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Epigenetics in Society

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EPIGENETICS IN SOCIETY

Windsor Epigenetics Study Group
Edited by Michael Crawford

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DEDICATION

We dedicate this book to the spirit of congenial and convivial teamwork that made the project such a joy to realize. Our families have a lot to do with this: they provided the time, patience, nurturing, and support. With love from us to you…

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PREFACE

We intend this book to serve several functions. First, we want to make the field of epigenetics accessible to lay readers. Second, and more importantly, we want to excite further interest and concern regarding the social, ethical, legal, health, and policy implications that this field will have for all arenas of our lives. Third, we want to arm our readers with knowledge and wariness so that they can understand and critique the nuanced debates that will inevitably arise when costs and benefits must be weighed: while the effects of epigenetics upon us as individuals may be subtle, the demographic implications and costs are huge.

The book is organized into discrete sections, each of which can be read on its own, although we recommend reading the first chapter as a primer to get the general gist of the picture, implications, our concerns, and agenda. Given the extensive ramifications of epigenetics to most realms of our lives, students of public health, ethics, law, medicine, biology and philosophy will find the contents useful, interesting, and engaging.

We have highlighted some of the technical terms and included a glossary, and the end of each chapter terminates with a non-comprehensive list of questions and solutions intended to stimulate thought and debate.

Each chapter has been reviewed and assessed by experts in the field. We have endeavored to check our facts and references rigorously, and to ensure the originality of our work. We hope you will find the effort of reading stimulating, provocative, and rewarding.

Chapter 1

Not Your Mendelian Genetics…
(Your genome is far from Mendel's pea plants)

Michael Crawford, Melissa Woghiren, Kendall Diemer, and Kaela Scott

Abstract

The DNA sequences that encode our genes interact with and are regulated by associations with many other molecules, and this constellation of players regulates how we respond to our environment. The sequence of DNA itself remains unchanged: the additional factors that package the genome into chromosomes create a dynamic architectural structure that modulates DNA activity and accessibility. More recently, it has become clear that the reverse is also true - our experience of our environment and social relations record and inscribe changes to the way that our genes behave, and this in turn has implications for our future health. In the years since the human genome was sequenced, it has become apparent that this higher order of architectural organization is much more complex and subtle than formerly acknowledged. An ever-expanding list of influences impinge upon it, and the changes that are installed are referred to as the: epigenome, epigenomic/epigenetic imprint, or sometimes simply as imprinting. Some of the packaging and behavioral changes are transient, and others may persist to affect our health decades later. Some might even transmit to our offspring. The diverse influences that modify

our epigenome include: eating habits, socio-economic status, education level, drugs, and toxin exposure to name but a few. Since an individual's epigenetic status can affect health decades - even generations later - the social, medical, ethical, and demographic impact of epigenetics are likely to be huge. We introduce the breadth of influences that need to be considered, and outline our agenda for discussion.

1.1 Introduction: What is Epigenetics?

Have you ever stopped to wonder why identical twins are never completely identical? When watched closely, unique characters become apparent. As individuals, they may differ in preferences of dress, foods, and what interests them most. They grow and develop their own quirks and differences that amplify with age. This is often written off as the result of different experiences, molding them through development… But what about variances in health? What can be said of the identical twin who develops bipolar disorder or is highly susceptible to depression while their counterpart is not? If they share the same genomes, then why are they not identical in *every* respect? For that matter, why does a 1700s painting of my father-in-law's ancestor look like the descendent managed to transport back through time to pose in dress-up clothes for the portrait? The genes for facial characteristics should have been diluted 1 in 32. Something has always been missing in our understanding of how genomes and inheritance function. These discrepancies present us with questions that may now be answered through the involvement of an additional layer of information encoding and inheritance: the epigenome.

For biologists, a surprising feature of the Human Genome project was the degree to which it required constant revision of our definition and understanding of what constitutes a gene. Nature is subtle, and the genome turned out to be much more richly

elaborate than originally understood. We discovered that genes were more varied, complicated, and surprising in their structure and function that anybody could have foreseen. Despite our fascination with these units of heredity, it is clear that another adjustment, this time of seismic proportions, is presently underway: biologists and physicians are coming to grips with an additional level and order of information encoding - the epigenome. The epigenome may explain familial tendencies to diabetes, obesity, addictions, or even certain social behaviors. The implications of this new science are vast. For example, *your* health might be affected by what your *grandparents* ate, or when they started smoking. Your future health disposition might be affected by the season of your birth, or the socioeconomic status of your parents. Even schooling matters…not only for your own sake, but perhaps also for your descendants.

The epigenome affects how DNA is architecturally bound into chromosomal form and how genes are regulated. This architectural modification of packaging turns out to have dynamic and interesting implications not merely for how our genes behave, but consequently for how we are primed to respond to stressors. Moreover, the complex code of modifying factors involved appear, in some instances, to be heritable, and this is forcing a reassessment of our appreciation of the intricacies of heredity.

The epigenome can be thought of as a malleable stamp that massages gene behaviour in pervasive but subtle ways. It is an integral part of the routine regulation of gene activity. The ink of this stamp comprises a complex mix of accessory protein and RNA molecules that decorate and reconfigure chromosomes in response to changing environmental demands. They alter chromosomal architecture so that specific combinations of genes are predisposed to be active or silent. Our genome responds to, and in some cases records its interaction with the environment through this dynamic epigenetic re-packaging. DNA sequence fidelity remains untouched, but the genes are induced to behave differently, turning

on or off in different patterns. When it is heritable, this change in DNA packaging is referred to as the epigenetic or epigenomic imprint. The bands that you see in images of chromosomes reflect attributes of this packaging – some large areas are densely packed, others less so. Importantly, epigenetic marks alter the way that your genome behaves without altering the sequence of DNA itself. For example, with the exception of the unmatched X and Y sex chromosomes in males, we all normally inherit paired chromosomes - one from each parent. By virtue of their location on paired chromosomes, our genes are similarly present as two copies. Even if you were to sequence the paired copies of a particular gene and found their DNA to be identical, they might behave differently depending upon which parent they were inherited from. The gene copy inherited via a maternal chromosome might be active, and the copy inherited via the paternal chromosome might be silent.

Many factors impinge upon the epigenome to turn genes on, off, or somewhere in between. We now know that the changes they install can have consequences for patterns of long term and even trans-generational health. Imagine the benefit for future generations if nutritional scarcity could prime human eggs and the consequent embryos (ie; subsequent generations) to grow larger and more efficient placentas without requiring genetic changes? Imagine if these changes could be installed within a generation? This, in fact, happens… While changes to our own long-term health might be subtle, when measured in aggregate, the thousands or millions of individuals that constitute a community potentially translates to huge demographic and societal costs. Consequently, our understanding of epigenomic health will have important implications for medicine, social justice, ethics, law, