancer compared to smokers without lung cancer, as analyzed by methylation-specific polymerase chain reaction (MSP) (Guzmán et al. 2012). Since many researchers are searching for potential DNA methylation-based biomarkers for early detection of cancer, understanding the differences in DNA methylation patterns between patients with and without cancer, in the context of smoking status and other confounders, is necessary.

A number of studies have shown that hypermethylation of the *CDKN2A* promoter is significantly associated with tobacco exposure in head and neck cancers (Hasegawa et al. 2002; Mani et al. 2012). Global hypomethylation was found to be significantly associated with smoking history in a study analyzing LINE-1 hypomethylation by pyrosequencing in noncancerous esophageal mucosae of ESCC patients ($P=0.014$ for smoking duration; $P=0.0017$ for number of cigarettes per day; $P=0.0002$ for tobacco pack-year) (Shigaki et al. 2012).

DNA methylation patterns in some cancers may constitute a cancer subtype. In fact, in recent years, a type of classification for colorectal cancers has emerged which is based on DNA methylation. CpG island methylator phenotype (CIMP)-positive colorectal tumors are tumors that are hypermethylated in at least three of a five-gene panel (CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1), whereas CIMP-negative tumors are hypermethylated in zero to two of the genes (Limsui et al. 2010). Interestingly, in a large prospective study of 37,399 women, smoking was associated with increased colorectal cancer risk for CIMP-positive tumors (RR = 1.88, 95 % CI = 1.22–2.90) but not CIMP-negative subtypes (RR = 1.02, 95 % CI = 0.81–1.30), suggesting that epigenetic modifications may be functionally involved in smoking-related colorectal carcinogenesis (Z). Smoking was also found to be associated with methylated MGMT gene in patients with sporadic colorectal cancer. Sixty-six percent of patients with MGMT methylation were smokers, whereas only 21 % of patients without MGMT methylation were smokers (Limsui et al. 2010).

In a population-based study on non-smoking patients with bladder cancer, using an array-based approach, a total of 33 CpG loci were found to be significantly associated with exposure to second-hand smoke, including FHIT and CDH3 (Wilhelm-Benartzi et al. 2011). Most of the methylated genes were associated with adulthood second-hand smoke exposure, and some were associated with childhood and occupational second-hand smoke exposure.

CDKN2A methylation has been found to be associated with cervical cancer in a number of studies (Lea et al. 2004; Jeong et al. 2006; Huang et al. 2011). For example, aberrant CDKN2A methylation was significantly higher in squamous cell cervical cancers (61 %) than in squamous high-grade dysplasia (20 %) or normal cytologic specimens (7.5 %). Interesting, aberrant CDKN2A methylation was also associated with smoking in patients with squamous carcinoma (odds ratio, 20.6; 95 % CI, 3.6–118; $P<.001$) and high-grade dysplasia (odds ratio, 4.57; 95 % CI, 1.63–12.78; $P=.002$) (Lea et al. 2004).

7.2.1.2 Associations Between Tobacco Smoke and DNA Methylation in Healthy Subjects

The vast number of reports on differences in DNA methylation between subjects with and without cancer have led to an increasing number of prospective studies on potential associations between DNA methylation and cancer risk in healthy individuals. One premise for studies on healthy subjects is that if aberrant DNA methylation is part of the cancer development process, detection of differences in DNA methylation early in the process, even before a cancer is clinically diagnosed, would enable the development of early detection markers. Another purpose for DNA methylation studies on healthy subjects is to increase our understanding of the sequence of molecular events that occur during cancer development.

Due to limited methods for relatively uninvasive specimen collection, studies on healthy subjects have largely been conducted using DNA from cells extracted from tissue like blood, saliva, and sputum. In addition, urine and fecal samples as well as cells from cancer screening procedures such as colonoscopies and cervical smears have also been studied. When methylation studies began, the panels of genes that were analyzed were limited and most often chosen for their potential roles in cancer development or tumor suppressive abilities in vitro. For example, a study on cells obtained from mouth and throat rinsing fluid samples in smokers and non-smokers revealed that p15 methylation was found in 3 of 37 healthy individuals (8 %) who did not drink or smoke compared to 15 of 22 healthy smokers and/or drinkers (68 %) (Chang et al. 2004). While global hypomethylation was observed in peripheral blood lymphocytes from 528 healthy control subjects by LINE-1 analysis in one study (Cash et al. 2012), another analysis on blood from 1,465 subjects found no associations with Alu or LINE-1 by pyrosequencing (Zhu et al. 2012).

At the dawn of availability of genome-wide methylation arrays, Breitling and colleagues analyzed methylation at 27,578 sites in more than 14,000 gene promoter regions in 177 current smokers, former smokers, and never-smokers, using the Illumina human methylation 27K BeadChip (Breitling et al. 2011). The most significant differentially methylated CpG site between smokers vs. non-smokers was identified to be coagulation factor II receptor-like 3 (F2RL3), which encodes the protease-activated receptor four gene, achieving Bonferroni-corrected significance threshold ($p < 1.81 \times 10^{-6}$) for multiple testing (Breitling et al. 2011). At this site, DNA methylation was significantly lower in smokers than in non-smokers (% methylation difference = 12 %; $p = 2.7 \times 10^{-31}$) and correlated negatively with the number of cigarettes smoked and positively with number of years after quitting. Results were validated in an independent cohort of 316 samples analyzed by mass spectrometry and Sequenom EpiTyper ($p = 6.33 \times 10^{-34}$) (Breitling et al. 2011). Similar exposure-related differences in the methylation of this gene were also seen in an independent study on 187 matched pairs of smokers and non-smokers using the next version of the Illumina methylation array, the 450K methylation BeadChip, which analyzes >450,000 CpG sites. In this study, the % methylation difference for F2RL3 was found to be 8 %; $p = 8.4 \times 10^{-11}$ (Shenker et al. 2013).

The other top loci that were found to be hypomethylated in smokers compared with former and non-smokers included the tumor suppressor gene aryl hydrocarbon receptor repressor gene (AHRR; cg05575921, $P = 2.31 \times 10^{-15}$; effect size = 14–17 %), a CpG island on 2q37.1 (cg21566642, $P = 3.73 \times 10^{-13}$; effect size = 12 %) and a region at 6p21.33 (cg06126421, $P = 4.96 \times 10^{-11}$; effect size = 7–8 %). These results were validated by bisulfite pyrosequencing in a further independent population of healthy individuals (n = 180) (Shenker et al. 2013).

Differences in DNA methylation have also been associated with exposure to prenatal tobacco smoke. For example, brain-derived neurotrophic factor-6 (BDNF-6) methylation was found to have higher methylation levels in blood samples from adolescents who were exposed to prenatal tobacco smoke compared to adolescents who were not exposed to prenatal tobacco smoke (Toledo-Rodriguez et al. 2010). BDNF-6 plays a role in brain development and has been implicated in adult neural plasticity, including learning and memory (Martinowich et al. 2003). Another study reported that prenatal tobacco smoke exposure was associated with slightly increased AXL, a receptor tyrosine kinase relevant in cancer and immune function, and PTPRO methylation in buccal cells from 272 kindergarten and first-grade children and with global hypomethylation in 348 children (Breton et al. 2009).

A limited number of studies on healthy subjects have also been conducted using tissue from more invasive procedures. For example, a study on colonic mucosal biopsies from 68 healthy subjects undergoing colonoscopy revealed that smokers had lower global DNA methylation than nonsmokers as measured by an in vitro methyl acceptance assay (p = 0.01) (Pufulete et al. 2005). In another study on a cohort of women aged 15–19 years, a causal link between smoking, aberrant DNA methylation, and cervical cancer was suggested by a study. In this study, women were recruited soon after they first had sexual intercourse. Cervical cytological samples and data on sexual and smoking history were collected at baseline and updated every 6 months. Among women who remained cytologically normal and who tested negative for human papillomavirus DNA in cervical smears during follow-up, those who first started to smoke during follow-up had an increased risk of acquiring CDKN2A methylation compared with never-smokers (odds ratio = 3.67; 95 % confidence interval 1.00–12.33; P = 0.04) (Ma). The median time to the first detection of *CDKN2A* methylation in those who acquired *CDKN2A* methylation was 266 days (inter-quartile range from 178 to 382). Follow-up studies are necessary to determine if those who had CDKN2A methylation were indeed at greater risk of developing cervical cancer.

7.2.1.3 Effect of Smoking Cessation on DNA Methylation Patterns

Even though promoter hypermethylation can persist for many years after smoking cessation (Zöchbauer-Müller et al. 2003; Bhutani et al. 2008), current smokers in general have a higher mean methylation index than former smokers, supporting the view that hypermethylation, unlike somatic mutations, is reversible (Yanagawa et al. 2011). In a combined analysis of 134,204 individuals, people who quit

smoking for 10–19, 20–39, and ≥40 years of were at lower risk of developing CIMP-high colorectal cancer compared with patients who currently smoked, with multivariate hazard ratios (95 % confidence intervals) of 0.53 (95 % CI, 0.29–0.95), 0.52 (95 % CI, 0.32–0.85), and 0.50 (95 % CI, 0.27–0.94), respectively ($P_{trend} = 0.001$), but not with the risk of CIMP-low/CIMP-negative cancer ($P_{trend} = 0.25$) ($P_{heterogeneity} = 0.02$, between CIMP-high and CIMP-low/CIMP-negative cancer risks) (S). These findings support the hypothesis that epigenetic modifications are functionally involved in smoking-related colorectal carcinogenesis (Limsui et al. 2010).

The reasons for differences in DNA methylation between smokers and non-smokers are not known. It would seem likely that, given the overall patterns of DNA methylation in smokers to be approaching those commonly seen in tumor tissue (global hypomethylation and gene-specific hypermethylation), that DNMT levels and/or function may have a role. That is, we would expect DNMT-1, primarily responsible for DNA methylation of newly replicated DNA (Bestor 1992), to be expressed at lower levels or exhibit lower activity, whereas DNMT-3, responsible for de novo DNA methylation at CpG islands (Okano et al. 1999), would be overexpressed or exhibit higher activity. However, studies on frozen autopsy cancer-free liver tissue, *DNMT1* mRNA levels were found to be higher in tissue from individuals who smoked compared to those who did not (Hammons et al. 1999), whereas *DNMT3a* and *DNMT3b* levels did not differ (Xiao et al. 2008). The mechanisms by which smoking leads to changes in DNA methylation are not known.

7.2.2 Air Pollution

Sources of air pollution include vehicles and industrial contaminants. The main two components are particulates and gases. While the gases include ozone, carbon monoxide, sulfur dioxides and nitrous oxides, the particulates include benzene, polycyclic hydrocarbons, metals, soil, and dust particles.

Particulate matter (PM) is commonly categorized by aerodynamic diameter, with the size of particles directly linked to their potential for causing health problems. Those that are less than 10 μm in diameter are generally able to pass through the throat and nose and enter the lungs. "Inhalable coarse particles," such as those found near roadways and dusty industries, are particles that are greater than 2.5 μm and less than 10 μm (PM_{10}), whereas "fine particles," such as those found in smoke and haze, are 2.5 μm in diameter and smaller ($PM_{2.5}$) (EPA website).

Associations between exposure to fine particles and lung cancer have been consistently observed (Dockery et al. 1993; Pope et al. 2002). In a large study by the American Cancer Society on approximately 500,000 adults in metropolitan areas throughout the United States, Pope et al. reported that for every 10 μg/cm^3 elevation in PM2.5 concentration there was an approximately 8 % increased risk of lung cancer related mortality (Pope et al. 2002). In a recent meta-analysis involving 312,944 subjects, the authors showed a statistically significant association between risk for lung cancer and PM10 [HR 1.22 (95 % CI, 1.03–1.45) per 10 μg/m(3)]. For PM2·5

the HR was 1·18 (95 % CI, 0.96–1.46) per 5 μg/m(3) (Raaschou-Nielsen et al. 2013).

Observational studies have linked PM exposures with blood DNA hypomethylation. PM-related hypomethylation has been repeatedly observed in transposable repeated elements, including *Alu* and LINE-1 (Tarantini et al. 2009; Madrigano et al. 2011) as well as in candidate proinflammatory genes like iNOS. In addition, some miRNAs related to oxidative stress and inflammation were found to be increased following occupational PM exposure in steel factory workers. Expression of miR-222 and miR-21 (using the 2-DeltaDeltaCT method) was significantly increased in postexposure samples (miR-222: baseline = 0.68 +/− 3.41, postexposure = 2.16 +/− 2.25, p = 0.002; miR-21: baseline = 4.10 +/− 3.04, postexposure = 4.66 +/− 2.63, p = 0.05) (Bollati et al. 2010). In a different cohort of subjects with different levels of benzene exposure, including 78 gas station attendants, 77 traffic police officers, and 58 unexposed controls in Italy, benzene exposure was found to be associated with global hypomethylation (−2.33 % in LINE-1 methylation, P = 0.009 and 1.00 % in AluI methylation, P = 0.027 for a tenfold increase in airborne benzene levels) and hypermethylation in the tumor suppressor gene p15 (+0.35 %; P = 0.018) (Bollati et al. 2007).

These observational studies prompted investigators to conduct controlled human exposure studies. A double-blind randomized cross-over trial was conducted on 15 healthy subjects between 18 and 60 years old wherein they were exposed to 120 min of concentrated ambient particles (CAPs). Blood was collected before the exposure and 1 h after exposure ended. Blood DNA methylation of LINE-1 and *Alu* repetitive elements and candidate proinflammatory genes (*TLR4*, *IL-12*, *IL-6*, *iNOS*) was measured by bisulfite pyrosequencing. After adjustment for multiple comparisons, fine CAPs exposure lowered Alu methylation (β-standardized = −0.74, adjusted-*P* = 0.03), and coarse CAPs exposure lowered TLR4 methylation (β-standardized = −0.27, adjusted-*P* = 0.04), a gene involved in inflammation and oxidative stress (Bellavia et al. 2013).

While most of the studies on air pollution and epigenetics have focused on cardiovascular effects, it is likely that air pollution can also contributes to cancer, given that inflammation and oxidative stress can contribute to cancer development (Elinav et al. 2013; Møller et al. 2008) and that global hypomethylation is also associated with cancer. A genome-wide search for epigenetic differences in genes related to cancer may reveal more associations with air pollution in addition to that found for p15 hypermethylation and benzene exposure (Bollati et al. 2007). There was one study in which DNA methylation was examined by the Inifinium 27K BeadArray in children living in two distinct cities in the Czech Republic: 100 children from the highly polluted Ostrava region and 100 children from the Prachatice control region. Demographics were similar between the two populations but exposures to air pollution were markedly different, up to sevenfold the concentrations seen in Ostrava for air components like PM2.5, PM10, benzene, and various metals. 9916 CpG sites were found to be significantly differentially methylation between children from Ostrava vs. Prachatice, and 58 CpG sites had differences >10 %. These 58 CpG sites were all found to be hypomethylated in samples from children in polluted Ostrava

and included sites located in genes related to immune system, DNA-protein binding, metabolism of xenobiotics or signaling pathways (Rossnerova et al. 2013).

Interestingly, PM exposure in mice and primary murine alveolar epithelial cells resulted in increased ROS production, expression of DNMT1, and methylation of the tumor suppressor gene p16 promoter (Soberanes et al. 2012).

7.2.3 Food and Water Pollution

Sadly, toxicants are often found in our food and water. Contaminants include pesticides, arsenic, cadmium, chromium, and nickel, and polycyclic aromatic hydrocarbons that are generated while cooking. The food packaging contaminant bis-phenol A has been discussed in a previous chapter.

7.3 Pesticides

Pesticide exposure has been repeatedly shown to be associated with cancers, namely, non-Hodgkin's lymphoma (Waddell et al. 2001; Cantor et al. 1992), brain cancer (Davis et al. 1993), and lung cancer (Alavanja et al. 2004; Pesatori et al. 1994). Because pesticide exposure is assessed via an extensive and detailed questionnaire about which chemicals were used, how often and for how long, it is possible to conduct analyses on individual chemicals.

Genomic DNA methylation content in blood leukocytes has been found to be inversely associated with plasma levels of pesticide residues in Arctic (Rusiecki et al. 2008) and Korean populations (Kim et al. 2010).

In vitro studies have shown that exposure to pesticides is associated with a genome-wide DNA methylation. Analyses on human hematopoietic K562 cells exposed to fonofos, parathion, terbufos, and diazinon using the Illumina Infinium HumanMethylation27 BeadChip revealed 1759, 1746, 1580, and 1069 CpG sites in at least 984 genes with significant methylation changes. Among the genes that were hypermethylated tumor suppressor genes TP53INP1 (3.0-fold, q-value <0.001), PTEN (2.6-fold, q-value <0.001), p53 inducible protein 11 (TP53I11) (4.0-fold for fonofos, q-value <0.001; 4.7-fold for parathion, q-value <0.001; 3.1-fold for terbufos, q-value <0.001; respectively) as well as histone deacetylase 3 (2.2-fold, q-value $=0.002$) (Zhang).

7.4 Arsenic

Arsenic occurs naturally in the earth's crust. Arsenic and arsenic compounds can be emitted into air and then deposited into water and soil during industrial operations such as ore mining and smelting, and during volcanic eruptions and forest fires

(U.S. Environmental Protection Agency 2007). The major source of human exposure to arsenic is contaminated food and drinking water. Inhalation and ingestion of arsenic have been linked to cancers of the skin, bladder, liver, lung, and prostate (IARC 2004b; Liu and Waalkes 2008).

Differences in global DNA methylation associated with exposure to arsenic have been reported in human studies across various tissue types, but the patterns of DNA methylation alteration associated with arsenic exposure have been inconsistent. For example, Wilhelm and colleagues studied global methylation in peripheral blood DNA of a New Hampshire population-based cohort of 465 healthy subjects. They found that levels of arsenic in the 90th percentile were associated with reduced LINE1 methylation ($P = 0.04$). Taken another way, subjects with greater than the 90th percentile of arsenic exposure (>0.20 μg/g arsenic) were at greater risk for LINE1 hypomethylation (OR 3.68; 95 % CI, 1.04–13.02) (Wilhelm et al. 2010). In another study, peripheral blood lymphocyte DNA from 16 females in Mexico exposed to variable amounts of arsenic in their drinking water were analyzed by Affymetrix Human Promoter 1.0R arrays, which represent >25,500 human promoter regions. Using spot urine samples to measure arsenic exposure, the researchers found that arsenic exposure was associated with differential methylation of 455 gene promoters, most of which were hypomethylated (Bailey et al. 2013). However, Niedzwiecki and colleagues found that water, blood, and urinary arsenic were positively correlated with global DNA methylation in blood of 320 healthy subjects in Bangladesh ($P < 0.05$) (Niedzwiecki et al. 2013). Still others have reported no change (Intarasunanont et al. 2012).

The differential findings may be explained by the studies using different methods for global methylation analysis, different preparations of biological samples, and modifiers like nutritional folate levels. For example, in a study on 581 elderly men in Boston environmental exposed to low levels of arsenic (measured in toenails), an association between arsenic exposure and blood DNA methylation in Alu repetitive elements was observed which varied by folate level. In subjects with plasma folate below the median (<14.1 ng/mL), arsenic was positively associated with Alu DNA methylation ($\beta = 0.08$ [95 % confidence interval = 0.03–0.13] for one interquartile range [0.06 μg/g] increase in arsenic), whereas a negative association was observed in subjects with plasma folate above the median ($\beta = -0.08$ [−0.17 to 0.01]) (Lambrou et al. 2012). Similarly, in a cohort of Bangladeshi adults chronically exposed to arsenic-contaminated drinking water, Pilsner and colleagues found that urinary arsenic, plasma arsenic, and plasma folate were positively associated with the methylation of blood DNA ($P = 0.009$, 0.03, and 0.03, respectively). However, contrary to Lambrou's findings, stratification of participants by folate nutritional status [<9 nmol/L ($n = 190$) or >or = 9 nmol/L ($n = 104$)] showed that the associations between arsenic exposure and methylation of PBL DNA were restricted to persons with folate concentrations >or = 9 nmol/L. These studies suggest a potential role for nutrition in arsenic toxicity but given the inconsistencies, this area warrants further investigation.

In the context of prenatal exposure to arsenic, two recently published studies of different cohorts in Bangladesh have reported positive associations between LINE-1 repeat element methylation in cord blood and arsenic exposure in utero

(Kile et al. 2010; Pilsner et al. 2007), but no significant associations were observed in a cross-sectional study conducted in Thailand (Intarasunanont et al. 2012).

Arsenic has also been associated with changes in histone modifications. For example, a global increase in H3K9 dimethylation and decrease in H3K9 acetylation were found in peripheral blood mononuclear cell DNA from subjects exposed to arsenic in their drinking water (Arita et al. 2012a). This is consistent with studies on arsenic exposure in human cells in culture, which showed increased H3K9 dimethylation and H3K4 trimethylation, and decreased H3K27 trimethylation (H3K27me3) (Zhou et al. 2008).

While most studies on arsenic exposure and epigenetics have focused on global alterations, a few studies have addressed gene-specific methylation. In a study on peripheral whole blood samples collected from residents of West Bengal, India, chronic arsenic exposure was associated with the hypermethylation of the tumor suppressor genes *p53* and *CDKN2A* (encoding p16INK4A) in 96 and 62 subjects, respectively (Chanda et al. 2006). Arsenic exposure was associated with hypermethylation of tumor suppressor genes *RASSF1A* and *PRSS3* (Marsit et al. 2006) and *DAPK* in bladder cancers (Chen et al. 2007) among individuals in arsenic-contaminated areas.

In utero exposure to arsenic was found to associated with promoter methylation of *CDKN2A* (Kile et al. 2010) and *TP53* (Intarasunanont et al. 2012) measured in human umbilical cord peripheral blood lymphocytes. Cord-blood derived DNA from 134 infants was analyzed using the Illumina Infinium Methylation450K array, and in utero arsenic exposure was estimated using maternal urine samples collected at 24–28 weeks gestation. 75 % of the most significantly differentially methylated CpG islands were hypermethylated, including *ESR1* (estrogen receptor 1) and *PPARGC1A* (peroxisome proliferator–activated receptor-γ coactivator 1-α), respectively. Results were validated by pyrosequencing (Koestler et al. 2013).

7.4.1 Mechanism

The molecular mechanisms behind the cancer-inducing property of arsenic are not well understood. Potential mechanisms include induction of oxidative stress, DNA–protein crosslinking, chromosomal aberrations (Rossman 2003), disruption of signaling pathways, and epigenetic dysregulation, particularly DNA demethylation (Reichard and Puga 2010). The first evidence demonstrating an association between arsenic tumorigenicity and global DNA hypomethylation was reported by Zhao and colleagues, who showed that arsenic exposure in rat liver epithelial TRL-1215 cells in vitro led to their malignant transformation and was paralleled by global DNA demethylation (Zhao et al. 2013). The extent of DNA hypomethylation was positively correlated with the tumorigenicity of the cells when the cells were implanted into nude mice, suggesting that loss of DNA methylation may be a causative factor in arsenic-induced carcinogenesis. Since then, global DNA hypomethylation was demonstrated in various in vitro and in vivo models of arsenic-induced tumorigenesis (Chen et al. 2004; Jensen et al. 2009; Ren et al. 2011).

Several possible explanations exist for the mechanism of DNA demethylation after exposure to arsenic. First, arsenic-induced DNA hypomethylation can be explained by the absolute requirement of S-adenosylmethionine (SAM) for both the detoxification of arsenic and DNA methylation reactions (Reichard and Puga 2010; Coppin et al. 2008). Thus, the two processes are in direct competition for SAM. The more arsenic there is the body, the higher its requirement for SAM, and the less its availability for DNA methylation. It was shown that arsenic treatment resulted in decreased SAM in human HaCat keratinocytes, and the expression of the DNA methyltransferase genes DNMT1 and DNMT3 decreased, coinciding with global DNA hypomethylation (Kann et al. 2005).

7.5 Cadmium

Cadmium occurs naturally in the earth's crust and also can be introduced into the environment by human activities such as coal burning and manufacturing. Cadmium and cadmium compounds are carcinogenic to animals and humans (IARC 1993; National Toxicology Program 2005). Ingestion, dermal contact, and inhalation are the main means of exposure for humans, but the primary source of cadmium exposure in the general population is through contaminated food (ATSDR 2012), largely fish and shellfish (Perelló et al. 2008). Plants may also absorb high levels of cadmium from contaminated soils (Kaneta et al. 1986). Cadmium exposure has been associated with cancers of the lung (Beveridge et al. 2010; Stayner et al. 1992), prostate, pancreas, renal, liver, stomach, bladder, and hematopoietic system (Waalkes 2000).

Very few studies have been done examining the relation between cadmium exposure and DNA methylation in humans. A study on a cohort of 202 non-smoking Argentinean women revealed that urinary cadmium was inversely associated with peripheral blood LINE-1 methylation as measured by bisulfite pyrosequencing ($\beta = -0.50$, $p = 0.007$; $\beta = -0.44$, $p = 0.026$, adjusted for age and coca chewing) but not associated with p16 or MLH1 methylation (Hossain et al. 2012). Another study on 892 women in Spain showed that arsenic toenail concentration was inversely associated with LINE-1 methylation ($\beta = -3.6$, $p = 0.003$) (Tajuddin et al. 2013). Another study conducted in 81 residents in Cd-polluted and non-polluted areas in China revealed that levels of blood and urinary cadmium correlated positively with levels of RASAL1 and KLOTHO methylation as measured by bisulfite pyrosequencing (Zhang).

Interestingly, effects of prenatal exposure to cadmium seem to be sex-specific. In a study on 127 mother-child pairs from rural Bangladesh, genome-wide cord blood DNA methylation was analyzed by the Infinium HumanMethylation450K BeadChip. Maternal cadmium exposure was associated with cord blood DNA methylation (p-value $< 10^{-16}$) overall. However, whereas in boys, 96 % of the top 500 CpG sites showed positive correlations (rS-values > 0.50), only 29 % of the top 500 CpG sites were positively correlated in girls ($rS > 0.45$). In girls, methylation changes in genes associated with organ development, morphology and mineralization of bone were

overrepresented, whereas changes in boys were found in cell death-related genes (Kippler et al. 2013).

Many more studies on cadmium exposure on DNA methylation have been done in experimental systems, but results have been mixed. While cadmium has been shown to result in global hypermethylation in some studies (Jiang et al. 2008; Yuan et al. 2013), other studies have shown that cadmium exposure results in global hypomethylation (Huang et al. 2008; Doi et al. 2011). The explanation for the observed differences may lie in the duration of cadmium exposure. Takiguchi and colleagues found that while 1 week of exposure of TRL1215 rat liver cells to 0–2.5 microM cadmium resulted in decreased DNMT activity and global hypomethylation, 10 weeks of exposure resulted in enhanced DNMT activity and global hypermethylation (Takiguchi et al. 2003). This result was replicated in human embryo lung fibroblast cells in which long-term, low-dose exposure increased DNA methylation and DNA methyltransferase activity (Jiang et al. 2008) and in human B lymphoblast cells in which 3 months of 0.1 μM cadmium resulted in global hypermethylation and hypermethylation of the p16 promoter as well as decreased p16 mRNA expression and increased cell proliferation. Addition of a DNA demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC), diminished Cd-stimulated cell proliferation associated with p16 overexpression, providing evidence that chronic exposure of low dose cadmium could induce p16 promoter hypermethylation which may contribute to Cd-induced carcinogenesis (Yuan et al. 2013). These results were further supported by a study on hens in which ingestion of a diet spiked with cadmium for 60 days resulted in global hypermethylation as measured by HPLC and increased expression of DNMT1 and DNMT3a as measured by semi-quantitative RT-PCR in liver and kidney tissues (Zhang et al. 2009).

7.6 Nickel

Nickel occurs at very low levels in the environment, although it is ubiquitous in air, water, and food. Environmental exposure occurs through inhalation, ingestion, and dermal contact. The general population takes in the most nickel via food, but exposure also can occur via tobacco smoke and nickel-plated materials such as coins, steel, and jewelry (National Toxicology Program 2005).

The study on 892 women in Spain showed that nickel toenail concentration was positively associated with LINE-1 methylation ($\beta = 0.02$, $p = 0.004$) (Tajuddin et al. 2013). In another study on 63 steel workers in Italy, cumulative exposure to nickel was positively correlated with both H3K4 dimethylation ($\beta = 0.16$; 95 % CI, 0.01–0.3) and H3K9 acetylation ($\beta = 0.27$; 95 % CI, 0.01–0.54) (Cantone et al. 2011). However, a study on 45 Chinese nickel refinery workers and 75 unexposed subjects found that urinary nickel was positively associated with H3K4 trimethylation ($r = 0.4$, $p = 0.0008$) but negatively associated with H3K9 acetylation ($r = 0.1$, $p = 0.01$) in peripheral blood cell DNA (Arita). The authors of the latter study attributed the differences to the type of exposure to the subjects. Nickel exposure in a

steel plant (Cantone et al. 2011) is associated with exposures to other metals which may also affect levels of histone modification. Furthermore, both studies are relatively small, indicating the need for more detailed investigations in humans of metal and epigenetic modifications.

In vivo and in vitro experiments have revealed changes in DNA methylation in response to nickel exposure. In nickel-induced tumors of mice injected with nickel sulfide, all the tumors exhibited hypermethylation in the promoter of the tumor suppressor gene p16 (Govindarajan et al. 2002). In a model to study epithelial-mesenchymal transition (EMT) in human bronchial epithelial cells, nickel induced EMT phenotype marker alterations including increased decreased E-cadherin mediated by hypermethylation. Treatment with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine restored E-cadherin expression (Wu et al. 2012).

In addition to gene silencing by DNA methylation, other nickel-induced epigenetic changes include the global loss of histone acetylation in H2A, H2B, H3, and H4, and increases in the ubiquitylation of H2A and H2B, decreases in H3K4 methylation (Arita and Costa 2009). In addition, nickel increases H3K9 mono and dimethylation, both of which are critical marks for DNA methylation and long-term gene silencing (Chen et al. 2006; Yan et al. 2003). It has been reported that the mechanism by which Ni exposure decreases histone acetylation is by inhibition of histone acetyltransferase activity, but has no effect on histone deacetylase activity.

The mechanisms by which changes nickel induces these changes in histone modifications may include the ability of nickel ions to inhibit a class of histone H3K9 demethylases (Chen et al. 2006). Since H3K9 methylation is important for DNA methylation and long-term gene silencing, the observed increase in DNA methylation after nickel exposure may be through this mechanism.

Changes in miRNAs may also be involved in nickel-induced carcinogenesis. Malignant transformation of immortal 16HBE cells with Ni_3S_2 compounds resulted in hypermethylation and decreased expression of miR-203, which exhibits tumor suppressive activity (Zhang et al. 2013a, b). It is unknown but likely that methylation of other miRNAs would also be affected by nickel exposure as well.

7.7 Chromium

Chromium is an essential trace element in mammals for maintaining normal carbohydrate metabolism. Chromium(III) is found in moderate amounts in a wide range of foods (Anderson and Kozlovsky 1985). However, hexavalent chromium compounds are carcinogenic to humans (IARC 1990; National Toxicology Program 2005). The general population may be exposed to hexavalent chromium compounds by inhalation, ingestion of water, or dermal contact with products that contain hexavalent chromium like steel or pressure-treated wood (National Toxicology Program 2005). Epidemiologic studies investigating the risk of lung cancer in occupational settings have consistently demonstrated the relationship between hexavalent chromium exposure and lung cancer (Gibb et al. 2000; National Toxicology Program 2005).

Evidence on the involvement of epigenetic mechanisms in chromium-related illness is sparse. In human lung tumors, hypermethylation of the *p16* promoter region was found in Japanese workers exposed to hexavalent chromium; the effect was time- and dose-specific (Kondo et al. 2006). Also among these workers, the DNA mismatch repair gene *hMLH1*, *APC*, *and MGMT* were hypermethylated (Sun et al. 2009). Significant differences between the methylation frequencies of APC and hMLH1 were observed between lung cancers in chromate-exposed individuals compared to non-chromate exposed individuals. While 86 % of chromate lung cancers exhibited APC methylation, 44 % of non-chromate lung cancers did. Twenty-eight percent of chromate lung cancers exhibited MGMT methylation, whereas none of the non-chromate lung cancers did. Based on these findings, the authors suggested that chromium carcinogenesis in the lung is linked to the progressive methylation of some tumor suppressor genes, which may be related to genomic instability (Ali et al. 2011).

Human B lymphoblastoid cells and human lung cancer A549 cells exposed to 5–15 μM potassium dichromate or 1.25–5 μg/cm^2 lead chromate for 2–24 h exhibited global hypomethylation and cell cycle arrest (Lou et al. 2013). In the A549 cells, 24 h exposure to 10 μM chromate resulted in global H3K4 trimethylation and H3K9 dimethylation (Zhou).

7.8 Conclusions

As the field of epigenetics expands, the role of epigenetics in the induction of carcinogenesis by environmental toxicants will become better understood. This chapter has provided a plentitude of examples of studies in which epigenetic aberrations have been associated with risk or phenotype of environmental toxicant-related cancers. While observational human studies provide clues on potential roles for epigenetic mechanisms, in vivo and in vitro experiments are needed to provide rigorous scientific support for the purported effects. The effects of exposure to specific substances, like arsenic, can be evaluated individually in experiments, whereas they must be interpreted in the context of other exposures that may be present in human studies. For example, it is impossible to evaluate the association between changes in global DNA methylation and exposure to drinking water contaminated with arsenic without knowing what other substances are also present in the drinking water and thus adjusting for them.

Another common limitation in human observational studies is that measures of DNA methylation are most often made using peripheral blood cell DNA. This is a problem for two reasons. First, peripheral blood contains more than one cell type that can contribute DNA, and we already know that different cell types have different epigenetic profiles. Thus, reported changes in global methylation levels in exposed individuals may be indicative of changes in the blood cell population and not changes in methylation within each cell type. Second, the epigenetic profile of peripheral blood cells may be largely irrelevant to cancer development in the target

tissue of an environmental toxicant. For example, arsenic exposure increases one's risk for lung cancer, but analysis of the methylation state of one's blood may not be relevant to the epigenetic state of the person.

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Chapter 8
The Epigenome and Aging

Sangkyu Kim and S. Michal Jazwinski

Abstract The epigenetics of aging is a relatively new field. Global DNA methylation has been examined for some time; however, only recently have age-related differentially methylated regions been elaborated. These regions encompass genes that in some cases interact with longevity and disease-associated genes. Histone modifications have now become of interest in aging studies. Model organisms have provided substantial evidence that some of the enzymes that are involved in histone modifications play a role in longevity, and direct evidence of such a role for one of the sirtuins has been gathered in mice. A number of studies examining expression of microRNA during aging in various organisms, including human, point to the possibility that these gene regulatory molecules may also be involved in aging. A recent study of one such microRNA in mice substantiates such a role in cardiac aging. All of these epigenetic mechanisms are responsive to environmental and lifestyle factors. Thus, the groundwork has been laid for an understanding of the interface between the genome and the environment that epigenetic mechanisms provide.

Keywords DNA methylation • Histone modification • microRNA • Aging • Longevity

8.1 Introduction

Epigenetics, in its current understanding, involves changes in gene expression involving mechanisms that do not depend on changes in the nucleotide sequence of DNA. Epigenetic changes are often heritable. The epigenome is the collection of epigenetic marks across the entire genome. This chapter is devoted to epigenetics and the epigenome in relation to biological aging.

Epigenetics is a relatively new field, and in terms of its application to aging research it is the product of the past few years. Part of the interest in the epigenetics of aging is associated with the realization that aging involves subtle changes in gene

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expression across the genome, which occur over a lifetime. Epigenetic regulation of gene expression has the potential for leading to such subtle change. In addition, epigenetics can link environmental influences to the aging process. This is particularly important as genetic variation contributes only about 25–35 % of the variation in lifespan in humans (Christensen et al. 2006).

DNA methylation has been widely appreciated as an epigenetic modifier of gene activity for some time. The methylation status both genome-wide and of specific genes can be modified by diet, exercise, or drug treatment (Kirchner et al. 2013). Recent studies of the acute effects of exercise on promoter methylation in human skeletal muscle explain how DNA hypomethylation results in activation of muscle biogenesis (Barres et al. 2012). Similar effects have been found for modification of histones on specific genes that alters their expression resulting in changes in glucose utilization (McGee and Hargreaves 2004). In fact, DNA methylation and histone modification often work in tandem to alter promoter activity. Amazingly, DNA methylation followed rapidly by demethylation is involved in memory formation and consolidation (Miller and Sweatt 2007). Thus, epigenetic changes are even associated with cognitive activity.

There are several potential mechanisms of epigenetic gene regulation that may be active during aging. Here, we will discuss the three that are best established. They include DNA methylation, histone modifications, and microRNA.

8.2 Epigenetics and Environmental Effects on Aging

8.2.1 Chromatin Organization

Eukaryotic DNA complexes with histone and non-histone proteins to form chromatin (Marushige and Bonner 1971; Elgin and Weintraub 1975). The basic repeating unit of DNA organization is the nucleosome, but nucleosome compaction into higher orders of chromatin structure is not constant throughout the genome. Early microscopic observations of interphase chromosomes from eukaryotic cells revealed two visibly contrasting chromatin types: the densely stained heterochromatin and relatively lightly stained euchromatin (Elgin and Weintraub 1975). Heterochromatin is a tightly packed form of chromatin that is resistant to a number of DNA transactions, including transcription, recombination, replication, and DNA repair. In this regard, heterochromatin represents a “closed” or “inactive” state of chromatin. On the other hand, euchromatin is a lightly packed form of chromatin that is responsive to such DNA transactions. Therefore, euchromatin represents an “open” or “active” state.

Heterochromatin is limited to genomic regions containing highly repetitive DNA sequences such as centromeres, telomeres, and other, transposable-element derived regions. The effect of heterochromatin on gene expression is well illustrated by the position-effect variegation in the activity of a gene located in the vicinity of a heterochromatic region (Spofford and DeSalle 1991; Henikoff 1990). A classic example of this is the variegated expression of the white eye-color gene in fruit flies

(*Drosophila melanogaster*) displaying mottled white eyes (Judd 1955). Expression of the white gene located across a heterochromatin and euchromatin border is usually suppressed in some cells in a stochastic manner. A number of epigenetic factors, including histone modifiers, are involved in the transcriptional silencing at the white locus (Elgin and Reuter 2013). Other genomic regions may alternate between the two states of chromatin in a cell-type specific or developmentally regulated way. These changes in chromatin structure also involve various epigenetic factors without any changes in DNA sequences.

Thus in addition to being driven by the typical genetic mechanisms, gene expression and other genetic processes can be further regulated by epigenetic modifications. In fact, in many studies, epigenetics has been providing attractive answers to various questions for which traditional genetics fail to account. A fascinating paradigm in studying epigenetics of aging and environment is as follows: (1) epigenetic modifications occur in response to environmental changes, (2) epigenetic modifications alter gene expression, and (3) altered gene expression is associated with aging.

8.3 DNA Methylation

8.3.1 Effect on Gene Expression

8.3.1.1 CpG Dinucleotides and CpG Islands

Genomic DNA contains modified DNA bases in addition to the four normal bases. The most frequently found is 5-methylcytosine (5-mC), and its oxidized form, hydroxymethyl-cytosine (5-hmC) also occurs at a lower frequency. DNA methylation generally refers to the covalent addition of a methyl group to the five position of the cytosine typically found in CpG dinucleotides. However, DNA methylation can occur in a non-CpG context, such as CpA, CpC, or CpT, and such non-CpG methylation can be a more influential form of DNA methylation in certain cell types (Lister et al. 2013). CpG dinucleotides are often found in clusters, and short stretches of DNA of about 1 kb enriched in CpG dinucleotides are called CpG islands (CGIs) (Jones 2012; D'Aquila et al. 2013).

8.3.1.2 Correlation Between DNA Methylation and Gene Expression

DNA methylation plays an important role in modulating gene expression during cell development and differentiation. Many studies have shown that hypomethylation (under-methylation) is correlated with active gene expression, whereas hypermethylation (over-methylation) is correlated with inactive gene expression (Calvanese et al. 2009). In accord with this generalization, transcription start sites are usually devoid of DNA methylation in the human genome (Straussman et al. 2009). For instance, increased expression of the brain-derived neurotrophic factor (*BDNF*)

gene in neurons is linked with decreased CpG methylation of its promoter (Martinowich et al. 2003). In the human prefrontal cortex, however, there are CpG sites whose hypermethylation correlates with active gene expression (Numata et al. 2012). Therefore, the relationship between DNA methylation and gene expression is likely to vary across the genome in different cell types, and DNA methylation alone may not be a good predictor of gene expression.

The effects of DNA methylation on gene expression are well studied for CGIs because of their high CpG content and frequent overlap with regulatory DNA sequences including sites for RNA polymerase II binding and transcription initiation (Deaton and Bird 2011). CGIs are found not only in or near 5′ promoter regions of genes, but also inside genes or between genes, and transcription can initiate from these intragenic and intergenic CGIs as well (Deaton and Bird 2011). In human brain, only 2 % of 5′ promoter CGIs are methylated, which is well below the average proportion of methylated CGIs (16 %) among all the human brain CGIs examined (Maunakea et al. 2010). On the other hand, the proportion of intragenic CGIs that are methylated in the brain is 34 %, much higher than the average (Maunakea et al. 2010). Interestingly, intragenic DNA methylation correlates with increased, not decreased, transcription (Rauch et al. 2009).

In mammals, intragenic DNA methylation tends to be tissue- and cell type-specific with highly conserved sequences (Eckhardt et al. 2006; Illingworth et al. 2008). During cell and tissue differentiation, transcription initiation from the 5′ promoters in certain genes is repressed as their promoter CGIs become hypermethylated (Stein et al. 1982; Shu et al. 2006; Mohn et al. 2008). Instead, transcription is driven from non-5′ promoter CGIs within the genes (Maunakea et al. 2010). The tissue-specific pattern of intragenic CGI methylation corresponds well to tissue-specific gene expression. The presence of these "bivalent" CGI promoters has been documented in a number of studies (Deaton and Bird 2011). Bivalent promoters can alternate between transcriptionally active and inactive states because they contain corresponding epigenetic signatures.

8.3.1.3 DNA Methylation and Chromatin Structure

The effect of an epigenetic modification on gene expression is through its impact on chromatin organization. A prevailing notion about DNA methylation and chromatin structure is that DNA methylation is associated with the "closed" or "inactive" state of chromatin that hinders binding of transcription factors and RNA polymerase II to the promoter (Boyes and Bird 1991). A transgene introduced in a methylated intragenic region is subject to a position effect, resulting in a decrease in the transgene expression (Lorincz et al. 2004). As we will discuss more below, the relationship between DNA methylation and chromatin status may not be straightforward because complex arrays of both *cis*-and *trans*-acting factors are involved in chromatin organization. For example, transcriptional repression of methylated CpG sites may employ a protein complex involving the 5-mC binding protein MeCP2, histone deacetylases (HDAC), and the transcriptional co-repressor mSin3A (Nan et al.

1998; Grzenda et al. 2009). The increased expression of neuronal *BDNF* associated with decreased DNA methylation also involves dissociation of the MeCP2-HDAC-Sin3 repression complex from the promoter (Martinowich et al. 2003). Transcriptional silencing by DNA methylation is often aided by deposition of repressive histone modifications including H3K9me3 or H3K27me3 (Table 8.1) (Lenhard et al. 2012; Harmston and Lenhard 2013). On the other hand, DNA undermethylation tends to occur together with tri-methylated H3K4 (H3K4me3), both of which are permissive to transcription.

8.3.2 Factors Affecting DNA Methylation

After DNA replication, de novo DNA methylation takes places on newly synthesized DNA strands, which is catalyzed by three main DNA methyltransferases DNMT1, DNMT3A, and DNMT3B in mammals (Denis et al. 2011). The methyl group from 5-mC can be actively removed by DNA demethylases through a series of biochemical reactions. The multi-step demethylation involves oxidation of 5-mC to 5-hmC by tet methylcytosine dioxygenases (TET1, 2, 3) and removal of the oxidized form by such DNA repair enzymes as activation-induced cytidine deaminase (AICD) and thymide-DNA glycosylase (TDG) (Ito et al. 2010; Guo et al. 2011a; Wu et al. 2011; He et al. 2011). Thus, 5-hmC is regarded as an intermediate product of the demethylation of 5-mC residues. Recent studies indicate that 5-hmC may serve as another type of epigenetic modification (Szulwach et al. 2011a, b).

Remarkably, epigenetic changes are known to be stable enough to be heritable for a number of generations (Slatkin 2009). This indicates that epigenetic changes brought about by environmental cues can reach the germline and be passed on to subsequent generations. However, studies show that the epigenome is reprogrammed during gametogenesis and early development (Cantone and Fisher 2013). This poses a tantalizing question of how newly acquired epigenetic states are re-established after erasure and transmitted to the next generation. One possibility is that not all the epigenetic marks undergo the reprogramming at the same time, and the remaining marks may signal and recruit other epigenetic modifiers to re-establish the previous epigenetic states. Supporting this possibility is the co-existence of different types of epigenetic marks that have similar effects on gene expression (Meissner et al. 2008). Also, epigenetic marks are not always erased during the reprogramming (Wu and Zhang 2010; Martin and Zhang 2007).

Non-genetic factors affecting epigenetic modifications are diverse and variable, resulting in epigenetic patterns that can change over the life course and across different types of cells and tissues. Non-genetic factors affecting DNA methylation include nutrition, environmental chemicals, lifestyle, and lifetime experience, and the resulting phenotypes range from relatively simple phenotypic traits to complex pathological conditions including obesity, reproductive defects, and cancer (Feil and Fraga 2011). In addition, recent studies showed that some of the genetic factors affecting DNA methylation are also subject to epigenetic control. DNMT3

Table 8.1 Major histone modifications that have been associated with modulation of transcription and longevity (0 = repressive, 1 = activating, 3 = implicated in longevity). The core histones are H2A, H2B, H3, and H4. Lysine residues (K) are listed according to the amino acid position from the N-terminus of the protein. The modifications shown are mono-methylation (me1), di-methylation (me2), tri-methylation (me3), and acetylation (ac). In addition, histone H2A.Z is associated with active transcription, and poised promoters contain H3K4me1 (Rada-Iglesias et al. 2011), H3K4me2 (Ernst et al. 2011), H3K4me3 (Zhou et al. 2011a), K3K27me3 (Zhou et al. 2011a), or H2A.Z (Zhou et al. 2011a)

	H3						H4		H2B
Type of modification	K4	K9	K14	K27	K36	K79	K16	K20	K5
me1	1 (Zhou et al. 2011a)	1 (Barski et al. 2007)		1 (Barski et al. 2007)		1 (Barski et al. 2007); Steger et al. 2008)		1 (Barski et al. 2007)	1 (Barski et al. 2007)
me2	1 (Zhou et al. 2011a)	0 (Zhou et al. 2011a)		0 (Rosenfeld et al. 2009)		1 (Zhou et al. 2011a)			
me3	1 (Zhou et al. 2011a); 3 (Greer et al. 2010)	0 (Zhou et al. 2011a)		0 (Zhou et al. 2011a)	1 (Zhou et al. 2011a)	0 (Barski et al. 2007); 1 (Steger et al. 2008)			0 (Rosenfeld et al. 2009)
ac		1 (Koch et al. 2007)	1 (Koch et al. 2007)	1 (Zhou et al. 2011a)			1 (Dou et al. 2005); 3 (Dang et al. 2009)		

methyltransferases are transcriptionally regulated by DNA methylation (Numata et al. 2012), and TETs and TDG mRNA levels are regulated by miR-29, which is a member of another class of epigenetic modifiers called microRNA (Zhang et al. 2013a).

8.3.2.1 Nutrition

Nutrition can have a profound effect on various traits through DNA methylation. The methyl group used in DNA methylation is derived from dietary methyl-donors, such as folate and choline (Anderson et al. 2012; Gueant et al. 2014). Folate, first isolated from spinach leaves, is reduced to the active form tetrahydrofolate, and its methylated form, methyltetrahydrofolate (MTHF), functions as an intermediate methyl carrier. The methyl group of MTHF is donated to homocysteine, resulting in the synthesis of the essential amino acid methionine. Methionine is then converted to the final methyl donor S-adenosyl-methionine (SAM) for DNA methyltransferases (and histone methyltransferases as well). The methionine synthesis involves flavin adenine dinucleotide and vitamin B12 as cofactors. Alternatively, methionine can be derived from betaine, which is an oxidized form of dietary choline. Thus, nutrition can have significant effect on DNA methylation levels and patterns (Chiacchiera et al. 2013).

In mice, the agouti gene is responsible for coat color development. In addition to its own promoter, transcription of the agouti gene can be driven from a cryptic promoter embedded in a retrotransposon inserted upstream of the transcription start site of the gene. This version of the gene whose expression is controlled by the cryptic promoter is termed A^{vy} (Wolff et al. 1998; Morgan et al. 1999). When the A^{vy} allele is expressed during a specific developmental stage, a/a homozygotes develop the brown (agouti) coat color instead of the black color. Interestingly, the extent of DNA methylation in and near the cryptic promoter correlates with the variation in coat color ranging from yellow (hypomethylated) to full agouti (hypermethylated). Furthermore, when female mice were fed a diet rich in methyl donors, the coat color distribution of the newly born A^{vy}/a offspring mice was significantly shifted toward the full agouti color compared with that of the offspring born from female mice without the diet supplementation (Wolff et al. 1998; Waterland 2003).

Further evidence for the effect of nutrition on the epigenome and gene expression has been provided by dietary supplementation with genistein, the major phytoestrogen in soy. Supplementation of maternal diet with genistein during gestation resulted in a significant shift of the coat color of the newly born A^{vy}/a offspring mice toward pseudoagouti (Dolinoy et al. 2006). This effect of dietary genistein is similar to that of dietary methyl donors and is significantly associated with increased methylation of the cryptic promoter in the retrotransposon. Contrary to the effect of genistein, bisphenol A induces DNA hypomethylation of the cryptic A^{vy} promoter (Dolinoy et al. 2007).

8.3.2.2 Environmental Chemicals

Effects of prolonged-exposure to tobacco and alcohol use on DNA methylation have been documented (Kaminen-Ahola et al. 2010; Joubert et al. 2012; Kutay et al. 2012; Zhang et al. 2013b; Perkins et al. 2013; Buro-Auriemma et al. 2013; Breitling 2013; Shenker et al. 2013; Lee and Pausova 2013). Even transient exposure to environmental endocrine disruptors can have profound and long-lasting effects. Such chemicals include vinclozolin, an anti-androgenic compound, and bisphenol A, another environmental estrogen. Exposure of gestating female rats during the period of fetal gonadal development to vinclozolin resulted in development of various tissue abnormalities, including tumors, in the male rats up to the F4 generation (Anway et al. 2006a, b). The transgenerational abnormalities were in part due to altered DNA methylation patterns in the male germ line. Treatment of newborn rats with estradiol or bisphenol A resulted in elevated susceptibility of prostates to neoplastic lesions (Ho et al. 2006). This induced susceptibility was associated with constitutively elevated expression of *PDE4D4*, in which promoter CpG sites are hypomethylated. *PDE4D4* encodes a phosphodiesterase responsible for the breakdown of cyclic AMP, an important signaling molecule. In untreated normal prostates, the *PDE4D4* promoter undergoes gradual age-dependent hypermethylation.

8.3.2.3 Occupation and Lifestyle

Changes in social settings and various events occurring during the life course are known to affect epigenetic patterns. Physical exercise induces genome-wide changes in DNA methylation (Ronn et al. 2013; Zhang et al. 2011). Early-life experiences, such as variations in socio-economic status, childhood abuse, or maternal care, can have a long-term impact on adult life by marking the genome with DNA methylation and histone modification (Borghol et al. 2012; Lam et al. 2012; Szyf and Bick 2013; Bagot et al. 2012; Suderman et al. 2012). Genes that are known to undergo differential epigenetic marking caused by early-life experiences include *BDNF*, *AVP* (arginine vasopressin), *GRM1* (metabotropic glutamate receptor 1), and *NR3C1* (nuclear receptor subfamily 3, group C, member 1; glucocorticoid receptor) (Bagot et al. 2012; Suderman et al. 2012; McGowan et al. 2009; Roth et al. 2009; Murgatroyd et al. 2009). High-work stress correlates with decreased methylation of the promoter of *SLC6A4*, which encodes a serotonin transporter (Alasaari et al. 2012). Significant quantitative change in DNA methylation occurs in the oxytocin receptor gene after acute pychosocial stress (Unternaehrer et al. 2012).

8.3.3 Aging and Age-Related Changes

8.3.3.1 Complexity of Aging

Aging is a complex biological phenomenon, in which both genetic and non-genetic factors are involved, resulting in diverse age-related changes. Age-related changes include physical and functional deterioration of various biological systems, with the concomitant increase in the incidence of disabilities and diseases. The heritability of human longevity is estimated to be somewhere between 0.15 and 0.35 (McGue et al. 1993; Herskind et al. 1996; Ljungquist et al. 1998; Gudmundsson et al. 2000; Kerber et al. 2001; Mitchell et al. 2001), which implies that the bulk of the phenotypic variation in human aging is dependent on non-genetic factors. The significant contribution of non-genetic factors to human aging is evident in relatively high degrees of discordance (phenotypic dissimilarity) of various traits between affected monozygotic (MZ) twins.

8.3.3.2 Age-Dependent Changes in DNA Methylation

There is mounting evidence showing that DNA methylation changes as chronological age advances. In a longitudinal follow-up of a cohort, DNA methylation status significantly changed at more than 10 % of the CpG sites in about a third of the individuals investigated (Bjornsson et al. 2008). Although less powerful compared with longitudinal studies, cross-sectional studies have been useful in compiling age-dependent differences in DNA methylation. In general, less genome-wide DNA methylation is found in older people across many tissues, although there can be locus-specific hypermethylation (Calvanese et al. 2009). DNA methylation content was significantly lower in centenarian and nonagenarian genomes than in more homogeneously methylated newborn genomes (Heyn et al. 2012). DNA methylation levels in such abundant repetitive elements as Alu and LINE-1 progressively decline over age (Bollati et al. 2009). Thus, hypomethylation seems to be a global process occurring in the aging genome.

What is the biological significance of genome-wide age-dependent loss of DNA methylation? If we simply apply the hypomethylation-higher gene expression notion, the age-dependent global hypomethylation will mean higher genome-wide gene expression at older ages, which, coupled with hypermethylation of tumor-suppressor genes (Esteller 2007), may underlie the higher incidence of age-related diseases later in life. In addition to its effect on gene dysregulation, global hypomethylation is likely to incur genome instability, another hallmark that is frequently associated with carcinogenesis. Interestingly, the genome-wide DNA hypomethylation is

delayed in centenarians' offspring compared with the offspring of short-lived parents (Gentilini et al. 2013). Offspring of long-lived parents are healthier than offspring of short-lived parents (Gentilini et al. 2013; Kim et al. 2013). Therefore, an attractive hypothesis is that the age-related loss of DNA methylation may be causally linked to the development of age-associated diseases (Gentilini et al. 2013). Further insight awaits more studies.

Not all the genomic sites undergo hypomethylation, and certain genomic regions may even become hypermethylated with age. In a cross-sectional study of 217 non-pathologic human tissues, DNA methylation of promoter CGIs increased with age, while non-CGI DNA methylation decreased (Christensen et al. 2009). In a genome-scale DNA methylation profiling of 93 females ranging from 49 to 75 years of age, some promoter CpGs showed increasing DNA methylation in older individuals, while other promoter CpG sites showed decreasing DNA methylation (Rakyan et al. 2010). Another recent study involving 421 subjects ranging in age from 14 to 94 also showed the age-dependent change in DNA methylation in either direction (Johansson et al. 2013). Therefore one can conclude that the aging genome tends to lose DNA methylation, but certain genomic regions may gain DNA methylation over time.

The loss of genome-wide DNA methylation over age could be due to decreased activity of DNMT1, which is the major maintenance methyltransferase (Lopatina et al. 2002; Casillas et al. 2003). Interestingly, the same studies found the activity of DNMT3b, a de novo methyltransferase, markedly increased in aged and immortalized cells. It was postulated that the increased activity of DNMT3 may be responsible for the locus-specific hypermethylation (Lopatina et al. 2002). Hyperactivity of DNMT3b in aged and immortalized cells could be a compensatory response to the loss of DNMT1 activity, resulting in aberrant hypermethylation with pathological consequences (Casillas et al. 2003; Fraga and Esteller 2007).

8.3.3.3 Age-Dependent Divergence of DNA Methylation

DNA methylation patterns in identical twins increasingly differ as a function of age, which is termed "epigenetic drift" (Fraga et al. 2005; Boks et al. 2009). MZ co-twins who are 50 years-old are markedly different from each other in the amount and distribution of 5-mC, whereas the differences in 3 year-old MZ twins are negligible (Fraga et al. 2005). DNA methylation differences are already apparent even in young MZ twins of ages between 5 and 10 years (Wong et al. 2010). Also older individuals exhibit significant tissue-specific variations in their overall DNA methylation patterns. Consistent with the epigenetic differences, differences in gene expression are larger in the older twins than in the young pairs (Kaminsky et al. 2009). These studies demonstrate that epigenetic changes accumulate during the lifetime of individuals resulting in divergent gene expression patterns (Fraga and Esteller 2007).

Both inherent genetic differences and exposure to different non-genetic factors contribute to the epigenetic drift (Fraga et al. 2005; Boks et al. 2009; Hannum et al.

2013). In the case of MZ twins, the degree of epigenetic drift between two MZ co-twins is ascribed mostly to the exposure to unshared environmental factors. As expected, the epigenetic difference between MZ co-twins is less extensive than the difference between DZ twins (Fraga et al. 2005; Gervin et al. 2012). In fact, in spite of the epigenetic drift that occurs at the whole genome level, old MZ twins show remarkably similar DNA methylation patterns at certain genomic sites (Talens et al. 2012). In addition, epigenetic differences may arise randomly from unidentifiable stochastic events, as shown using experimental organisms with the identical genetic background in the same environment (Xie et al. 2011).

8.3.3.4 Functional DMRs

Our ultimate interest is to establish the causal relationship between epigenetic changes and aging. One approach to this is to find age-related differentially methylated regions (aDMRs) and determine whether these aDMRs are functionally associated with biological aging. In a study of 172 female twins, only 0.2 % of aDMRs were associated with age-related phenotypes (Bell et al. 2012). The age-related phenotypes included in this study were telomere length, blood pressure, lung function measures, hand grip strength, several clinical laboratory tests, and parental longevity status. In a separate study involving larger numbers of subjects and methylation probes, about 10–15 % of the CpG marks examined were age-associated (Hannum et al. 2013). In this latter study, however, diabetes status was used as the sole age-related phenotype, and this is probably the reason for the much higher frequency of aDMRs compared to the aforementioned study above (Bell et al. 2012). Among the age-associated CpG marks, a set of 71 methylation marks was found to be highly associated with age and predictive of chronological age with high accuracy. Most of these marks lie within or near genes whose functions have been linked to aging-related conditions, such as Alzheimer's disease, cancer, tissue degradation, DNA damage, and oxidative stress.

One category of age-related changes is the appearance of age-related diseases, notably cancer. Therefore, many aDMRs are expected to be associated with this age-related pathology. In pluripotent stem cells, there are genes whose expression is required for differentiation but are repressed by Polycomb group (PcG) proteins (see below) (Lee et al. 2006). Promoters of these PcG target genes become hypermethylated not only in older people (Teschendorff et al. 2010) but also in various types of cancer cells (Ohm et al. 2007; Widschwendter et al. 2007). This hypermethylation coincides with lower expression of the genes in cancer cells (Ongenaert et al. 2008; Ben-Porath et al. 2008). Interestingly, a number of these PcG target genes in human embryonic stem cells bear similar DNA methylation patterns to those present in various preneoplastic tissue samples. This finding lends support to the hypothesis that cancer originates from aging stem cells (Teschendorff et al. 2010).

What is the biological role of the genes in aDMRs? In a comprehensive protein interaction network generated for the human genome, genes in aDMRs largely

occur in the peripheral positions with low centrality and connectivity, whereas longevity-associated genes occupy more central positions (West et al. 2013). Thus, genes in aDMRs may be regarded as less important for survival compared with longevity-associated genes. However, many of the genes in aDMRs form larger subnetworks with longevity and disease-associated genes than expected by change alone, indicating that these genes in aDMRs may frequently interact with the longevity- and disease-associated genes.

8.4 Histone Modification

8.4.1 Histones in Chromatin

Histones are the major chromatin proteins responsible for structural organization of long DNA molecules within nuclei (Campos et al. 2010). The basic repeating unit of DNA organization is the nucleosome, which consists of approximately 147 base pairs of DNA wrapped around a core of 8 histones (2 copies of histones H2A, H2B, H3, and H4) and a stretch of linker DNA and a linker histone (H1) (Luger et al. 1997). The N-terminal tails of the core histones are prone to various post-translational modifications, which can alter the DNA-protein interactions in nucleosome assembly as well as the protein-protein interactions in higher-order chromatin structures (Smolle and Workman 1829; Cosentino and Mostoslavsky 2013). Thus, histone modification, along with DNA methylation, constitutes the molecular basis for heterochromatin organization.

Most of the histone-tail modifications occur at lysine residues, especially in the highly basic segments of the tails, and these lysine modifications include acetylation, methylation, ubiquitination, and sumoylation (Zentner and Henikoff 2013). Other amino acids are also subject to modification, such as serine/threonine/tyrosine phosphorylation and proline isomerization. Certain arginine residues in H2B are required for normal silencing and longevity in yeast, but the molecular basis for this is not clear (Dai et al. 2010). Nucleosomes may incorporate variants of the common histones, such as H2A.Z and H3.3. These variant histones are known to have distinct nucleosome assembly pathways with specialized functions (Luger et al. 1997; Henikoff et al. 2004; Subramanian et al. 2013).

8.4.1.1 Promoter Settings

A fruitful approach to understanding the roles of various epigenetic modifications in transcriptional modulation is to profile epigenetic marks in promoter regions and see how they relate to transcription initiation. Promoters of multicellular organisms can be classified into three main types depending on the settings of epigenetic features around the core, proximal elements, and distal enhancers (Lenhard et al. 2012;

Harmston and Lenhard 2013). Type I promoters display narrow transcription start sites with "less organized" or "disordered" nucleosomes (Rach et al. 2011). Devoid of CGIs nearby, these promoters are regulated only by their core and proximal promoter elements and typically found in genes that yield tissue-specific expression (Roider et al. 2009). Type II promoters display broad transcription start sites with "well-positioned", "ordered" nucleosomes (Rach et al. 2011). They are located near CGIs and are found in ubiquitously expressed house-keeping genes. Type III promoters are associated with developmentally regulated genes. Epigenetic marks found in these promoters are more variable to accommodate differential gene expression. Type III promoters often contain both active and inactive histone domains together (Zhou et al. 2011a). These bivalent promoters can switch from "poised" to an active or inactive state or from active/inactive to inactive/active state during different developmental stages. They typically have long or multiple intragenic CGIs and are often further regulated by distal enhancers that also interact with many different transcription factors and chromatin modifiers.

8.4.1.2 Histone Marks, Chromatin Structure, and Gene Expression

The promoters described above are associated with discernible patterns of histone modifications. The hypothesis of a "histone code" predicts that distinct histone modifications, individually or in combination, dictate structural and thus functional states of chromatin (Strahl et al. 1999; Strahl and Allis 2000; Jenuwein and Allis 2001). Some examples of the histone code are mono- or tri-methylation of H3K4 (H3K4me1 or H3K4me3) found in actively transcribed promoters and H3K9me3 and H3K27me3 in constitutively repressed promoters (Table 8.1). Demethylation of these sites reverses the effect of the modification on transcription of the gene. For example, demethylation of H3K4me1 by KDM1A (lysine-specific demethylase) in embryonic stem cells is essential for full repression of the genes associated with the modification (Whyte et al. 2012). Demethylation of H3K9me2 (or me3) results in activation of androgen-receptor target genes (Metzger et al. 2005). Interestingly, phosphorylation of threonine 6 of H3 (H3T6) by protein kinase C beta I blocks demethylation of methylated H3K4, while a knockdown of the kinase enhances demethylation of methylated H3K4, resulting in inhibition of androgen receptor-dependent transcription (Metzger et al. 2010). The latter study also provides an example of interdependence of histone modifying enzymes. All the known histone-tail acetylations, such as H3K4ac, H3K9ac, and H3K14ac, are found in active promoters.

One notion that emerges from this complex array of epigenetic modifications, especially from those found in Type III promoters, is that expression of a gene in a particular cell type at a particular moment is an outcome of the concerted action of a multitude of *cis*- and *trans*-acting factors, both genetic and epigenetic, on its chromatin state (Cedar and Bergman 2009). Epigenetic protein factors involved in DNA and histone modifications are often found together in larger protein complexes. As seen from the histone code, histone deacetylases and DNA methyltransferases

interact together to augment transcriptional silencing (Fuks et al. 2001). In fact, DNA and histone modifying enzymes are part of large multiprotein complexes. There are a number of such well-known complexes that play critical roles for appropriate development and differentiation.

8.4.1.3 The NuRD (Nucleosome Remodeling and Histone Deacetylases) Complex

This complex contains six core subunits, and two of them are an ATPase and a histone deacetylase (Allen et al. 2013). The other four subunits are non-enzymatic but important for DNA and protein interactions. Of these, the MBD2/3 subunit is for 5-mC DNA binding. Therefore, the NuRD complex is thought to recognize acetylated histones and methylated DNA and actively modify chromatin to activate or repress gene transcription. The histone lysine demethylase KDM1A (also known as LSD1) is physically associated with the NuRD complex.

8.4.1.4 The SWI/SNF Complex

The SWI/SNF complex uses ATP to reposition nucleosomes (Euskirchen et al. 2012; Shain and Pollack 2013). SWI/SNF complex purified from a human cell line contains about ten polypeptides and has a molecular mass of ~2 MDa (Wang et al. 1996). Initial association of SWI/SNF with chromatin is thought to be aided by epigenetic modifications. Some subunits including the ATPase subunits contain bromodomains that are known to recognize acetylated lysines in histones, and others contain chromodomain-related residues (Brehm et al. 2004). Proteins containing chromodomains are known to bind methylated histones, such as H3K9me (Nielsen et al. 2002; Jacobs and Khorasanizadeh 2002).

8.4.1.5 The Nucleolar Remodeling/Silencing Complexes

The ribosomal DNA locus consists of several hundred repeats of transcription units and is subject to transcriptional silencing (Strohner et al. 2004; Santoro et al. 2002). The epigenetic modulation of the mammalian rDNA locus is controlled by protein complexes, such as NoRC (nucleolar remodeling complex) and eNoSC (energy-dependent nucleolar silencing complex). These protein complexes contain histone deacetylases and methyltransferases, and of the HDACs, SIRT1, a NAD^+-dependent histone deacetylase, is the key component in the eNoSC complex (Salminen and Kaarniranta 2009).

8.4.1.6 Polycomb Group (PcG) Proteins

Polycomb group (PcG) proteins function as transcription repressors through chromatin modification and compaction (Simon and Kingston 2013; Aloia et al. 2013). PcG proteins are found in several multiprotein complexes (Simon and Kingston 2013), the best characterized of which are Polycomb repressive complexes 1 and 2 (PRC1 and PRC2) (Margueron and Reinberg 2011). PRC1 represses transcription by mediating ubiquitylation of histone H2A (Stock et al. 2007) or by chromatin compaction (Francis et al. 2004). PRC2 is responsible for methylation of lysine 27 of histone H3 (H3K27me2/me3), which act as repressive epigenetic marks (Cao et al. 2002; Czermin et al. 2002; Kuzmichev et al. 2002; Muller et al. 2002). Studies also indicate that recruitment of PcG proteins to their target may be guided by non-coding RNAs (Brockdorff 2013).

8.4.1.7 Trithorax Group (TrxG) Proteins

TrxG proteins are known to function in opposition to PcG proteins (Schuettengruber et al. 2007, 2011). PcG proteins are typically associated with gene silencing, whereas TrxG proteins are most commonly linked to gene activation. The TrxG proteins can be arranged into three groups: one group contains the SET domain and methylates histone tails, another group consists of proteins capable of ATP-dependent chromatin-remodeling, and the third group contains proteins that can bind specific DNA sequences and displays some histone and chromatin modifying functions. Most of these TrxG proteins exist in large protein complexes. The histone modifications by TrxG proteins include trimethylation of H3K4 (H3K4me3), which is often reinforced by acetylation of histone H4 (H4K16) by a histone acetyltransferase (Dou et al. 2005).

The effect of epigenetic modification is not limited to transcriptional modulation by affecting the binding of the transcriptional machinery to the chromatin. For instance, histone modification can affect alternative splicing of pre-mRNA (Luco et al. 2010). Also, epigenetic modification has the potential to influence other cellular processes. As mentioned above, TET proteins oxidize 5-mC to several oxidized forms including 5-hmC, and these oxidized derivatives attract proteins involved not only in transcription but also in DNA repair (Spruijt et al. 2013). The histone acetylase GCN5 is involved in DNA replication, DNA damage response, as well as in nucleosome assembly (Burgess and Zhang 2010; Burgess et al. 2010).

8.4.2 Histone Modifications and Aging

Many genetic and environmental factors affect histone modification. Most of the environmental factors that are associated with DNA methylation are likely to have an effect on histone modification. This is particularly true with the dietary methyl

donor folate and related cofactors because the same biochemical pathways are involved in both DNA and histone methylation (Chiacchiera et al. 2013). Metabolic intermediates that influence histone acetylation status include nicotinamide adenine dinucleotide (NAD^+) as a cofactor for histone deacetylases and acetyl coenzyme A (acetyl-CoA) as the acetyl donor (Cosentino and Mostoslavsky 2013). In addition, as described above, there are many factors directly or indirectly involved in post-translational modifications of histones (Huidobro et al. 2013), and their altered functions have the potential to affect chromatin structure. For example, small interfering RNAs (siRNA) can induce chromatin modification through histone methylation at H3K9 (Gu et al. 2012).

8.4.2.1 Histone Methylation

Histone methyltransferases can transfer up to three methyl groups to the target lysine residue, and the number of methyl groups may have different consequences. Thus, H3K9me1 is transcription-activating, but both H3K9me2 and H3K9me3 are repressive (Table 8.1) (Barski et al. 2007). Also, as mentioned above, H3K4me3 is a mark associated with active chromatin and is generated by the TrxG complex. A defect in this TrxG complex results in lifespan extension in the soil nematode *Caenorhabditis elegans*, indicating that loss of active chromatin is conducive to longevity (Greer et al. 2010, 2011; Larson et al. 2012). Consistent with this observation, histone levels decrease with normal aging, and increased histone expression extends yeast replicative lifespan (Feser et al. 2010). Interestingly, genetically wild-type descendants from the parents defective in the TrxG complex also showed extended lifespan for several generations, providing an example of transmission of epigenetic marks for longevity (Greer et al. 2011).

8.4.2.2 Histone Acetylation

Histone lysine acetylation by histone acetyltransferases (HATs) is associated with an open chromatin state, allowing enhanced transcription (Roth et al. 2001; Szerlong et al. 2010). HATs can be grouped into two families based on their cellular locations: the nuclear GCN5-related N-acetyltransferases (GNATs) family and the cytoplasmic MYST family. The MYST family contains the founding members MOZ, Ybf2, Sas2, and Tip60 (Lee and Workman 2007). Another feature that separates these two groups is that GNATs contain a bromodomain, while MYST HATs contain zync fingers and chromodomains. GNATs usually interact with a number of transcription activators and participate in coordination of cell growth with mitochondrial metabolism (Cosentino and Mostoslavsky 2013).

A good example of a GNAT is Gcn5 associated with SAGA or SLIK (SAGA-like) complexes along with a number of other transcription-regulatory subunits (Grant et al. 1997; Pray-Grant et al. 2002). The role of *GCN5* in lifespan was examined in yeast: Deletion of *GCN5* suppresses the replicative lifespan extension resulting

from the retrograde response, through which mitochondrial dysfunction leads to changes in nuclear gene expression (Kim et al. 2004; Jazwinski 2005). On the other hand, deletion of *SAS2*, one of the MYST family members, decreases the level of acetylated H4K16, with a concomitant increase in lifespan (Dang et al. 2009). The effect of *SAS2* deletion fits the general theme that failure to maintain appropriate levels of heterochromatin and transcriptional silencing is deleterious to cell survival and longevity. The effect of *GCN5* deletion, however, doesn't appear to fit the theme, and one possible reason for this is that Gcn5 functions in multi-subunit complexes in which individual subunits are dependent on each other, so the functions of other subunits are affected by the absence of Gcn5.

8.4.2.3 Histone Deacetylation

Sir2 in Model Organisms Removal of the acetyl group from lysine residues in histones is catalyzed by histone deacetylases, leading to heterochromatization and transcription repression. The yeast *Saccharomyces cerevisiae* chromatin modifier Sir2 has drawn much attention to HDACs, since it was shown to be involved in yeast replicative lifespan and found to be an HDAC (Kaeberlein et al. 1999; Imai et al. 2000; Kim et al. 1999). Sir2, silent information regulator 2, was originally identified as one of the regulators of transcriptional silencing (Rine and Herskowitz 1987). Sir2 has an NAD^+-dependent deacetylase activity, and this activity is required for transcriptional silencing, suppression of rDNA recombination, and lifespan extension (Imai et al. 2000; Lin et al. 2000). The presence of NAD^+ as a cofactor is also essential for the effects of Sir2, and overexpression of Pnc1 or Npt1 involved in NAD^+ biosynthesis increases silencing and lifespan (Anderson et al. 2002, 2003). These results indicate that Sir2 activity can be modulated by cellular levels of NAD^+.

Sir2 is one of the proteins that are abundantly expressed in long-lived yeast strains (Guo et al. 2011b). The Sir2 level decreases with replicative age, which is accompanied by an increase in H4K16ac and loss of histones at specific subtelomeric regions in old cells (Dang et al. 2009). Related to the loss of histones in old cells, increased expression of histones extends lifespan (Feser et al. 2010; Feser and Tyler 2011). These results indicate that heterochromatin formation is beneficial to longevity (Larson et al. 2012). The effect of Sir2 through histone deacetylation on yeast replicative lifespan is antagonized by Sas2, the major H4K16 acetyltransferase (Dang et al. 2009).

The function of Sir2 seems conserved across different species, but its role in longevity needs to be further corroborated. In *C. elegans*, a duplicated copy of a yeast *SIR2* homolog extended lifespan through the insulin/insulin-like growth factor 1 (IGF1) pathway (see below) (Tissenbaum and Guarente 2001). A similar lifespan extension was observed by overexpression of the Sir2 homolog of the fruit fly *Drosophila melanogaster* (Rogina and Helfand 2004). However, these results from the nematodes and flies were not repeatable when the genetic background of the

experimental strains was adjusted and proper controls were used (Burnett et al. 2011). More recent studies found that the effect of Sir2 in these model organisms is highly dose-dependent. Increased expression of *Drosophila* Sir2 by two to fivefold extended lifespan, but higher expression over the range actually caused cellular toxicity and decreased lifespan (Hoffmann et al. 2013; Whitaker et al. 2013). More studies are needed to gain insight into the discrepancies.

Many nematode mutations that result in lifespan extension are weak mutations that lower the activity of the insulin/IGF1 pathway (Kenyon 2001). The lifespan extension by mutations in the insulin/IGF1 pathway requires activation of a downstream transcriptional regulator DAF-16, which is a member of the Forkhead box-O transcription factor (FOXO) family (Murakami and Johnson 1996; Lin et al. 1997). DAF-16 regulates expression of many genes including those involved in metabolism and stress-protective responses (Sampayo et al. 2003; Murphy 2003). Interestingly, DAF-16 forms a complex with the SWI/SNF chromatin remodeler at the DAF-16 target promoters, and the complex activates transcription presumably by chromatin remodeling (Riedel et al. 2013).

The effect of histone deacetylation is not limited to Sir2 and its closely related homologs. Unlike other types of HDACs, NAD^+-dependent HDACs are not inhibited by an antifungal antibiotic called trichostatin A (TSA) (Yoshida et al. 1990). Of the HDACs that are sensitive to TSA, the budding yeast has Rpd3, whose activity is not dependent on NAD^+ (Rundlett et al. 1996). Deletion of *RPD3* extends yeast lifespan (Kim et al. 1999), and partial reduction of the Rpd3 level increases lifespan in fruit flies (Rogina et al. 2002).

Sirtuins Mammals contain seven homologs of Sir2, each containing a highly conserved NAD^+-dependent sirtuin core domain (Frye 2000; Haigis and Sinclair 2010). Of the seven, the nuclear SIRT1 has been most extensively studied so far. SIRT1 is involved in various biological pathways, encompassing energy metabolism, regulation of oxidative damage, DNA repair, genome stability, and cell survival (Park et al. 2013). Regulation of these pathways involves transcriptional modulation of genes by deacetylation of histones near the gene promoters or by direct deacetylation of gene products.

There are a number of important protein targets of SIRT1. For example, calorie restriction (CR) leads to elevated NAD^+ levels and activation of SIRT1, which in turn activates transcriptional co-activator PGC-1α encoded by PPARGC1A (peroxisome proliferator-activated receptor gamma, coactivator 1 α) by deacetylation. PGC-1α plays an important role in energy metabolism by upregulating expression of genes involved in mitochondrial biogenesis and function, for increased respiration and ATP production (Nemoto et al. 2005; Rodgers et al. 2005). Interestingly, PGC-1α is acetylated and deactivated by GCN5 (Lerin et al. 2006). Thus, the activity of PGC-1α is controlled by SIRT1 and GCN5, the activities of which are in turn modulated by the cellular energy status; SIRT1 is regulated by the intracellular level of NAD^+ and GCN5 by that of acetyl-CoA (Jeninga et al. 2010). Other important targets of SIRT1 include the FOXO transcription factors and p53 (Frescas et al. 2005; Luo et al. 2001; Vaziri et al. 2001). FOXO3A has been shown to be associated

with human longevity in several population studies (Willcox et al. 2008; Flachsbart et al. 2009; Anselmi et al. 2009; Li et al. 2009).

SIRT1 also deacetylates histones. It binds to the promoter of the p66Shc gene and downregulates its expression by deacetylating histone H3 (Zhou et al. 2011b; Chen et al. 2013). The p66shc protein is a cytoplasmic protein that converts oxidative signals into apoptosis (Migliaccio et al. 1999). It regulates intracellular levels of reactive oxygen species (ROS) and oxidative-stress-induced apoptosis (Migliaccio et al. 1999; Trinei et al. 2002; Nemoto and Finkel 2002). Homozygous deletion of p66shc in mice induces stress resistance and prolongs the lifespan by 30 % (Migliaccio et al. 1999).

SIRT1 also facilitates DNA repair. Mouse embryos with SIRT1 deficiency by mutation undergo early death, with impaired DNA damage response and repair function (Wang et al. 2008). Upon DNA damage, SIRT1 relocates to damaged sites and promotes damage repair and genome stability (Oberdoerffer et al. 2008). This is reminiscent of Sir2 relocating from its usual binding sites to DNA break sites in yeast (Martin et al. 1999; McAinsh et al. 1999; Mills et al. 1999).

SIRT1 is also associated with circadian physiology. CLOCK, one of the core circadian regulators, is a histone acetyl transferase sharing homology with the MYST HATs, which indicates that chromatin remodeling is associated with circadian physiology (Doi et al. 2006). CLOCK dimerizes with a transcription factor BMAL, and the heterodimers display enhanced activity for acetylation of the circadian promoters of CLOCK targets. Interestingly SIRT1 is also recruited to the circadian promoters via the CLOCK-BMAL heterodimer. Knockout of SIRT1 results in dysregulated circadian cycles and disturbed acetylation of BMAL (Nakahata et al. 2008).

In addition, there is a wealth of reports showing the protective role of SIRT1 against various age-related changes including such pathological conditions as diabetes, cardiovascular disease, neurodegenerative disorders, and various types of cancer (Herranz et al. 2010; Herranz and Serrano 2010). In spite of its role in various cellular processes of high importance, the function of SIRT1 in mammalian longevity is not definitive yet (Park et al. 2013). In one population study, an allele of a *SIRT1* SNP was significantly associated with protection against telomere shortening and longevity (Kim et al. 2012a). Recently *SIRT6*, another nuclear sirtuin required for transcriptional silencing (Tennen et al. 2011), was shown to extend lifespan of transgenic mice overexpressing Sirt6 (Kanfi et al. 2012). The lifespan extension was accompanied by lower serum levels of insulin-like growth factor 1 (IGF1). It is puzzling, however, that all these effects were limited to male mice (Kanfi et al. 2012).

8.5 Micro-RNAs and Aging

8.5.1 The Non-coding RNA World

Non-coding RNAs (ncRNA) have been around for a long time. After all, ribosomal RNA (rRNA) and transfer RNA (tRNA), along with the coding messenger RNA (mRNA), are the stuff of classic molecular biology. However, these well-known

entities have been joined by an expanding plethora of ncRNA in recent years. There is a vast expanse available in the human genome to accommodate this 'dark world' of RNA, as it has been called. The human genome contains some 20,000–25,000 protein-coding genes that have a coding length of approximately 34 Mb (Qu and Adelson 2012). This constitutes less than 2 % of the genome. The non-coding transcriptome is about 50-times larger, occupying some 60 % of the genome, and by some estimates as much as 85 %. It has become apparent that the new denizens of this transcriptome possess regulatory functions.

Among the inhabitants of the genomic 'dark world' are the easily recognizable small interfering RNA (siRNA) and micro-RNA (miRNA). They, together with PIWI-interacting RNA (piRNA), span the 20–30 nucleotide length range, and they anchor the small ncRNA category (Qureshi and Mehler 2012). These small ncRNA are involved in post-transcriptional regulation of mRNA via RNA interference (RNAi). In the case of siRNA and piRNA, this includes the silencing of transposons. However, the diversity of small ncRNA does not end there. They have been joined by numerous other species that have either regulatory roles, structural roles at heterochromatic regions of chromosomes, or whose roles are unknown.

Long non-coding RNAs (lncRNA) have begun to draw more interest in the past few years. These are classified as molecules of >200 nucleotides in length (Qureshi and Mehler 2012). Some are complementary to coding regions of the genome, and they may possess antisense activity. Others appear to act over a long distance in epigenetic regulation of chromatin or as scaffolds for nuclear macromolecular complexes. They are encoded in intergenic regions of the genome. Recently, lncRNA have been implicated in aging (Abdelmohsen et al. 2013). This result was obtained in a population-doubling paradigm of senescence of normal human cells in tissue culture (Hayflick and Moorhead 1961). It employed RNA sequencing (RNA-seq) to identify differentially expressed lncRNA species of various categories in young and old cells. A novel lncRNA was found among several known species, and it was shown to play a causal role in delaying senescence. This study opens up the lncRNA arena to aging studies. Here, we will, however, review the better elaborated roles of miRNA in longevity and aging.

8.5.2 miRNA Biogenesis and Activity

miRNA were discovered in *Caenorhabditis elegans* in 1993 (Lee et al. 1993). They are ubiquitously present throughout phylogeny in plants, animals, and fungi. In humans, over 1,000 different miRNA have been found (Watanabe and Kanai 2011). miRNA are often first identified from genomic DNA sequence using computational methods, and not all such miRNA have been functionally validated.

miRNA coding sequences occur in clusters throughout the genome, in intergenic regions, although some can also be found in introns. The regulation of their transcription is not well understood. Several well-known transcription factors that regulate miRNA transcription have been identified. They include c-myc, p53, HIF-1,

and NFκB, which can variously have positive or negative effects on transcription (Sun et al. 2010). The promoters for miRNA can be within a few kb or as much as over 50 kb upstream of the coding region for the miRNA cluster. Among the genes regulated by miRNA are transcription factor genes. Thus, the possibility of intricate regulatory networks springs to mind.

miRNA are transcribed as primary transcripts (pri-miRNA) by either RNA polymerase II or III, depending on the particular miRNA. This is processed by an RNase III called Drosha, which exists in a complex with another protein called DGCR8 or Pasha (Qureshi and Mehler 2012; Sun et al. 2010). Drosha/DGCR8 cleaves the surrounding RNA to release a 60–80 nucleotide hairpin stem-loop from the pri-miRNA, yielding a pre-miRNA with a 22 bp stem-loop and a 2-nucleotide 3′-overhang (Fig. 8.1). This pre-miRNA is exported to the cytoplasm. There, it is further processed by another RNase III called Dicer resulting in an approximately 22 bp miRNA. This double-stranded molecule is unwound. One strand becomes the mature miRNA, while the other strand is rapidly degraded. The mature miRNA is packaged into a ribonucleoprotein complex called miRNA-induced silencing complex (miRISC) that also contains a member of the Argonaute (Ago) protein family. The regulation of all of these processes is under active investigation.

miRNA target the 3′-untranslated regions (3′-UTR) of mRNA (Fig. 8.1). This is achieved through base-pairing of a two to eight nucleotide seed sequence close to the 5′-end of the miRNA, followed by stabilization due to base-pairing of the nucleotides in the 3′-half of the miRNA with the 3′-UTR of the mRNA (Sun et al. 2010). In animals, there are mismatches in the duplex formed by the miRNA and the 3′UTR of the mRNA, often resulting in loops. However, these cannot occur in the region of contact with Ago within the miRISC. An individual miRNA can target many different mRNA, and a given 3′UTR can have more than one binding site for a particular miRNA. Conversely, a given 3′-UTR can be targeted by more than one miRNA.

Ago in the miRISC can cleave the miRNA-mRNA duplex, but it does not attack the single-stranded mRNA. It can also repress protein translation (Sun et al. 2010). Plants display a high level of complementarity between the miRNA and its target 3′-UTR. This leads to cleavage of the mRNA by the Ago in miRISC. In animals, the preferred mode of action is the repression of protein synthesis. This can occur directly by preventing ribosome assembly during initiation of translation. It can also occur at later stages of translation, including termination. In some cases, miRISC promotes the removal of the poly A tails from the mRNA, which can lead to the degradation of the mRNA or to its sequestration in cytoplasmic processing bodies (P bodies).

8.5.3 miRNA and Cell Senescence

The limited population doublings of normal diploid human fibroblasts in tissue culture have been used as a model of aging (Hayflick and Moorhead 1961). Cells that stop proliferating at the limits of their replicative potential enter a senescent state.

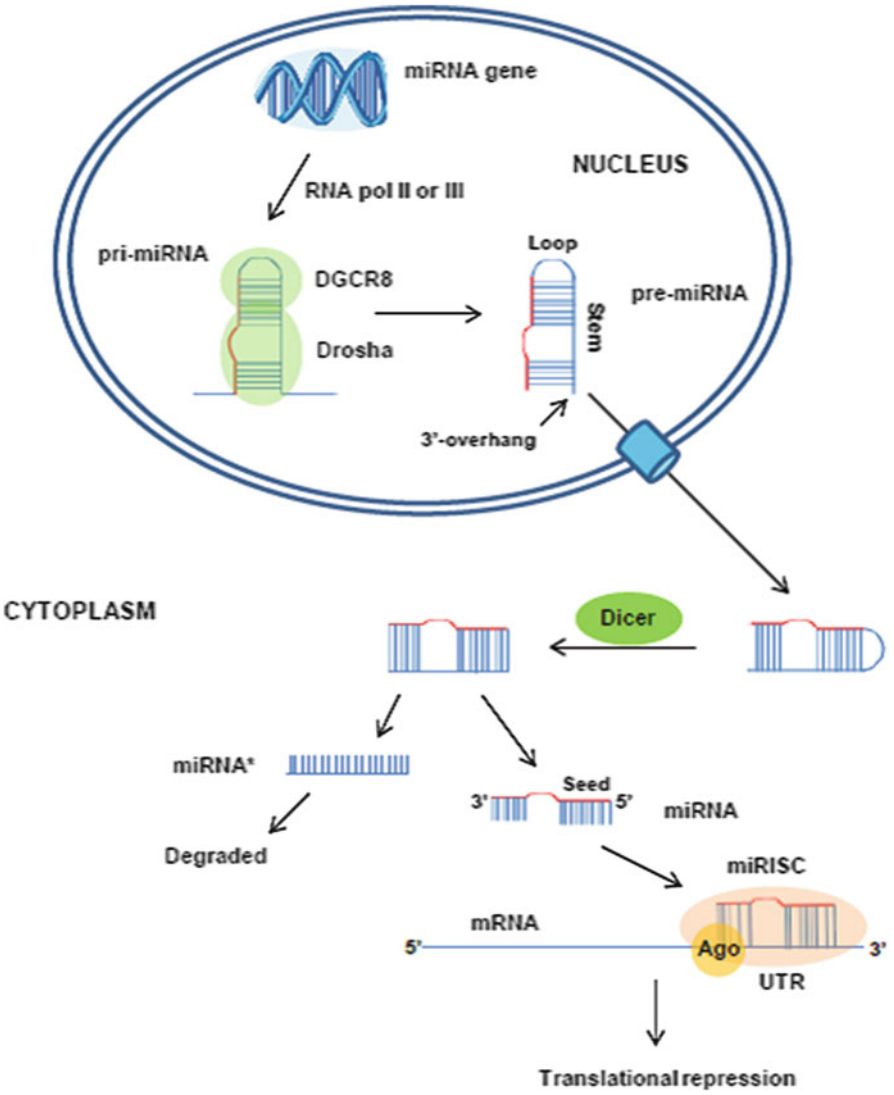

Fig. 8.1 miRNA biogenesis and activity. The long pri-miRNA transcript, which can contain sequences corresponding to several miRNA, is transcribed by either RNA polymerase II or III, depending on the particular miRNA gene or gene cluster. This pri-miRNA is processed by the RNase III called Drosha, aided by its partner DGCR8. This results in a 22-base pair stem, a loop, and a two-nucleotide 3′-overhang, together called the pre-miRNA, which is transported out of the nucleus. In the cytoplasm, another RNase III, Dicer removes the overhang and the loop, to yield the duplex miRNA. This is unwound by a helicase, resulting in the miRNA*, which is degraded, and the mature miRNA. The miRNA forms a structure called miRISC at the 3′-UTR of its target mRNA. miRISC contains the Argonaute protein (Ago) that possesses endonuclease activity directed towards the miRNA-mRNA hybrid, but not to other parts of the mRNA. The miRISC ribonucleoprotein complex, containing Ago, represses translation of the target mRNA, by both direct and indirect mechanisms. This is the primary mechanism that silences translation in animal cells, while in plants the primary mechanism is the degradation of the mRNA. Recognition of the target mRNA by the cognate miRNA involves a seed region of two to eight nucleotides close to the 5′-end of the miRNA that are complementary to sequences in the 3′-UTR of the mRNA. This is stabilized by complementarity between nucleotides in the 3′-half of the miRNA with the UTR

This occurs due to the shortening of the telomeres at the ends of the chromosomes with each cell division (Gorospe and Abdelmohsen 2011). The DNA damage response is triggered, resulting in a series of events that culminate with the activation of the tumor suppressor p53, among others. The target of p53, $p21^{Cip1}$, is a cyclin-dependent kinase inhibitor whose induction locks the cell into the non-proliferating state. Another mechanism that leads to the same overall response is through activation of the retinoblastoma tumor suppressor Rb, which induces another cyclin-dependent kinase inhibitor $p16^{INK4a}$. Various cell insults, such as oxidative stress, that do not result in telomere attrition can also trigger senescence. This premature senescence depends on either p53 or p16 and cannot be rescued by ectopic expression of telomerase. Senescent cells are recognized by their shorter telomeres, expression of senescence-associated β-galactosidase, and expression of DNA damage response proteins.

Senescent cells have been identified in human tissues by the same criteria as those used in tissue culture (Gorospe and Abdelmohsen 2011). Their presence can affect the phenotype of surrounding cells because the senescent cells secrete an array of chemokines and cytokines, such as interleukin-6 (IL-6), IL-8, IL-1α, GM-CSF, and others, as well as matrix metalloproteinases and insulin-like growth factor 1-binding proteins. This senescence-associated secretory phenotype (SASP) (Coppe et al. 2008) promotes cancer cell migration. SASP has been implicated in atherosclerosis, diabetes, neurodegeneration, and COPD, all of which may be related to the chronic inflammation associated with aging.

A substantial effort to identify miRNA that are differentially expressed during cell senescence has been made during the past 5 years (Gorospe and Abdelmohsen 2011). In several cases, specific miRNA have been causally implicated in cell senescence. The miRNA-34 (miR-34) family was shown to be a downstream effector of p53. Key mRNA repressed by miR-34a include E2F, SIRT1, Cdk4, and Cdk6, explaining its effect on cell proliferation. It has also been shown that the miR-34 promoter is hypermethylated in various cancer cell types, which would block its synthesis (Lodygin et al. 2008). Other mRNA that are key for cell senescence are also conspicuous targets of various senescence-associated miRNA. This includes components of the SASP.

A recent study has shown that among several up-regulated miRNA during replicative or premature senescence of normal human endothelial cells was miR-21 (Dellago et al. 2013). Transfection of miR-21 into early passage cells induced senescence, up-regulation of $p21^{Cip1}$, and down-regulation of CDK2. Stable knockdown of miR-21 expression extended the replicative lifespan of the endothelial cells. This study establishes a causal role for a miRNA in cell senescence.

A novel miRNA-associated mechanism in cell senescence was demonstrated through implication of Wig1 (Kim et al. 2012b). This protein controls the formation of the miRISC containing Ago2 with $p21^{Cip1}$ mRNA. Down-regulation of Wig1 increased p21 mRNA levels, resulting in premature senescence. It will be interesting to see whether Wig1 plays a role in adjusting p21 expression during aging in vivo.

8.5.4 miRNA, Aging, and Longevity

Differential expression of miRNA during human aging has garnered considerable attention recently. An interesting comparison of several human in vitro replicative senescence models (endothelial cells, $CD8^+$ T cells, renal proximal tubule epithelial cells, skin fibroblasts) with human in vivo aging (foreskin, mesenchymal stem cells, $CD8^+$ T cells) has been carried out (Hackl et al. 2010). Four specific miRNA (miR-17, miR-19b, miR-20a, miR-106a) were down-regulated during aging in most of these models. One of these, miR-17, was down-regulated in all seven. This down-regulation correlated with an increase in $p21^{Cip1}$ mRNA levels, consistent with a broad role for miR-17 in cell senescence, both in vitro and in vivo.

Centenarian studies have begun to examine miRNA expression profiles. One such study found a significant overlap of miRNA profiles in circulating mononuclear cells between centenarians and young individuals but not with octogenarians, by principle component analysis (Serna et al. 2012). The small ncRNA included 7 species up-regulated in centenarians compared to young individuals and 102 as compared to octogenarians. Of these, the up-regulation of miR-21 and miR-103a were validated by quantitative PCR. Of particular interest was miR-19b which showed a similar level in young individuals and in centenarians, but a considerably lower level in octogenarians by quantitative PCR. This is a novel finding. miR-21 has been widely associated with aging phenotypes, and miR-19b is associated with $p21^{Cip1}$ levels, as discussed above.

Another study utilized immortalized B cells from centenarians to identify differentially expressed miRNA in comparison to young individuals (Gombar et al. 2012). This study was carried out by deep sequencing rather than using microarrays as above. Twenty-two miRNA were up-regulated and two were down-regulated in centenarians. Of these, miR-363* showed a decline in expression across age groups. However, its expression in centenarians was similar to that in individuals 50–60 years-old. This profile matches that of miR-19b above. miR-363* belongs to the group of miRNA degraded strands that may have a functional role. Some of the predicted, though not validated mRNA targets of miR-363* have been implicated in longevity. One peculiar aspect of this study is the fact that five of the miRNA up-regulated in centenarians are down-regulated in cell senescence.

The long-lived Ames dwarf and growth hormone receptor gene knockout (GHRKO) mouse models have been analyzed for a role of miRNA in longevity (Liang et al. 2011). These models showed up-regulation of three miRNA (miR-470, miR-669b, miR-681) that are involved in the regulation of growth hormone/IGF-1 signaling pathway genes. The expression of these miRNA is located primarily in the brain (hippocampus), and it correlates with decreased expression of IGF-1, IGF-1R, and PI3 kinase. This hypothetical function in regulation of these molecules was validated in cell culture by transfection of cells with miRNA.

The *klotho* gene plays a role in determining murine lifespan (Kuro-o et al. 1997). Its knockout results in copious senescence. A recent study has shown that these mice display increased expression of miRNA in the miR-29 family (Takahashi et al. 2012). Normal mice also show increased miR-29 levels as they age. This correlates

with lower expression of Type IV collagen, whose mRNA is a target of miR-29. One of the phenotypes of *klotho* knockout mice is connective tissues abnormalities to which up-regulation of miR-29 would contribute.

In a study of cartilage aging, it was shown that expression of miR-199-3p and miR-193b was up-regulated, while that of miR-320c was down-regulated (Ukai et al. 2012). Chondrocytes were transfected in tissue culture to over-express or repress the relevant miRNA. The effects of these manipulations on predicted mRNA targets of the miRNA were examined. These targets, Type II collagen, aggrecan, ADAMTS5, and SOX9, play important roles in cartilage growth and maintenance. The results point to involvement of miR-199-3p and miR-193b in cartilage senescence, and the function of miR-320c in maintenance of youthful properties of cartilage.

A striking recent study has implicated *Dicer* in aging and longevity (Schaefer et al. 2007). As mice age, there is a down-regulation of *Dicer* and of miRNA processing in general in adipose tissue, which is duplicated in *C. elegans*. Calorie restriction (underfeeding), which extends lifespan in numerous aging models, (Masoro 2006) prevents this in both mice and worms (Mori et al. 2012). Old humans also show decreased *Dicer* expression in preadipocytes, in this study. Knockdown of *Dicer* in tissue culture cells causes premature senescence. Similarly, mutations in *Dicer* in the worm reduce its lifespan, while overexpression extends it. This study suggests that miRNA processing in adipose tissue may be an important contributor to metabolic syndrome with attendant effects on longevity.

The research reviewed briefly above suggests important roles for miRNA in human aging. It is also worthwhile considering the genes and pathways that have been implicated in aging for potential targets of miRNA, to further support a role in human aging. In this regard, miR-27a, miR-140, and miR145 have multiple targets, among which are IGF-1R, IGFBP-1, and IRS1, all components of the aging and longevity associated insulin/IGF-1 pathway (La Rocca et al. 2009a, b; Miyaki et al. 2009; Tardif et al. 2009). Mammalian target of rapamycin (mTOR) is targeted by miR-100 in human (Nagaraja et al. 2010). Several *SIRT1* expression-regulating miRNA have been described. They include miR-34a, miR-132, and miR-217, in human (Lee et al. 2010; Strum et al. 2009; Menghini et al. 2009). A broad analysis of replicative senescence in a variety of human endothelial cells and angiogenic cells resulted in a detailed miRNA profile. Among the validated miRNA showing the highest up-regulation during senescence were miR-9, miR-146a, miR-204, and miR-367 (Olivieri et al. 2013). miR-146a was targeted for inhibition and expression analyses that demonstrated its association with cell senescence. This miRNA down-regulates expression of the IRAK1, the toll-like receptor associated kinase 1 which is involved in signaling in the innate immune response. However, this did not abrogate the pro-inflammatory SASP in the senescent cells. The miR-27a and miR-140 results described above were obtained in an osteoarthritic cartilage model, which suggests a direct relationship to an aging phenotype. Such a relationship can be contrived for miR-146a, as well. In all of the other cases, the relationship to aging is indirect because it is solely based on the potential role of the mRNA target in human aging.

8.5.5 miRNA in Age-Related Disease

Aging is the major risk factor for the development of chronic diseases. Changes in miRNA expression levels have been associated with various diseases, including cancer, cardiovascular disease, neurodegenerative disorders, and metabolic diseases. Although these associations are persuasive, it is not clear whether the miRNA have a causal role to play or are simply biomarkers of disease. We have already alluded to the potential of miRNA changes in the development of cancer and metabolic diseases, as well as inflammatory states. Here, we will add cardiovascular disease and neurodegenerative disorders. Most of the studies have been carried out in mouse models, as might be expected.

miR-29 family members were shown to be up-regulated in mouse aortas during aging (Boon et al. 2011). This miRNA is known to suppress the expression of matrix proteins, and it is associated with a decrease in collagen and elastin expression in these aortas. miR-34a is associated with endothelial cell senescence, as mentioned earlier. Recently, it has been found to regulate cardiac aging and function (Boon et al. 2013). This miRNA is induced in the aging murine and human heart. This correlates with cardiac fibrosis, telomere shortening and cell death of cardiomyocytes. These changes can be ameliorated by inhibition of miR-34a in vivo in mice. Such treatment also restores contractile function in aged animals. miR-34a was shown to target the 3′-UTR of PNUTS mRNA. PNUTS protein is responsible for inhibition of apoptosis of cardiomyocytes, and it prevents the impairment of cardiac contractile function. The multiple effects of miR-34a appear to be mediated by PNUTS, which requires the telomere binding protein TRF2. PNUTS expression is reduced during aging, and it regulates telomere maintenance and the DNA damage response. This study provides the clearest example of a role of a miRNA in aging and age-related physiologic decline, albeit in an animal. Nevertheless, correlates with human aging were also demonstrated.

miR-22 is also up-regulated during cardiac aging in mice. Suppression and overexpression studies have shown that it induces senescence and promotes migration of cardiac fibroblasts (Jazbutyte et al. 2013). This miRNA exerts its effects at least partially by targeting mimecan/osteoglycine, but it also induces hypetrophy through other targets.

Cerebellar degeneration has been associated in mice with loss of Dicer. Conditional knockout of Dicer in Purkinje cells resulted in cerebellar degeneration and ataxia in vivo (Schaefer et al. 2007). On the other hand, its ablation in the forebrain caused abnormal tau phosphorylation and neurodegeneration. The miR-29 cluster has been implicated in sporadic Alzheimer's disease. The expression of these miRNA decreases with age, which correlates with enhanced expression of β-secretase (Hebert et al. 2008, 2009), which initiates amyloid precursor protein processing to yield Aβ. Brains of Alzheimer's disease patients show increased levels of β-secretase. miR-34c is up-regulated in the hippocampus of aged mice and in Alzheimer's disease patients (Zovoilis et al. 2011). Inhibiting this miRNA improves mouse cognitive function. One of the targets of miR-34c is SIRT1 whose decreased levels cause memory impairment in mice.

8.5.6 Circulating miRNA

miRNA have been found circulating in body fluids, either in protein coated particles or in microvesicles. They have been proposed as biomarkers of various disease states, although they may serve as a means of communication throughout the body similar to hormones. They have recently become the subject of aging studies. In one study, plasma miRNA were examined in healthy subjects in various age groups (20, 80, and 100 years), and validated in healthy adults (20–105 years-old) as well as cardiovascular disease patients and healthy centenarian offspring (Olivieri et al. 2012). Factor analysis showed up-regulation of certain miRNA in octogenarians and in centenarians, as separate groups. miR-21 was higher in cardiovascular disease patients and lower in healthy, age-matched controls. It was correlated with C-reactive protein and fibrinogen levels, suggesting enhanced signaling in the TGFβ pathway. In fact, TGFβ mRNA is a miR-21 target, whose levels increase in leukocytes from certain octogenarians. Thus, miR-21 may be a useful biomarker of inflammation.

A recent feasibility study for the use of plasma miRNA to detect mild cognitive impairment showed promising results (Sheinerman et al. 2012). Two miRNA families, miR-132 and miR-134, discriminated between subjects who showed cognitive impairment from controls, with high sensitivity and specificity. They were capable of predicting subsequent impairment before subjects became symptomatic with mild cognitive impairment or Alzheimer's disease.

Endothelial cells secrete microvesicles containing miR-143 and miR-145 that prevent vascular smooth muscle cell de-differentiation (Hergenreider et al. 2012). Injection of these microvesicles into $APOE^{-/-}$ mice reduced atherosclerotic lesion formation. Microvesicles have been isolated from cerebrospinal fluid of Alzheimer's disease patients, and they show enrichment for some 60 miRNA compared to healthy controls (Cogswell et al. 2008). It will be of interest to determine whether these are purely biomarkers or whether they possess signaling function.

8.6 Food and the Epigenome

The realization that food not only has nutritional value but also is the source of signaling molecules that possess hormone-like effects has dawned recently (Ryan and Seeley 2013). Many of these molecules directly affect signaling pathways, such as mTOR, which are known to play an important role in aging and in the development of age-related phenotypes. Various bioactive dietary agents have been found to modulate tissue levels of several miRNA that are associated with cancer development, though the mechanisms are not clear (Shah et al. 2012). This has not as yet been extended to aging studies.

The case for dietary/metabolic and even exercise effects on DNA methylation profiles is well established, and this extends to histone modifications (Kirchner et al. 2013). This sort of control has far reaching consequences. Suboptimal nutrition during early rat development results in epigenetic silencing of the Hnf4a enhancer.

This weakens its interaction with the promoter, resulting in a permanent reduction in Hnf4a expression in pancreatic islets (Sandovici et al. 2011). On the other hand, aging results in progressive epigenetic silencing across the entire Hnf4a gene, and this is further augmented due to poor maternal nutrition. The transcription factor Hnf4a has been implicated in type 2 diabetes. Thus, these results suggest an epigenetic mechanism that regulates health during the lifespan. This mechanism lies at the interface of the environment and the epigenome.

8.7 The Gut Microbiome at the Environmental Interface with the Epigenome

The human gastrointestinal tract is host to a vast bacterial flora. These microbes outnumber 100-fold in gene number the human genome. This numeracy is matched by the species diversity the gut microbiome encompasses. Next generation sequencing and sophisticated bioinformatics have allowed access to this enormous diversity in recent years. Three robust clusters or enterotypes of gut bacteria have been identified (Arumugam et al. 2011). These are neither nation nor continent specific. Although certain species are abundant, as determined by the 16S rRNA sequences found in the large intestine, they are not necessarily the source of the abundant molecular activities, which are instead contributed by less abundant species of bacteria. The enterotypes are not correlated with host phenotypes, such as body mass index, age, or gender. However, certain marker genes that can be viewed as functional modules in the gut microbiome are associated with age and body mass index. Recent studies point to a large degree of genetic variation in the gut microbiome. This variation easily exceeds host genetic variation (Schloissnig et al. 2013). The genetic variation in the microbiota is specific for individual hosts, and it remains largely constant over longer periods of time despite changes in the actual composition of bacterial species. On the other hand, enterotypes can fluctuate over shorter periods at the abundance level, and they are responsive to dietary and antibiotic intake (Tiihonen et al. 2010).

The gut microbiome has been associated with disease (Tiihonen et al. 2010) and inflammation (Maslowski and Mackay 2011). Evidence for a causal role in metabolic disease has been provided in twin studies (Ridaura et al. 2013). Fecal matter from twins concordant or discordant for obesity was transplanted into germ-free mice fed diets mimicking human diets that either promote obesity or leanness. The microbiota from obese twins favored the development of obesity in the mice, which displayed an interaction with the diet they were fed. In contrast, the microbiota from lean twins worked in the opposite direction. These changes were associated with specific differences in bacterial fermentation, as well as host gene expression and metabolic differences.

A remarkable degree of inter-individual differences are found in gut microbiome populations (Claesson et al. 2011). Centenarians are distinguishable from young

and healthy elderly individuals (Biagi et al. 2010). These differences may be correlated with host inflammatory status. A recent large study of the elderly points to the potential role of the gut microbiome in frailty and other clinical measures (Claesson et al. 2012). Age, diet, and medication can contribute to the magnitude of this effect, but they do not alter the association.

The fermentation products of the gut microbiota are a source of macro- and micronutrients, such as vitamins that affect methylation reactions. They also can contain harmful substances. Some of these products affect signaling pathways, much as hormones do (Ryan and Seeley 2013). Short-chain fatty acids, in particular, can have profound effects on energy and glucose metabolism (Ryan and Seeley 2013), as well as on immune function (Maslowski and Mackay 2011). Butyrate can directly impact histone deacetylases. Thus, these fermentation products potentially play a role in modulating the aging process. Future research will likely uncover how diet and the microbiome interact at the interface with the epigenome to impinge on aging.

8.8 Concluding Remarks

Epigenetic regulation of gene expression is clearly associated with human aging. Until recently, the causal effect on aging and longevity had not been established. However, we now have good evidence that this is the case based on animal studies. It is abundantly clear that the environment and lifestyle impact the epigenome. This bodes well for strategies to postpone aging-related phenotypes and thus to improve function and quality of the later years of life. We can expect further elaboration of the causal role for epigenetic regulation. However, clinical trials that employ safe environmental influences must be tested for their salutary effect on age-related disorders and for promotion of healthy aging.

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Chapter 9
Environmental Contributors to Epigenetics and Cardiovascular Disease Risk

Lawrence C. Park, Paul J. Turgeon, and Philip A. Marsden

Abstract Cardiovascular diseases are the leading causes of death and disability worldwide. With the advent of high throughput genome analysis tools, the scientific community has begun the process of associating heritable SNPs with genetic predispositions for disease. However, models for how the environment interacts with the static DNA code are now needed. The exciting new field of epigenetics allows for a newer perspective of how a cell's DNA sequence dynamically responds to environmental stimuli and insults. How does smoking evoke chromatin-based changes to alter gene expression? What non-coding RNAs are responsive to hypercholesterolemia in atherogenesis? This chapter will highlight emerging epigenetic concepts in cardiovascular health in relation to a range of environmental stressors from starvation to microfluidic hemodynamics in our vasculature. Epigenetics provides unique insights into the pathogenesis of vascular diseases and suggests alternative therapies and diagnostic tools for physicians.

Keywords Epigenetics • miRNA • Cardiovascular disease • Atherosclerosis • DNA methylation • Histone code • Long non-coding RNA • Endothelial cells

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9.1 Introduction

All of the somatic cells of the body, whether it is endothelial cells, neurons or keratinocytes, share the same ACGT DNA code that is faithfully replicated as cells divide in mitosis. The genomic sequence is effectively stable and serves as the template off of which diverse cell types are derived and maintained in homeostasis. This phenomenon led early geneticists to explore the regulatory mechanisms and chromatin based modifications on our genome, which eventually opened the door for epigenetic research. Epigenetics encompass chromatin-based mechanisms that regulate gene expression without changes in the DNA sequence per se. Epigenetic pathways are much more dynamic and responsive to the environment, serving as a form of cellular memory to early-life insults (Jaenisch and Bird 2003). Indeed, the epigenome is less faithfully maintained during mitosis and the rate of evolution via epimutations is greater than the rate of genetic evolution (Schmitz et al. 2011; Becker et al. 2011). Surprisingly, the stark difference between humans and other primates lies not within the static genetic code but in its differential regulation (King and Wilson 1975). With accumulating evidence supporting the importance of our epigenome, we are challenged to define the consequences of its dysregulation. Do perturbations to the established epigenome pose as a net liability to an organism? In turn, are aberrant epigenetic modifications a part of disease pathogenesis? Several international initiatives, including the Human Epigenome Project and the International Human Epigenome Consortium, were among the first to catalogue our epigenome (Abbott 2010; Bradbury 2003; Rakyan et al. 2004). Notably, the Encyclopedia of DNA Elements (ENCODE), launched by the US National Human Genome Research Institute (NHGRI), serves as an invaluable tool for the newer field of epigenetics (Maher 2012). Notwithstanding these extensive databases, the mysteries of epigenetic functions, especially in disease, remains a growing and important area of research. In this chapter, we will highlight DNA methylation/hydroxymethylation, histone post-translational modifications, long non-coding RNAs and the more controversial short non-coding RNAs, in the context of cardiovascular diseases.

As noted by the famed Canadian physician William Osler in 1892, "Longevity is a vascular question, and has been well expressed in the axiom that 'a man is only as old as his arteries (Osler 1892).'" Cardiovascular diseases (CVD) are the leading causes of death and disability worldwide (Libby 2002). In particular, coronary artery disease (CAD) is the most common type of heart disease and accounts for one in every six of total deaths in the USA. CAD follows complex atherosclerotic plaque formation and progressive narrowing/occlusion of vessels carrying oxygen-rich blood to the heart (Go et al. 2013). We refer interested readers to a recent review on epigenetics and the risks of stroke (Matouk et al. 2012). Atherosclerosis is a well-characterized chronic inflammatory disease with defined genetic predispositions as well as environmental risk factors. Fittingly, epigenetic research has greatly enhanced our understanding of its pathophysiology through discordance between monozygotic twins, sexual dimorphism, parent-of-origin dependent clinical differences, and a relatively late age of onset of disease manifestation (Webster et al. 2013; Yan et al. 2010). This chapter will highlight the emerging epigenetic concepts in cardiovascular health in relation to a range of environmental stressors from

starvation to microfluidic hemodynamics in our vasculature. We first present three examples wherein epigenetics provides a newer perspective on key aspects of how the environment influences the risks of CVD.

9.1.1 Twin Studies: Assessing the Genetic Contribution to Cardiovascular Diseases

The classical twin studies proved to be a valuable tool in assessing the genetic contribution in many diseases, including CAD (Marenberg et al. 1994; Fraga et al. 2005). These studies are based on the fundamental principle that monozygotic twins (MZ) are genetically identical, whereas dizygotic twins (DZ) only share approximately 50 % of their genome. Therefore, if the concordance rate for a trait or disease is greater in MZ twins compared to DZ twins, a genetic component can be hypothesized for the disease phenotype. A 1994 study on 10,502 Swedish twin pairs revealed a genetic component in CAD as the mortality of a co-twin led to doubling of the vulnerability for death by CAD in MZ twins relative to DZ twins (Marenberg et al. 1994). Similarly, a separate study on Danish twins also observed a strong genetic influence on mortality caused by CAD (Wienke et al. 2001). With respect to CAD, heritability of disease was 0.55 and 0.53 for males and females respectively. Although a genetic component is apparent, considerable discordance among MZ twins for death due to CAD still exists notwithstanding whether the twin pairs were raised together verses apart. How can this discordance for CAD within MZ twin pairs be explained? Here we argue that gene-environment interactions are also important. In 2005, Fraga et al. reported that MZ twins carry nearly identical epigenetic patterns early in their life. However, epigenetic differences accumulate with age, different lifestyles and in response to different environments. Surprisingly, our understanding of the mechanistic links between environmental risk factors and CAD is poorly understood.

9.1.2 The Missing Heritability of Cardiovascular Diseases

Twin studies highlighted an evident genetic contribution to CAD but did not pinpoint which allelic differences are important for disease risk. When we successfully completed the Human Genome Project in the early 2000s, the scientific community anticipated specific genetic predisposition to disease to be a powerful diagnostic tool. Indeed, genome-wide association studies (GWAS) that correlate genetic variation with phenotypic variation has shed light on novel loci associated with diseases such as CAD (Manolio et al. 2009). This method identifies single-nucleotide polymorphisms (SNPs) that are prevalent in populations burdened by disease. This model assumes the “common disease, common variant” hypothesis which postulates that common diseases are attributable to common risk variants found within at least 1–5 % of the total population (Manolio et al. 2009). However, for most human diseases or complex traits, susceptibility risk variants only account for only a

modest increase in risk of 1–1.5-fold, and explain only a small proportion of the estimated variability (Marenberg et al. 1994). For example, Maremberg et al. demonstrated that between 30 % and 60 % of inter-individual variability for CAD risk is attributable to genetic factors.

What factors account for this "missing heritability" in GWAS of CAD? Epigenetic theory provides an alternative paradigm that, at least in part, explains this discrepancy. Ironically, GWAS, a method designed to identify "hot-variants" of the ACGT code, has instead pointed to epigenetic effectors as the major determinants of CAD liability. In 2007, multiple research groups identified a genomic susceptibility locus for CAD and myocardial infarctions that out-trumps all other predictive risk factors including smoking and diet. These studies pointed to the intergenic non-coding region of chromosome 9p21, which contains a long non-coding RNA (lncRNA), *ANRIL* (further discussed in Sect. 9.3.3) (Holdt and Teupser 2012). The finding that a lncRNA, involved in epigenetic functions, is relevant to CAD pathogenesis was surprising and lends insight into the missing heritability of CVD.

9.1.3 The Dutch Hunger-Winter Famine: Exploring the Epigenetic Responses to the Environment and Its Heritability

Conceptually, it may not be surprising that identical twins diverge with age and exposure to different environments. Even before epigenetics came to light, we were well aware of the adaptive potential of our genome. However, heritability of acquired traits was heresy to the classical model of genetics. Transgenerational epigenetics is attracting a lot of attention. However, the exact mechanism by which epigenetic marks are transmitted via meiosis is still poorly understood (Whitelaw and Whitelaw 2008). Nevertheless, it is indisputable that environmental factors can affect an offspring even if the stimulus occurred several generations prior. The legacy of the Dutch Hunger-winter famine of 1944–1945 serves as a quintessential example in studying epigenetic inheritance (Heijmans et al. 2008). Barring its tragic history, this unique famine attracted scientists' attention because it struck a developed country and is thus well documented.

Briefly, a German-occupied area of the Netherlands suffered an imposed famine during World War II from November 1944 to May 1945 due to a blockage of all transport of food combined with an unusually early and harsh winter. Understandably, children who were conceived by mothers during this period had higher susceptibility to low birth weight, growth-defects and mental disorders (Lumey et al. 2007). The first hint to the long-term affect of the famine was found in studies of males subjected to the famine in utero, suggested those exposed to famine early and mid-gestation was associated with a doubling in the prevalence of obesity (Heijmans et al. 2008). Further and more in depth analyses revealed individuals exposed to the famine during the periconceptional and early gestation periods showed reduced fertility and fecundity, growth retardation, and a higher risk of CVD, including CAD and schizophrenia (Heijmans et al. 2008). Interestingly, they also showed a higher

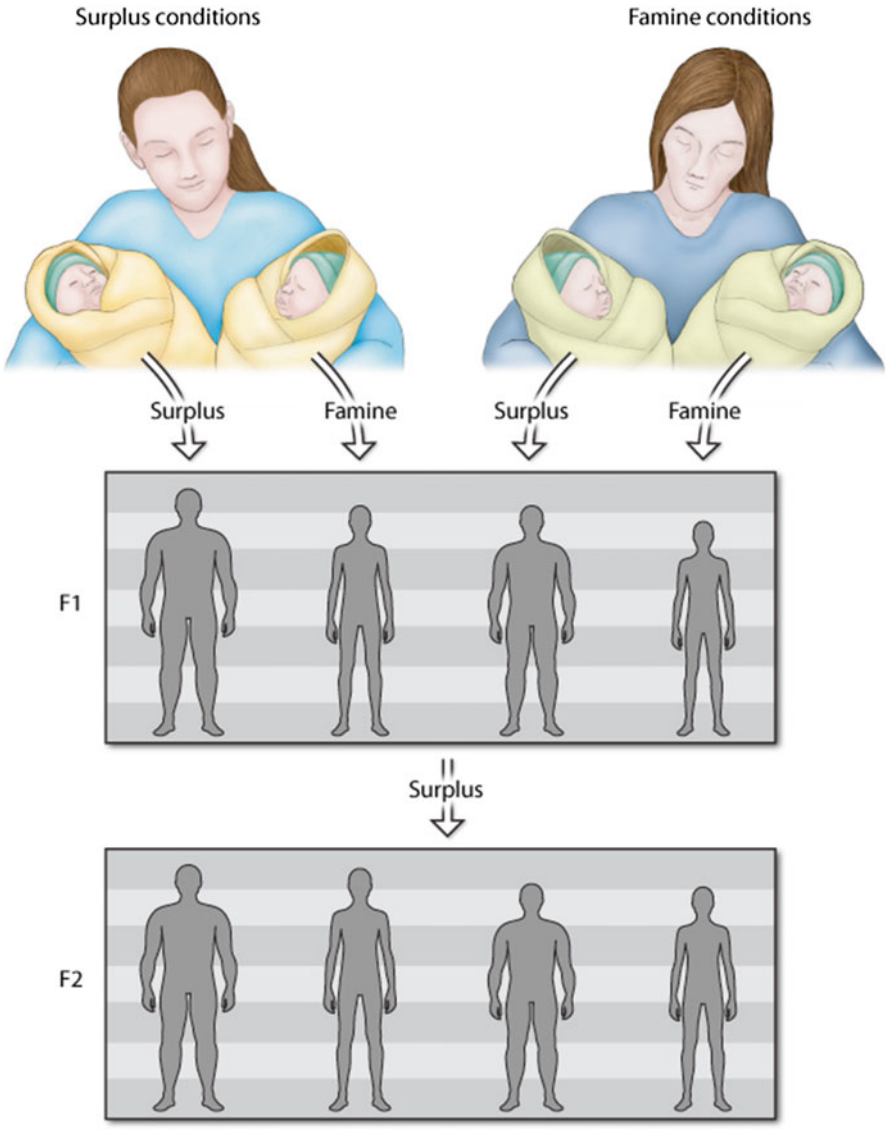

Fig. 9.1 Heritable interaction between the environment and our genome. The Dutch Hunger-winter famine and the Twin-studies showed that our genome interacts with the environment through epigenetic mechanisms. Importantly, epigenetic modifications can be retained throughout life and inherited transgenerationally

rate of births with congenital neural defects, indicating germ cell alterations. This suggests that altered epigenetic states of primary gonadal germ cells of grandparents, as well as the gametes that are derived from them, contribute to the epigenome of their grandchildren (Fig. 9.1).

Interestingly, 60 years post-famine, individuals who were exposed in utero to the famine were analysed at the molecular level using whole blood extracts. Those exposed periconceptually were found to show reduced methylation compared to control at the maternal copy of the insulin-like growth factor 2 gene (*IGF2*) (Heijmans et al. 2008; Painter et al. 2006). IGF2 is a crucial factor for early embryonic growth and development, and under normal conditions contains a maternally imprinted differentially methylated region (DMR). Decreased methylation of the IGF2 DMR can lead to bi-allelic expression of the gene, commonly found in Beckwith-Wiedemann syndrome (Tobi et al. 2009). Further analysis found several other genes showed differences in methylation, such as interleukin-10 (IL10), ATP-binding cassette subfamily A member-1 (ABCA1) and maternally expressed 3 (MEG3) that were also affected. Some associations were sex-specific such as Leptin (LEP), GNAS antisense RNA (GNAS-AS) and INS-IGF2 readthrough (INSIGF). Interestingly, LEP is an important adipokine which plays a key role in energy intake and expenditure while ABCA1 is important in cholesterol transport to HDL. Notably, MEG3, GNAS-AS and ININSIGF are predicted to have non-coding function (further discussed below), which may be important in establishing proper epigenetic marks. The legacy of the famine led us to appreciate transgenerational epigenetics but the mechanisms and depth of study is still young. We will discuss other facets of epigenetics (please see Sect. 9.3) that should be explored in this model. Most importantly, it will be critical to examine whether the grandchildren of the Dutch Hunger-winter famine will continue to transmit their epigenetic memory to their children.

9.2 Endothelial Cells and Vascular Smooth Muscle Cells: Key Regulators of Vascular Homeostasis

This chapter will mainly focus on the epigenetic mechanisms by which gene expression is regulated in endothelial cells and vascular smooth muscle cells, the two most abundant cell types in our vascular tree.

The innermost lining of our vasculature is composed of endothelial cells (ECs), which are the first cells to interact with external stimuli in our blood and maintain vascular homeostasis in both vessels and capillaries. The vascular endothelium is a key intermediary between environmental factors, such as hyperlipidemia, and progressive stages of atherosclerotic plaque formation (Sumpio et al. 2002). Typically, endothelial dysfunction and lesion initiation is thought to be triggered by risks factors such as elevated blood pressure, diabetes, dyslipidemia, and smoking. The resulting endothelial cell "dysfunction" represents a net liability to the host through the disruption of its homeostatic roles including vasomotor tone, leukocyte trafficking, and hemostasis. A cardinal role of healthy ECs is robust nitric oxide (NO) bioavailability via endothelial nitric oxide synthase (eNOS, *NOS3*), often dubbed the quintessential endothelial gene (Marsden and Brenner 1991). NO is a potent vasodilator that can also alter gene expression and biochemical signaling pathways

in a paracrine and autocrine manner. Given NO's anti-inflammatory and anti-atherosclerotic role, reduction of eNOS has been documented to exacerbate the inflammatory response, setting the stage for the onset of atherosclerosis (Won et al. 2007). Interestingly, eNOS expression is highly responsive to environmental stimuli. The combination of epigenetic pathways will be explored below in further detail.

Vascular smooth muscle cells (VSMCs) provide structural and homeostatic functions, especially in large vessels. Adult VSMCs are non-terminally differentiated cells that undergo phenotypic changes to switch between contractile and synthetic states to define vasomotor tone and vascular permeability (Doran et al. 2008). During development, VSMCs are highly proliferative and migratory in their synthetic state but differentiate into quiescent and contractile VSMCs in healthy adult vasculature. However, VSMCs in diseases dedifferentiate to undergo a phenotypic switch to the synthetic state, which express significantly lower levels of smooth muscle markers such as myocardin, alpha smooth muscle actin (*α-SMA*), calponin and smooth muscle protein 22 (*SM22-α*) (Zeiffer et al. 2004; Ross and Glomset 1973; Takaichi et al. 1993; Zhao et al. 2011). Synthetic VSMCs proliferate and migrate through the internal elastic lamellae into the neointima, depositing aberrant levels of extracellular matrix (ECM) and takes on a myointimal phenotype that is very similar to that of fibroblasts. Synthetic VSMCs are functionally relevant to atherosclerosis, in stent restenosis and post-angioplasty restenosis. The phenotypic modulation of VSMCs is highly dependent on intercellular signaling, especially from endothelial cells. To date, several VSMC-transforming factors such as platelet-derived growth factor (*PDGF*), endothelin-1 (*ET-1*), thrombin, interleukin-1 (*IL-1*), and secreted EC-derived miRNAs have been shown to affect VSMC proliferation (Zhao et al. 2011; Scott-Burden et al. 1991; McNamara et al. 1993; Porreca et al. 1993).

ECs and VSMCs are highly responsive to environmental stimuli. They play a concerted role in inflammation, immune responses, vascular permeability and tone, in both healthy and diseased tissue. Basally, distinct epigenetic signatures contribute, at least in part, to the phenotypic and transcriptomic differences between the two cell types. Surprisingly, master transcription factors that drive expression of cell-specific genes and define cell-types (e.g. PPARγ in adipocytes) is not present in endothelial cells. Instead, recent work identified epigenetic mechanisms to be a major contributor to endothelial-specific genes expression (Shirodkar et al. 2013). We will further explore how the environment communicates with ECs and VSMCs in the context of CVD.

9.3 Epigenetic Mechanisms and the Vasculature

The haploid human genome consists of ~3.3 billion DNA base pairs. Difference in length is largely attributed to interindividual copy number variation and sex (i.e., length of X versus Y chromosome). Indeed, linearized DNA from a single cell

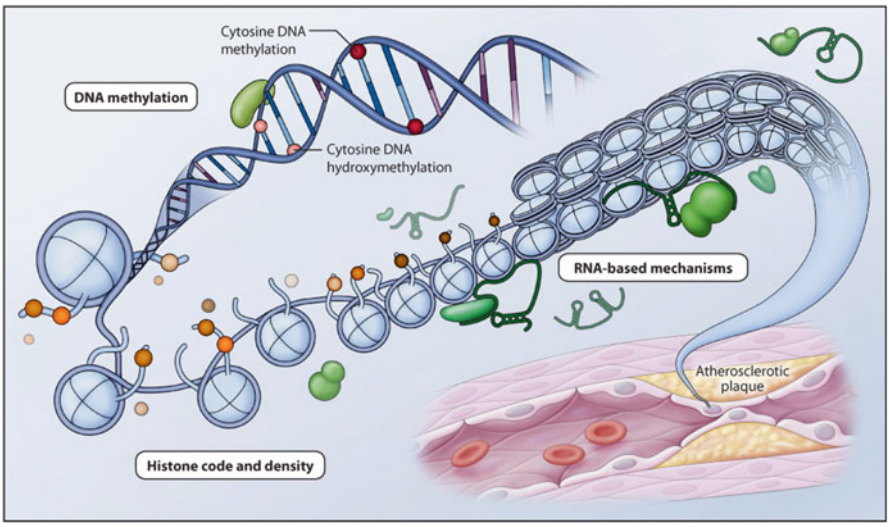

Fig. 9.2 Overview of epigenetic mechanisms. DNA methylation refers to the addition of a methyl group to the 5′-position of cytosine, which occurs symmetrically almost exclusively in the context of CpG dinucleotides in mammals. 5-hydroxymethylation also occurs at CpG dinucleotides and is thought to act as an intermediate for active demethylation. Histone density and post-translational modifications refers to the abundance of histones and N-terminal histone tail post-translational modifications, respectively. These changes alter histone protein-DNA interactions and change chromatin structure to affect accessibility of DNA binding elements, modulating gene transcription. RNA-based mechanisms interact with chromatin and chromatin modifying complexes in cis or trans, to modulate gene expression (Adapted from Turgeon et al. (2014). With permission from Karger and Basel)

would span approximately 2 m in length. Packaging genomic DNA into the nucleus of a eukaryotic cell is a marvel achieved by compact DNA-protein complexes called chromatin. Epigenetic theory encompasses the molecular effectors that regulate the structure and accessibility of chromatin without altering the underlying ACGT genetic sequence per se (Fig. 9.2). These mechanisms are highly malleable and evolutionarily conserved for regulated gene expression and will be individually elaborated in the following sections.

9.3.1 The "Fifth" and "Sixth" Base Pairs of Our Genome

Since its initial discovery in mammals by Hotchkiss in 1948, our knowledge of the establishment and function of DNA methylation has largely changed our understanding of gene regulation (Hotchkiss 1948). DNA methylation refers to the covalent modification of the 5-position of cytosine to generate 5-methylcytosine (5mC), commonly referred to as the "fifth base of DNA" (Miranda and Jones 2007). Although the abundance of 5mC is highly tissue-dependent, it is generally believed

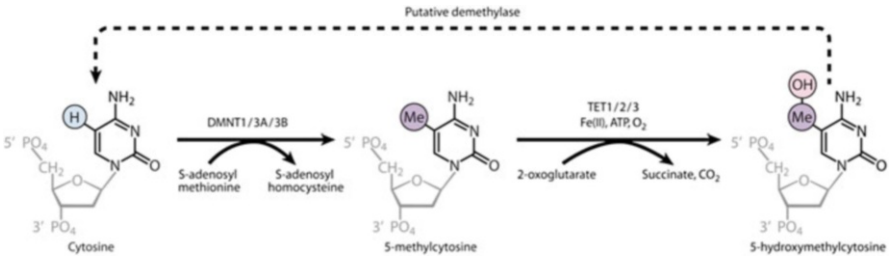

Fig. 9.3 DNA methylation and hydroxymethylation. The addition of a methyl group at the 5-position of cytosine is mediated by the DNA methyltransferase (DNMT) family, where S-adenosylmethionine serves as the methyl donor. The TET family of enzymes is able to oxidize 5-methylcytosine to 5-hydroxymethylcytosine in an oxygen-dependent reaction requiring ATP and 2-oxoglutarate. Cytosine can then be generated from the action of putative demethylases on 5-hydroxymethylcytosine, but has not yet been fully described (Reprinted with permission from Dove Medical Press Ltd.: Matouk et al. (2012))

to encompass 0.6–1.5 % of the human genome (Li and Liu 2011). Early work found that 5-methylcytosine base pairs with guanine, is transmitted semi-conservatively during DNA replication, and that it was highly restricted to CpG dinucleotides in mammals. DNA methylation is traditionally viewed as a repressive mark associated with inhibition of transcriptional initiation when found adjacent to promoters. It is therefore especially important in development and disease including genomic imprinting, X chromosome inactivation, mammalian embryogenesis and cellular differentiation (Scarano et al. 1967). It also serves as a protective mechanism to silence repetitive DNA elements, which make up 25–40 % of mammalian genomes (Miranda and Jones 2007). Dysregulation of methylation, both at single genomic loci as well as globally, has been associated with cancer (Stenvinkel et al. 2007). However, its contribution to CVD has yet to be fully explored.

Generally, DNA methylation is a stable covalent DNA modification that can be inherited mitotically and to a lesser degree, filially via meiotic transmission. The error rate for DNA methylation transmission is significantly greater than that of the ACGT static DNA code (Becker et al. 2011). Nevertheless, DNA methylation maintains the most stable epigenetic modification to be characterized. In mammals, DNA methylation occurs almost exclusively in the context of CpG dinucleotide palindromes in a healthy genome. The DNA methyltransferase (DNMT) family of enzymes catalyzes the addition of a methyl group to the 5-position of cytosine using a cofactor, S-adenosyl methionine (Fig. 9.3). During mitosis, DNA replication transmits existing 5mCpG sites semi-conservatively. DNMT1 is the maintenance methyltransferase that recognizes and methylates hemimethylated DNA to replicate the methylation pattern of the parent cell before mitosis (Dhe-Paganon et al. 2011; Liang et al. 2002). De novo methylation of CpG sites is catalyzed two enzymes: DNMT3a and DNMT3b (Okano et al. 1999). They are thought to exert their main function in establishing methylation patterns on unmethylated DNA during embryogenesis and early development.

Methylation studies revealed an interesting phenomenon in genomic evolution. The spontaneous deamination of cytosine yields uracil, which is recognized and restored to cytosine by DNA repair enzymes. However, deamination of 5mC results in thymine, causing a CpG to TpG mutation. Therefore, the CpG dinucleotide is relatively depleted in the mammalian genome, occurring at a much lower frequency than statistically predicted based on overall nucleotide content (Miranda and Jones 2007; Saxonov et al. 2006). Genomic regions called CpG islands lack 5mC and are thus spared from this CpG depletion. CpG islands occur within regulatory elements of 40–60 % of human genes (Scarano et al. 1967). The genome of cancer cells is characterized by global hypomethylation but paradoxical hypermethylation of CpG islands that are associated with promoters of tumor suppressor genes (Kron et al. 2009; Tan et al. 2013). Surprisingly, a similar pattern is observed on the inactivate X-chromosome in XX female mammalian cells (Sarma et al. 2010). Orphan CpG islands that are not associated with a promoter show higher frequency of 5mC, further suggesting that evolution has carefully monitored sites of DNA methylation to regulate gene expression (Maunakea et al. 2010).

DNA methylation is believed to participate in gene repression via three distinct mechanisms. First, the presence of the methyl group disrupts the 3-D orientation of the double helix, sterically inhibiting the binding of trans-factors to cis- DNA-binding elements, such as described transcription factors CTCF, c-Myc, and HIF-1α (Matouk and Marsden 2008). Second, the methyl group can also influence other epigenetic pathways, namely histone modifications. Recent evidence suggests that DNA methylation inhibits the formation of the activating histone 3 lysine 4 (H3K4) di- and tri-methyl marks (Miranda and Jones 2007). Lastly, specific methyl-binding proteins (MBP) can bind to 5mCpGs (Voo et al. 2000). The recruitment of these proteins is directly mediated by the presence of DNA methylation. Although the mechanism of repression is not fully understood, the members of this family (MBD1, MBD2, MBD3, MeCP2 and Kaiso) are thought to recruit repressor proteins and histone deacetylases, which mediate inactive chromatin signatures. Non-canonical DNA methylation also occurs outside the CpG context, although the mechanisms for establishment, maintenance and consequences have not been fully elucidated (Meissner et al. 2008).

Although a stable mark, removal of methyl groups can occur via passive or active mechanisms. Passive DNA demethylation can occur in primordial germ cells and pre-implantation embryos, where reduction in DNMT activity may be observed (Kagiwada et al. 2013). Consequently, passive demethylation is dependent on mitosis, DNA replication, and dilution of the existing 5mC throughout the daughter cells. An area of more active research is active DNA demethylation, the site-specific removal 5mC (Oswald et al. 2000). This replication-independent mechanism can quickly change levels of DNA methylation, sometimes within hours. Active DNA methylation has been proposed to occur through several pathways, but the most exciting is the recent discovery of the oxidative formation of 5-hydroxymethylcytosine (5hmC), now known as the “sixth base of DNA”. The 10-11 translocation (TET) enzyme family (TET1, TET2 and TET3) oxidize 5-methylcytosine to form 5-hydroxymethyaltion and the subsequent oxidative products, 5-formylcytosine or

5-carboxycytosine (Fig. 9.2) (Tahiliani et al. 2009; Ito et al. 2011). The TET family may work in conjunction with the family of base excision repair glycosylases, to regenerate the initial cytosine residue (Bhutani et al. 2011). The function of 5hmC is currently under active study as growing evidence suggests a role outside of an intermediate in demethylation. Most notably, accumulation of 5hmC in various cell types, especially Purkinje neurons, and its specific recognition by protein complexes, indicate that it also serves a purpose in gene regulation (Wang et al. 2012; Yildirim et al. 2011; Wu and Zhang 2011a). In particular, hydroxymethylation has been implicated in the activation of genes associated with pluripotency (Wu and Zhang 2011b). A complex story is anticipated, as unlike 5mC, 5hmC is associated with both transcription repression and activation (Wu and Zhang 2011a).

Clinically, synthetic VSMC of advanced atheroma display global hypomethylation in both patients and animal models of atherosclerosis (Turunen et al. 2009). Further studies, however, are required to monitor global changes in DNMTs or TETs in early stages of CVD. The human estrogen receptor α and β accumulate 5mC with age even in healthy tissues (Baccarelli et al. 2010). Accordingly, denser methylation at the estrogen receptor α and β promoters has been observed in atherosclerotic patients. Furthermore, classical risk factors for CVD such as air pollution and second hand smoking, cause reduced global methylation levels, highlighting the dynamic potential of DNA methylation in relation to the environment (Baccarelli and Bollati 2009). At a pragmatic level, however, the two classical laboratory techniques for assessment of DNA methylation are bisulfite sequencing and methylation-sensitive restriction enzyme digests – neither of which is ideal for distinguishing between 5mC and 5hmC. Therefore, the previously described functions of 5mC must now be re-evaluated using more sensitive techniques such as antibody-based immunoprecipitation protocols (e.g. MeDIP, hMeDIP). These fundamental discoveries poise the field for re-thinking 5mC and 5hmC dynamics in the context of treating CVD and possible diagnostic or prognostic tools.

9.3.2 The Histone Code

The second layer of epigenetic gene regulation resides in the modification of histone proteins, around which the DNA double helix is wound. These evolutionarily conserved proteins are comprised of a globular domain and N-terminal tail. In particular the histone tails provide a robust platform for a myriad of post-translational modifications that regulates gene expression in health and disease (Wang et al. 2007). Allis proposed the histone code hypothesis in 2000, that post-translational histone modifications encrypt a language that dictates chromatin accessibility (Strahl and Allis 2000). To date, more than 100 distinct modification sites have been characterized (Tan et al. 2011). Well-studied histone post-translational modifications include lysine acetylation and lysine methylation, among others (Table 9.1). Many of these marks are mutually exclusive for any given histone amino acid residue. For example, histone 3 lysine 9 (H3K9) can either be acetylated or methylated,

Table 9.1 Known epigenetic modifications and their effect on gene transcription (Reprinted from Turgeon et al. (2014). With permission from Karger and Basel)

Mechanism	Transcriptional effect
DNA modifications	
Methylation (CpG dinucleotides)	↓
Hydroxymethylation (CpG dinucleotides)	?
Histone posttranslational modifications	
Histone H3	
Acetylation (K9, K14)	↑
Methylation	
K4, K36	↑
K9, K27	↓
Phosphorylation (S10)	↑/↓
Histone H4	
Acetylation (K5, K8, K12, K16)	↑
Methylation (K20)	↓
Long noncoding mRNA (lncRNA) mechanisms	↑/↓
E.g. Xist, ANRIL, HOTAIR	↓
E.g. HOTTIP	↑
microRNA (miRNA) mechanisms	?

K lysine, *S* serine

but not both. As alluded to earlier, the histone code synergizes with DNA methylation to provide a dynamic epigenetic landscape superimposed on the underlying static genetic code.

A series of histone code "writers", "readers" and "erasers" are known (Wang et al. 2007). Each modification is catalyzed by an increasing catalogue of "writers" that can then be interpreted by a distinct group of "readers". Histone lysine acetylation is one of the most well characterized permissive epigenetic marks that are strongly correlated with transcriptional activation. The negatively charge acetyl group neutralizes the positive charge on the histone tails, loosening the electrostatic interaction with the negatively charged DNA molecule. Therefore, promoters containing lysine acetyl modifications are sterically accessible to transcription factors and activators. Unexpectedly, classical transcriptional co-activators contain histone acetyltransferase (HAT) activity. The removal of histone lysine acetylation is accomplished by four families of histone deacetylases (HDAC classes I-IV) or "erasers". These HDACs are further categorized experimentally by their sensitivity to inhibition by the pharmacological agent, trichostatin A (TSA). Class I and II HDACs are TSA-sensitive, while class III and IV HDACs are TSA-insensitive. Interestingly, HATs and HDACs demonstrate poor specificity for individual lysine residues and are recruited to target promoters in large, multiprotein complexes through a group of acetyl-lysine "readers" that contain a bromodomain. Importantly, HDAC inhibitors have found early clinical purpose in the treatment of various cancers, neurodegenerative disease and inflammation (McKinsey 2012). In mouse model of atherosclerosis, the addition of TSA accelerated macrophage infiltration

and fatty streak development (Turunen et al. 2009). These conflicting ideas demonstrate the complexity of epigenetic modifications in both health and disease that must be overcome before translational applications can be made.

Unlike histone lysine acetylation, lysine methylation can have diverse residue-specific and context-dependent effects gene expression. Lysine methylation follows a complex biology due to its mono-, di-, or tri-methylation capacity, each with profoundly distinct effects on mammalian gene expression (Kouzarides 2007). For example, H3K27 tri-methylation is a repressive transcription mark leading to heterochromatin, while H3K4 mono-methylation is an activating transcription mark. Proteins containing the Tudor domain, MBT domain, or PHD domain reads specific histone methylation marks and exerts the respective downstream effects of the code (Kouzarides 2007). The functional consequence of these modifications may be related to their differential localization within the genome. For example, it has been proposed that H3K4 mono-methylation preferentially localizes to enhancers and H3K4 tri-methylation to active promoters (Heintzman et al. 2007).

Histone post-translational modifications have been proposed to epigenetically regulate gene expression through two broad mechanisms. First, histone post-translational modifications can alter the physical structure of chromatin and its accessibility to DNA-binding proteins, such as transcriptional regulators (Kouzarides 2007). As previously mentioned, the higher-order compaction of chromatin is prevented by histone lysine acetylation. More important than the direct physical effect on the structure of chromatin, perhaps, is the regulatory information contained within the specific combinations of histone marks. The "histone code hypothesis" provides the mechanistic concept that a given combination of modifications is read by a combination-specific protein or protein complex to effectuate a specific gene expression outcome (Kouzarides 2007; Patel et al. 2011). Given the diversity of histone modifications, there are a myriad of permutations that can give rise to distinct functions. Therefore, deciphering the histone code remains a daunting, yet top priority for the epigenetics research community.

In addition to considerable complexity of histone post-translational modifications, important roles for nucleosome density, ATP-dependent chromatin remodeling, and the regulated, replication-independent incorporation of histone variants in the control of mammalian gene expression (Faast et al. 2001; de la Serna et al. 2006). These additional layers of epigenetic control provide further complexity and sophisticating the highly responsive system superimposed on the static genetic code. Altogether, tremendous strides have been made in understanding the impact of specific histone post-translational and density modifications in the control of mammalian gene expression.

9.3.3 Long-Noncoding RNAs

Historically, the central dogma of genetics describes a linear pathway of DNA being transcribed into an RNA intermediate to ultimately translate effector protein molecules. This dogma, however, has been shown to describe only a small proportion of

our genome. Long non-coding RNAs (lncRNA) encompass the third layer of epigenetic regulation. Although the nuclear function of lncRNAs is the least understood epigenetic regulator, the discovery of lncRNAs has highlighted RNA function outside of the central dogma of genetics. The field is still in its infancy and general criteria to be catalogued as "lncRNA" has not yet been established. Henceforth, we will conservatively define lncRNA as a single stranded >200 nucleotide long transcript with an RNA function. lncRNAs can mediate activation or repression of target genes through DNA methylation and histone posttranslational modifications, as described above. Interestingly, unlike protein-coding genes, lncRNAs often lack strong conservation across species, suggesting that they may be under rapid evolutionary divergence and key for speciation (Rinn and Chang 2012).

One of the earliest characterized lncRNAs is X-inactive specific transcript (XIST), which is involved in one X-chromosome inactivation in female mammals. This lncRNA works in conjunction with repressive histone marks and DNA methylation to silence one of the two X chromosomes in female somatic cells (Lee and Bartolomei 2013). Initially, Xist and other long non-coding RNAs were thought to be rare examples of this functional group of RNAs. A global and unbiased approach using chromatin state mapping identified unique signatures of H3K4 tri-methylation at promoters and H3K36 tri-methylation, termed K4–K36 domains at actively transcribed genomic regions (Guttman et al. 2009). This led to the identification of 1,600 human intergenic lncRNAs and has further expanded to more than 8,000 lncRNAs that may exist near or antisense to protein coding genes since 2007. Most early work has focused on the functional identification of lncRNAs and their interaction with other epigenetic modifying machinery, but the effects of the genomic context has also become a prominent area of research (Wang et al. 2011).

Two excellent examples that encompass some of these newer facets of lncRNA function come from the *HOX* gene clusters (Rinn et al. 2007). HOTAIR is expressed from the *HOXC* cluster on chromosome 12 in humans and acts *in trans* to regulate the expression of developmental genes in the *HOXD* locus on chromosome 2. It silences the *HOXD* cluster, in part, by recruiting polycomb repressive complex 2 (PRC2) which facilitates the addition of H3K27 tri-methylation marks. In general, this is a labile repressive histone mark. The mechanism for how this targeting occurs remains unclear. A second lncRNA found in the *HOX* gene cluster is HOTTIP, which is expressed at the distal tip of the *HOXA* locus and promotes transcription, in *cis*, of the adjacent *HOXA* genes. It activates these genes, in part, through its interaction with the histone methyltransferase MLL, which mediates H3K4 trimethylation. These two examples of lncRNAs provide good examples of lncRNAs with contrasting function, namely ones that act either in *cis* or *trans* to either activate or repress transcription, respectively. More complicated models exist, where the lncRNA overlaps or are present within introns of coding genes. Two notable examples exist in CVD, namely, sONE and ANRIL, which will both be discussed below. In general, the study of lncRNAs is just beginning. Their roles must be distinguished functionally from the biological effects of the adjacent protein coding genes.

9.3.3.1 Epigenetic Mediator: ANRIL

GWAS have identified lncRNAs that may be relevant to the genetic predisposition to CVD, especially ischemic stroke and CAD (please see 10.1.2). lncRNA Antisense Non-coding RNA in the INK4 Locus (ANRIL, CDKN2B antisense RNA), as its name suggests, is found within the *INK/ARF* gene cluster (Pasmant et al. 2007). The ANRIL genomic locus spans approximately 42 kb on human chromosome 9p21. Only months after its discovery, several SNPs associated with augmented risk for CAD were found in a genomic region devoid of protein coding gene but in, or near, the ANRIL lncRNA (Helgadottir et al. 2007; McPherson et al. 2007). Several disease associated SNPs: rs1333049, rs10757274, rs10757278, rs2383206 and rs2383207, showed modest effects size in various aspects of CVD. SNP rs10757274 is the strongest genetic predictor of early myocardial infarction, independent of the classical CAD risk factors such as lipoproteins or hypertension, making ANRIL a key candidate for linking genotype with phenotype in CVD (McPherson et al. 2007). In CAD, rs10757278 shows a modest effect size but was also identified in GWAS studies for increase risk of atherosclerosis (Liu et al. 2009).

The molecular characterization of the encoded RNA has been complex due to the generation of multiple splice variants, both linear and circular (Burd et al. 2010). Importantly, ANRIL expression occurs in cells known to play a prominent role in atherosclerosis/CVDs, namely ECs, VSMCs and immune cells. Functional studies have found that ANRIL aids in *cis* recruitment of chromatin-modifying complexes, specifically, polycomb repressive complexes 1 and 2 (PRC1 and PRC2), to neighboring genes of the INK4b/ARF/INK4a locus. ANRIL regulates these genes by recruiting PRC2 to add H3K27 tri-methylation repressive methylation marks. H3K27 tri-methylation is then recognized by PRC1 which establishes H2AK119 mono-ubiquitylation to silence transcription (Yap et al. 2010). Of note, these protein-coding genes were known to serve as tumor suppressor genes through the regulation of cellular division and senescence (Congrains et al. 2013). It is believed that ANRIL transcription and secondary structure formation are able to interact and direct PRC1 and PRC2 to the adjacent INK4/ARF locus to mediate the establishment of these repressive marks. The presence of these epigenetic marks is associated with chromatin compaction and transcriptional repression of these protein-coding genes (Yap et al. 2010). One model suggests that the presence of the disease-associated SNP allele changes the abundance or function of ANRIL splice variants, resulting in their reduced ability to regulate the INK4/ARF locus SNPs within the risk locus (Fig. 9.4) (Burd et al. 2010).

The clinical implications and the basic science discovery of lncRNAs are just emerging. ANRIL is a specific example of a lncRNA which was identified through unbiased GWAS studies and is associated with a non-Mendelian human disease and offers an exciting newer perspective on CAD pathophysiology. Moreover, many unbiased GWAS studies have identified a significant proportion of hits that fall into genomic regions with no protein coding genes, termed “gene deserts” (Hindorff et al. 2009). This raises a key concept, that a gene can be implicated in the genetic cause of disease because it produces a functional RNA as an epigenetic modifier.

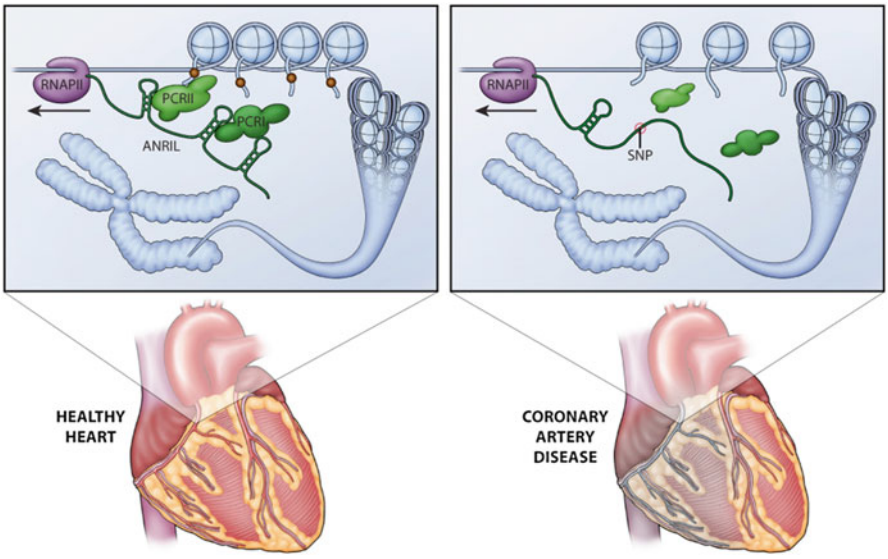

Fig. 9.4 ANRIL: potent predictor of CAD. Long noncoding RNAs can act in cis or trans to activate or repress gene transcription through modification of chromatin. In cis, the lncRNA is transcribed and recruits chromatin-modifying complexes to the site of transcription. For example, ANRIL recruits PRC2, which serves to repress the transcription of adjacent genes that have histones with the mark H3K27me3

9.3.4 miRNAs

Although the distinction between small and long non-coding RNAs is an arbitrary length, small non-coding RNAs can play a role gene regulation. For example, they have been implicated in epigenetic mechanisms in yeast and plants but evidence in mammals are still preliminary (Kim et al. 2008). MicroRNAs (miRNAs), a subgroup of small non-coding RNAs, are short (~22 nucleotides) non-coding single stranded RNAs. Although not directly involved in epigenetic regulation, many would argue their involvement in gene regulation and importance in disease through the regulation of translation of mRNAs and abundance of RNA species. In mammals, their most well characterized role is in the RNA interference (RNAi) pathway where miRNAs repress expression of target genes post-transcriptionally by binding to target RNAs via partially complementary seed sequences (~7 nucleotides) (Kim et al. 2009). Mature miRNA sequences, especially the seed sequence, are highly conserved between species. This short sequence is required to repress expression and allows one miRNA to silence multiple genes. Indeed, there are 2042 annotated human miRNAs on miRBase v.19 (http://mirbase.org) that are predicted to control greater than 60 % of all human protein coding genes, making miRNAs one of the most abundant gene regulatory pathways in a cell (Friedman et al. 2009). The discovery of RNAi by Craig and Mello led to their Nobel Prize in 2006, but its receipt

in the field of epigenetics was controversial (Fire et al. 1998). Importantly, a clear interplay between miRNAs and other epigenetic pathways have been described, especially in human diseases. For example, miRNA-26a is downregulated in prostate cancer due to hypermethylation of its promoter, leading to the overexpression of *EZH2*, a H3K27 methyltransferase (Dang et al. 2012; Borno et al. 2012). Moreover, interactions between lncRNAs and miRNAs have been described in complex gene regulatory loops. Specifically, lncRNA molecules can act as "molecular sponges" for miRNAs. This prevents these miRNAs from repressing target protein-coding miRNAs via post-transcriptional processes. Additionally, miRNAs contribute to concepts which can be applied to long non-coding RNAs, such as intercellular trafficking and as biomarkers in disease. Therefore, we will discuss miRNAs in this chapter, but further studies are required to unveil direct chromatin-based effects in mammals.

Biogenesis of miRNAs follows a highly conserved pathway (Ho and Marsden 2014). In mammals, primary miRNAs (pri-miRNAs) are mainly transcribed by RNA polymerase II, and to a lesser extent by RNA polymerase III. Drosha, a nuclear RNase III enzyme, cleaves pri-miRNAs, forming premature miRNAs (pre-miRNA) hairpins. RNase III-type enzymes cleave asymmetrically, leaving a 2 nt 3′ overhang which is recognized by the nuclear transmembrane protein Exportin 5 that shuttles pre-miRNAs into the cytoplasm in a Ran-GTP dependent manner. A cytoplasmic RNase III enzyme, Dicer, further processes pre-miRNAs into mature miRNAs and load them onto effector Argonaute proteins to form the RNA induced silencing complex (RISC) (Fig. 9.5). Growing evidence suggests that miRNAs are crucial for vascular homeostasis, the dysregulation of which has been shown to at least partially contribute to CVD pathogenesis (Bernstein et al. 2003; Merritt et al. 2008; Pan et al. 2011).

9.3.4.1 miRNA-126 in Endothelial Biology

miRNA-126 is highly enriched in endothelial cells and plays a unique role in regulating endothelial homeostasis and angiogenesis (Fish et al. 2008). It is derived from intron 7 of a protein coding gene, namely endothelial growth factor like 7 (*Egfl7*), which is also involved in angiogenesis (Wang et al. 2008). It is not uncommon for a miRNA and its host transcription unit to share similar function (e.g. miRNA-33a and *SREBP2*), but a miRNA can downregulate its host transcription unit in a negative feedback loop (e.g. miRNA-133b and *linc-MD1*) (Marquart et al. 2010; Legnini et al. 2014). miRNA-126 promotes vascular growth by inhibiting expression of VEGF (vascular endothelial growth factor) signaling antagonists, PIK3R2 (phosphoinositide kinase 3 regulatory subunit 2) and SPRED-1 (sprouty related EVH1-domain containing 1). This contributes to an overall regulation in the amount of VEGF signaling through translational regulation. Accordingly, knockout of miRNA-126 without disruption of Egfl7 expression in a mouse model revealed impaired angiogenesis after myocardial infarction (Fish et al. 2008). Furthermore, miRNA-126 has very specific functions in ECs such as attenuation of inflammation by

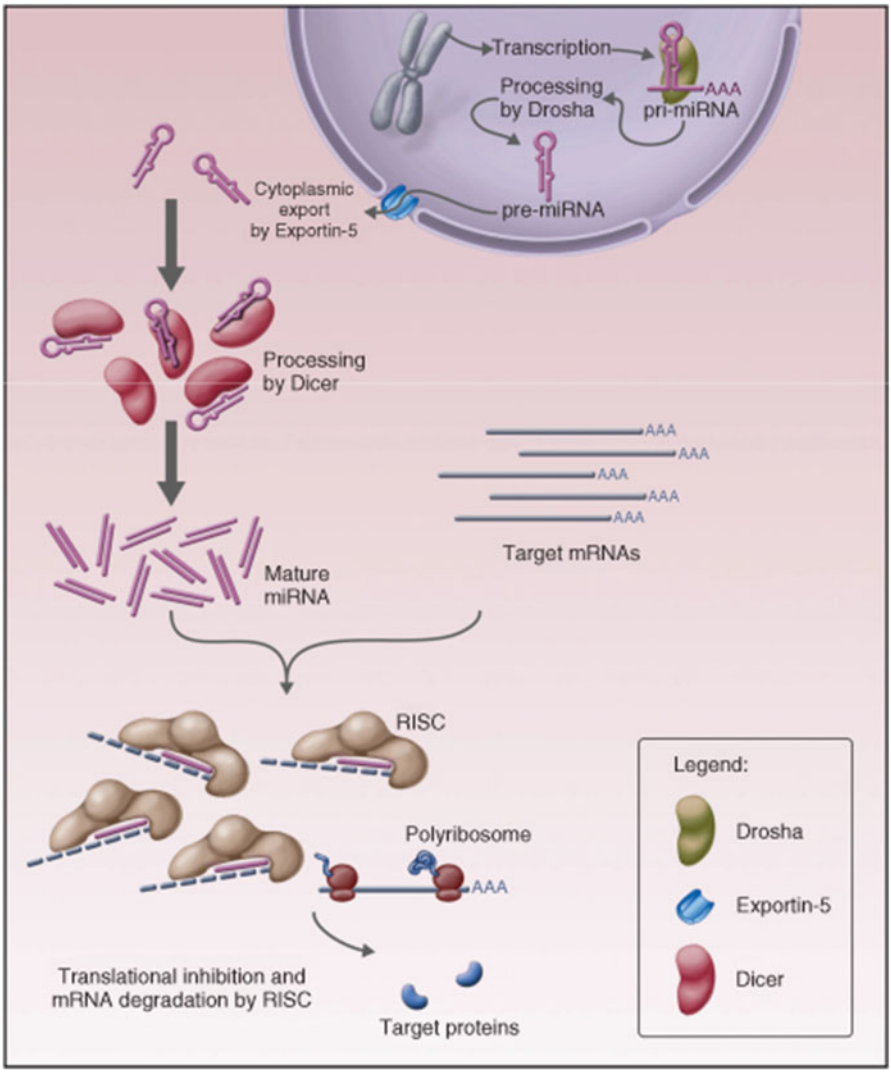

Fig. 9.5 Schematic of the miRNA processing machinery. pri-miRNAs are transcribed in the nucleus and cleaved by Drosha into hairpin pre-miRNAs. pre-miRNAs are shuttled to the cytoplasm via Exportin 5 where it is finally processed by Dicer into mature miRNAs and loaded onto effector Argonautes to form the RNA-induced silencing complex. RNAs that contain regions of complementarity to miRNAs are targeted for degradation and or translational repression (Reprinted with permission from Wiley et al. (2014))

directly targeting vascular cell adhesion molecule 1 (VCAM1), which recruits lymphocytes and monocytes and initiates fatty streak formation in early atherosclerotic plaques. More recently, serum-starved ECs were shown to form apoptotic bodies that can mediate the transmission of miRNA-126 to nearby ECs, but whether other

cell types such as adjacent VSMCs may also take up these apoptotic bodies have not yet been elucidated (Fichtlscherer et al. 2010).

9.3.4.2 miRNA-145 Carries Therapeutic Potential for Cardiovascular Diseases

miRNA-145 is one of the most abundant and well-studied miRNAs in VSMCs. It is characterized as a housekeeping VSMC miRNA and is often viewed as the VSMC equivalent of miRNA-126 in ECs (Cheng et al. 2009). In contrast to miRNA-126, pri-miRNA-145 is transcribed bicistronically with miRNA-143 independent of a host gene (Cheng et al. 2009; Elia et al. 2009). Of note, miRNA-145 targets Kruppel-like-factor 4 (KLF4), one of the four Yamanaka factors that induce pluripotency (Okita et al. 2007). Desuppression of KLF4 in synthetic VSMCs leads to the downregulation of smooth muscle markers such as myocardin in CVD, including atherosclerosis. In vitro cultured VSMCs overexpressing pre-miRNA-145 showed significantly lower proliferation and migration, highlighting the role of miRNA-145 in maintaining the contractile phenotype (Cheng et al. 2009). miRNA-145 knockout mice exhibit high vascular abnormalities including aneurysms and atherosclerosis-like lesions within the vasculature without hypercholesterolemia (Elia et al. 2009). VSMC dysfunction was apparent and canonical VSMC markers and contractility of the vessel were lower in these mice. Reduced levels of miRNA-145 were also observed in balloon-injured and atherosclerotic mice, two disease models where VSMC proliferation can be observed (Cheng et al. 2009; Lovren et al. 2012). Rescue of miRNA-145 via lenti-virus significantly reduced the disease burden in both models, foreshadowing a therapeutic potential for miRNA-145 in human vascular dysfunctions. miRNA-145 downregulation represents a critical and targetable pathway for CVD in the future development of RNA-based therapeutics.

The two aforementioned miRNAs are very relevant to disease prognosis and outcome, particularly in cardiovascular diseases. They are responsive to the circulating microenvironment and overall function of the cell. Importantly, although there is no current evidence for these microRNAs in epigenetic regulation of genes, they have provided scientists with a unique concept, gene regulation through RNA trafficking. This concept may be applicable to lncRNAs where epigenetic regulation occurs in cells distant from the site of synthesis. Moreover, the use of miRNAs as biomarkers for disease may be expanded to encompass lncRNAs.

9.4 Hemodynamics: Internal Stimuli Driving Vascular Epigenetics

Although risk factors such as hyperlipidemia affect the vascular endothelium in a systemic manner, the development of atherosclerotic lesions displays a focal distribution (Won et al. 2007). This pattern is not arbitrary, as numerous studies have

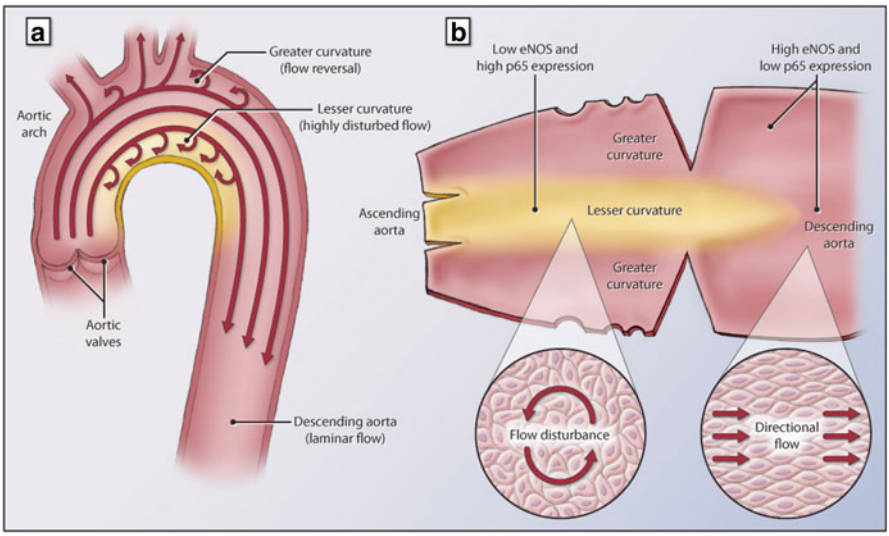

Fig. 9.6 Hemodynamics effect gene expression. (**a**) Flow patterns of the aortic arch display both laminar flow in the greater curvature, and disturbed flow in the descending thoracic aorta. (**b**) En face dissection of the aortic arch reveals interesting phenotypic changes in endothelial cells (ECs). ECs of the greater curvature experiences laminar flow, exhibit eNOS expression and decreased p65 expression, an inflammatory mediator in ECs, and are less prone to atherosclerosis. These cells show a directional cell shape, which correspond with directional flow. ECs of the lesser curvature of the aortic arch are prone to atherosclerosis, decreased eNOS expression and p65 enrichment. These ECs also experience disturbed flow and exhibit a disorganized pattern (Adapted from Turgeon et al. (2014). With permission from Karger and Basel)

identified a role for hemodynamics in priming certain areas of our vasculature for plaque formation. The physical forces of blood circulation are dependent on the size and geometric orientation of the host vessel and thus are not consistent across all ECs. The endothelium is exposed to different forms of hemodynamic forces, but most attention has been on the influence of shear rate and shear stress on EC biology. Blood flow can be modeled as an infinite series of concentric layers called laminae (Papaioannou and Stefanadis 2005). Shear rate (s^{-1}) is the rate of relative movement among the adjacent layers of fluid (Papaioannou and Stefanadis 2005). Shear stress (dynes/cm^2) is defined as the tangential frictional force per area generated at the surface of the endothelium by blood flow. Largely, arterial flow patterns are categorized into two broad subgroups: atheroprotective and atheroprone flow (Chatzizisis et al. 2007). Atheroprotective flow is characterized by laminar flow at straight segments of the arterial network (e.g., descending thoracic aorta) and relatively higher levels of shear rate/stress (Fig. 9.6a). In contrast, atheroprone flow occurs at geometrical irregularities, such as bifurcations or curvatures (e.g., lesser curvature of aortic arch, human coronary artery), resulting in flow turbulence, flow reversal and lower average levels of shear rate/stress (Chiu and Chien 2011).

Accordingly, EC gene expression is not uniform across the vasculature (Fig. 9.6b) (Won et al. 2007). Atheroprotective flow induces expression of atheroprotective genes such as Kruppel-like factor 2 (KLF2). KLF2 is a major transcription factor in ECs that is upregulated by flow to activate down-stream flow-responsive genes such as eNOS. KLF2 and eNOS expression is significantly higher in endothelial cells exposed to laminar flow in vitro and in vivo. On the other hand, atheroprone flow predisposes the site for development of atherosclerosis via expression of inflammatory cascade priming the NF-κB pathway. The NF-κB complex is a transcription factor that activates immune-response genes such as VCAM1, which is a known player in the early stages of atherogenesis (Chiu and Chien 2011). Emerging ideas in the field infer chronic exposure to different patterns of flow elicits epigenetic changes to reflect the respective endothelial gene expression. More interestingly, recent work showed EC-VSMC communication in response to hemodynamic forces via epigenetic pathways, namely miRNAs.

9.4.1 Intercellular Communication Via miRNAs in Response to Flow

Accumulating data suggest that secreted miRNAs may have paracrine downstream effects on neighboring cells during development and disease. Circulating miRNAs have previously been proposed as non-invasive biomarkers for diagnosis of various malignancies including cardiovascular diseases and cancer, but the functionality of these miRNAs was still unclear (Fichtlscherer et al. 2010). Since RNA is highly labile, circulating miRNAs are generally secreted in microvesicles or as a miRNA-protein (microRNP) complex. Two paradigm-shifting studies revealed intercellular communication from ECs to VSMCs via secreted miRNAs in a flow-responsive manner (Fig. 9.7).

Several miRNAs (e.g. miRNAs-10a, -19a, -23b, 92a, etc.) have been shown to be regulated by flow and exert effects on endothelial gene expression (Wu et al. 2011; Hergenreider et al. 2012). A landmark study in 2012 by Hergenreider et al. unexpectedly found miRNA-145 to be upregulated by KLF2 in endothelial cells where no known targets of miRNA-145 have been annotated. Surprisingly, the upregulated miRNA-145 in ECs are packaged and secreted in microvesicles in a flow-dependent manner as paracrine signals to neighboring cells (Hergenreider et al. 2012). In a VSMC-EC co-culture assay, labeled miRNA-145 was actively transported from ECs to VSMCs. This study was the first to show that secreted miRNAs may be used in intercellular signaling cascades and opened up the idea that the functional location of a miRNA does not necessarily dictate its origin. If we extrapolate this finding to assume that atheroprone flow reduces miRNA-145 secretion by ECs, this reduction may contribute to the synthetic phenotype of VSMCs in that region. Therefore, atheroprone flow not only primes ECs to the development of atherosclerosis, but also VSMCs via EC-derived miRNA-145 – a key modulator of VSMC phenotype during development and disease.

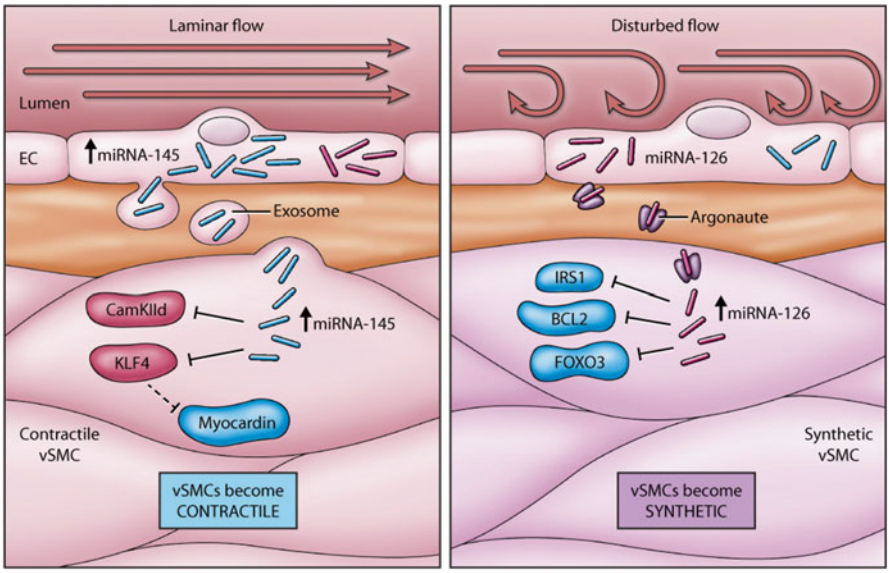

Fig. 9.7 Intercellular communication via miRNAs in response to different patterns of flow. Endothelial cells secrete different miRNAs in response to the hemodynamic forces of flow. The secreted miRNAs exert downstream effects on vascular smooth muscle cell phenotype, especially in disease settings

The targets of miRNA-126 were mainly studied in the context of endothelial cells, until it was recently shown to control VSMC turnover via secreted miRNA-126-Ago2 complex (Zhou et al. 2013). Unlike miRNA-145, laminar flow attenuated the endothelial secretion of miRNA-126. However, disturbed flow increased miRNA-126 uptake by VSMCs and downregulation of its target genes – BCL2, FOXO3 and IRS1. These proteins are key molecules that induce cell cycle progression, proliferation and apoptosis. Increased cell proliferation of VSMCs and decreased VSMC markers were observed in a co-culture system; these effects were rescued by application of laminar flow or inhibition of miRNA-126 in ECs or VSMCs. In vivo ligation-induced-neointimal-growth model in miRNA-126 knockout mice showed increased levels of neointimal growth and VSMC proliferation when miRNA-126 was delivered locally to VSMCs (Zhou et al. 2013). It is interesting that ECs can selectively transfer miRNAs to VSMCs in response to different shear forces of blood flow. Under laminar flow, miRNA-145 is secreted to promote the contractile phenotype whereas under disturbed flow, miRNA-126 is secreted to promote the synthetic phenotype. Further studies are required to discover secreted lncRNAs and their putative roles in vascular homeostasis and disease. Although the study of paracrine RNA signaling is still young, it would be important to consider the intercellular communication between ECs and VSMCs in developing RNA-based therapies for vascular diseases.

9.5 eNOS, the Model Epigenetic Gene

Given the role of EC dysfunction in the initiation, progression and clinical phenotype of atherosclerosis, investigating how gene expression is regulated in these cells and how these processes are perturbed in disease will provide novel insight into the pathogenesis of CVD. Historically, endothelial dysfunction is defined as perturbation of eNOS expression or function (Marsden and Brenner 1991; Wilcox et al. 1997). eNOS is the most abundant source of NO in the vasculature and its expression is basally restricted to ECs. Soon after the cloning of the eNOS gene, the promoter was characterized and examined for mechanisms of endothelial specificity (Marsden et al. 1992, 1993). A plethora of classical *cis*/*trans* elements were identified on the eNOS promoter as thoroughly reviewed in Tai et al. but the prevailing regulatory mechanism has been shown to be epigenetics (Tai et al. 2004).

The initial discovery of the role of epigenetics in eNOS gene expression came from the use of episomal eNOS promoter-reporter constructs (Chan et al. 2004). Unlike the native gene, the episomal construct was expressed in all cell types examined in vitro, implicating the importance of chromatin in cell-specific expression of the eNOS promoter. Indeed, stably integrated eNOS promoter-reporter constructs into the genome of transgenic mice demonstrated similar expression patterns to the native eNOS gene (Teichert et al. 2000). These results suggested that eNOS specificity is not governed solely by classical *cis*/*trans* elements, but also by epigenetic chromatin-based mechanisms. The first direct evidence for eNOS epigenetic regulation was the functional differential DNA methylation at the eNOS promoter (Chan et al. 2004). DNA hypomethylation at the eNOS promoter was observed in ECs in contrast to dense DNA methylation in non-expressing cells, such as VSMC. This idea of endothelial-enriched expression due to differential DNA methylation was recently expanded to encompass several other important EC-enriched genes such as *CD31*, *vWF* and *ICAM2* (Shirodkar et al. 2013). Interestingly in human atherosclerotic lesions, eNOS protein expression was blunted in ECs; in contrast, VSMCs showed upregulation. It will be important to examine epigenetic dysregulation in atherogenesis that may have led to this irregularity.

When examined in depth, endothelial chromatin structure at the eNOS promoter harbors activating histone marks such as acetyl H3 and H4 and tri-methylated H3K4, while VSMCs lack these marks (Fish et al. 2005). Histone modifications and DNA methylation play a synergistic role in endothelial eNOS specificity as inhibition of both pathways in non-expressing cells can rescue eNOS expression to a degree that is greater than the sum of each respective inhibition. The environment can regulate the histone marks at the eNOS promoter. Hypoxia, a model for cellular stress that is highly relevant to CVD, causes a decrease in histone activating marks such as acetylation, H3K4 tri-methylation as well as histone eviction under acute conditions (4 h). Under chronic hypoxia (24 h), the histone octamers returned to normoxic levels, but the basal activating marks were not present (Fish et al. 2010). Moreover, in response to laminar flow, there is increased acetylation of histones H3 and H4, which is indicative of transcriptional activation (Huddleson et al. 2005). Downregulation of HDACs in laminar sheer stress can also indirectly induce KLF2

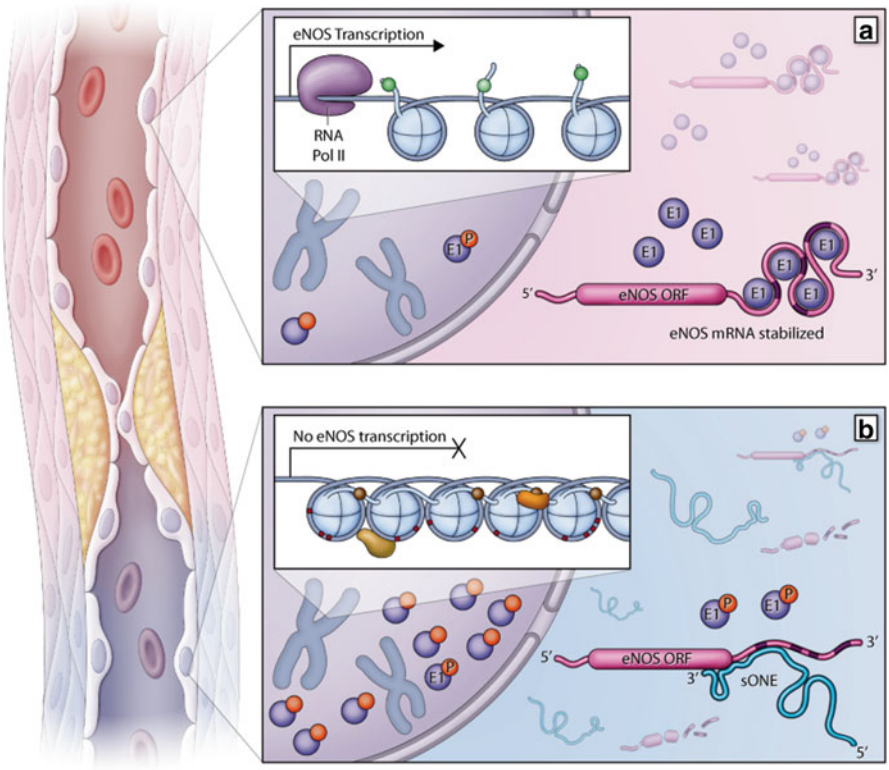

Fig. 9.8 eNOS, a model epigenetic gene. eNOS is highly responsive to environmental stress through epigenetic mechanisms. Under chronic hypoxia, histone remodelling occurs to repress eNOS transcription. Furthermore, reduced stabilizing agents on eNOS mRNA leads to susceptibility to post-transcriptional mechanisms including sONE and miRNA based degradation (Adapted from Ho and Marsden 2014)

and eNOS transcription (Burd et al. 2010). Taken together, these data suggest that histone modifications are highly responsive to environmental stimuli in regulating eNOS expression.

It is also worth highlighting that hypoxia upregulates the expression of a lncRNA, sONE (eNOS antisense, *NOS3AS*), which overlaps the 3′ end of the eNOS gene (Fish et al. 2007). sONE has been shown destabilize eNOS mRNA at the post-transcriptional level as a natural antisense transcript. Under basal conditions, however, a stabilizing complex, hnRNP E1, binds to eNOS mRNA 3′UTR and protects it from both sONE and complementary miRNAs (Ho et al. 2013). However, under cellular stress such as hypoxia, hnRNP E1 is unable to bind to the 3′UTR and repressive factors are able to target eNOS mRNA for degradation. Therefore, eNOS is highly regulated both epigenetically and post-transcriptionally in response to environmental stimuli. Further studies to assess possible roles of sONE at the chromatin level will add a novel epigenetic mechanism to the model epigenetic gene, eNOS (Fig. 9.8).

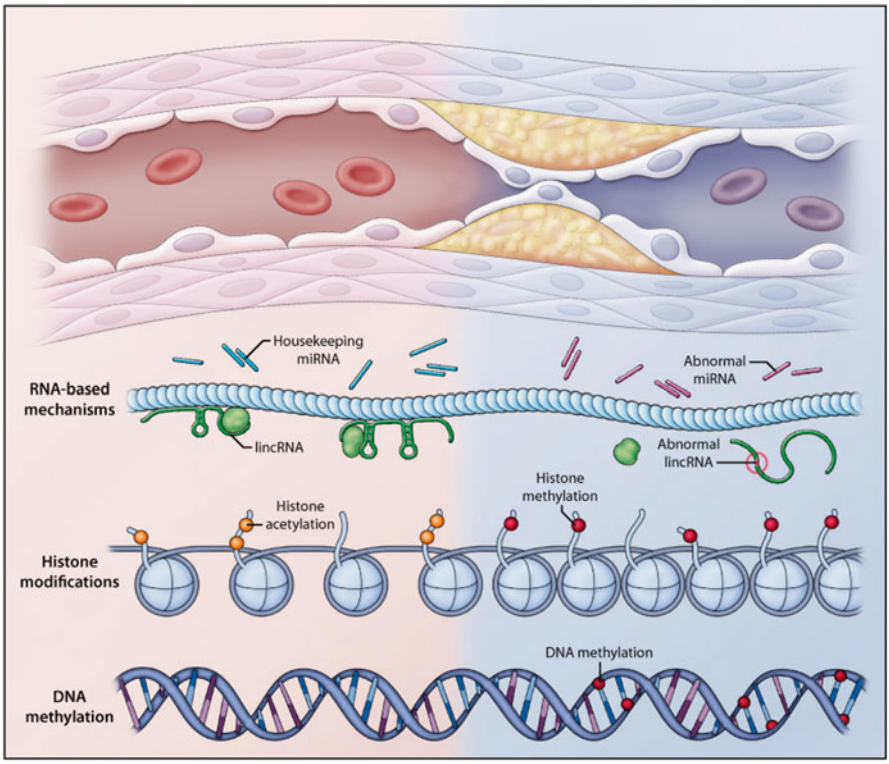

Fig. 9.9 Environmental contributors to epigenetic dysregulation in cardiovascular diseases. Epigenetic mechanisms, including RNA based mechanisms, histone modifications and DNA methylation, serve as an important regulatory tool in gene regulation. All facets of epigenetics have been correlated to development of cardiovascular diseases. Although translational aspects of epigenetic research are still in its infancy, therapies that target epigenetic dysregulation are starting to emerge and will be the focus of future research

9.5.1 Future Directions

Therefore, our epigenome is comprised of a complex network of modifications that synergize with each other and the static DNA code to control gene expression and cellular phenotype. Most notably, the epigenome is dynamic and highly responsive to environmental stimuli. Recently, several epigenetic mechanisms have been correlated with CVD and studies have shown its importance in disease pathophysiology (Fig. 9.9). The use of epigenetic modifiers in the clinic has shown promise. DNMT inhibitors have been approved for myelodysplastic syndrome and acute myelogenous leukemia, while various HDAC inhibitors are being used for certain T-cell lymphomas. Alternatively, the epigenetic modifiers can also be used for prognostic purposes. For example, HOTAIR expression was found to be predictive of breast cancer metastasis and death. In the future, lncRNAs may serve a prognostic

purpose and remain a therapeutically targetable candidate in CVD. It would not be surprising if other epigenetic mechanisms may be translationally applied to vascular dysfunction and allow us to modulate important players such as eNOS and ANRIL in diseased vessels.

Our previous knowledge on gene control, specifically the *cis*/*trans* paradigm, has provided invaluable models of transcription regulation in the vasculature during health and disease. Epigenetic theory unveils a novel molecular basis for understanding inter-individual differences outside the static code. Furthermore, it serves as an intermediary between environmental cues and genetic reactions, initiating adaptive responses or eliciting disease susceptibility. It is well established that our environment has chronic effects on gene expression. As life expectancy increases, scientists must use new paradigms to help understand disease progression, especially in vascular diseases such as CAD. New therapies and approaches for non-Mendelian diseases, CAD in particular, are now urgently needed to help decrease the tremendous socioeconomic burden caused by this spectrum of diseases. We look towards epigenetic theory to fill these gaps in knowledge in the future.

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Chapter 10
Gene-Environment Interactions in Human Health

Leah E. Mechanic and Carolyn M. Hutter

Abstract Risk of most complex diseases is determined by a combination of environmental and genetic factors. By studying gene-environment interactions, it may be possible to describe disease mechanisms, discover novel genetic variants associated with disease, better understand heterogeneity between populations, identify populations with higher risk from exposure, and target preventive and therapeutic interventions. However, there are several challenges to the study of gene-environment interactions. As technologies and analytical tools improve, opportunities to better understand the complex interplay between genes and environment may result in improved insights in disease and treatment outcomes.

Keywords Gene-environment interactions • Germline genetic variation • DNA sequence • Single nucleotide polymorphisms (SNPs) • Somatic mutation • Pharmacogenomics • Pharmacogenetics • Genome-wide association study • GWAS • Epigenetic alterations • Rare variants • Environment • Common genetic variation • Interaction • Study design • Case-control • Nested case-control • Case-only • Family designs • Family-based designs • Recall bias • Biomarkers • Reverse causality • Cohort • Independence assumption • Collaborative cross • Relative risk (RR) • Risk difference (RD) • Odds ratio (OR) • Multiplicative scale • Additive scale • Synergistic • Antagonistic • Interaction p-value • Measurement error • Misclassification • Environmental exposure assessment • Exposome • Power • Alpha • Multiple comparisons • Biological interactions • GEWIS • GE-Whiz • Personalized medicine

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10.1 Why Study Gene-Environment Interactions?: Implications for Human Health

Not everyone exposed to an environmental risk factor develops disease. There are clear examples that show how modification of environmental exposure may reduce impact of genetic mutations. A classic example is phenylketonuria (PKU), which is a syndrome resulting from a genetic deficiency in phenylalanine hydroxylase. For children with PKU, reduction or elimination of exposure to phenylalanine will prevent adverse effects (National Institutes of Health Consensus Development Panel 2001). In another rare genetic syndrome, xeroderma pigmentosum (XP), patients are 10,000 times more likely to develop skin cancer before age 20 due to germline mutations in the DNA excision repair pathway. These effects are reduced by avoiding exposure to sunlight (DiGiovanna and Kraemer 2012). These two examples focus on rare diseases. For the majority of human diseases and disorders, including common complex diseases (e.g., cardiovascular disease, cancer, and diabetes) it is also believed that susceptibility involves the combination of multiple genetic and environmental factors (Bookman et al. 2011). Even in a complex disease like lung cancer, where smoking is a strong established risk factor, lung cancer does not develop in everyone who smokes, suggesting that genetic predisposition could play a role in disease risk (Alberg et al. 2005).

Given the known roles of both environmental and genetic influences on disease, several motivating factors are often cited for studying gene-environment interactions (GxE) (for review (Thomas 2010)). For example, these studies may assist in elucidating biological pathways to understand mechanisms which cause complex diseases as has been done for genetic variation in N-acetyltransferases (NATs), exposure to aromatic amines (AA) polyacyclic aromatic hydrocarbons (PAHs) and bladder cancer (Kadlubar and Badawi 1995) (Fig. 10.1). In addition, by studying genetic and environmental factors together, it may be possible to increase the ability to discover genetic variants associated with disease (Manolio et al. 2009). Moreover, studies of GxE may explain heterogeneity of effects across studies. In a study examining the effect of genetic variation in adiposity associated genes on waist circumference, the relation of variation in the *FTO* (fat mass and obesity associated protein) gene and physical activity with waist circumference differed depending on the geographic region. The differing effects were correlated with variation in the levels of physical activity in the geographic regions (Moore et al. 2012). Furthermore, GxE studies may allow identification of susceptible populations to better target interventions (Rothman et al. 1980). For example, a recent paper indicates that the impact on bladder cancer of eliminating smoking may be higher for individuals with a higher genetic risk (Garcia-Closas et al. 2013). Finally, understanding GxE involved in complex disease outcomes may also improve performance of risk prediction models for disease prevention and treatment and is the basis of the field of pharmacogenomics as discussed in more detail below (Thomas 2010).

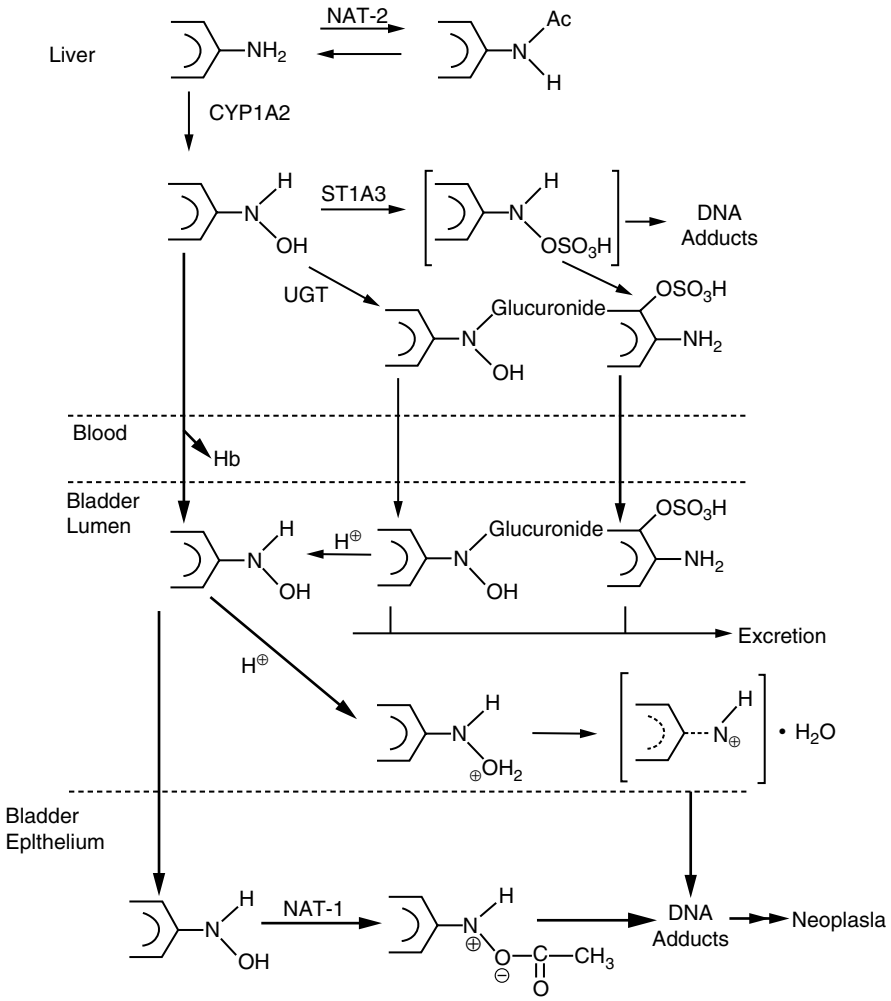

Fig. 10.1 Proposed mechanism of metabolic activation and detoxification pathways in bladder cancer. Genetic variation in N-acetyltranferases (NATs), which reduce NAT activity, are thought to alter levels of DNA adducts and modulate bladder cancer risk (Reprinted from Kadlubar and Badawi 2010, Copyright (1999), With permission from Elsevier)

10.2 What Is Meant by Gene-Environment Interaction?

Genetics and environment, and the interplay between, may have unique meanings depending on the context. Typically, in studies of GxE, investigators exploring genes are examining differences in germline genetic variation, or changes in DNA sequence, often in the form of single nucleotide polymorphisms (SNPs), though other forms of genetic variation exist (e.g., copy number variation). Germline

genetic variation, or genotypes, typically remain constant during an individual's lifetime and are presumed to precede exposure and phenotypes (for review (Burton et al. 2005)). In contrast, somatic genetic variation, usually described as somatic mutations, occur during mitotic divisions within an individual. Somatic mutations are most commonly studied in the context of tumor cells, although they have also been observed in neurons (Poduri et al. 2013) and other tissues (O'Huallachain et al. 2012). Some researchers are investigating the relationship between these somatic genetic alterations and response to therapy (Hertz and McLeod 2013), a form of pharmacogenomics. Another form of genetic variation which may be investigated is epigenetic variation. Epigenetic alterations are not due to changes in the DNA nucleotide sequence, but are modifications in DNA methylation and chromosome structure which influence gene activity and expression. Understanding the timing of exposure and disease occurrence is critical for studying somatic mutations and epigenetic alterations, as these could be altered by factors including exposure and aging. Epigenetic alterations can also be altered by disease, thereby influencing interpretation. The focus of this chapter is on germline genetic variation, but both somatic and epigenetic variation will be discussed.

Studies of germline genetic variation may focus on a small number of candidate variants or may be more agnostic and survey variation genome wide through genome-wide association studies (GWAS). The International HapMap project (International HapMap Consortium et al. 2007), and similar other activities, have helped identify and catalogue SNPs across the genome. These projects have provided researchers with information regarding the linkage disequilibrium (LD) structure within the genome, thereby allowing for selection of haplotype tagging single nucleotide polymorphisms (tSNPs) which resulted in creation of genotyping arrays that made large-scale GWAS practical. These studies succeeded in identifying hundreds of genetic markers associated with common diseases (Hindorff 2014). Findings from GWAS to date have been primarily limited to variants represented on commercially available genotyping arrays, or common genetic variation (Zuk et al. 2014). Therefore, the contribution of other classes of genetic variation to complex diseases, including less common or rare variants and structural variants, has not been well-studied but is a growing area of research (Manolio et al. 2009; Eichler et al. 2010; Lander 2011). For illustrative purposes, the focus on the chapter is in the simplified case of considering a single variant, but genome wide interaction studies will also be discussed.

In study of risk factors for disease, and GxE in particular, a broad definition of "environment" is often used to include all physical, chemical, biological, as well as social and economic factors which may affect health. In many cases these "environmental" factors themselves have a partial genetic basis. Given that a key goal of GxE research is to understand disease etiology and identify modifiable risk factors, for the purposes of this chapter, the broader definition of environment is used.

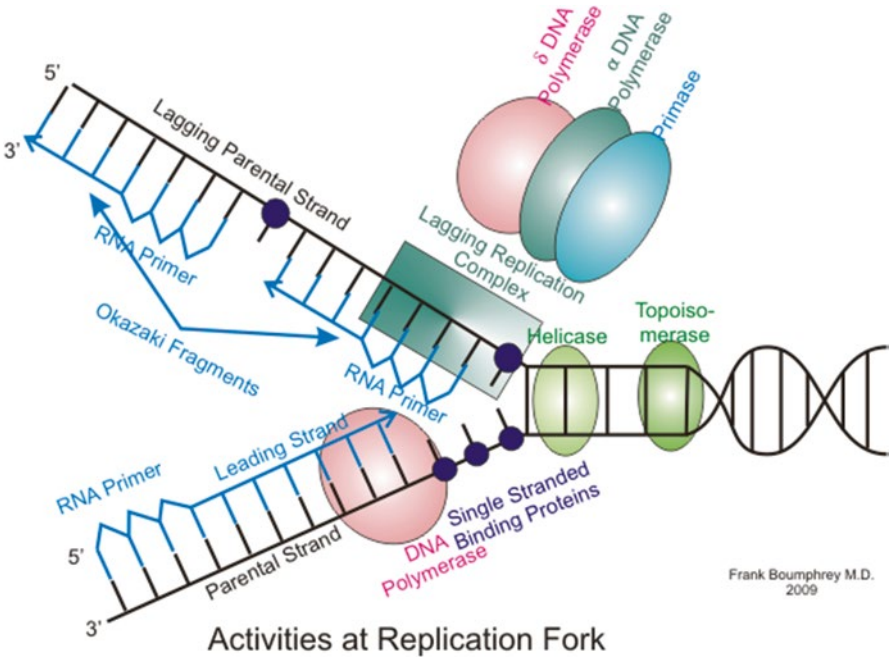

Fig. 10.2 The multi-protein complex which makes up the replication fork is illustrated. These proteins include the helicase, topoisomerase, DNA polymerases, and single stranded binding proteins which function together to support DNA replication (Image created by Frank Boumphrey, MD in 2009 and used in compliance with Creative Commons Attribution-Share Alike 3.0 Unported license)

The term "interaction" also has several interpretations. For biologists, interaction between factors typically implies a physical association or one molecule functioning directly with another molecule (see discussion in (Thomas 2010; Moore and Williams 2005)). To illustrate what biologists mean by interaction, one can consider the process of DNA replication. In DNA replication, several proteins form a complex and work together to copy DNA as the cell divides (Fig. 10.2). In contrast, in epidemiology studies, the term interaction often used to describe the situation where the association of an environmental and genetic factor (or factors), in combination, with disease is either stronger or weaker than predicted by the associations of each factor independently. This type of interaction is often also called effect modification or, more precisely, effect measure modification (Rothman and Greenland 1998). In this context, the definition of interaction may be dependent on the selection of analytical scale (see discussion below about scale and interpretation). The focus of this chapter is in the context of epidemiology research studies. An overarching model to consider how genes and environment may alter disease risk is presented in Fig. 10.3.

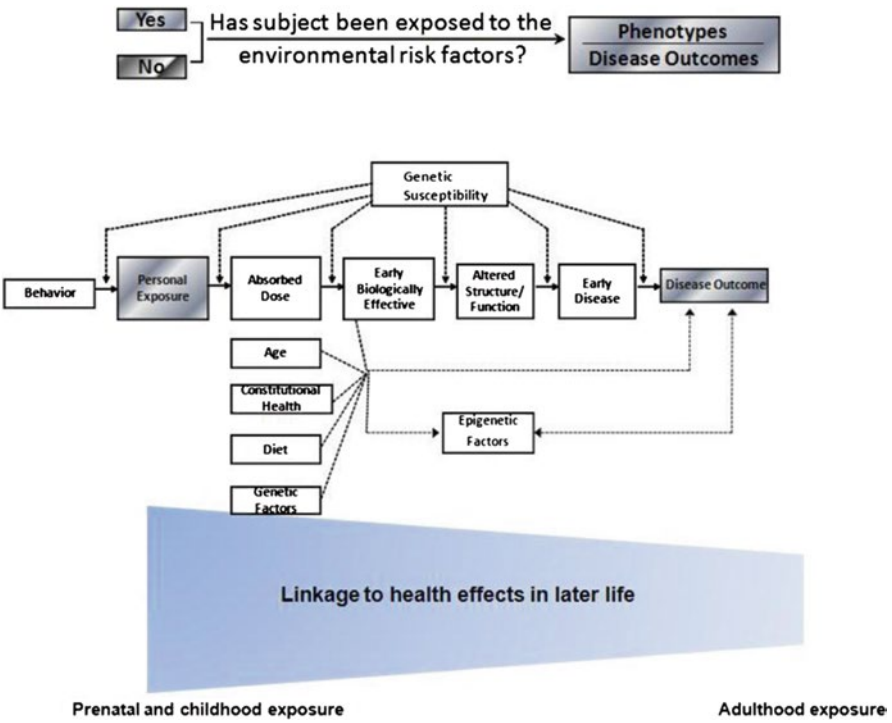

Fig. 10.3 Expanded paradigm of genetic susceptibility and disease related to environmental exposure in different life stages. In this model, germline genetic variation "Genetic Susceptibility" modulates environmental exposure at various stages. "Epigenetic Factors" correlate with environmental exposure and disease outcome (Image created by Liu et al. 2012; Copyright (2012); used with permission from Creative Commons (http://creativecommons.org/licenses/by/2.0/). Original image may be found (http://www.ehjournal.net/content/11/1/93))

10.3 Designs and Approaches for Study of GxE

10.3.1 Study Designs

In epidemiology research, a variety of study designs are used for investigating GxE (for review (Liu et al. 2012; Kauffmann and Demenais 2012)); a general overview of observational study designs may be found in (Gordis 2000). The most common designs for GxE are case-control and nested case-control studies, but other common designs include case-only and family designs.

In a case-control study, individuals with disease or outcome (cases) are compared with individuals without the disease or outcome (controls) (Gordis 2000). In this design, as cases and controls are selected at the time of case diagnosis, data on exposures is obtained retrospectively. Therefore, case-control studies are often subject to bias related to the recollection of exposure, or recall bias, i.e., cases may

differentially recall exposures compared with controls. Biomarkers, or substances measured in biological specimens, could be used to supplement exposure assessment performed using questionnaires (Vineis and Perera 2007; Mechanic et al. 2012). However, in the case-control design biological specimens are collected at the time of diagnosis or case ascertainment. As a result, biomarkers measured in these samples may be potentially altered by disease (e.g., reverse causality). This is not considered an issue with germline DNA as it is essentially unchanged over the course of a lifetime.

In a nested case-control study, cases and controls are selected from within a cohort study population. In cohorts, classification of individuals is based on whether they are exposed or non-exposed and both groups are followed over time to compare the rate of disease. Cohort studies can be prospective or retrospective. In a nested case-control study based in a prospective cohort, the assessment of environmental exposure by questionnaires and biomarkers are typically performed prior to case diagnosis and this design is less subject to issues of recall bias or reverse causality (See discussion about case-control and cohort studies in (Rothman and Greenland 1998)). The advantage of a nested case-control study over an analysis of the entire cohort, is that only a subset of the subjects needs to be genotyped and assayed for exposure. This allows for efficiency and cost-savings. Case-cohort studies can be used if multiple outcomes are of interest in the same cohort.

A third design is the case-only approach. In this design, under specific strong assumptions, the power, or ability to detect GxE is improved in comparison to case-control designs (Piegorsch et al. 1994). The key underlying assumption in the case-only design is that the genetic and environmental factors are independent in the source population, this is referred to as the independence assumption. If the assumption is violated, case-only studies are susceptible to bias and large type I error (Albert et al. 2001; Cornelis et al. 2012; Mukherjee et al. 2012; Thomas et al. 2012). Case-only studies also have the advantage that only cases need to be collected. In the case-only design, only the interaction between the genetic and environmental factors is examined. Because of the lack of controls, it is not possible to examine the main effects of either the genetic or environmental factor alone.

Case-control, nested case-control, and case-only studies are population based designs, and are subject to biases including population stratification. Family-based designs may provide some advantages since they are robust to population stratification, make genetic inferences by comparisons to family members, in certain settings have increased power for assessing GxE interactions (Witte et al. 1999), and are resistance to biases due to self-selection of controls (Shi et al. 2011; Weinberg 2012).

Family based designs were most prominent in the 1990s and early 2000s, when there was large concern about population stratification and other biases in candidate gene association studies. Most studies exploring the association of genetic variation with genome-wide have been performed in case-control and nested case-control designs (Hutter et al. 2013), and methods were developed to account for population stratification using the genome wide genetic data on GWAS platforms (Price et al.

2010). As research in genetic variation begins to focus on less common and rare genetic variations, there may be a return to emphasis on family designs.

Model organism systems have been developed to facilitate the study of GxE. One such effort is the Collaborative Cross (CC), a large common set of genetically defined mice, which serves as a resource for the study of complex interactions among genes and environments (Churchill et al. 2004). In addition to animal models, lymphoblastoid cell lines generated from large populations of individuals which were studied to characterize genetic variation by sequencing and genotyping, e.g., 1,000 genomes project (The 1000 Genomes Project Consortium 2012) and HapMap (International HapMap Consortium et al. 2007) may be used to study combination of genetic and environmental factors *in vitro*. In particular, these cell lines have been used to examine the correlation between genetic variation and drug response (Zhang and Dolan 2010).

10.3.2 Analytical Approaches

In general, the analytical approaches used to study GxE in epidemiology attempt to answer the question whether there is effect modification, where the environmental factor has a differential effect on disease risk depending on an individual's genetic background (or germline genotype) or, alternatively, whether the effects of genetic variation on disease is dependent on environmental exposure. A recent review summarizes current analytical approaches (Hutter et al. 2013) and chapters from these texts provide further details (Rothman and Greenland 1998; Austin 2013).

10.3.2.1 Measures of Association

To measure whether an exposure, or genetic variation, has an effect on outcome of a disease, one typically considers one of the measures of association from the epidemiologist tool box. These include consideration of relative risks (RR), risk differences (RD), and odds ratios (OR). The risk ratio is an estimate based on the multiplicative scale, i.e., it assesses how much risk is multiplied in the exposed. The RR is defined as the risk in the exposed ($\text{Risk}_{\text{exp+}}$) divided the risk in the unexposed ($\text{Risk}_{\text{exp-}}$):

$$\text{RR} = \text{Risk}_{\text{exp+}} / \text{Risk}_{\text{exp-}}$$

The risk difference is based on an additive scale, or how much risk is added in the exposed, and is defined as the risk in the exposure minus the risk in the unexposed population:

$$\text{RD} = \text{Risk}_{\text{exp+}} - \text{Risk}_{\text{exp-}}$$

An alternative measure on the multiplicative scale, used often in case-control studies, is the odds ratio. Odds are defined as probability/(1-probability). So the odds in exposed individuals is $\text{odds}_{\text{exp+}} = \text{risk}_{\text{exp-}} / 1 - \text{risk}_{\text{exp+}}$. The odds ratio is

Table 10.1 Layout of case-control study. The number of cases exposed (a) or unexposed (c) and controls exposed (b) or unexposed (d) are provided in the table. The odds ratio is calculated by the cross product ratio or ad/bc

	Exposed (exp+)	Unexposed (exp−)
Cases	a	c
Controls	b	d
OR	ad/bc	

defined as the odds in the exposed ($Odds_{exp+}$) divided by the odds in the unexposed ($Odds_{exp-}$).

$$OR = Odds_{exp+} / Odds_{exp-}$$

The odds ratio may be calculated using the cross-product ratio based on counts in a 2 by 2 table comparing number of exposed and unexposed cases and controls (Table 10.1):

$$OR = \left(\text{Number Cases Exposed} \times \text{Number of Controls Unexposed}\right) / \left(\text{Number of Controls Exposed} \times \text{Number of Cases Unexposed}\right)$$

An odds ratio is used often in case-control studies because the relative risk cannot be calculated directly in this design. The odds ratio can be used as an estimate of the relative risk, when the disease is rare. Another reason odds ratios are frequently used is the ease of calculating by using the cross-product ratio or logistic regression modeling (Hutter et al. 2013).

10.3.2.2 Estimates of Interaction Effects

In the exploring the question of whether there is a differential effect of an environmental exposure (E+) on disease risk, based on germline genotype, one must compare the risk ratio, risk difference, or odds ratio for the exposure in participants with the specific genotype (G+) to the same risk measure in participants without the specified genotype (G−). Note that in this situation, presence of environmental exposure (E+) or specific genotype (G+) is considered as "exposed" (exp+) using the formulas described for measures of association. Another way of expressing the evaluation of possible interaction is that the population is stratified according to genotype and the effect of the exposure is examined in each genotype. This strategy is exemplified in the case-control setting, provided in Table 10.2 (Botto and Khoury 2001). In Table 10.2, the odds ratios (ORs) for the combined effect of genetic and environmental factors (OR_{G+E+}), genetic factors (OR_{G+E-}), or environmental factors (OR_{G-E+}) may be estimated. A similar strategy may be used if exploring whether the effect of genotype was differential depending on environmental exposure.

In order to quantify the possible interaction effect, one explores whether the OR_{G+E+} is greater or less than expected based on the individual OR_{G+E-} (OR for

Table 10.2 Case-control study layout for analysis of gene-environment interaction (Adapted from Botto and Khoury 2001). By stratifying the population according to both genotype and environmental exposure, the odds ratios (ORs) for the combined effect of genetic and environmental factors (OR_{G+E+}), genetic factors (OR_{G+E-}), or environmental factors (OR_{G-E+}) may be estimated. Numbers of cases or controls in each cell are represented by a, b, c, d, e, f, g, and h

	High risk genotype present (G+)		High risk genotype absent (G−)	
	Environment exposure (E+)	Environment unexposed (E−)	Environment exposure (E+)	Environment unexposed (E-)
Cases	a	c	e	g
Controls	b	d	f	h
ORs	$OR_{G+E+} = ah / bg$	$OR_{G+E-} = ch / dg$	$OR_{G-E+} = eh / fg$	1.0 (reference)

Table 10.3 Interpretation and expectations in case-control study for different scales (Adapted from Melissa Austin; Genetic Epidemiology: Methods and Applications)

Measurement scale and interaction effect	Expectations for OR_{G+E+}
Multiplicative scale	
No interaction	$OR_{G+E+} = OR_{G+E-} \times OR_{G-E+}$
Synergistic interaction	$OR_{G+E+} > OR_{G+E-} \times OR_{G-E+}$
Antagonistic interaction	$OR_{G+E+} < OR_{G+E-} \times OR_{G-E+}$
Additive scale	
No interaction	$OR_{G+E+} = OR_{G+E-} + OR_{G-E+} - 1$
Synergistic interaction	$OR_{G+E+} > OR_{G+E-} \times OR_{G-E+} - 1$
Antagonistic interaction	$OR_{G+E+} < OR_{G+E-} \times OR_{G-E+} - 1$

genetic variation) or OR_{G-E+} (OR for environmental exposure). Assuming the multiplicative scale, this, in the presence of no interaction on the multiplicative scale, the expected $OR_{G+E+} = OR_{G+E-} X OR_{G-E+}$. In the presence of no interaction on the additive scale, the expected $OR_{G+E+} = OR_{G+E-} + OR_{G-E+} - 1$.

If the OR_{G+E+} is greater than the expectation under the model of no interaction for a specified scale, the interaction is considered synergistic. Meanwhile, if the OR_{G+E+} is less than the expectation under the model of no interaction for the specified scale, the interaction is considered antagonistic. The interpretation of the ORs_{G+E+} for the different measurement scales is summarized in Table 10.3.

Examining Table 10.3 more closely, the decision about whether interaction is observed is dependent upon the scale which is explored. Importantly, when neither ORs for exposure (OR_{G-E+}) or genotype (OR_{G+E-}) alone is equal to 1 the expectations under multiplicative scales and additive scales are different. When these expectations are different, the interpretation, interaction or not, may be different depending upon the scale. More discussion about the challenges of studies GxE and the issue regarding scale are provided later in the chapter.

Often when testing for interaction using case-controls studies, investigators implement logistic regression modeling and incorporate a product term for the genetic and environmental factors. The p-value associated with the product term in

the logistic regression model is often considered the interaction p-value and may be used to evaluate whether the association of the environmental factor differs depending on strata of genotype (or vice versa), although some argue against this interpretation (Austin 2013). Testing in this manner is similar to applying a test of homogeneity to evaluate the different strata (Rothman and Greenland 1998).

Statistical methods have been developed for examining additive interactions from logistic regression, and can be applied to case-control and nested case-control studies. These include the relative excess risk due to interaction (VanderWeele and Vansteelandt 2014) and the synergy index (Knol et al. 2011).

The focus of this discussion has been using ORs, since case-control studies are most commonly used for GxE at this time. However, the general approach of comparing exposures in individuals in presence or absence of particular genotypes is also used when examining risk differences or risk ratios.

10.4 Challenges in Study of GxE

10.4.1 Exposure Assessment

As with all risk factor epidemiology, critical considerations must be given to assessing the risk factors accurately and at the appropriate time. If there is non-differential measurement error or misclassification, in ordinary circumstances, the ability to detect the association is reduced due to influence on the power of the test (see power discussion below) and bias towards the null. Although in some circumstances the direction of non-differential misclassification could produce bias away from the null. If there is differential misclassification, there may be bias and increased false positive or negative findings. Further, risk factors may only modulate risk at particular time periods relative to disease onset. If exposure is assessed at the incorrect time point there will again be misclassification. Importantly, for the risk factor to influence disease, the individual must be exposed prior to the onset of the disease (Rothman and Greenland 1998; Gordis 2000).

In recent years, the technology for the assessment of genetic variation has advanced rapidly with applications of GWAS arrays and next generation sequencing technologies enabling higher quality and agnostic evaluation of genetic variation. However, methods for environmental exposure assessment have not kept pace (Hutter et al. 2013; Wild 2005). Traditionally, in large population based studies, environmental exposure assessment is performed using questionnaire methods (for overview of challenges and considerations of exposure assessment, see (Rothman and Greenland 1998)). These approaches are based on the recollection of the participant, and therefore are subject to error (Spiegelman 2010). More sophisticated quantitative methods of exposure assessment are often expensive and difficult to apply in large studies. Several recent reports outlined clear need for developing improved measures of environmental exposure (Balshaw and Kwok 2012).

Moreover, in studies of complex diseases, often neither the critical exposure nor the route of exposure associated with disease is known.

One strategy to improve exposure assessment is to incorporate the use of biomarkers within epidemiology studies. These markers can either be direct measurements as biomarkers of exposure, or measures of early effect (Vineis and Perera 2007). Exposure biomarkers are direct measurements of the toxin or chemical, while measures of early effect reflect the underlying biological process. Epigenetic alterations in response to an environmental exposure are being explored as measures of early effect (for review (Jirtle and Skinner 2007)).

Another challenge with environmental exposure assessment, is that unlike germline genetic variation, which is considered to remain constant in an individual's lifetime, environmental exposures are dynamic and change over time and an individual's lifespan (Spiegelman 2010). There is uncertainty in environmental data stemming from our lack of knowledge of the timing of effects. For instance, even if we could measure a person's pack years of smoking without error for a study of breast cancer risk, we might still need to know whether smoking was initiated prior to her first birth, and how long ago she quit smoking. These considerations are compounded for studies of the impact of *in utero* and early childhood exposures on a disease with late onset, and highlight the need for methods and designs, such as longitudinal studies, that consider interactions with over time and time-varying exposures (Hutter et al. 2013). Taken together, the lack of certainty regarding the timing of exposure and need for agnostic measures of the environment, has led to some researchers suggesting that studies need to examine the life-course of environmental exposures, or 'exposome' to enable agnostic evaluation of environmental exposure (Wild 2005; Rappaport 2011) (Fig. 10.4).

A challenge in epidemiologic studies of GxE is that populations differ in patterns of genetic variation and some populations have unique or rare environmental exposures (e.g., high levels of arsenic). Therefore, in some situations it is difficult to confirm epidemiology associations in a second population (Hutter et al. 2013).

10.4.2 Power

The power of a statistical test is probability that one rejects the null hypothesis given that the null hypothesis is false. In the case of examining the association of either a genetic or environmental factor with disease, the power of the statistical test for this association is the probability to conclude there is an association of the factor with disease given that this the factor is truly related with disease (Rothman and Greenland 1998; Gordis 2000). Therefore, power can be considered the ability to detect the association, when the alternative hypothesis is true. Typically, studies aim for at least 80 % power to detect the association.

The power of the test is dependent on the strength of the association, the frequency of the risk factors, the alpha (α) (or threshold for determining statistical significance) and the population study size (example power curves for

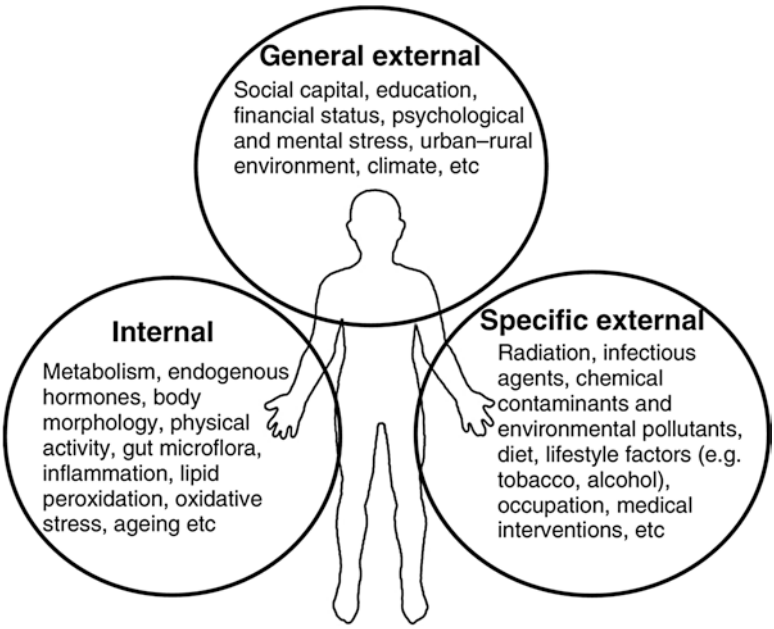

Fig. 10.4 The Exposome. The exposome is the combined exposures from all sources that reach an individual's internal environment (Wild 2012, by permission of Oxford University Press)

gene-environment interactions) (Fig. 10.5). The α value is selected when designing a study and traditionally is selected at 5 %. In significance testing, to decide whether the test is statistically significant, the calculated p-value for the test must be below the selected α. Therefore, at a given α, frequency of risk factors, and strength of association, the sample size must be increased to improve the power to detect an association.

The selection of the α value, as described above, is based on the assumption of a single independent test. When performing multiple tests, such as testing several different genetic variants, the likelihood of detecting an association by chance (type I error) increases. In GWAS for main effects, investigators are examining hundreds of thousands to millions of genetic variants associated with disease. Performing multiple hypothesis tests in this manner is considered making multiple comparisons. There are several strategies that may be implemented to handle hypothesis testing when performing multiple comparisons (Chanock et al. 2007). In GWAS studies, a strategy commonly used by researchers is a more stringent α of 5×10^{-7} to 10^{-8} to indicate "genome-wide significance" and account for multiple testing and reduce the likelihood of following up on false positive associations (Chung and Chanock 2011). Another factor impacting power in genetic association studies is that the observed effect sizes, or strength of association, for genetic variants with common diseases have been modest. For example, the observed odds ratios for most common genetic variants assessed using GWAS arrays (minor allele frequencies >5 %) with

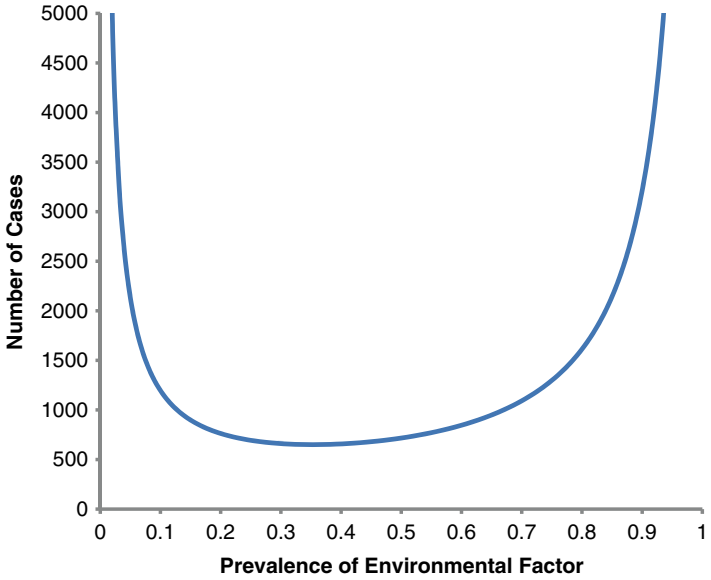

Fig. 10.5 Example power curve demonstrating the relation between sample size and prevalence of environmental factor. The minimum number of cases required to detect a twofold interaction on multiplicative scale ($OR_{G+E+} = 8, OR_{G+E-} = 2$ and $OR_{G-E+} = 2$), assuming the prevalence of the genetic factor, or minor allele frequency of 0.5. Figure was generated using Power v3.0 Software (Garcia-Closas et al. 1999)

cancer were reported between 1.1 and 1.4 (Chung and Chanock 2011). In contrast, for highly penetrant, rare mutations in genes such as BRCA1/2 are associated with a 20-fold increased risk of breast cancer. However, these types of mutations are less common, requiring large sample sizes to detect as well. Meanwhile, while other less common or rare variants may have strong effect sizes, it is not currently clear if this is the case as suggested by recent results in breast and prostate cancer (Haiman et al. 2013).

Thus far, this discussion regarding power has focused on genetic associations. However, to explore GxE interactions, the situation is even more challenging because one must consider the frequency of genetic factor, frequency of environmental factor, strength of associations and the magnitude of interaction. A rule of thumb is that at least four times the sample size required for main effect is needed to detect an interaction of similar magnitude (Smith and Day 1984). As a result, it has been estimated that the sample sizes needed for genome-wide studies of GxE may range from the thousands for candidate genetic variants to tens of thousands (Thomas 2010). Even in studies with sufficiently large samples for analyzing interactions with common exposures, sample size issues may arise when examining gene-environment interactions if a particular exposure or genotype of interest is relatively rare. In these situations there may be small cell counts where asymptotics break down, leading to unstable results. Finally, because of small effect sizes,

multiple testing corrections, and the need to model more parameters, studies of GxE interactions need to have large sample sizes. Lack of power for tests of interaction is an important limitation in many GxE studies, particularly GEWIS (Mukherjee et al. 2012; Dempfle et al. 2008) and developing analytical methods to increase power to study GxE is an active area of research.

10.4.3 Additive and Multiplicative Scale and Interpretation

The interpretation of interaction effect estimates is another challenge in studies of GxE. A primary challenge is the lack of explicit links between statistical and biological interactions which limits the ability to characterize GxE findings and provide biological insights (Weinberg 2012; Cordell 2002; Siemiatycki and Thomas 1981; Thompson 1991; VanderWeele 2011). The discordance is due in part to the fact that detection of a statistical interaction refers to departure from a model on a particular scale (i.e., additive, multiplicative, etc.) (Thomas 2004). Selection of the most appropriate scale is a highly debated concept in genetic epidemiology. Since most interaction methods are based on testing using multiplicative logistic models and epidemiologists frequently analyze data using risk ratios or odds ratios, the multiplicative scale is easier to implement. Further, these measures can be interpreted using standard epidemiologic interpretations for multiplicative interactions. However, some people argue additive models have clearer biological interpretations, and as noted earlier additive methods are seeing increased use. When examining small effects and rare outcomes, multiplicative and additive risk models are approximately equivalent (Weinberg 2012), therefore in GEWIS of most complex diseases, where genetic loci and many environmental factors likely have modest effects on disease risk, distinguishing between additive and multiplicative tests may be less critical. However, the similarity between multiplicative and additive models breaks down when one of the main effects is large or the environmental exposure is continuous. In this situation, selection of the scale should depend on expected model of joint effects, which may be based on empirical data. In most situations, when performing discovery to identify new factors, the most appropriate model is not known and model free approaches could be considered (Hutter et al. 2013).

10.5 Genome Wide GxE (GEWIS)

In describing the design and approach for the analysis of GxE (Sect. 10.3), this chapter has focused on the case of single genetic and environmental factors. However, for many common diseases, risk of disease is likely due to combinations of several genetic and environmental factors. With the success of GWAS studies at identifying common genetic variants associated with diseases (Hindorff 2014; Chung and Chanock 2011), studies have begun looking at GxE on a genome wide

scale in order to identify additional genetic and environmental factors associated with disease. By applying GxE to data obtained using GWAS arrays, researchers may 'discover' new findings. These studies are often referred to as to as gene-environment-wide interaction studies ("GEWIS" (Khoury and Wacholder 2009) or "GE-Whiz" (Thomas et al. 2012)). The focus of these types of studies is to identify novel factors which may contribute to the etiology of disease, either new genetic variants or environmental factors.

There have only been a small number of GEWIS publications to date, but the field is growing (Hutter et al. 2013). In one study large study, several genetic variants were identified to interact with alcohol in esophageal squamous cell carcinoma (Wu et al. 2012). Another study identified variants in *GRIN2A*, associated with Parkinson's disease by considering interaction with coffee drinking (Hamza et al. 2011). There have been several reports where consideration of environmental factors or GxE interactions contributed to the discovery of novel genetic loci for other complex diseases (Cornelis et al. 2012; Hancock et al. 2012; Manning et al. 2012).

Many of the challenges for the study of interaction of single genetic variants and environmental factors are magnified when performing genome wide interaction testing due to the number of tests being performed and modest effect sizes. In particular, the lack of power of statistical tests of interaction is an important limitation to studies of GEWIS, and novel methods and approaches are being developed to improve power in this setting (Mukherjee et al. 2012; Dempfle et al. 2008).

10.6 Pharmacogenomics

Pharmacogenetics and pharmacogenomics are the study of the role of genetic variation with drug response (for review (Meyer 2004)). In this type of GxE study, the drug could be considered the environmental exposure. Individuals in a population vary in the range of responses to drugs in treatments, with some individuals requiring more of a drug to respond while others may be more sensitive and likely to have negative effects at a given dose. Understanding the impact of an individual's genetics on drug response is one of the goals of 'personalized medicine'(Hamburg and Collins 2010).

There are several examples of GxE in pharmacogenomics research. One of the most well-known examples is the observation that individuals with specific germline mutations in *NAT2*, an acetylation enzyme which functions in drug metabolism, were more likely to suffer toxicity after treatment with isoniazid for tuberculosis (Meyer and Zanger 1997). Several genetic variants in another enzyme, cytochrome P450 monoxygenase 2D6 (*CYP2D6*) (Meyer and Zanger 1997; Zanger et al. 2004), mediate drug metabolism phenotypes which may alter the treatment response for numerous drugs. Finally, in studies of targeted cancer therapies, somatic mutation profiles or genetic variation/mutations in tumors have been observed to modulate treatment response (Rahman 2014). For example, patients with mutations in EGF

receptor (*EGFR*) were shown to have improved therapeutic response to targeted small-molecular inhibitors (Mitsudomi et al. 2006).

Obtaining sufficient population sizes to study the role of genetic variants on treatment outcomes is often a challenge impacting power to detect associations (Innocenti et al. 2011). However, if these studies are performed in the context of randomized control intervention trial, the exposure in the intervention group is known with precision, reducing potential for measurement error (Huang et al. 2011) and benefiting study of GxE. In addition, it is anticipated that effect sizes for pharmacogenetic studies of toxicity may have larger effect sizes than those studies of disease risk (Innocenti et al. 2011).

Often the relationship between genes (or genetic variants) and therapies is not a simple relationship and it is likely that multiple genes or pathways modulate the outcome of therapy (Innocenti et al. 2011; Plenge et al. 2013). The field has focused on studying targeted genes, but genome wide approaches have been emerging in pharmacogenetic studies. As experienced in genetic studies of disease risk, more genome wide approaches may provide insights into biological mechanisms of drugs and eventually lead to more effective targeting of medications.

10.7 Epigenetics

The focus of this chapter on GxE has been the interplay of germline genetic variation and environmental exposure on disease, however, as discussed in other sections of this textbook, the risk of disease because environmental exposures may be mediated by epigenetic alterations. Therefore, in considering the inter-relationship between genetic and environmental factors with disease, epigenetic variation could be another contributing to disease mechanisms (for review (Bjornsson et al. 2004)).

Germline genetic variation may influence several epigenetic pathways (Foley et al. 2009). Genetic variants in DNA methyl transferases (DNMTs), such as C to T substitution in at nucleotide 677 mehylenetetrahydrofolate reductase (*MTHFR*) was associated with lower levels of DNA methylation (Turek-Plewa and Jagodzinski 2005). By influencing methylation of genes, germline variation and corresponding epigenetic changes could modulate response to environmental exposure.

Another way to consider the role of epigenetics in GxE, relates to response to environmental factors. Several environmental factors are thought to modify epigenetic profiles (Cortessis et al. 2012). In particular, studies suggested that diet could alter epigenetic profile. These epigenetic changes could result in alteration of gene expression, affecting disease.

Finally, epigenetic variation may modulate a GxE response, by impacting expression of DNA containing the genetic variant. For instance, in considering drug metabolism pathways, if a particular genetic variant results in reduced activity of a metabolism enzyme, the reduction of activity compared to wild-type variant would depend on relative gene expression, or levels of the gene. In this way, epigenetic changes may mediate any GxE response. As an illustrative example how genetic

and epigenetic variations may function in combination in GxE, *BRCA1* and *BRCA2* genes are inactivated in serous ovarian cancer by germline and somatic mutation (22 %), and epigenetic silencing of *BRCA1* (11 %) of cases (Cancer Genome Atlas Research Network 2011). Cancer cells which lost *BRCA1/2,* either through mutation or expression, are more responsive to PARP inhibitors (Fong et al. 2009; Veeck et al. 2010).

It should be recognized, in contrast to measurements of germline genetic variation, timing of assessment of the epigenetic variation is more challenging due to the dynamic nature of the epigenome. The epigenome changes with age and exposure. Moreover, epigenome profile may differ depending on source of tissue (e.g., blood, saliva, organ tissue, tumor tissue). It is critical that biological specimens for measurement of epigenetic variation are collected at the appropriate time period to ensure accurate interpretation (Bjornsson et al. 2004; Foley et al. 2009; Cortessis et al. 2012).

10.8 Summary

In summary, studies of GxE are important for our understanding of disease risk and will likely play a key role in translation and application of genetic association findings to human health. However, it is important to understand and consider the challenges and limitations when using this approach. GxE research and application is a rapidly evolving field, requiring multi-disciplinary knowledge including epidemiology, biostatistics and biological sciences. New, more sensitive, sophisticated and powerful methods are being developed as genome-wide approaches are considered for common and rare DNA variants, and for epigenetic variants.

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Chapter 11
Environmental Epigenomics: Applications of Epigenetic Biomarkers to Investigate Epigenetic Alterations from Environmental Exposures

Lee E. Moore, Sara Karami, and Jennifer A. Rusiecki

Abstract Over the past decade, evidence has shown that epigenetic mechanisms play an important role in human disease susceptibility and cancer. Studies of potential human carcinogens have been expanded to include those that do not appear to damage DNA directly but rather alter gene expression patterns through epigenetic mechanisms such as alterations in DNA methylation, histone modifications/chromatin alterations, and microRNA. Given that almost 25 % of all human diseases are estimated to be caused by environmental exposures, here we review current studies of xenobiotic exposures for which there is growing mechanistic evidence for indirect DNA alteration through epigenetic mechanisms. Also described are common variations in genes that may modify epigenetic alterations in blood and tumor DNA. Epidemiological methods used to incorporate epigenetic alterations into studies of disease etiology as well as current methods used to apply and critically evaluate study results reporting associations between environmental exposure and disease are described.

Keywords Environmental exposure • Methylation • Histone proteins • miRNAs • Epigenetic biomarkers

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11.1 Introduction

Several types of epigenetic alterations have been observed in cancer and other diseases such as diabetes, obesity, cardiovascular diseases, hypertension, renal dysfunction, and adverse reproductive outcomes (Moore et al. 2003; Baccarelli and Bollati 2009; Ulrich and Grady 2010; Hou et al. 2012; Nelson et al. 2011). Although epigenetic alterations do not alter DNA directly, they appear to be correlated with a phenotype of altered gene expression, cell signaling, chromosome instability, and control pathways leading to cellular proliferation, differentiation, and disfunction. In this way, epigenetic alterations from environmental exposures appear to favor survival of damaged cells, thus leading to neoplastic transformation, cancer, and heritable reproductive disorders which will be a primary focus of this review.

DNA Methylation DNA methylation has been the most frequently studied epigenetic alteration in epidemiologic studies. DNA methylation is involved in many cellular functions including gene transcription, imprinting, maintenance of chromatin structure/remodeling, chromosome stability, and X-chromosome inactivation. Approximately 2.5 % of cytosines in the genome are typically methylated, mainly at CpG dinucleotides (Bestor and Tycko 1996; Costello and Plass 2001; Bird 2002). Within gene promoter regions, CpG site methylation suppresses DNA transcription because methyl groups physically block the binding of DNA polymerases and transcription factors to the gene promoter, thereby inhibiting transcription. The role of methylation of CpGs located within gene bodies and shores is less understood (Brennan and Flanagan 2012).

Because total genomic 5-methyl cytosine (5-MeC) content is commonly altered in tissue and blood from cancer patients, the first assays used to investigate associations between exposures and disease risk in epidemiological studies measured total %5-MeC in blood and tumor DNA. These included studies of global genomic DNA methylation using 5-MeC specific antibodies, methyl acceptance assays, high-performance liquid chromatography (HPCE), and treatment with restriction enzymes (such as *HpaII*), which selectively cleave DNA at specific sequences observed at high frequency within CpG dense regions. Levels of cleaved and uncleaved DNA are quantified using methods such as densitometry (Fraga et al. 2002; Moore et al. 2008). More recent methods require bisulfite treatment of DNA, which causes deamination of unmethylated but not methylated cytosines, converting them to urocil. When regions of interest are sequenced, methylated cytosines remain present whereas unmethylated cytosines will be observed as thymidine bases. Methylated and unmethylated loci are quantified subsequent to sequencing by comparing the proportion of unaltered cytosines and thymidines at loci of interest. High-throughput methods have been developed as surrogate measures of total %-MeC content (frequently referred to as "global methyation") that focus upon repeat sequences throughout the genome that include approximately 90 % of all genomic 5-MeC in humans (Bollati et al. 2007). These include transposable sequences of long interspersed nuclear repeat elements that represent approximately 17 % of the genome (i.e. *LINE1* assay), short interspersed nuclear elements which represent approximately 11 % of the genome (i.e. *Alu* assay), and "satellite" short

tandem repeated noncoding sequences (such as Sat2) located in centromeric and heterochromatin regions on chromosome 1 (Yang et al. 2004; Laird 2010). More recently, epigenome-wide investigation studies (EWAS) have been conducted to examine specific CpG loci in relation to disease and exposures (Rakyan et al. 2011). Due to the development of array-based technologies, it is now possible to examine methylation at over 450 k CpG sites that comprehensively span the genome. Regions that demonstrate variability in methylation levels are termed differentially methylated regions (DMR). Although some studies have found variation in human blood DNA located at CpG islands within gene promoters, recent evidence has demonstrated that most inter-individual epigenetic variation is concentrated within gene bodies and shores located adjacent to coding regions (Rakyan et al. 2011; Finer et al. 2011). These regions display higher variation in methylation compared to islands, despite having a lower density of CpG sites (Rakyan et al. 2011; Flanagan et al. 2013). Recently, analyses are focusing upon specific CpG loci demonstrating high between-person variation, and the least laboratory and temporal variation to improve statistical power for epidemiologic studies.

Histone Protein Modification Histones are globular proteins, around which DNA is coiled for compaction and regulation of gene transcription. Histone proteins can be modified via acetylation, methylation, phosphorylation, glycolization, sumoylation, and ADP ribosylation (Suganuma and Workman 2008). Enzymes such as histone methylases, acetyl transferases, and deacetylases can alter gene expression by changing histone structure. Chromatin remodeling factors enable opening of chromatin to facilitating gene expression (Kouzarides 2007). The most common alterations described in epidemiologic research are those occurring through acetylation and methylation of lysine residues in the amino terminal regions of histone 3 (H3) and histone 4 (H4). When acetylation is increased, transcription is activated; when acetylation is decreased transcription is repressed. Histone methylation can be associated with either activation or repression of transcription, depending on which lysine residue is methylated (Yan and Boyd 2006).

microRNAs (miRNAs) are small single stranded RNAs (~21–23 nucleotides in length) that are transcribed from DNA and cleaved to become mature miRNA that are complementary to one or more messenger RNA (mRNA). In the cytoplasm, the main function of miRNA molecules is to down-regulate gene expression through interference and transcriptional repression of mRNA molecules. Annealing of miRNA to mRNA inhibits protein translation because the mRNA is no longer available for translation (Jackson and Standart 2007; Pillai et al. 2007).

11.2 Epigenetic Alterations in Toxicologic Studies of Environmental Exposures

The first evidence that exposures can alter DNA methylation patterns came from studies of chemotherapeutics and micronutrients involved in methylation reactions, like those required for one-carbon metabolism reactions (Fig. 11.1). Genomic

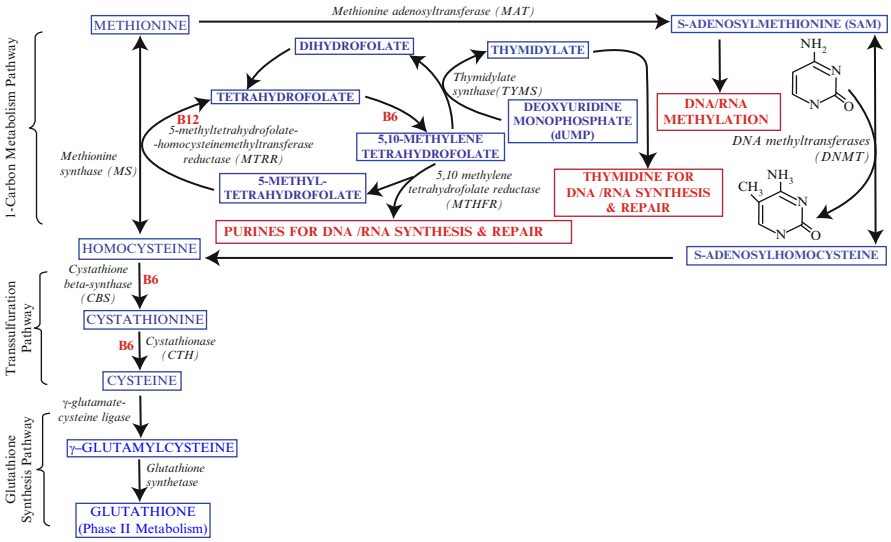

Fig. 11.1 Relationship between the one-carbon metabolism, transulfuration, and glutathione synthesis pathways. The one-carbon metabolism pathway is central to DNA methylation, thymidylate and purine synthesis. Homocysteine can be remethylated to methionine and the methyl donor SAM. Alternatively homocysteine can be converted to cysteine, the rate limiting step for glutathione synthesis. Glutathione is important in Phase II metabolism and excretion of xenobiotics via glutathione S-transferase enzymes

instability in tumor cells is thought to play a major role in tumor progression, metastasis, and resistance to chemotherapeutics (Nyce 1989, 1997; Nyce et al. 1993). Researchers have demonstrated that a variety of commonly used chemotherapeutics induce profound changes in DNA methylation patterns in human tumor cells in vitro. Drug-induced DNA hypermethylation silences gene expression during the period of drug-induced toxicity but can also lead to drug resistance through random inactivation of genes encoding for proteins required for activation of cancer therapeutics to reactive intermediates (Nyce 1989, 1997; Nyce et al. 1993). Chemotherapy induced epigenetic changes have been observed in vitro and in vivo. For example, cisplatin, a DNA cross-linking agent, is a very potent inducer of DNA hypermethylation, possibly inducing conformational changes in the enzyme DNA cytosine methyltransferase. Exposure to the drug 5-deoxyazacytidine, an enzyme involved in methylation reactions, has been shown to inhibit methyltransferase activity causing hypomethylation of DNA. Exposure to alkylating agents induces DNA hypomethylation, possibly by inactivating DNA methyltransferase because of an affinity to bind to sulfhydryl groups within the enzyme (Wilson and Jones 1983). DNA methylation is dependent upon the one-carbon metabolism pathway (Wagner 1985). Therefore, low dietary intake of protein and other micronutrients such as choline, methionine, and folate can contribute to cancer risk by demethylation of DNA due to uracil misincorporation during DNA synthesis (Herbert 1986). Folate is essential

for *de novo* biosynthesis of purines and thymidylate but also acts as an important mediator of methyl group transfer, which is necessary to maintain DNA integrity and synthesis of S-adenosyl methyltransferase (SAM), the universal methyl donor (Wagner 1985). Folate deficiency has been associated with chromosomal damage in human lymphocytes in vitro and in human lymphocytes and buccal cells in vivo (Crott et al. 2001; Titenko-Holland et al. 1998). Cofactors such as B_6 and B_{12} are also important components responsible for efficient pathway functioning and are derived from the diet (Ames 1998, 2001).

11.3 Epigenetic Alterations and Environmental Exposures

Tobacco Tobacco is the most frequently investigated exposure in etiological studies of cancer. The relationship between global methylation levels and smoking remain unclear, although the association has been examined in several studies of tobacco-associated cancers such as bladder, renal, and head and neck. Bladder cancer is the most studied tobacco-associated cancer with respect to global methylation. Smoking does appear to modify the association between genomic methylation and bladder cancer risk but findings have been mixed. Two case-control studies of bladder cancer found stronger associations for total and interspersed nuclear element sequences (*LINE1*) %5-MeC and bladder cancer risk among never compared to ever smokers; no association was observed with smoking status among controls (Moore et al. 2008; Cash et al. 2012). In another case-control study, no association was observed between *LINE1* methylation levels and bladder cancer risk (Wilhelm et al. 2010). Findings from two nested case-control studies conducted within the Prostate, Lung, Colorectal, Ovarian Cancer Screening Trial (PLCO) and the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Cohorts reported that higher levels of *LINE1* methylation were associated with bladder cancer risk using prediagnostically collected blood DNA (Andreotti et al. 2013). In the PLCO study (a study that included male/female smokers/nonsmokers), smoking did not modify the association between high compared to low *LINE1* methylation levels and future bladder cancer risk, but the magnitude of risk was significant in males, particularly male smokers. When the hypothesis was tested in a replication study in the ATBC cohort (a study of Finish male smokers), findings were strengthened and highly significant when results were pooled. Although in both cohorts smoking was associated with increased risk of bladder cancer, smoking status was not associated with methylation levels among controls. Similarly, in one case-control and two nested case-control studies of PLCO Trial and ATBC cohort kidney cancer case-control subjects, not only were *LINE1* methylation levels higher among cases compared to controls, but the association was stronger among current smokers compared to former or never smokers (Liao et al. 2011; Karami et al. 2015). In studies of head and neck cancer, a comparison of *LINE1* methylation in blood DNA was compared among Human Papillomavirus (HPV) positive cases and tobacco associated disease.

Lower levels of *LINE1* methylation were observed with smoking duration among smoking-associated cases, but not among HPV positive cases (Furniss et al. 2008; Houseman et al. 2012). Other studies of tobacco exposure conducted among healthy control subjects reported lower levels of *LINE1* methylation in leukocyte DNA among subjects who smoked blond tobacco (Tajuddin et al. 2013).

Recently, EWAS scans have been conducted using the Infinium HumanMethylation450 bead chip to identify whether differences in methylation of specific CpG loci are associated with tobacco exposure. At the US National Institutes of Environmental Health Sciences (NIEHS), investigators evaluated the impact of maternal smoking on epigenome-wide methylation profiles measured in 1,062 umbilical cord blood DNA samples from a prospective birth cohort [Norwegian Mother and Child Cohort Study (MoBa) study] (Joubert et al. 2012). Highly significant changes in methylation were identified at 26 CpGs located in ten genes. Altered methylation levels at CpG loci within three genes (*AHRR*, *CYP1A1*, *GFI1*) were replicated in a second US-based Newborn Epigenetic Study Chord Blood Study (NEST). Smoking-induced methylation changes (often called delta beta) can be both positive (hypermethylation) and negative (hypomethylation). Independently, hypomethylation of *AHRR* was associated with current smoking in adults in the European Prospective Investigation into Cancer and Nutrition (EPIC) and the Breakthrough Generations Cohort (BGC); hypomethylation of *AHRR* was also associated among former smokers and with time since last smoked (Shenker et al. 2013). Philbert and colleagues independently reported that demethylation of two distinct loci within *AHRR* was associated with serum cotinine levels (Philibert et al. 2013). Combined, these findings provide consistent evidence that demethylation of *AHRR* could be a good biomarker of current and prior tobacco exposure in humans. This finding has biological significance since *AHRR* acts as a negative regulator of the aryl hydrocarbon receptor, which mediates metabolism of tobacco smoke components.

11.4 Metals

Several studies have observed associations between environmental exposures to metals such as arsenic, cadmium, chromium, and nickel and epigenetic alterations in cells exposed in vitro, in vivo and in epidemiologic studies conducted in exposed populations. Previously it was hypothesized that metals caused oxidative stress and production of reactive oxygen species (ROS) that physically damage DNA through direct oxidation. However recently, metal exposures have been shown to react through a number of epigenetic mechanisms. Arsenic exposure has been the most studied "metalloid" element to date since millions of people worldwide are exposed to levels above the recommended levels (10 ug/L) set by the World Health Organization (WHO) and the U.S. Environmental Protection Agency (EPA) for drinking water (International Programme on Chemical Safety 2013; Smith et al. 2012).

Arsenic (As) Animal studies have shown that As has the potential to epigenetically alter the genome through several mechanisms. Epigenetic alterations, like genetic alterations, result in heritable changes in gene expression patterns. Epigenetic mechanisms that contribute to the carcinogenic potential of metals and metalloids include alterations in cytosine methylation patterns across DNA sequences, structural histone post-translational and chromatin modifications, alterations in the presence of non-histone chromatin-binding proteins, non-coding RNAs, and gene silencing of tumor suppressor and DNA repair genes. A mechanism through which As is thought to influence gene expression is through alteration of cytosine methylation patterns throughout the genome (National Research Council 1999). Interestingly, methylation is the main mechanism of As metabolism and excretion (National Research Council 1999). Upon ingestion and absorption, As is enzymatically methylated, primarily in the liver, consuming SAM in the process. The observation that DNA methyltransferases also require SAM as a methyl donor has suggested a role for DNA methylation in As-related cancer and other diseases. The first hypothesis-generating studies were conducted using cell lines followed by studies of exposed human populations. A study conducted using rat-liver epithelial cell lines exposed to chronic low dose As showed that malignant transformation was associated with depressed SAM levels, genomic DNA methylation, and DNA methyltransferase activity (Zhao et al. 1997). Following this finding, several studies have shown that As exposure is associated with gene-specific hypermethylation, and with global alteration of repetitive elements across the genome (Chanda et al. 2006; Chen et al. 2001; Zhong and Mass 2001; Mass and Wang 1997; Reichard et al. 2007; Sciandrello et al. 2003). A study of 294 adults from Bangladesh chronically exposed to As in drinking water reported a positive relationship between global methylation and As exposure levels measured in urine and blood. The positive associations observed were modified by folate intake, particularly among subjects with blood folate levels >9 nmol/L, suggesting a dependence upon methyl group availability for As metabolism in humans (Pilsner et al. 2007). In the same population, elevated risk of As-related skin lesions was reported among individuals with low folate, hyperhomocysteinemia, or low leukocyte DNA methylation levels (Pilsner et al. 2009). Each of these factors was associated with decreased methylation capacity and/or urinary excretion of inorganic As (Pilsner et al. 2009). In a study conducted in India, blood DNA samples were used to evaluate promoter regions of two important tumor suppressor genes, *p53* and *p16*. Each promoter region was more highly methylated in blood DNA from high compared to low As-exposed subjects indicating a possible relationship between exposure and epigenetic silencing of cell-cycle control genes in blood. Alterations in miRNA expression were reported in human lymphoblastoid cells grown and treated with sodium As (Marsit et al. 2006). The miRNAs altered in response to As exposure were also involved in one-carbon metabolism (methylation) reactions. An epidemiologic study conducted in Bangladesh, reported significant associations between As exposure and global post-translational histone modification in peripheral blood mononuclear cell (PBMC) DNA from a subset of participants (N = 40) enrolled in a clinical trial of folate supplementation (Chervona et al. 2012). Arsenic levels in drinking water were positively associated with several

post-translational histone modifications that were uniquely observed in either exposed males or females. For example, H3K4me3 and H3K27me3 were positively associated with exposure among female subjects but negatively associated among males. In contrast, water levels were inversely associated with H3K27ac and H3K18ac among females but positively associated among males. Urinary As levels were positively associated with H3K9me2 and inversely with H3K9ac. Although the above findings require replication, they support the hypothesis that As exposure from drinking water is associated with alteration of the human epigenome. A recent exposure study conducted in Spain found that of 12 metals measured in toenails, only As exposure was inversely associated with *LINE1* methylation. In contrast, iron and nickel were positively associated with *LINE1* methylation (Tajuddin et al. 2013).

Nickel and Nickel Compounds Nickel (Ni) is considered a Group 1 Known Human Carcinogen by International Agency for Research on Cancer (IARC) (2014). Like other metals, the mechanism through which Ni exposure alters gene expression has not been related to direct genotoxicity. Early findings suggested that Ni-related carcinogenicity was caused by inhibition of DNA repair enzymes. In experimental animals, Ni compounds were not mutagenic although they produced high cancer rates at the site of administration (Broday et al. 2000). More recently, epigenetic investigations have found that Ni induces DNA hypermethylation in vitro by blocking expression of transcription factors important in cancer (Lee et al. 1995). Exposure to $NiCl_2$ has been shown to modify histone acetylation, increase demethylation of H2K9, and monoubiquitination of H2A and H2B in vitro (Ke et al. 2006). Broday and colleagues studied Ni effects at nontoxic levels and found a decrease in histone H4 acetylation at lysine 12 in mammalian cells and all H4 lysines in yeast (Broday et al. 2000). In human lung cells exposed to soluble Ni, loss of acetylation of histones H2A, H2B, H3, and H4, increased H3K9 demethylation, and increased ubiquitination of H2A and H2B was observed (Baccarelli and Bollati 2009; Broday et al. 2000; Lee et al. 1995; Chen et al. 2006; Karaczyn et al. 2006; Golebiowski and Kasprzak 2005; Govindarajan et al. 2002). Because Ni is a known human carcinogen and has been shown to interact and significantly alter histone proteins through several mechanisms, epidemiologic studies of human exposed populations are warranted.

Cadmium/Chromium Cadmium and chromium also demonstrate low direct genotoxicity as mutagens, but both have been classified as Group 1 Known Human Carcinogens by IARC (2014). Like other metals, exposure is associated with creation of ROS, aberrant gene transcription and expression patterns, and altered DNA methylation (Huang et al. 2008). Takiguchi and co-authors demonstrated that cadmium inhibits DNA methyltransferase activity and alters genome methylation possibly through interaction with the DNA methyltransferase-binding domain (Takiguchi et al. 2003). It also inhibits DNA methylation at oncogene promoter regions, leading to increased expression of proteins related to cell proliferation and

differentiation (Takiguchi et al. 2003). Hexavalent chromium (Chromium VI) is a Group I Human Carcinogen primarily associated with lung cancer risk in occupational studies (IARC 2014). Examination of lung cancer patients with past occupational exposure to chromate found hypermethylation of tumor suppressor *p16* in tumor tissue (Kondo et al. 2006). Exposed workers also demonstrated microsatellite instability and protein expression of the DNA mismatch repair gene *hMLH1* (Takahashi et al. 2005). More recent in vitro studies conducted in human lung A549 cell line have shown that exposure was related to histone protein alterations, methyltransferase, and demethylase activity which would affect both histone methylation and gene silencing.

11.5 Drinking Water Disinfection

Disinfection byproducts (DBP) are complex mixtures of undesirable chemicals that are formed subsequent to the disinfection of drinking water, through processes utilizing chlorine, ozone, and bromine to disinfect drinking water. Upon reaction with organic matter, by-products are formed that include some possible human carcinogens including chloroform, dichloroacetic acid, and trichloroacetic acid (IARC 2014). These chemicals are known mouse liver carcinogens have shown an ability to alter DNA methylation (Pereira et al. 2001). Trihalomethanes (THMs) are the most prevalent group of DBPs and are used as surrogate estimates of overall exposure in epidemiologic studies. Lifetime exposure to THMs in drinking water but also through showering and swimming pools has been associated with bladder cancer (Costet et al. 2011; Villanueva et al. 2007). The carcinogenicity of DBPs is attributed to genotoxic and non-genotoxic mechanisms. The suspected mechanism of metabolism and bioactivation of halogenated compounds is primarily through the glutathione S-transferase theta enzyme (GSTT1) (Cantor et al. 2010). Recent reports have shown that epigenetic mechanisms may also be altered through exposure. Experiments in rodents showed that THM and haloacetic acid exposures induced alterations in global genomic DNA methylation, increased mRNA expression of proto-oncogenes *c-MYC* and *c-JUN*, and induced kidney and liver tumors (Pereira et al. 2001). A Spanish hospital-based case-control study reported an association between THM exposure and bladder cancer and examined risk modification with blood *LINE1* %5-MeC levels (Salas et al. 2013). When stratified by high and low *LINE1* methylation levels, only the group with lowest methylation levels demonstrated an increased risk of bladder cancer compared to those with higher THM levels. No association was observed between THM exposure and bladder cancer risk among subjects with the highest methylation levels in blood DNA (Salas et al. 2013).

11.6 Trichloroethylene (TCE), Dichloroacetic Acid (DCA), and Trichloroacetic Acid (TCA)

Trichloroethylene (TCE) is a chlorinated hydrocarbon that had been commonly used as an industrial solvent but is also found in drinking water. It is a known human carcinogen (Karami et al. 2012; Guha et al. 2012). Its metabolites, dichloroacetic acid (DCA), and trichloroacetic acid (TCA) are known mouse liver carcinogens (Bull et al. 1990; Herren-Freund et al. 1987; Latendresse and Pereira 1997; Pereira 1996; Pereira and Phelps 1996; Pereira et al. 1997; Chiu et al. 2013). Increased expression of mRNA and decreased methylation of the *c-jun* and *c-myc* genes have been reported in liver tissue in mice treated with these chemicals (Latendresse and Pereira 1997; Nelson et al. 1990; Tao et al. 1999, 2000a). Another study that administered these same DBP to female B6C3F1 mice found that pre-treatment with methionine prevented methylation and mRNA expression in both genes in mouse livers. The prevention by methionine of TCE-, DCA- and TCA-induced DNA hypomethylation supports the hypothesis that these carcinogens may act in part by depleting the availability of S-adenosylmethionine (SAM) (Tao et al. 2000b).

11.7 Endocrine Disruptors

Endocrine-disrupting chemicals are synthetic or natural compounds that affect the function of the endocrine system. They have profound effects on development and fertility (Fowler et al. 2012; Giwercman 2011; Knez 2013; Meeker 2012). Both DNA methylation and histone modifications have been reported to be involved in the mechanisms related to endocrine disruption (De Coster and van Larebeke 2012). Although not yet adequately studied in humans, evidence from animal models indicates that exposure to these chemicals during critical periods of development can induce trans-generational disease states or abnormalities associated with changes in epigenetic states, particularly altered DNA methylation patterns in the germ line (Anway et al. 2005, 2006a, b; Anway and Skinner 2006; Skinner and Anway 2005). Methoxychlor is an estrogenic endocrine-disrupting pesticide derived from dichlorodiphenyltrichloroethane (DDT) (Anway et al. 2005; Anway and Skinner 2006). Treatment of pregnant mice with methoxychlor altered the methylation pattern of sperm imprinted genes tested in F1 offspring. Effects were transgenerational, though disappeared gradually from F1 to F3 offspring (Stouder and Paoloni-Giacobino 2011). Similar results were found in rodents after treatment with another endocrine disruptor vinclozolin, an anti-androgenic chemical (Anway et al. 2005; Guerrero-Bosagna et al. 2012). Further study of F1–F4 offspring reported various disease states in adulthood, including prostate and kidney disease, immune system and testis abnormalities, and tumor development, which the authors hypothesized appeared to be due in part to epigenetic alterations in the male germ line (Anway et al. 2006a; Anway and Skinner 2006). In animal models, environmental influences

on epigenetic mechanisms have been transmitted trans-generationally. A targeted genome-wide methylation study in rats using Nimblegen 3x720K CpG Island Plus RefSeq Promoter Arrays found that key molecules in specific pathways, including PTEN, IGF-1, or rapid estrogen signaling were epigenetically altered in methoxychlor-exposed ovaries (Zama and Uzumcu 2013). It has also been reported that early life exposures to endocrine disruptors may alter gene expression in hypothalamic nuclei via DNA methylation and histone acetylation (De Coster and van Larebeke 2012; Gore 2008). Human studies demonstrating the possibility that environmental exposures can transmit epigenetic alterations trans-generationally are described in subsequent studies.

Bisphenol A (BPA) BPA is a chemical produced for use primarily in the production of epoxy resins (used in lacquers to coat metal products such as food cans, bottle tops, and water supply pipes) and polycarbonate plastics (found in water and infant bottles, compact discs, impact-resistant safety equipment, and medical devices). Some dental sealants and composites may also contribute to human BPA exposure. Developmental exposure to BPA and estradiol increased susceptibility to prostate carcinogenesis and regulated phosphodiesterase type 4 variant 4 expression via changes in DNA methylation (Ho et al. 2006). A study in a rat exposure model found that maternal exposure to BPA altered methylation at specific loci (A^{vy} and $Capb^{IAP}$) (Dolinoy et al. 2007). The study also found that maternal supplementation with folic acid or genistein, a phytoestrogen, at levels comparable to humans consuming high soy diets, prevented the change in methylation and affiliated change in coat color of the exposed animals (Dolinoy et al. 2007). Avissar-Whiting and colleagues investigated the effect of BPA on miRNAs in human placental cells. Microarray analysis revealed several miRNAs to be significantly altered in response to BPA treatment in two cell lines. Real-time PCR results confirmed that *miR-146a* in particular was strongly induced (Avissar-Whiting et al. 2010). Another study that examined epigenetic changes in breast epithelial cells treated with low-dose BPA found DNA hypermethylation in the lysosomal-associated membrane protein 3 (*LAMP3*) CpG island, which correlated with gene silencing (Weng et al. 2010). Pregnant CD-1 mice treated with BPA in utero had hypomethylation of the *Hoxa10* gene and corresponding increased mRNA and protein expression in the reproductive tract (Bromer et al. 2010). A study by Dolinoy et al. showed that maternal BPA exposure altered the coat color of viable yellow agouti (*Avy*) mouse offspring toward yellow by decreasing CpG methylation in the *Agouti* and the CDK5 activator-binding protein (*CabpIAP*) genes (Dolinoy et al. 2007). Maternal dietary supplementation, with either methyl donors like folic acid or the phytoestrogen genistein, negated the DNA hypomethylating effect of BPA (Finer et al. 2011). Global DNA hypomethylation in hepatic tissue preceded development of insulin resistance induced by perinatal BPA exposure in female Wistar rats (Ma et al. 2013).

Persistent Organic Pollutants (POPs) POPs are also considered to be endocrine disruptors. There are a few human studies investigating the association between environmental POP exposures and DNA methylation. Rusiecki and colleagues reported *Alu* and *LINE1* hypomethylation based on pyrosequencing results, with

increasing POPs concentrations including dichlorodiphenltrichloroethylene (DDT), dichlorodiphenyldichloroethylene (DDE), beta-hexachlorocyclohexane (β-BHC), oxychlordane, chlordane, mirex, several polychlorinated biphenyls (PCBs), and the sum of all PCBs in plasma of highly exposed Greenlandic Inuit (Rusiecki et al. 2008). Another study of healthy Koreans, with low POP measurements, also reported and inverse association with *LINE1*, measured via Pyrosequencing, with increasing serum levels of POPs (Kim et al. 2010). A recent study of an elderly white population (N = 524) in Sweden observed that high serum levels of PCB126, OCDD, and p,p′-DDE as well as high toxic equivalence quotient (TEQ) were related to global DNA hypermethylation using the Luminometric Methylation Assay, which is based on restriction enzymes (Lind et al. 2013). Exposure to DDT altered the methylation pattern in the hypothalamus of young male rats, as found in a study which indicated that six CpG islands in *Sst*, *Gal*, *Arf1*, *Ttr*, *Msx1*, and *Grifin* genes were significantly hypomethylated compared with controls (Shutoh et al. 2009). A mixture of organochlorine pesticides, PCBs, and methylmercury chloride administered to pre-pubertal female Sprague-Dawley rats resulted in reduced hepatic global genome DNA methylation as well as in particular CpG sites in the tumor suppressor *p16INK4a* promoter (Desaulniers et al. 2009). The mechanisms underlying the process of altered methylation from POPs exposures is still unclear, and it has been suggested that low levels of oxidative stress appear to correlate positively with hypomethylation (Shutoh et al. 2009).

11.8 Pesticides

Organophosphorus pesticides or organophosphates (OPs) are designed to inhibit the enzyme acetylcholinesterase, yet they can also disrupt DNA methylation through regulation of miRNAs. Exposure of porcine kidney epithelial PK15 cells to the organophosphate dichlorvos resulted in the up-regulation of 16 miRNAs and down-regulation of 14 miRNAs (Li et al. 2011). Genome-wide DNA methylation analyses using the Illumina Infinium HumanMethylation27 Bead Chip on DNA from a human hemoatopoietic K562 cell line exposed to several OPs reported that pesticides, such as fonofos, parathion, terbufos, and diazinon induced methylation changes in the promoter regions of numerous genes involved in carcinogenic pathways (Zhang et al. 2012a, b). Investigators identified 1,069 CpG sites in 984 genes with significant methylation changes in diazinon-treated cells (Zhang et al. 2012b). Exposure of N27 dopaminergic cells to paraquat induced histone H3 acetylation, but did not alter H4 acetylation (Song et al. 2011). In a similar experiment, N27 cells were exposed to the organochlorine pesticide dieldrin, which induced a time-dependent increase in both H3 and H4 acetylation (Song et al. 2010). An experimental study of hepatic miRNA activity in mice after sustained feed administration containing two tumorigenic conazole fungicides (triadimefon, propiconazole) and one non-tumorigenic conazole fungicide (myclobutanil), reported significant altered

expression of 19 miRNAs in both the triadimefon- and propiconazole-treated animals but not in myclobutanil-treated animals. All but one of the altered miRNAs was down-regulated, compared to controls (Ross et al. 2010). In a study of the epigenetic effects of exposures to tetrachloromethane, a component of Halon, Freon, some cleaning agents, and chlorophos (an OP pesticide), a preventive injection of the phytosteroid BTK-8L prior to intoxication of rats with tetrachloromethane and chlorophos showed a partial protective effect on transcriptionally active and repressed liver chromatin. BTK-8L was found to bind with chromatin histone proteins, thus changing the structure of the nucleoprotein complex. This resulted in reduced accessibility of the chromatin fraction components to the damaging action of the two chemical exposures (Levitskii et al. 1996). A pesticide mixture of permethrin and N,N-Diethyl-meta-toluamide (DEET) administered to gestating F0 generational female rats exposed during the period of fetal gonadal sex determination promoted epimutations in 363 methylated regions in the sperm epigenome in F3 generation animals. This observation provides supportive evidence that exposure to a pesticide mixture could promote trans-generational inheritance of potential sperm epigenetic alterations from ancestral environmental exposures (Manikkam et al. 2012).

11.9 Air Pollution

Benzo[a]pyrene (BaP) and other polycyclic aromatic hydrocarbons (PAHs) exert both genetic and epigenetic toxicity. To date, there have been a few human studies evaluating the epigenetic effects of various components in air pollution. A longitudinal cohort study of approximately 700 children in New York City investigated whether epigenetic markers associated with trans-placental PAH exposure and/or childhood asthma risk could be identified in fetal tissues. Methylation of the gene *ACSL3* 5′-CGI was significantly associated with maternal airborne PAH exposure exceeding 2.41 ng/m^3 (Perera et al. 2009). The effects of exposure to particulate matter (PM) and PM metal components on the expression of candidate miRNAs (miR-222, miR-21, and miR-146a) were compared post-exposure to baseline in a study among 63 workers at an electric-furnace steel plant (foundry workers) (Bollati et al. 2010). Exposure to metal-rich PM modified the expression of candidate mi-RNAs (miR-222, miR-21, and miR-146A) in peripheral blood leukocytes (Bollati et al. 2010). Among the same 63 steel plant workers, histone modifications were apparent after long-term exposure to inhalable Ni and As particulates. Both H3K4me2 and H3K9ac expression in blood were positively associated with years of employment in the plant. H3K4me2 expression increased in association with air levels of Ni, As, and iron (Fe) (Cantone et al. 2011).

Baccarelli and colleagues investigated whether traffic particulate pollution modified DNA methylation in heavily methylated sequences with high representation throughout the human genome in 718 elderly men in the Normative Ageing Study.

Blood leukocyte DNA methylation measured via pyrosequencing in repetitive elements, represented by *LINE1* methylation, decreased after recent exposure to both higher black carbon and higher $PM_{2.5}$ (Baccarelli et al. 2009). No differences were found using *Alu* as a biomarker of repetitive element methylation. In the study of 63 foundry workers described above, both short- and long-term blood leukocyte DNA methylation in *Alu* and *LINE1* repetitive elements was investigated via pyrosequencing, as well as promoter region methylation of inducible nitric oxide synthase (*iNOS*), a gene expressed in mice and rats exposed to PM. Blood DNA was obtained from workers on the first day of a workweek (baseline, after 2 days off work) and after 3 days of work (post-exposure). Methylation in *Alu* and *LINE1* did not show changes in post-exposure measures compared with baseline PM measures, however hypomethylation of *Alu* and *LINE1* were positively associated with increasing exposure to PM10 while *iNOS* promoter DNA methylation was inversely associated in post-exposure compared to baseline DNA (Tarantini et al. 2009). An in vitro study was conducted in which BALB/c mice were exposed to inhaled diesel exhaust particles for 3-weeks. Inhaled diesel exhaust particles induced hypermethylation at CpG sites in the *IFN-gamma* promoter and hypomethylation at one CpG site in the *IL-4* promoter. This finding correlated significantly with increased IgE protein levels (Liu et al. 2008). In another study, an increase in global methylation was found in sperm DNA from mice exposed to polluted ambient air, compared to those exposed to high-efficiency particulate absorption (HEPA) filtered air using both the cytosine extension assay (CEA) and the methyl-acceptance assay (MAA) (Yauk et al. 2008).

11.10 Benzene

High exposures to benzene have been associated with increased risk of acute myelogenous leukemia (AML) (Chemico-Biological Interactions 2005). A study among healthy gas station attendants and traffic police officers evaluated whether epigenetic changes were induced by low-level exposure to benzene. Airborne benzene was associated with a significant reduction in *LINE1* (−2.33 % with a tenfold increase in airborne benzene levels) and *Alu* (−1.00 %, also with a tenfold increase in benzene levels). Promoter methylation was elevated in *p15* (+0.35 %) and reduced in *MAGE-1* (−0.49 %) with increasing airborne benzene levels (Bollati et al. 2007). An in vitro study using human TK6 lymphoblastoid cells found that the active benzene metabolite hydroquinone induced global DNA hypomethylation (Ji et al. 2010). In another study using lymphoblastoid cell line F32, benzene dramatically decreased mRNA expression and increased methylation of *PARP-1*, a DNA repair gene (Gao et al. 2010).

11.11 RDX

RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine), also known as "Royal Demolition Explosive", is a secondary explosive that is used extensively by the U.S. military in explosive manufacturing. A synthetic product, it belongs to a class of compounds known as explosive nitramines. Major manufacturing of RDX began in the U.S. in 1943 during World War II and was produced in enormous quantities (USEPA 2014). Though it is still used worldwide and produced for military use in the U.S., it is no longer produced commercially in the U.S. (USDHHS, PHS, ATSDR 2012). In 2006, 6.9 million pounds were produced at the Holston Army Ammunition Plant in Tennessee. In the U.S. RDX and its metabolites have been identified in soils (Jenkins et al. 2006). Residues of energetic compounds have been identified and distributed at Army live-fire training ranges (Jenkins et al. 2006) and groundwater (Beller and Tiemeier 2002). High concentrations of RDX, using liquid chromatography/tandem mass spectrometry to detect distinctive indicators of in situ RDX transformation, have been detected in contaminated groundwater (Beller and Tiemeier 2002). A class C potential human carcinogen (EPA), exposure to RDX has been associated with neurotoxicity and immunotoxicity in humans and increased risk of cancer in mice (Zhang and Pan 2009). In a recent study, miRNA expression in mouse liver and brain was measured via expression microarrays after exposure to RDX. Several miRNAs were differentially expressed in the exposed mice, and some of the miRNA expression profiles altered were in gene pathways related to cancer (Zhang and Pan 2009).

11.12 Early Life Exposures

Human studies provide strong biological evidence connecting in utero and early-life environmental exposures to later-life disease and suggest that epigenetic mechanisms are involved (Boekelheide et al. 2012). Few direct human data are available to support the hypothesis that early life exposures may affect health later in life with the exception of rare exposures (such as atomic bomb radiation), medications, and rare cancers (such as vaginal carcinoma from diethylstilbestrol in utero), and more prevalent exposures such as tobacco smoke for which historical exposure assessment is challenging (Nilsson 2001; Preston et al. 2008). Well-documented examples in large populations include those of maternal malnutrition during famine, maternal infection during pandemics, and environmental As exposure from drinking water. Cohort studies conducted of Dutch famine victims who were malnourished during pregnancy gave birth to children who were small for gestational age (SGA) and susceptible to obesity, cardiovascular diseases, diabetes, and renal dysfunction during adulthood. The children exposed in utero born to parents in this SGA cohort demonstrated a heritable passage of susceptibility (Painter et al. 2005, 2008). Children born to mothers who contracted the Spanish flu during the 1918 pandemic

demonstrated lower economic performance, socioeconomic status, and increased prevalence of physical disabilities compared to those who were not (Almond 2006). Children born to mothers who contracted the Brazilian flu (which occurred soon after the Spanish pandemic) demonstrated reduced educational and occupational attainment compared to children of unexposed mothers (Nelson 2010). Environmental As exposure from drinking water in Chile is a third example. Beginning in 1958 with the development of a new water supply, the town of Antofagasta in Region II of northern Chile was exposed to very high levels of As in drinking water (~800 ppb) (Ferreccio et al. 2000). Exposure was reduced to <10 ug/L in 1970 with the introduction of filtration, however, during a 13-year period over 100,000 people were exposed to average As concentrations greater than 800 ug/L. The cohort of individuals who were exposed to arsenic in utero or childhood was later found to have significant deficiencies in lung function and higher cardiovascular mortality compared to subjects unexposed to these high levels (Smith et al. 2006, 2012; Dauphiné et al. 2011; Yuan et al. 2007). A more recent study examined lung and bladder cancer cases diagnosed from 2007 to 2010 and matched controls from unexposed regions of Chile. Residents exposed in utero or during childhood during the high exposure time period were compared to those exposed only during adulthood over the same exposure period. The authors concluded that lung and bladder cancer incidence was markedly increased in adulthood when As exposure occurred in utero or childhood, even up to 40 years after the high exposures ceased (Steinmaus et al. 2013). Odds ratios (ORs) in residents only exposed in utero or childhood to As water concentrations ranging from <110, 110–800, and >800 ug/L were 1.00 (reference), 1.79 (95 % confidence interval (CI): 0.94–3.42), and 4.80 (95 % CI: 2.86–8.06; p-trend < 0.001) for lung cancer and 1.00 (reference), 2.30 (95 % CI: 1.05–5.05), and 7.76 (95 % CI: 4.22–14.25; p-trend < 0.001) for bladder cancer. ORs for individuals exposed during adulthood were 1.00 (reference), 1.08 (95 % CI: 0.54–2.17), and 1.37 (95 % CI: 0.79–2.36; p-trend = 0.24) for lung cancer and 1.00 (reference), 2.42 (95 % CI: 1.12–5.23), and 4.88 (95 % CI: 2.72–8.75; p-trend < 0.001) for bladder cancer. The highest As exposures (greater than 800 ug/L) began 49–52 years and ended 37–40 years before cancers were diagnosed. This was one of the first human studies to identify such associations with a large-scale environmental exposure other than those due to famine or infection, but suggest that humans may be extraordinarily susceptible to early life As exposure, and raise the possibility that susceptibility may be transferred to future generations.

11.13 Common Human Genetic Variation and Epigenetics

Single-nucleotide polymorphisms (SNPs) are common heritable changes that are found throughout the human genome. Depending upon the type of sequence alteration, they may be silent or result in alterations in structure, quantity, or stability of the associated protein product. The first studies to investigate the role of inherited

genetic variation and epigenetic alterations focused upon functional polymorphisms in genes involved in the one-carbon metabolism pathway (Fig. 11.1). Some common functional inherited variants result in the production of enzymes that can be unstable, less efficient, or produced in lower quantities than those required when stressed by a xenobiotic exposure, (i.e. when the enzymes and necessary co-factors for metabolism are limited). The functional polymorphisms most commonly investigated to date in relation to epigenetic mechanisms are located within the methylene tetrahydrofolate reductase gene (*MTHFR*). *MTHFR* variants may modify the bioavailability of methionine, a precursor of the universal methyl donor SAM, which is required for most methylation reactions. One of the earliest reported cases in which insufficient enzyme activity was shown to increase the toxicity of an environmental exposure was reported in a case study of a 16-year old girl previously diagnosed with homocysteinuria. Although homocysteinuria is generally caused by deficient activity of the cystathionine-*B* synthase gene (*CBS*) (involved in the one-carbon metabolism pathway), this patient's homocysteinuria resulted from deficient MTHFR activity, an enzyme active in the same pathway. The metabolic differences became apparent when all family members were exposed to the pesticide copper arsenate. Only the daughter with deficient MTHFR activity demonstrated signs of As poisoning because the enzyme deficiency severely reduced her ability to methylate, and eliminate As through urinary excretion. Family members with normal enzyme activity remained symptomless (Brouwer et al. 1992). The C>T variant at codon 667 of *MTHFR* has been examined in relation to disease susceptibility. The variant allele has been protective in studies of colon and bladder cancer risk and associated with lower genomic methylation levels in the presence of low plasma folate (Castro et al. 2004; Friso et al. 2002, 2013). In a case-control study of renal cancer, a second *MTHFR* variant (rs180113) modified cancer risk but associations were not observed in studies of gastric or bladder cancer (Wilhelm et al. 2010; Hou et al. 2010; Moore et al. 2007). Other one-carbon metabolism pathway genes of functional importance and that modify enzyme expression and disease risk include: methionine synthase (*MS*) 5-methyltetrahydrofoltate-homocysteine reductase (*MTR*), *CBS*, and thymidylate synthetase (*TYMS*); however, associations with epigenetic alterations have been mostly inconclusive and most studies were underpowered to identify gene-environment interactions. The largest genetic susceptibility study conducted to date, that examined 515 genetic variants in 24 one-carbon metabolism genes among 892 subjects. This study assessed modification of *LINE1* methylation levels and heavy metal exposure in toenails and blood DNA (Tajuddin et al. 2013). Interestingly, polymorphisms in DNA methyl transferase 3A alpha (*DNMT3A*), transcobalamin II (*TCN2*), arsenic methyltransferase (*AS3MT*), solute carrier family 19 (folate carrier-*SLC191A*), and 5,10-methenylenetetrahydrofolate synthase (*MTHFS*) were associated with *LINE1* methylation in blood DNA after adjustment for multiple comparisons. Moreover, a significant interaction was observed between As exposure and *AS3MT* with *LINE1* methylation levels. Phase II metabolic enzymes such as *GSTM1* and *GSTT1* have been included in several studies of epigenetic alterations and disease because of they are functional, important in xenobiotic metabolism of many occupational an environmental exposures, in addition to their

biochemical proximity and reliance of cysteine substrates generated from for 1-C metabolism reactions (Cash et al. 2012). Common variation in genes encoding for enzymes regulating DNA methylation, histone modification, chromatin remodeling, and miRNAs expression are also candidate markers that may be modified by genetic variation in human populations environmentally exposed to the substances included in this review. Genome wide association study (GWAS) data is available for many large genetic susceptibility studies in which an agnostic approach could be applied to identify gene regions as well as epigenetic alterations associated with exposure and disease.

11.14 Inherited Propensity Towards Epigenetic Inactivation

In addition to common metabolic genetic variants, a few reports have identified inherited genomic DNA variants associated with gene-specific promoter methylation in somatic and/or tumor tissue. Promoter hypermethylation generally results in reduced gene and protein expression in somatic tissues. This inherited propensity toward epigenetic variation also referred to as "facilitated epigenetic variation" has been documented in a few specific cases in the scientific literature (Hitchins and Ward 2009; Chen et al. 2007; Ogino et al. 2007; Hawkins et al. 2009; Raval et al. 2007; Murrell et al. 2004, 2011). For example, heritable cases of non-polyposis colorectal cancer caused by hypermethylation and gene silencing of *MLH1* and *MLH2*, have been associated with constitutional (germline) mutations and are also referred to as "epimutations" (Hitchins and Ward 2009; Chen et al. 2007). In colorectal cancer, an *MGMT* germline polymorphism (rs16906252 C>T) located within the transcriptional enhancer region was strongly associated with CpG island methylation and gene silencing in tumor tissue (Ogino et al. 2007; Hawkins et al. 2009). Epigenetic silencing and transcriptional suppression of the death associated protein kinase 1 gene (*DAPK1*), a key underlying determinant in familial B cell chronic lymphatic leukemia, is associated with germline SNP (c.1-6531 A>G) located upstream from the *DAPK1* promoter. Presence of this SNP in genomic DNA results in higher binding affinity for the HOXB7 protein (Raval et al. 2007). In Beckwith-Wiedemann syndrome, an *IGF2* polymorphism is associated with loss of imprinting and methylation of the maternal *KCNQ1* gene allele (Murrell et al. 2004). In a recent study of renal cancer, *VHL* tagging SNPs and two haplotypes spanning the *VHL* gene region were strongly associated with promoter hypermethylation in renal tumors compared to genetically inactivated *VHL* cases or wild type tumors (Moore et al. 2011). *VHL* inactivation is an early event in renal carcinogenesis; somatic *VHL* alteration and chromosome 3p loss are observed in approximately 90 % of clear cell renal cell carcinoma tumors. Together, these findings demonstrate unique examples of epigenetic mechanisms through which common inherited genetic variants appear to directly influence epigenetic alterations in somatic tissue that are directly related to disease risk.

11.15 Application of Epigenetic Biomarkers to Epidemiological Studies of Environmental Exposures and Disease

Epigenetic alterations in blood and somatic tissues have been associated with environmental exposures and used as intermediate endpoints hypothesized to modify or directly impact pathways to disease. In this review, several applications of epigenetic biomarkers applied in human population studies have been described. The outcome measures included alterations in DNA methylation, histone alteration, chromatin remodeling, and miRNA expression. These types of biomarkers are recent contributions to molecular epidemiology that have extended understanding of exposure disease relationships. Yet, to date, only a few well-designed methods studies have been conducted to provide information necessary to estimate differences between comparison group means and sources of variance that are required to design high-quality, sufficiently powered studies (Nelson et al. 2011; Brennan and Flanagan 2012; Andreotti et al. 2013; Chen et al. 2001). Therefore, many study results presented must be interpreted with caution when studies were underpowered or when insufficient patient data or potential sources of variation were not evaluated. Studies of environmental exposures conducted in human populations not only require exposure variation, but the timing of biological specimen collection must be relevant to the epigenetic biomarker of interest (White 2011; Garcia-Closas et al. 2011). Similarly, the assays employed must be biologically relevant to exposure and intermediate markers must be relevant to disease. Second, the epigenetic biomarker assays employed require validation in pilot studies to assess assay sensitivity, specificity, accuracy, and understanding of factors contributing to biomarker variation. Results of pilot studies are rarely published alone or described along with study results. Pilot studies provide important information to determine estimates of intra- and between-person sources of variability, expected background levels in healthy subjects, and provide information necessary to estimate expected differences between comparison groups. Some publications provide the average coefficient of variation (CV) per subject, calculated as the ratio of the standard deviation of repeat measurements divided by the mean which is multiplied by 100 and reported as a percent. A low CV can be indicative of high assay reproducibility of replicate samples. However, the CV can be artificially influenced by the mean (i.e. a CV will be low when the mean is high and elevated when a mean is low). This measure does not reflect biomarker validity. The intra class correlation coefficient (ICC) is another important measure rarely reported in epigenetic biomarker studies because it compares the variability observed between subjects to all sources of variability (i.e. the sum of between- and intra-individual variation of repeat measurements specific to the type of assay employed, batch effects, sample storage conditions, or other unknown factors). It is a ratio of the between subject variation, over the sum of all sources of variation (i.e. between subject variation and within person variation divided

by the number of repeat samples per individual). Recently, the *LINE1* pyrosequencing assay has come under scrutiny because: (i) it has not been consistently correlated with overall CpG methylation levels using assays such as HPCE, or other surrogate repeat element methylation measures using *ALU* or *SAT2* sequences; (ii) the low between person variation observed in some populations has been small in comparison to the total between- and within-person variance and results in low ICCs measurements that have ranged from 0 to 0.50 when provided, (iii) when whole blood has been analyzed and the proportion of cell subtypes had not been considered (Nelson et al. 2011; Brennan and Flanagan 2012; Andreotti et al. 2013; Shenker et al. 2013). Because of the low population variance and small differences reported between comparison groups, population studies must be large to obtain sufficient study power to identify significant differences between comparison groups. In some instances, differences in *LINE1* methylation levels between comparison groups have been within the range of assay technical variation. In this situation, differences observed would be driven towards the null because the variability would increase the likelihood of false negative results.

To critically evaluate and utilize new epigenetic biomarkers, additional pilot work will be essential to estimate parameters required for sample size calculations to ensure statistical power and reduce risk of Type I and Type II error (Rothman et al. 1998). A Type I error is the incorrect rejection of a true null hypothesis and is reported as a false positive. A Type I error leads one to conclude that a supposed effect or relationship exists when in fact it does not. A Type II error is the failure to reject a false null hypothesis, resulting in a false negative finding (Rothman et al. 1998). For all statistical tests, there exists some probability of Type I or Type II errors.

EWAS studies present additional study design challenges. Because a large number of CpG loci are analyzed, false positive findings are likely due to multiple comparisons. To avoid false positive reporting, multiple comparisons must be considered in statistical analyses. Initial studies must also plan for subsequent replication studies of significant findings, ideally conducted in independent study populations and evaluated using complementary laboratory assays. Although EWAS studies include >450 k CpG sites for analysis, recent methods work has shown that preliminary analyses will require focus upon CpG loci that demonstrate minimal temporal and/or within person variation and high between person variability. Recent methods studies of EWAS data have identified specific probes on the 450 k array that demonstrate high between subject variability, temporal stability, and low assay variability. These include CpG sites with individual ICC measurements of at least 0.50. For example, the *AHRR* CpG loci associated with tobacco use met these criteria and associations have shown consistent, independent replication in several populations (Flanagan et al. 2013; Joubert et al. 2012; Shenker et al. 2013; Philibert et al. 2013).

All epidemiological study designs have strengths and weaknesses and the consistency of results across studies provides evidence of a relationship between exposure, biomarker, and/or disease. The cross-sectional study design is used to examine

a population at a single time of exposure. High quality data of exposure, covariates as modifiers of exposure, and potential confounders can be obtained including collection of biological materials from healthy subjects related to exposures. Intervention studies can be conducted to determine if interventions or exposure reduction results in alteration of intermediate biological effects. In case-control studies, assessment of epigenetic biomarkers with respect to historical exposure levels estimated from questionnaires or occupational records as well as personal data and potential confounders related to disease risk can be evaluated at the time biological samples are collected. A disadvantage is that the biological samples and biomarkers are measured at enrollment, and often do not reflect historic levels to which the subjects may have been exposed or the epigenetic alteration at time of and/or post-exposure, prior to disease. There are also questions of reverse causality (i.e. whether the epigenetic biomarker occurred prior to, or as a result of disease) (Nelson et al. 2011; Brennan and Flanagan 2012). In addition, biases can be introduced when cases are enrolled post diagnosis due to biases in reporting information, behavioral changes, and treatment. Recently, prospective cohorts are considered the most efficient way to eliminate risk of reverse causality and examine temporal relationships between exposure, biomarkers, and disease, because exposure and other patient information was collected while subjects were healthy. For studies using epigenetic biomarkers within nested case-control studies, utilization of samples collected prior to cancer diagnosis are considered the "gold standard" because the risk of reverse causality is eliminated, the temporal relationship between epigenetic biomarker and disease can be examined, and intermediate endpoints related to disease can be examined. On the other hand, cohort studies are very costly, and collection of high quality data and/or targeted questions related to exposures and diseases is difficult. Biological sample collection cannot be conducted to the extent as is possible in cross-sectional or case-control studies. They are also not optimal for rare disease studies as the number of cases will be small.

In summary, this chapter provides a review of current research conducted to date that has provided evidence for associations between environmental exposures and epigenetic alterations in cells, animals, and humans through a number of mechanisms. The need for additional pilot and methods studies have been described which are necessary for designing, conducting, and critically evaluating such studies. Additional research is merited to further examine and elucidate mechanisms to explain the relationship between environmental exposures that have provided evidence of causing alterations in the human epigenome and their role in disease risk.

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Chapter 12
Interplay of Epigenetics, Genome Rearrangement, and Environment During Development

Yih-Horng Shiao

Abstract Genome rearrangement, characterized by insertion, deletion, amplification, inversion, and/or transposition of DNA segments, during development has been observed in multiple cell types, such as, B-cell and T-cell lymphocytes, and non-mammalian organisms, such as ciliates. These genome restructuring processes are driven by epigenetic markings, including DNA methylation status, histone modification, and/or noncoding RNA transcription, which are modifiable by external and internal environments. In turn, the genome rearrangement establishes a new state of genomic control via epigenetic marks regulating expressions of genes in specific cell types or stages. Epigenetic reprogramming occurs during mammalian development. Direct evidence of ontogeny-driven genome rearrangement in mammals at organismal level is still missing. It is arguable that genome rearrangement, which introduces genome diversity, is a driving force of mammalian development. There are some indirect evidence supporting that such genome rearrangement occurs at organismal level.

Keywords Epigenetics • Genome rearrangement • Interplay • Environment • Ontogeny • Tissue differentiation • Reprogramming

12.1 Introduction

Genome rearrangement is programmed for development in selected cell types, such as mating-type switch in yeasts, diversification of variant surface glycoprotein in trypanosomes, amplifications of chorion protein genes for eggshell production in *Drosophila*, and recombinations of variable (V), diversity (D), and/or joining (J) segments of the immunoglobulin gene during maturation of B and T lymphocytes in humans (Bostock 1984). Transposition of the repressed *a* gene from the *HMRa* locus to replace the expressed α gene at the *MATα* locus converts *α* yeast cell to *a*

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cell type by derepressing the *a* gene along with elimination of the *α* gene, and deletion/inversion of V(D)J gene segments activates an upstream promoter in the V segment by bringing the upstream promoter to close proximity of a downstream enhancer located between J and constant (C) segments (Borst and Greaves 1987). The *rDNA* gene, tandemly repeated in multiple chromosomes in many unicellular and multicellular organisms (Long and Dawid 1980), is a hotspot area for homologous recombination (Eickbush and Eickbush 2007; Stults et al. 2008). Amplification of the *rDNA* repeats is observed during oogenesis in *Xenopus* and macronuclear development in *Tetrahymena* (Bostock 1984). Direct allelic reduction of the *rDNA* repeats in *Drosophila* lessened heterochromatin formation and gene silencing in unlinked genes elsewhere in the genome (Paredes and Maggert 2009).

Epigenetic reprogramming and programming have been observed throughout mammalian development (Hemberger et al. 2009). The reprogramming erases DNA methylation marks and methylations of histone 3 at lysine 9 and 27 in selected genomic loci at two stages, primordial germ cells and early embryo before blastocyst. This erasure allows the subsequent reset of epigenetic marks to maintain the cell lineage fates. The programming is controlled by developmentally determined expressions of ontogenic proteins that support the progression of different cell lineages. Exogenous stimulants, such as nutrient or environmental exposure, are known to modulate epigenome during development and may in some case increase plasticity and survival (Hochberg et al. 2011). Identification of tissue-specific differentially methylated regions, variably methylated regions among individuals, and ontogenic genes in close proximity leads to the proposal that mammalian ontogeny and evolutionary adaptation are driven by stochastic epigenetic mechanism (Feinberg and Irizarry 2010). Furthermore, pluripotency factors (Nanog, Oct4, Sox2, and Sall4) and epigenetic modifiers (Ezh2, Dnmt3A, Dnmt3B, Dnmt3L, etc.) that catalyze DNA methylation and histone modifications are mutually regulated (Hemberger et al. 2009), strengthening the idea that epigenetic reprogramming and programming control the developmental processes in mammals.

Environmental stresses are essential in organismal evolution. Organisms respond to stresses by altering their epigenotypes, genotypes, and phenotypes (McClintock 1984; Rando and Verstrepen 2007; Badyaev 2005). Some of these stress responses are transmissible to next generation(s) in many organisms, including bacteria, fungi, plants, invertebrates, and mammals (Rando and Verstrepen 2007; Badyaev 2005; Jablonka and Raz 2009; Molinier et al. 2006; Barber et al. 2002). The mode of this transgenerational effect is often interpreted as Lamarckism, Darwinism, or modified theories (Koonin and Wolf 2009).

Developmentally determined and environmentally influenced genome rearrangement is well documented in ciliates and B-cell/T-cell lymphocytes, which are discussed first to illustrate that the genome rearrangement is initiated by epigenetic mechanisms. Indirect evidence of ontogeny-driven genome rearrangement in mammals is provided next and is drawn in parallel with the epigenetic mechanisms identified in the ciliates and B/T cells to support that genome rearrangement is also an integral part of mammalian organismal development. Furthermore, such genome rearrangement in mammals is modifiable by parental exposure.

12.2 Developmental Genome Rearrangement in Ciliates

Ciliates, eukaryotes and mostly unicellular organisms, are characterized by the presence of hair-like structures called cilia and nuclear dimorphism (Prescott 1994). Nuclear dimorphism segregates two kinds of genome into micronucleus, containing germline sequences which are transcriptionally silent during vegetative growth, and macronucleus, performing somatic functions of transcription and translation for vegetative growth and asexual reproduction. During starvation, sexual conjugation is activated between two compatible mating types, in which diploid micronucleus undergoes meiosis to generate haploid micronucleus for exchange (fertilization). Each of the two fertilized cells carries new diploid (zygotic) micronucleus and old (maternal) micronucleus, and the zygotic micronucleus enters mitotic division to replicate micronucleus for subsequent differentiation to new (daughter) macronucleus, followed by degradation of maternal macronucleus (Nowacki et al. 2009). The differentiation of daughter somatic macronucleus involves developmentally determined gene rearrangement to remove internal eliminated sequences (IESs) in post-mitotic micronucleus guided by maternal somatic macronucleus-derived non-protein-coding RNAs. Two consensus models have been proposed as follow: (a) scanRNA (scnRNA) subtraction in *Tetrahymena* (Fig. 12.1a) and (b) piRNA retention in *Oxytricha* (Fig. 12.1b) (Bracht et al. 2013).

In *Tetrahymena*, the noncoding RNAs are transcribed from both strands (antisense and sense directions) of the entire germline micronuclear genome and form complementary double-stranded RNAs, recognized by a Dicer-like Dcl1p protein to generate small RNAs of 26–31 nucleotides (Duharcourt et al. 2009). The resulting small RNAs are scnRNAs and are stabilized by 2′-O-methylation catalyzed by Hen1p RNA methyltransferase. The scnRNAs bound to Twi1p proteins are transported into maternal somatic macronucleus to hybridize with homologous sequences (possibly nascent noncoding RNAs). The hybridization induces degradation of scnRNAs that are complementary to maternal macronuclear genome and in this way to enrich scnRNAs for later targeting to IESs in the post-mitotic micronucleus. The enriched scnRNAs are then transported into the post-mitotic micronucleus and upon recognition of complementary IESs triggers serial epigenetic modifications, including Ezl1p-catalyzed methylation of histone H3 at lysine 9 (H3K9me3) and lysine 27 (H3K27me3), indicative of silent chromatin, which recruit Pdd1p and Pdd3p chromodomain proteins to establish a heterochromatic structure. The PiggyBac transposase-related protein Tpb2p recognizes the heterochromatic structure and catalyzes the IESs excision process to produce daughter somatic macronuclear genome (Mochizuki 2012). The excised DNAs are repaired by the nonhomologous end-joining (NHEJ) pathway involving Ku80.

In *Oxytricha*, transcription of either strand of the entire maternal somatic macronuclear genome generates long noncoding RNAs flanked by telomere sequences during sexual conjugation. Some of the long noncoding RNAs are processed into piRNAs of 27 nucleotides and after forming complex with the Piwi-related Otiwi1 proteins are transported into the post-mitotic micronucleus to hybridize with and to

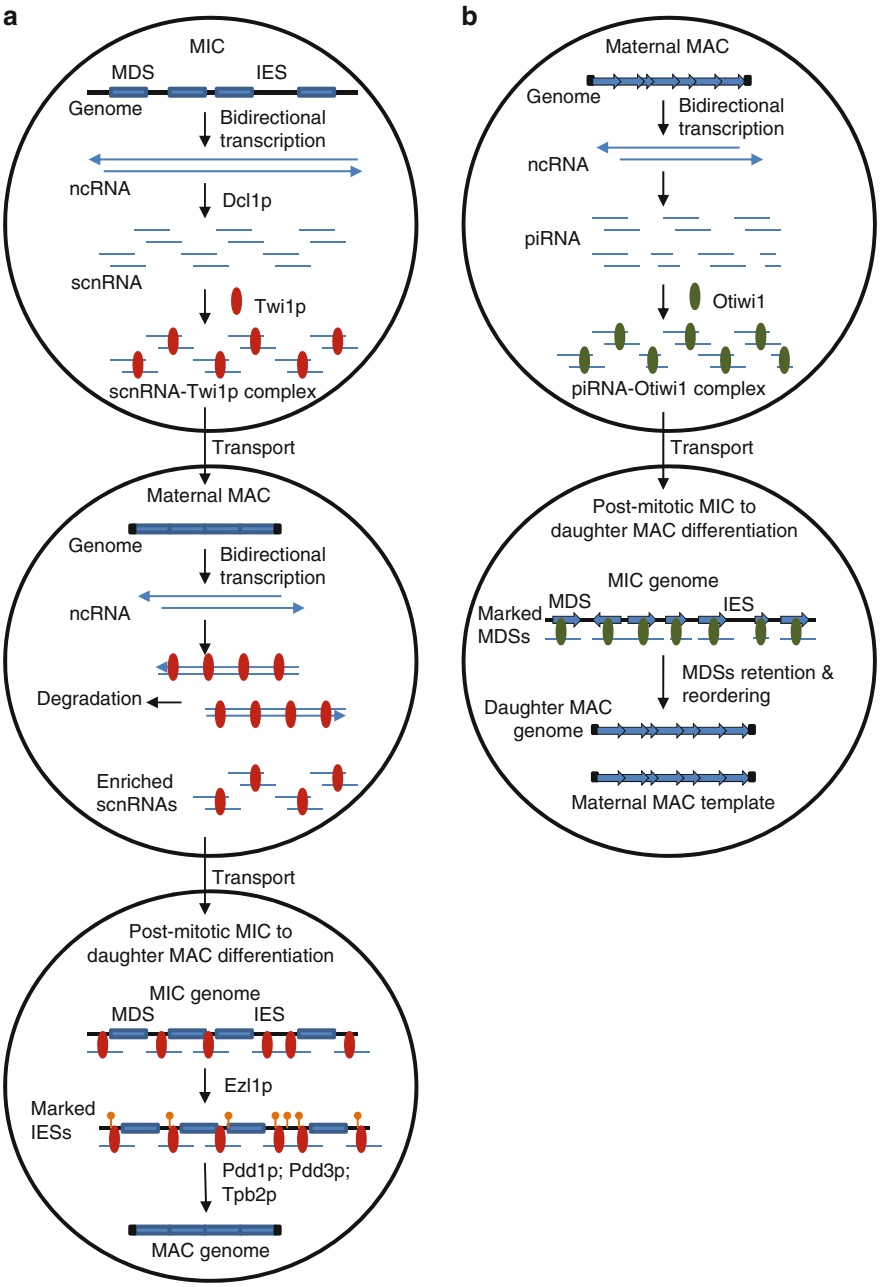

Fig. 12.1 Two models of noncoding RNA-mediated genome rearrangement in ciliates. (**a**) scanRNA (scnRNA) subtraction in *Tetrahymena* and (**b**) piRNA retention in *Oxytricha*. *MIC* micronucleus, *MAC* macronucleus, *ncRNA* noncoding RNA, *black rectangle*: telomere, *MDS* macronuclear-destined sequences, *IES* internal eliminated sequence, *orange lollipop*: H3K9me3 and/or H3K27me3

mark the macronuclear-destined sequences for retention. A portion of long noncoding RNAs from the maternal somatic macronucleus is also imported into the postmitotic micronucleus and serves as a template to guide the assembly of ordered or unscrambled macronuclear-destined sequences while removing IESs, followed by DNA repair and capping of short telomeres.

12.3 Recombinations of the Immunoglobulin Gene in B Cell and the T-Cell Receptor Gene in T Cell

Upon antigen exposure, such as bacteria and viruses, mammalian adaptive immune system attack antigens by two types of antigen-responsive cells: B and T cell lymphocytes, in which B cells secrete immunoglobulin antibodies to neutralize antigens and T cells produce cytokines to recruit other immune cells, including macrophages, neutrophils, mast cells, and natural killer cells (Keim et al. 2013). During maturation, syntheses of antigen receptors in B and T cells require a serial genome rearrangement known as V(D)J recombination, which occurs in the loci of immunoglobulin heavy (IgH) and light (Igκ and Igλ) chains for B cells inside bone marrow and in T-cell receptor loci (TCRα, TCRß, TCRδ, and TCRγ) for T cells at thymus (Jaeger et al. 2012). The IgH, TCRß, and TCRδ loci consist of V, D, and J segments, whereas Igκ, Igλ, TCRα, and TCRγ loci contain only V and J segments. The IgH gene rearranges first, followed by Igκ or Igλ gene; likewise, TCRß gene precedes TCRα gene for rearrangement. For IgH and TCRß genes, D-to-J rearrangement completes before V-to-DJ recombination. In IgH, Igκ and TCRß loci, the V-to-DJ or V-to-J recombination follows the allelic exclusion fashion, whereby antigen receptor chains are encoded from one of two parental alleles. The B cells, called naive B cells, after completion of the V(D)J recombination enter blood stream and when contact with antigens continue further genome modifications to enhance antibody affinity, namely, class switch recombination (CSR) and somatic hypermutation (SHM) in the constant (C) segment of the IgH locus at germinal center in lymphoid tissues (Keim et al. 2013). These serial recombinations are cell-type and lineage specific and the immunoglobulin loci only recombine in B cells whereas the TCR rearrangement occurs exclusively in T cells. How are these orderly genome rearrangements regulated? Several epigenetic mechanisms, including noncoding RNAs, histone marks, and genome folding have been identified and are discussed below.

12.3.1 V(D)J Recombination

Through genetic manipulations to inhibit or to enhance functions of gene segments or cellular molecules, several epigenetic mechanisms are deemed to directly contribute to an opened chromatin which initiates recombination process and allows accessibility of the recombination activating gene (RAG) recombinase (Matheson and Corcoran 2011; Schatz and Ji 2011; Jaeger et al. 2012). First, sense noncoding RNAs (originally termed germline transcript for its transcription from unrecombined gene loci) are expressed from the intronic enhancer Eμ (Iμ transcript) and from the PDQ52 promoter/enhancer upstream to the most 3′ D segment (μ0 transcript) in the IgH locus prior to the D-to-J recombination (Fig. 12.2a). Antisense noncoding RNAs transcribed upstream to the intronic enhancer Eμ are also detected in the D and J regions of germline alleles at this stage. This polymerase II-catalyzed Eμ-dependent antisense transcription leads to acquisition of trimethylation of histone 3 at lysine 4 (H3K4me3, a histone mark for active chromatin), which is also known to be a preferred binding site for RAG2 recombinase. Targeted deletion of Eμ, which depletes both sense and antisense noncoding RNAs, results in a defective D-to-J recombination. After completion of D-to-J recombination, new sense (Dμ) and antisense noncoding RNAs are transcribed from D and V segments of IgH (Fig. 12.2a). Experimental deletion of the IgH locus between the V and D segments leads to extension of antisense transcription from the D into the V region and V-to-DJ recombination in T cells, which do not normally undergo recombination in IgH locus. The functional requirement of noncoding RNA transcription is further supported by insertion of a transcription terminator into two locations in the Jα cluster of the TCRα-TCRδ locus (Fig. 12.2b). This genetic manipulation blocks the transcription in the Jα region and V-to-J recombination downstream to the terminator.

Second, the requirement of chromatin remodeling for recombination is demonstrated in in vivo studies of the TCRß locus (Fig. 12.2c). The TCRß enhancer (Eß) in conjunction with PDß1 promoter and PDβ2 promoter have been determined to be required for transcriptions and recombinations of Dß1-Jß1 and Dß2-Jß2 clusters, respectively. Recombination of Dß1-Jß1 is initiated by recruiting SWI–SNF chromatin remodeling complex into the Dß1-Jß1 region to form a DNA loop and a complex containing Eß, PDß1, transcription factors and components of the transcriptional machinery. Even the PDß1 promoter is deleted, binding of the chromatin remodeling factor BRG1 to a site immediately adjacent to Dβ1 region is able to restore transcription and recombination of Dß1-Jß1 (Osipovich et al. 2007). In addition, SWI/SNF remodeling complexes are also required for V region antisense transcription, and Eμ-dependent Iμ, μ0, and D antisense transcription in IgH locus, described above (Osipovich et al. 2009).

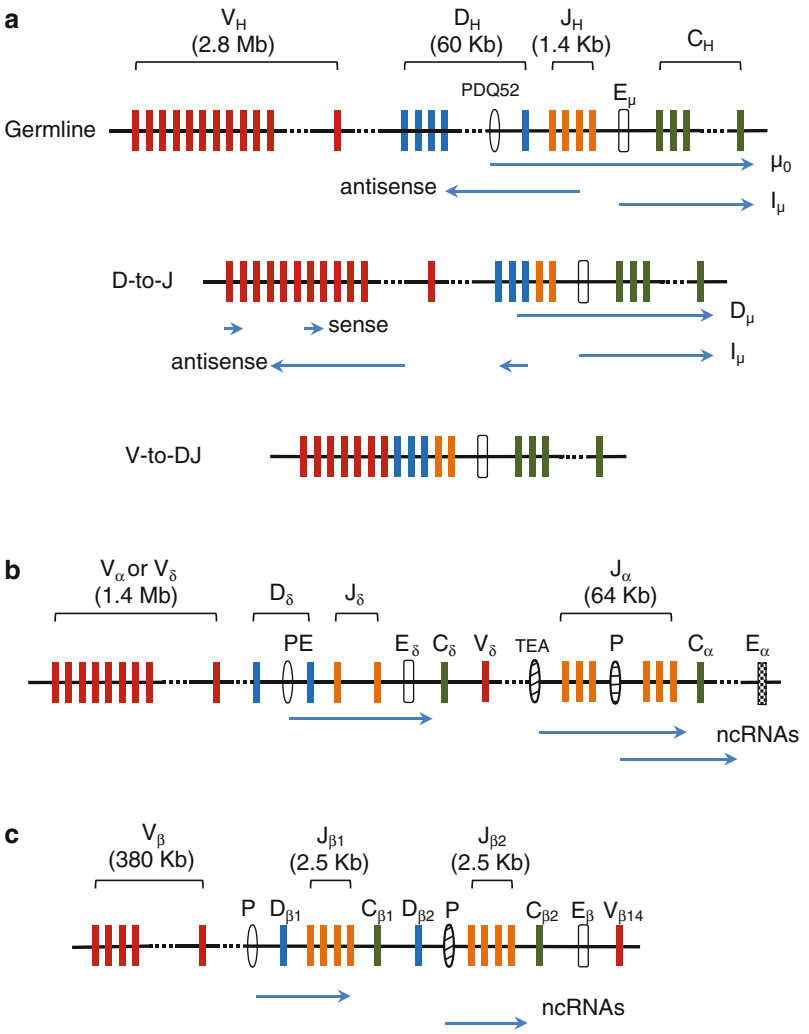

Fig. 12.2 The structures of antigen receptor genes. (**a**) IgH locus; (**b**) TCRα-TCRδ locus; (**c**) TCRß locus. See description in the main text. *ncRNAs* noncoding RNAs

12.3.2 CSR and SHM

CSR, specific for the C segment of IgH locus, is a deletion-type gene rearrangement occurring between the most upstream switch sequence (Sμ) and one of the downstream switch sequences, whereas SHM introduces point mutations in the recombined V(D)J of IgH, Igκ, or Igλ to increase the affinity of antibody to a specific antigen; both CSR and SHM are carried out by activation-induced deaminase (AID), a DNA cytidine deaminase (Keim et al. 2013; Li et al. 2013). Five IgH classes are Igμ (IgM), Igδ (IgD), Igγ (IgG), Igε (IgE), and Igα (IgA), and space out across the

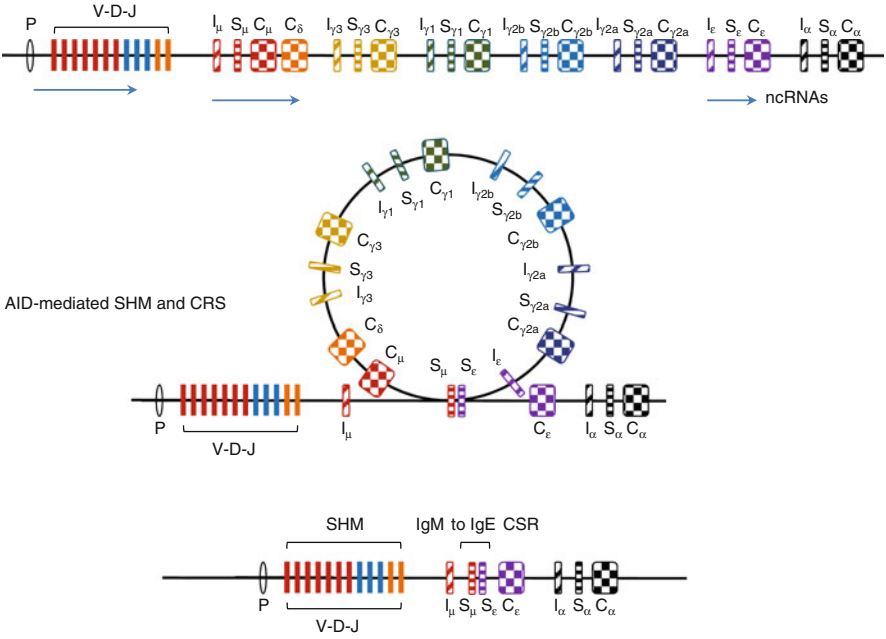

Fig. 12.3 The structure of C segment of the IgH locus and activation-induced deamiase AID)-mediated class switch recombination (*CRS*) and somatic hypermutation (*SHM*). See description in the main text. *ncRNAs* noncoding RNAs

C segment in clusters each consisting of a promoter-containing exon I, a switch sequence S, and a class exon except Cδ which shares the same Iμ and Sμ with Cμ (Fig. 12.3). Similar to V(D)J recombination, transcription is required for initiating CSR and SHM. The Iμ-Sμ-Cμ cluster is constitutively expressed and displays active histone marks, including H3K4me3, H3K9ac/K14ac, H3K36me3, H4K8ac, and H2BK5ac. Transcription from other class is directed by specific cytokines, such as IL-1 for Iγ1-Sγ1-Cγ1 and Iε-Sε-Cε, IFN-γ for Iγ3-Sγ3-Cγ3 and Iγ2a-Sγ2a-Cγ2a, TGF-ß for Iγ2b-Sγ2b-Cγ2b and Iα-Sα-Cα, and IFN-α for Iγ2a-Sγ2a-Cγ2a, in which I is a noncoding exon, S is intron, and C is exon. Noncoding transcripts are produced by splicing to fuse I and C exons. Targeted deletion of splicing acceptor and donor sequences disrupts CSR, indicating that RNA processing is also required during CSR. Furthermore, these transcriptions in I-S-C clusters allow histone-modifying enzymes and Spt5-bound AID enzyme riding on the RNA polymerase II to reach the S region and upon RNA polymerase II stalling, likely due to secondary DNA structure in the S region, histone is further modified to open the chromatin for the access of CSR machinery. The same RNA polymerase II stalling mechanism is also required for introducing SHM.

12.4 Ontogeny-Driven *rDNA* Rearrangement and Methylation

The mammalian *rDNA* (*rRNA*) is a multicopy gene of about 45,000 nucleotides in length in each copy and is a tandemly repeated gene decorated with many types of transposable elements in the intergenic regions and in some cases at the 28S *rRNA* loci (Grozdanov et al. 2003; Melen et al. 1999). The long repetitive sequences pose many technical challenges for the genome sequencing project, while large parts of other genome regions have been completed (Mouse Genome Sequencing Consortium et al. 2002). Single nucleotide polymorphisms (SNPs) are identified among *rDNA* copies and SNP-defined copy variants are detected in outbred Cr:NIH Swiss mice (Shiao et al. 2011). Changes in the frequency of and/or DNA methylation in either copy variant throughout different developmental stages are surrogates for ontogeny-driven genome rearrangement and/or epigenetic modification. Since DNA samples from different developmental stages in a single individual are unobtainable for direct monitoring of those genetic and epigenetic changes, a population-based study is carried out and statistical variance within litter (siblings born to the same parents) is used for comparison. A significant difference in the within-litter variance between developmental stages from multiple litters represents genome rearrangement and/or epigenetic modification because the within-litter variance would remain constant throughout development if the genome and epigenome are permanently established after fertilization. Strikingly, statistically significant increases of these genetic and epigenetic variances in tissues from 93 litters of 6-week adult offspring are observed compared with 98 litters of day-8 (E8) embryos (Fig. 12.4). The within-litter variances in one or more *rDNA* copy variants and *rDNA* methylation are further modulated at various stages of development and tissue differentiation following paternal treatment with Cr(III) or acid saline. Direct comparisons of *rDNA* copy variants and *rDNA* methylation among lung, liver, and/or sperm from the same mouse reveal that frequencies of several copy variants and methylation levels of the *rDNA* repeats are unequal among fully differentiated tissues (Fig. 12.5), providing compelling evidence for ontogeny-driven genome rearrangement and epigenetic modification in mammals at organismal level. These ontogeny-related genetic and epigenetic diversifications are further modified by paternal injection of Cr(III) or acidic saline.

How is the genome rearrangement controlled during mammalian development? The requirement of noncoding RNA transcription to initiate genome rearrangements in ciliates and in B-cell/T-cell lymphocytes is highlighted in the above section. Although noncoding RNA-initiated *rDNA* rearrangement has not been reported in mammal cells, sense and antisense noncoding RNAs transcribed from intergenic sequences are detected in *rDNA* loci and are discussed below.

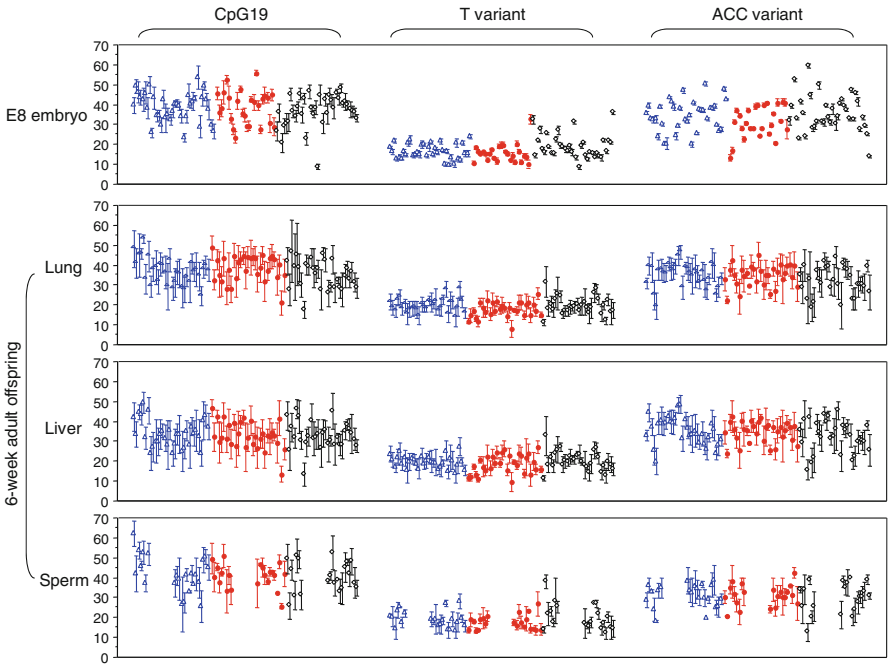

Fig. 12.4 Increase of littermate-to-littermate variation during ontogeny. Within-litter variation, represented by error bars, of *rDNA* copy variant frequencies (%, T and ACC) and CpG19 methylation (%) widened from E8 embryos to 6-week adult tissues in three paternal treatment lineages (genders combined; *blue*: acidic saline; *red*: $CrCl_3$; *black*: untreated). The average pup number in each litter is 9 at E8 embryo and 8 at 6-week adult stage. The plots are displayed as mean ±1× standard deviation to illustrate the degree of variation (Shiao et al. 2011)

12.4.1 Intergenic Noncoding rRNAs

Intergenic noncoding rRNA transcripts have been observed in rodents and humans (Shiao et al. 2009; Henderson and Sollner-Webb 1986; Grummt and Kuhn 1987). A segment of the noncoding rRNA has been shown to regulate transcription of the primary rRNA in mouse fibroblast cells (Mayer et al. 2006). The primary rRNA transcript, precursor of the 18S, 5.8S, and 28S rRNAs, undergoes serial steps of processing that begins with excision of the Leader sequence from the 5′external transcribed spacer at about +650 nucleotide downstream of the transcription start site in mice (Gurney 1985; Eichler and Craig 1994). Proliferating cells exhibit high levels of ribosome production as well as increased rRNA biogenesis, including transcription and subsequent processing, that are orchestrated by many oncogenes and tumor suppressor genes (Ruggero and Pandolfi 2003; White 2008).

Furthermore, mouse cells express bidirectional noncoding rRNAs including at least three fragments of antisense noncoding rRNAs and the most upstream tran-

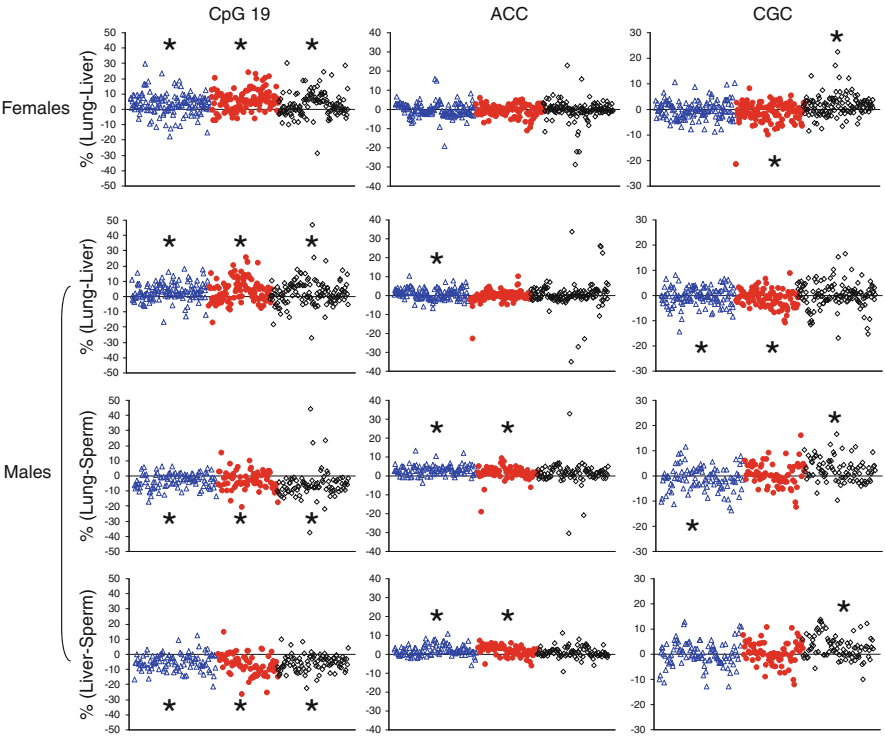

Fig. 12.5 Genetic and epigenetic differences among adult offspring tissues. The arithmetic differences (y-axis) of *rDNA* copy variant frequency (%, ACC and CGC) and CpG19 methylation (%) between two 6-week adult tissues of the same animals were plotted. Deviations of the differences from the zero reference line are frequently detected in three paternal treatment lineages (*blue*: acidic saline; *red*: Cr; *black*: untreated). *Denotes statistical significance in paired difference between tissues of the same mice in each treatment group and identifies positive/negative mean differences by its location above/below the zero reference line (Shiao et al. 2011)

scription start site was located outside the 28S region (Fig. 12.6) (Gee et al. 2012). Sense noncoding rRNAs are transcribed several hundreds to thousands of nucleotides upstream from the primary transcription start site, where two sense transcripts are detected with approximately 10 kb in length, starting at about 300 nucleotides upstream of the transcription start site and ending in the ITS2 region. Some of these sense noncoding rRNAs undergo a cleavage-ligation process to generate transcripts joining upstream sequences with downstream segments. The frequent cleavage-ligation sites occur between the −226 and −44 nucleotides in the upstream and two downstream hot-spot regions, +273 to +556 and around +3,999. Upregulation of bidirectional noncoding rRNAs via antisense technology perturbs rRNA biogenesis and induces apoptotic cell death. Targeting to specific sites of bidirectional noncoding rRNAs with antisense oligonucleotides enables preferential killing of human lung cancer cells via apoptosis (Hwang et al. 2011).

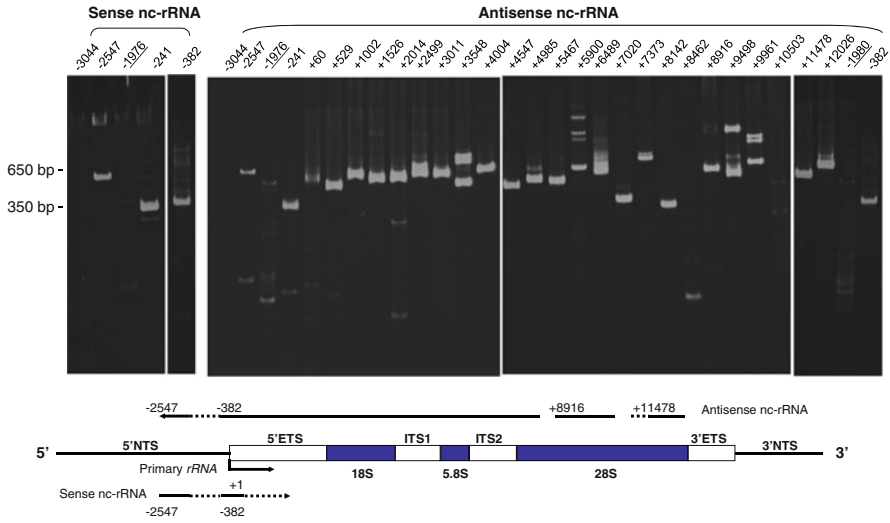

Fig. 12.6 Bidirectional sense and antisense noncoding rRNA transcripts within an *rDNA* locus. The gel lanes show amplified transcripts by reverse transcription-polymerase chain reaction and their locations in the rRNA are identified by their 5′-terminal nucleotides. Those lanes labeled with underlined nucleotide identifiers, also presented as *dotted lines* in the *bottom* figure, represent regions that are not detected. *NTS* Nontranscribed spacer, *ETS* External transcribed spacer, *ITS* Internal transcribed spacer (Gee et al. 2012)

12.5 Conclusion

Noncoding RNA-mediated genome rearrangement has been demonstrated in sexual conjugation of ciliates and maturation of B-cell/T-cell lymphocytes in response to starvation and antigen, respectively. This is achieved through complete characterization of micronuclear and macronuclear genome sequences in ciliates and genetic loci of immunoglobulin in B cells and T-cell receptor in T cells. The entire mammalian *rDNA* repeats have not yet been fully sequenced and thus the sites of gene rearrangement cannot be determined unambiguously. In addition, DNA samples from tissues at different developmental stages in a single mammal are not available. Determination of genome rearrangement relies on indirect measurements of surrogates in a population-based study; however, such study requires a design to distinguish individual variability from true rearrangement. The indirect evidence of *rDNA* rearrangement during mammalian development, which is also modifiable via paternal exposure, and detection of noncoding RNAs in *rDNA* loci set a foundation for future examination of noncoding RNA-mediated genome rearrangement in mammals at organismal level. To characterize precisely the sites of *rDNA* rearrangement, a new sequencing technology needs to be developed to identify which copy variant(s) among several hundred mammalian *rDNA* copies are targeted for rearrangement.

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Chapter 13
Environment, Epigenetic, and Disease: The Modifiable Trilogy

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Abstract From conception, to gestation, birth, and throughout the life course maternal behaviors (e.g. nutrition, exercise, social interactions), and exposure to pathogens, define how the body responds to stimuli and the susceptibility to complex diseases like asthma, obesity, cardiovascular disease, and cancer. This chapter summarizes the relationship between the environment, epigenetics and disease. In the last 20 years, this relationship has been the focus of intense research. Epigenetic alterations due to environmental insult may be reversible through novel treatment options. By changing the epigenetic "landscape" through simple lifestyle modification, disease risk may be ameliorated, thus improving healthspan. As we advance our understanding of the "epigenome", the portion of human genome that is either expressed or repressed in disease based on epigenetic status, we may be able to modify this profile to reduce potential sequelae. New technologies have become available allowing scientists to study epigenetic processes targeting specific genes, genomic regions, and even entire genomes. Further advances in this field are crucial to understanding the role of epigenetic modifications in normal biological functions and disease states. Because epigenetics is implicated in growth and development, cell differentiation, tumorigenesis, and many other important cellular processes

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involved in both normal function and disease states, the need to understand and measure epigenetic changes is becoming increasingly important.

Keywords Environment • Epigenetics • Disease • Methylation • Diet • Pollutants

13.1 Introduction

The capacity to modulate the level of gene expression has the potential to make an individual more or less susceptible to chronic diseases ranging from asthma, obesity, cardiovascular disease to cancer. What unites these apparently disparate diseases is the role of genes involved in the inflammatory response at multiple stages. Gene expression is modulated by several mechanisms. Single nucleotide polymorphisms (SNPs) may affect the rate of transcription by either directly modifying the binding site of transcription factors, or by generating cytosine-phosphate dinucleotide (CpG) sites prone to methylation; mRNA rate of transcription and degradation; and translational mechanisms and post-translational phenomena that ultimately affect the level of production of a specific protein. Post-translational modifications including methylation and acetylation of histones, nucleosome positioning and micro RNA (miRNA) together with DNA methylation (reviewed in Sharma et al. 2010), comprise much of what is known as epigenetic mechanisms of gene regulation. The term "epigenetics" refers to acquired, heritable, and potentially reversible ways of controlling gene expression without modifying the DNA sequence (Waterland and Michels 2007). These epigenetic "marks" are crucial in cell development and tissue differentiation through what is known as "epigenetic reprogramming" in which genes are turned on or off as needed during early development (Reik 2007). Today, epigenetic modifications of cellular responses can be modified by a variety of intracellular interactions, leading to gene activation or repression. Studying the environmental role in modifying the risk of disease has been the current focus of intense research and debate, and in particular, the role of environmental stressors in causing epigenetic changes that influence the inflammatory response.

Hans Selye (Selye 1936) was the first to recognize that exposing laboratory animals to a variety of noxious environmental stressors resulted in the same physiologic "stress" response characterized by activation of the Hypothalamic Pituitary Adrenal Axis (HPA). Since that time, an array of measures have been used to assess the construct of psychosocial stress. In animal studies, manipulation of environmental stressors, as well as, maternal care and separation methods have been the measures of psychosocial stress that dominate research designs. For example, the first study by Francis et al. (1999), demonstrated an epigenetic change on the glucocorticoid receptor gene in rodents stressed by lack of maternal care, once again involving the HPA axis and the binding of transcription factors to their specific DNA binding sequences (Weaver et al. 2004). Epigenetic researchers posit that early exposure to various stressors, even during the gestational period, may lead to reprogramming of

the epigenetic "landscape" in DNA, thus leading to increased or reduced expression of specific cellular pathways. In humans, characterizing psychosocial stress is a decidedly more complex endeavor. For clear ethical reasons, characterizing psychosocial stress at levels sufficient to cause epigenetic changes must be obtained observationally, rather than experimentally. Consequently, measures derived from exposure to adverse life events represent a major means of characterizing psychosocial stress in human populations. Two constructs are typically used to characterize the environmental stressors that have the epigenetic potential to cause the inflammatory states associated with chronic diseases, which are believed to be socially determined: (1) psychosocial stress and (2) nutritive stress (Thayer and Kuzawa 2011). For example, bereavement, history of a suicide attempt, history of abuse, isolation and lack of food, have all been established as psychosocial stressors, which modulate epigenetic changes. Heijmans et al. (2008) showed that subjects exposed to famine prenatally during a German food embargo to The Netherlands at the end of World War II, presented a differential profile of gene methylation when compared to same-sex relatives or age matched unrelated individuals not exposed to the famine. Twin studies have revealed that from the moment of birth, and during the aging process, monozyogous twins grow different in their epigenetic profile likely due to differential exposure to environmental stressors through the life course (Talens et al. 2010; Fraga et al. 2005).

The hypothesis that environment modifies the risk of disease has been the focus of extensive research across a variety of disciplines. Elucidating these processes requires a trans-disciplinary perspective, ranging from public health to molecular genetics. This chapter contends that public health provides a process and molecular genetics a mechanism, to understand how environment and genetics interact to make people more or less prone to develop diseases. Epigenetics has emerged to provide mechanisms that explain variation in phenotype given the same genotype. These mechanisms allow for variation in phenotypic expression due to environmental stressors.

It has been noted that the goal of epigenetic research involves "determining the specific molecular pathways that mediate the biological impact of adverse social condition" (Cole et al. 2011). At the molecular level the pathways that alter the immune response, which results in a chronic inflammatory state, appear to explain the disparity in chronic disease outcomes associated with environmental stressors. These pathways have been revealed in studies of disease susceptibility during adolescence and adulthood involving the influence of the childhood environment. Cohen et al. (2004) reported that the susceptibility to rhinovirus was inversely correlated with familial socioeconomic status (SES) during childhood. It is also clear that these pathways must originate within molecules associated with the hypothalamic-pituitary-adrenal (HPA) axis (Hawkley et al. 2012; Norman et al. 2012; Cole et al. 2009). Exposure to environmental stressors during various periods of the lifecycle leads to characteristic epigenetic changes (Braveman 2006; Gluckman et al. 2008; Kuzawa and Sweet 2009). Furthermore, the chronicity of exposure to environmental stressors appears to result in the sustained biologic changes associated with epigenetic mechanisms, such that in some cases acute exposure can have

an inverse effect on the inflammatory response. Finally, it is widely acknowledged that characterizing the role of environmental stressors initiating epigenetic variation is challenging, given that our measures of the social environment in general, and environmental stressors in particular, have relatively low resolution.

13.2 Early Life and Fetal Programming

The human placenta is susceptible to a variety of complex actions in response to maternal behaviors (e.g. nutritional and physical activity habits; weight status and gain) and environmental exposures. Thus, external influences may have a significant effect on the future health of not only the mother, but also the fetus. The Barker Hypothesis of fetal programming focuses on the critical prenatal period when the mother provides nutrients and other life-sustaining elements. Barker theorizes that such a crucial time in development of the fetus is highly dependent on the mother's health and nutrition behaviors (Sanchez-Muniz et al. 2013; Barker 1992a, b, c; Eberle and Ament 2012; Hales and Barker 1992). Prenatal conditions associated with social or nutritive stress, including malnutrition (i.e. diets unbalanced in macronutrients and deficient in micronutrients); weight status, exercise habits; and exposure to viruses, diseases, and chemicals may have adverse health outcomes on fetal development during gestation, infancy, and later in life (Barker 1992b, c; Hales and Barker 1992, 2001; Barker et al. 2013; Chen et al. 2013). Research in gravid women suggests the existence of a thrifty phenotype which affects the future health of the fetus (Hales and Barker 2001). The thrifty phenotype hypothesis postulates that the effects of poor nutrition in utero and in early life promote negative health outcomes, including permanent changes in glucose-insulin metabolism that perpetuate into adulthood. The reduced capacity for insulin secretion and insulin resistance in the children of malnourished mothers may be a result of the intrauterine influences on development of the fetus (Hales and Barker 2001). Specifically, recent epidemiological data indicates that impaired intrauterine growth and adult metabolic and cardiovascular disorders, including coronary heart disease, type 2 diabetes, and insulin resistance are associated with an inhospitable fetal environment (Sookoian et al. 2013). Initially, adverse health outcomes later in life were explored in low birth weight babies; however, due to the dramatic increase in obesity, research has further expanded to the study of large-for-gestational age (LGA) babies.

An understanding of the fetal growth cycle and involved maternal organs will serve as the foundation to understanding more recent literature regarding the impact of epigenetic factors. Gestation begins with fertilization, where fetus subsequently develops to approximately 38 weeks in the mother's uterus, followed by birth. Epigenetic properties of developing germ cells may affect the growth of the developing zygote. A totipotent zygote forms at the moment of gamete union in fertilization. This cell can generate all cell types. A set of developmentally critical genes

which are maintained in an epigenetically poised state through the end of meiosis dictates this change from a highly differentiated and specialized cell, to a totipotent zygote. The condition of these developmental genes is an essential property of the mammalian germ-line nucleus, allowing differentiated gametes to stimulate the initiation of a totipotent program following fertilization. This totipotent program provides the basis for the embryo and extra-embryonic tissue (Dhobale et al. 2013).

Brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are involved in the development of the placenta and fetal brain at the onset of the gestation period (Dhobale et al. 2013). Before fetal maturation can occur, the placenta joins the fetus to the uterine wall allowing gas and nutrient exchange as well as waste elimination through maternal blood supply. The intrauterine environment in which the fetus matures is a developmental incubator evolutionarily designed to aid in the growth and support of the fetus, as well as to protect the fetus throughout the gestation period.

Due to the epigenetic pathway, negative health outcomes for infants, into childhood, and throughout the life course are frequently attributed to poor maternal health status and unhealthy behaviors prior to and throughout pregnancy. From 2001 through 2010, the prevalence of pre-gravid women who were overweight or obese increased from 48.0 % to 53.4 % (Dalenius et al. 2012). Furthermore, racial and ethnic differences are contributing factors to maternal obesity and weight gain, which may be indicators for the racial disparity plaguing childhood obesity. Leddy et al. (2008) found that pre-gravid African American women are more likely to be overweight and were more likely to gain excessive weight during pregnancy than non-African American women. Women who are obese prior to pregnancy will experience greater challenges, both behaviorally and physiologically. Challenges in particular include, but are not limited to, poorer nutritional intake during pregnancy, management of gestational diabetes mellitus (GDM) and the women's ability to adhere to Institute of Medicine (IOM) weight gain recommendations.

13.3 Malnutrition to Low Birth Weight

Compelling epidemiological studies linking under-nutrition in pregnancy with maternal, fetal, and infant morbidity and mortality has prompted extensive animal studies to identify the biological mechanisms for nutritional regulation of fetal growth and development long term health consequences. Malnutrition causes metabolic adaptations that are detrimental to growth and development in limited environmental and energetic conditions (Sanchez-Muniz et al. 2013). The uterus expands with fetal growth; however, underweight mothers do not have adequate space for this expansion which constricts fetal development (Phan et al. 2013). Recent studies show micronutrients and omega-3 fatty acids are interlinked in the one carbon cycle, of which any alterations of components will lead to changes in methylation patterns. Such alterations have been associated with lower concentrations of BDNF and NGF mRNA in preterm placentae. These relatively low

concentrations affect gene expression at critical periods of development, resulting in complications during pregnancy which may contribute to risk for neurodevelopmental disorders as well as poor vascular development in children born preterm (Dhobale et al. 2013).

13.4 High Fat Intake and GDM

Diet-induced obesity (i.e. a high fat diet during gestation) elevates expression of inflammatory-mediator genes in both the ovary and surrounding adipose depot, potentially negatively affecting ovarian function (Nteeba et al. 2013). Gestational Diabetes Mellitus (GDM) has epigenetic effects on genes preferentially involved in metabolic diseases pathway. In a study conducted to analyze the consequences of GDM, around 3,200 and 3,800 genes in placenta and cord blood, respectively, were differentially methylated between mothers who had or did not have GDM. Consequences on fetal growth and development in mothers with GDM provide supportive evidence that DNA methylation is involved in fetal metabolic programming (Ruchat et al. 2013). Recent research revealed the insulin-like growth factor 2 (IGF2) gene, located within a cluster of imprinted genes on chromosome 11p15, encodes a fetal and placental growth factor affecting birth weight of the newborn. DNA methylation at the IGF2/H19 genes locus may act as a modulator of fetal growth and development. IGF2/H19 DNA methylation in the placenta correlate to fetal birth weight, which may link birth weight and fetal metabolic programming of late onset obesity (St-Pierre et al. 2012). Insulin levels may have a reversible effect on the fetus. Hyperglycemia alters the epigenetic mechanisms in neural stem cells, resulting in altered expression of some development control genes, which may form the basis for some neural tube defects. Since epigenetic changes are reversible, once can argue their value as therapeutic targets in order to improve fetal outcomes in diabetic pregnancy (Shyamasundar et al. 2013).

Just as fluctuating blood glucose levels contribute to negative health outcomes due to decreased levels of insulin, insufficient protein intake may too result in negative birth outcomes. Investigations in mice have shown that either protein or calorie restriction during pregnancy leads to glucose intolerance, increased fat mass, and hypercholesterolemia in adult male offspring (Chen et al. 2013).

13.5 Maternal Obesity and Weight Gain

Despite recommendations, PRAMS data revealed that only one in three women had weight gain consistent with the IOM's 2009 recommendations. In a study consisting of 26,028 women, more women gain an amount of weight above the IOM guidelines (43.3 %) than either within (36.6 %) or below (20.1 %) the guidelines (Stotland et al. 2006). Furthermore, pre-gravid women who are overweight or obese should

have the lowest percentage of weight gain within 2009 IOM guidelines. Almost 56 % percent of obese women and 58.8 % of overweight women gained more weight than recommended compared to only 38.6 % of women of normal weight (Dalenius et al. 2012).

Excessive weight gain may have a significant impact on the intrauterine environment and therefore, fetal development the health of the child later in life (Leddy et al. 2008). Consequences of pre-gravid obesity and weight gain above IOM guidelines include, but are not limited to, preeclampsia, cesarean delivery, macrosomia, low 5-min Apgar Score, hypoglycemia, large for gestational age (LGA) and obesity later in life for both the mother and baby. A study that examined the effect of gestational weight gain and pregnancy outcomes in 120,170 obese women found that as pregnancy weight gain increased the risk of preeclampsia, cesarean delivery and LGA increased (Kiel et al. 2007). Another study of 2,080 obese women in which 18 % gained below IOM guidelines, 25 % within and 57 % above reported increased odds of macrosomia (adjusted odds ratio [OR] 3.36; 95 % CI 1.74–6.51) and LGA (adjusted OR 1.80; 95 % CI 1.36–2.38) (Vesco et al. 2011). Furthermore, regardless of the mother's pre-gravid weight, gestational weight gain above IOM guidelines increases negative health outcomes (Stotland et al. 2006). Current research shows maternal over nutrition may be associated with susceptibility of offspring to future cardiovascular disease. In one animal study, maternal over nutrition was shown to promote fetal and placental inflammatory responses, specifically a marked increase in TLR4, IL-1α, IL-1β, and IL-6 expression as well as promoted phosphorylation of IκB, decreased cytoplasmic NF-κB levels and increased neutrophil and monocyte infiltration. These results suggest fetal heart inflammation and altered fetal cardiac development are associated with maternal overfeeding prior to and throughout gestation (Kandadi et al. 2013). Vital pathways for placental growth and function are likely affected by epigenetic alterations due to obesity, which in turn may contribute to an increase in the newborn's risk of future metabolic sequela (Nardelli et al. 2013). One study from Center for the Study of Fetal Programming in Wyoming found maternal obesity induced inflammation and enhanced expression of pro-inflammatory cytokines in fetal and offspring large intestine, which correlated with increased TGFβ and IL17 expression. These data show that maternal obesity may predispose offspring gut to Inflammatory Bowel Diseases (Yan et al. 2011). Research shows that the epigenome is sensitive to maternal nutrition around conception. It has been shown, for example that the level of methylation of fetal leptin and adiponectin genes change depending on the levels of maternal glycemia (Bouchard et al. 2010, 2012). Thus, there is an increasing need for dietary interventions that maximize metabolic benefits and minimize metabolic consequences for the next generation (Nicholas et al. 2013).

As previously stated, the body of knowledge concerning the future health status of the fetus due to the mother's behaviors throughout the gestation period is expanding; however, evidence shows the expecting mother's malnutrition; weight status; exposure to viruses, diseases, and chemicals; eating habits; and exercise habits are associated with positive versus negative health outcomes.

13.6 Effect of Diet

Undoubtedly, the environment in which all living things interact is a major modifier of the responses to challenges faced every day. In today's world stress, life style, food, and industrialization have a huge impact on the way we, as humans, respond to mutagens and disease. It is now proposed that early life nutrition, at gestation and lactation, has a major role in defining the risk of developing complex diseases like asthma, obesity, cardiovascular disease, and probably cancer (reviewed in Junien 2006). One of the best examples of the role of diet in changing the methylation status of the DNA came from a study published by Heijmans et al. (2008) showing that subjects exposed to famine prenatally during a German food embargo to The Netherland at the end of World War II, had a significant reduction in the fraction of methylation in four different CpG sites in the insulin-like growth factor II (*IGF2*) gene, even six decades later and contrary to their same-sex siblings and unrelated individuals not exposed to the famine. Interestingly, there seems to be a gender-specific effect of diet on gene methylation since males had increased hypomethylation of *IGF2*, as well as leptin (*LEP*) when compared to females (Tobi et al. 2009). Females, on the contrary, showed increased methylation of the gene *GNASAS* while both genders showed hypermethylation of the *IL10* gene (Tobi et al. 2009). A recent study has shown that human embryonic stem cells treated with retinoic acid change the pattern of global and specific methylation (Cheong et al. 2010), suggesting that diet can influence gene expression profiles at germinal centers by altering the pattern of DNA methylation. A recent study in school-age children has reveal a differential correlation between the levels of methylation of a repetitive sequence in the DNA (LINE-1) and the circulating levels of vitamin A (retinol) and with C-reactive protein (Perng et al. 2012), suggesting that methylation of this repeated DNA element may play a role in modifying inflammatory responses and that vitamin intake may help modifying those responses.

13.7 Early Life Environment and Immunity

It has been extensively suggested that in uterus life and early life circumstances influences the response of one individual to diseases later in life. For example, it has been shown that smoking during pregnancy leads to hypomehtylation on several regions of the genome (Flom et al. 2011). Larcombe et al. (2011) has shown that in offspring of mice exposed to tobacco smoke during pregnancy are born with significantly lower body weight and reduced lung volumes leading to impaired respiratory function. In addition, several lines of evidence suggest that hardship during childhood, measured as socioeconomic status, higher risk of coronary heart disease (Kittleson et al. 2006) and increased susceptibility to rhinovirus and influenza viruses infections later in life, both in humans and mice (Cohen et al. 2004; Avitsur et al. 2006). The reason for the latter is not known but Cole et at (Cole et al. 2012)

has elegantly shown in a model of Rhesus Macaques that early life adversities are associated with a significant reduction of gene expression in B lymphocytes. This event is paired with a significant increase in gene expression in T cells and monocytes, suggesting that innate immune responses, as well as acquired responses may be affected, including those essential to develop immunity against viral threats. Interestingly, these inflammatory responses appear to be associated with a differential response of the HPA axis to stress. Specifically, signaling through the glucocorticoid receptor (GR) may act as a regulatory mechanism, through a negative-feedback circuit, of the activation of the HPA axis (Avitsur et al. 2006; Bailey et al. 2003). Recent research in mice suggest that early life adversities may increase the level of methylation of the GR in the hippocampus in maternally separated C57BL/6 J mice (Kundakovic et al. 2013). This specific early life adversity may lead to differential methylation in the promoters of genes associated with the regulation of the HPA axis. At the transcriptional level, the activation and binding of transcription factors like NFkB, CREB/ATF, JAK/STAT, CEBP, and GATA pathways are observed to be associated with differential gene expression in response to neural and endocrine stimulation under social stress (Cole et al. 2007). Interestingly, these neuroendocrine pathways appear to be connected directly to the activation of inflammatory pathways through interleukin (IL)-6 (IL-6) production by the mediation of GATA1 (Cole et al. 2010) after the stimulation of mononuclear cells latently infected with herpervirus 8 and stimulated with nor-epinephrine.

In summary, early life environment plays an essential role in shaping the immune response to stressors. It also seems possible that these responses, if not reprogrammed, modulate ones risk to disease.

13.8 Cardiovascular Disease

The rates of cardiovascular disease (CVD) around the world differs by geographical location but, in general, a sustained reduction has been documented worldwide (?) (Roger et al. 2012; Levi et al. 2002). Despite reduced CVD-related mortality, one American dies of CVD every 39 s, accounting for about 33 % of all deaths in the country in 2008 (Roger et al. 2012), and indicating that there are still sizeable gaps in the understanding of the origins and progression of disease. Diet has been shown to modulate the risk of CVD, with ingestion of sodium, calcium potassium, magnesium and fat, among others, having a significant role in changing the level of blood pressure (He et al. 1991). These changes are, in general, associated with changes in lifestyles and migration from areas with differential levels of environmental stressors (He et al. 1991; Chakraborty et al. 2003; Elford et al. 1989; Wannamethee et al. 2002; Worth et al. 1975). Interestingly, the risk of CVD adverse intrauterine and early life circumstances may modulate the risk of CVD later in life, as has been demonstrated in human and animal studies (Alkemade et al. 2007; Barker 2002).

Altered patterns of methylation have been described in patients with CVD (Castro et al. 2003; Chang et al. 2008; Movassagh et al. 2011). Several reviews have

elucidated the correlation between CVD and epigenetics (Shirodkar and Marsden 2011; Ordovas and Smith 2010; Webster et al. 2013). However, discrepancies between the level of methylation in the atherosclerotic plaques and the levels of methylation in peripheral blood cells have been reported (Castro et al. 2006; Lee and Wang 1999; Sharma et al. 2008; Zaina et al. 2005). Interestingly, these methylation profiles are highly dependent on the quality of the environment in which ones lives. Air quality is a major influence on the development of CVD. Airborne particulates have varying capacities to penetrate deeper in the body, modify the risk of CVD (Katsouyanni et al. 2001; Samet et al. 2000a, b) and contributing to mortality levels, depending on their size (Pope et al. 2002; Pope 2004). For example, ultrafine particulates (UFP) can reach and be absorbed by the alveoli, entering the circulatory system, and consequently causing various types of health complications (reviewed in Bhatnagar 2006). The effect of particulate matter (PM) on the heart cardiac autonomic dysfunction, however, is modified by the ingestion levels of vitamin B6 and B12, and methionine (Baccarelli et al. 2008). At the genomic level, Baccarelli et al. (2009) have shown that exposure to air pollutants induce hypomethylation of LINE-1 sequences and this associated with changes in VCAM-1, ICAM-1, C-reactive protein, and fibrinogen (Bind et al. 2012; Baccarelli et al. 2010). In addition to air pollutants, exposure to metals has been also associated with CVD (Nawrot et al. 2002; Chen et al. 1988).

13.9 Asthma

Maternal behaviors appear to increase asthma susceptibility more than the influence of paternal inheritance (Cookson et al. 1992; Litonjua et al. 1998; Sears et al. 1996). It is now clear that maternal diet and habits during pregnancy mediate the risk of asthma in her developing fetus. For example, increased gestational intake of methyl donors including folic acid, vitamin B_{12}, choline, L-methionine, zinc, and betain in animal models, modifies the pattern of methylation of specific genes associated with the risk of allergy and asthma (Hollingsworth et al. 2008). As with many other human diseases, smoking is directly associated with the development of asthma. A significant correlation ($p < 0.05$) between the appearance of asthma (within 1 year of age) and the mother's smoking habits during the last trimester of pregnancy has been documented (Sears et al. 1996; Li et al. 2008; Xepapadaki et al. 2009). In addition, Liu et al. (2008) have shown that exposure to air pollutants like diesel fumes is associated with increased production of Ig-E and IL-4, promoters of Th2 responses characteristic of allergic reactions. Miller et al. (Miller et al. 2004) have also demonstrated that exposure to polycyclic aromatic hydrocarbons (PAH) is associated to asthma development. Liu et al. (2008) and Perera et al. (2009) have shown that these exposures are related to a differential level of methylation of genes involved in immune response and fatty acid metabolism, including hypermethylation of CpG sites in the interferon-gamma (*IFNG*) and the acyl-CoA synthetase 3 (*ACSL3*) genes and hypomethylation of the *IL-4* gene.

13.10 Cancer

Environmental factors have long been known to play a significant role in cancer susceptibility. Social support, for example, has been shown to mediate health outcomes in individuals under stressful conditions (Ell et al. 1992; Cohen and Wills 1985). In cancer, the interaction between behavioral stress and disease outcome has been shown in several cancer models (Ell et al. 1992; Lutgendorf et al. 2012, 2013; Hassan et al. 2013; Clevenger et al. 2013). These responses are associated with activation of the HPA axis and the SNS leading to the release of stress associated glucocorticoids and epinephrine and nor-epinephrine (Antoni et al. 2006; Nagaraja et al. 2013a, b; Armaiz-Pena et al. 2009, 2013; Thaker et al. 2007). Collectively these relationships appear to be mediated through the activation of several cellular pathways that lead to the increase of inflammatory responses. Shahzad et al. (2010) has demonstrated that siRNA inhibition of either *IL-8* or *FOSB* genes inhibited chronic stress-related growth of ovarian carcinoma. In addition, Lutgendorf et al. (2008) and Costanzo et al. (2005) have shown that increased levels of depression in advanced stage-ovarian cancer are associated with increased circulating levels of cortisol and IL-6. Further pathways include the activation of the signal transducer and activator of transcription-3 (STAT3) in response to epinephrine and norepinephrine, leading increased production of matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) which are contributing to invasion and metastasis in ovarian carcinoma (Landen et al. 2007).

In addition to hormone-mediated responses to stress, diet has also been described as a modifier of cancer risk through epigenetic mechanisms described above. Several lines of evidence show this interaction between diet, and specifically micronutrients, epigenetics and disease (reviewed in Davis and Ross 2007; Duthie 2007; Ho et al. 2011; Riscuta and Dumitrescu 2012). The levels of circulating folates have been associated with significantly higher levels of LINE-1 ($r=0.21$, $p=0.007$) (Zhang et al. 2012). Interestingly, hypomethylation of LINE-1 has been found associated with increased risk of colorectal cancer and seems to be an early biomarker of disease since histologically normal tissue adjacent to the malignant lesions, also had hypomethylation of this DNA sequence (Suter et al. 2004). It was shown recently that the level of LINE-1 methylation on the right colon was significantly lower than that on the left colon (Figueiredo et al. 2009) and this may help explain variations in risk of site-specific colorectal cancer (Caldarella et al. 2013; Faivre et al. 1989). The latter suggests the potential use of these compounds to help in the treatment of malignancies.

One of the most studied relationships in disease risk and outcome is the connection between heavy metals, air pollutants and cancer. Data from basic and epidemiological research suggest, for example, that long term exposure to cadmium through tobacco smoke and contaminated foods (Waalkes et al. 1992) is associated to malignant transformation and cancer development in animals and humans (Waalkes et al. 1992; Abshire et al. 1996; Joseph et al. 2001; McElroy et al. 2006). These events may be mediated by the capacity of cadmium to interact with sulfhydryl groups and

alter cellular functions, including but not limited to oxidative respiration, and inhibition of DNA mismatch repair (Singh and Dogra 2009; Elez et al. 2001; Banerjee and Flores-Rozas 2005; Rikans and Yamano 2000). Cadmium exposure has different effects on the methylation of the DNA. Interestingly, even in areas with low industrialization or pollution, long term exposure to cadmium, likely through drinking water, has been associated with DNA hypomethylation (Hossain et al. 2012). Prostate epithelial and B lymphoblast cell lines exposed to low dose cadmium show increased expression of DNA methyltransferases (DNMT) 1 and 3b, and a parallel hypermethylation of tumor suppressor genes *p16* and *RASSF1A* (Benbrahim-Tallaa et al. 2007; Yuan et al. 2013). Similarly, bronchial epithelial cells treated with cadmium had a progressive hypermethylation of DNA repair genes over time (Singh et al. 2009; Zhou et al. 2012).

However, cadmium is not the only heavy metal associated with carcinogenesis. Nickel, chromium and arsenic have also been associated with increased incidence of cancer through changes in both global methylation and at specific sets of genes involved in cell cycle regulation, DNA repair, and other crucial cellular functions (Arita and Costa 2009; Martinez-Zamudio and Ha 2011; Oller et al. 1997; Pogribny and Rusyn 2013; Reichard and Puga 2010).

13.11 Technologies Used to Study Epigenetic Changes

In the next section we list some of the many techniques currently used to determine the status of epigenetic changes in biological samples. This small review of technologies does not intend to provide details of each technique but rather show the current possibilities for epigenetic analysis, specifically DNA methylation. Several books have recently been published with detailed protocols in many of these techniques (Tollefsbol 2004, 2011a; Dumitrescu and Verma 2012).

Bisulfite-Based Analysis This analysis is based on the deamination of cytosine to uracil after the treatment of DNA with sodium bisulfite (Frommer et al. 1992). Thus, following PCR amplification, non-methylated cytosines are identified as thymines, and 5-methylcytosines are identified as cytosines. The advent of bisulfite methylation sequencing set the stage for the debut of many additional methylation analysis techniques that expanded upon this method including Methylation Specific PCR, Combined Bisulfite Restriction Analysis.

Methylation Specific PCR (MSP) This technique allows for interrogation of methylation patterns in CpG islands. The advantages this technique offers include requirement of only a small amount of DNA, which can even be obtained from paraffin-embedded samples (Lasabova et al. 2010; Herman et al. 1996), and no need for cloning or methylation-sensitive restriction enzymes. To determine whether a specific sequence is methylated or non-methylated, two pairs of primers are synthesized using bisulfite-treated DNA as template – one pair is specific for methylated DNA and the other for non-methylated DNA. PCR amplification using the primer

specific for methylated DNA indicates methylated DNA, whereas amplification using the non-methylated specific primer indicates non-methylated DNA. The PCR product is then run in an agarose gel and visualized under UV light.

Bisulfite Pyrosequencing Bisulfite pyrosequencing is an extension of the bisulfite methylation sequencing principle that uses PCR amplification of bisulfite treated DNA and quantifies the incorporated nucleotides using light emission detection techniques. Despite this technique's inability to determine methylation patterns at a single allele, it is able to identify heterogeneous methylation patterns. Although this method bears the disadvantage of limited DNA length coverage (approximately a 100 base pairs), serial bisulfite pyrosequencing overcomes this drawback by allowing the assessment of longer PCR products (Tollefsbol 2011b). In addition, when analyzing DNA methylation at specific loci, the need to remove PCR products can be circumvented by performing amplification and analysis of bisulfite-treated DNA in one tube, which is sometimes referred as a closed-tube method. An example of this approach is real-time analysis-methylation specific PCR (SMART-MSP), MethyLight, and methylation-sensitive high resolution melting (MS-HRM) (Tollefsbol 2011b; Wojdacz and Dobrovic 2007; Eads et al. 2000; Kristensen et al. 2008). SMART-MSP implements melting curve analysis and a fluorescent dye that binds to double-stranded DNA, while MethyLight uses a fluorescent hydrolysis probe. MS-HRM can be used when the methylation patterns of the DNA of interest is unknown as a pre-screening technique.

Methylation Specific-Endonuclease Digestion The restriction endonuclease *HpaII* and its isoschizomer *MspI* are methylation-sensitive enzymes commonly used to determine the methylation status of DNA sequences (Brunner et al. 2009; Oda et al. 2009). Interestingly, while activity of the first is inhibited by CpG methylation in its DNA target sequence (C/CGG), the second is not. Then, these two enzymes are very useful to define global methylation status of genomes which is very important when analyzing and comparing normal versus non-normal conditions, including human tumors (Bhattacharyya et al. 2013; Heuck et al. 2013). This technique, as a subsequent step to the bisulfite treatment of the DNA, makes the combined bisulfite restriction analysis (COBRA) (Xiong and Laird 1997), which analyzes methylation patterns within specific regions of the DNA. However, not all CpG sites in the human genome can be interrogated using this technology (Schumacher et al. 2006).

Restriction Landmark Genome Scanning (RLGS) Although determining DNA methylation statuses at specific loci or within particular regions is important, assessing DNA methylation patterns of very large segments or even the entire genome may be preferred in some studies. Several techniques have been developed to allow researchers to perform such assessments, each employing various techniques and analytical methods. One of those methods is the RLGS which couples endonuclease digestion of the DNA with 2-dimensional electrophoresis to determine the genome-wide methylation status (Costello et al. 2002a; Okuizumi et al. 2011). This methodology has been improved and utilized to increase detection of mutations and other

genetic alterations in cancer, integration of methylation and copy number analyses, study of methylation and gene expression and several other types of studies (Costello et al. 2002a, b; Okuizumi et al. 2011; Plass et al. 1999; Takamiya et al. 2006; Allegrucci et al. 2007; Asakawa 2008).

Infinium Methylation This is a high throughput microarray-based methylation covering CpG site throughout the human genome (Bibikova et al. 2009). The technique developed by Illumina Inc. (www.illumina.com) involves treatment of the DNA with sodium bisulfite, DNA amplification and fragmentation and hybridization onto chips that were initially designed to interrogate 27,000 CpG sites in more than 14,000 genes and that was later upgraded to detect the methylation status of more than 450,000 CpG sites covering 99 % of annotated genes.

Bisulfite-Based Genome-Wide Methylation Analysis by High Throughput Sequencing Several companies have developed different methodologies to carry out a whole genome analysis of methylation. Genome-wide methylation analysis involves fragmentation of the DNA either mechanically as in the whole genome bisulfite sequencing (WGBS) or enzymatically as in the reduced representation of bisulfite sequencing (RRBS), initially described by Meissner et al. (2005). In both techniques the DNA is later bound to adapters, size selected by gel electrophoresis, treated with sodium bisulfite, PCR-amplified using primers specific to the adapters, quantitated and finally subjected to deep sequencing following protocols providers by the specific vendor.

Enrichment of Methylation-Rich Regions After DNA fragmentation the methylated DNA imunoprecipitation (MeDIP) technique exploits the use antibodies to pull down methylated cytosines (5-methylctosine, 5-mC), while the enrichment of methyl binding domains (MBD) is achieved by the high affinity interaction of the MeCP2 protein with methylated regions. In both cases the methylated DNA is of purified methyl-binding domains (MBD) which are later precipitated. Li et al. (2010) have estimated that at least 3 GB of 45-basepair paired-end reads would be the minimum for a comprehensive cost-effective methylome analysis.

Inhibition of DNA Methylation DNA methylation is one of the two major areas of study within epigenetics, an integral process in epigenetic control of gene expression, and enzymatically catalyzed by DNA methyltransferases (DNMTs). As such, possessing the abilities to inhibit DNMTs and measure their activities is vital to the study of epigenetics. Inhibiting the activity of DNMTs in somatic cells enables researchers to determine the specific roles that DNMTs and DNA methylation serve in gene expression, cellular differentiation, nuclear reprogramming, and various other cellular processes. Inhibiting the activity of DNMTs is the most commonly used approach to inhibit DNA methylation and is accomplished by pharmacological agents such as 5-azacytidine, 5-aza-2'deoxycytidine, and procainamide. Small interfering RNA (siRNA) molecules have also been employed to reduce DNA methylation and offer the advantage of specifying which of the three DNMTs the researcher wishes to knock down (reviewed in (Giraldo and Bondioli 2011)).

13.12 Concluding Remarks

The last 20 years of research on environmental influences on human health in different populations has led to the conclusion that our lifestyle and industrialization are key modifiable factors that may influence our risk to develop complex diseases like asthma, obesity and cancer. The gestational environment is crucial in shaping how we respond to stress, external insults and infections throughout the lifecourse. These effects are mediated by changes in the expression of genes involved in the control of inflammatory responses not through changes in DNA sequence but by modifications that lead to interference or promotion of gene transcription. Fortunately, environmental effects are largely modifiable. Improving nutrition, increasing exercise, eliminating tobacco consumption, as well as limiting exposure to environmental insults, help to mitigate the deleterious effects on epigenetic pathways. The pharmaceutical industry continues to develop drugs targeting a number of enzymes involved in the generation of these epigenetic changes. With the development of innovative technologies, sequencing of the human genome, understanding the molecular bases of the relationship between environment and disease, and identifying targets to modify that relationship, the scientific and medical communities have made great strides towards reducing the impact of epigenetically-derived sequela. However, environmental concerns, such as the effects of industrialization, may require greater trans-disciplinary collaboration with policy makers to develop comprehensive, macro-level solutions to epigenetic disparities.

Several methodologies have been described to analyze the "epigenome", the fraction of the genome that is modified by epigenetic changes. Here we refer the reader to several techniques used to study DNA methylation. While outside the scope of this chapter, other epigenetic changes include chromatin modifications like histone acetylation and methylation as well as nucleosome positioning, and microRNA signatures.

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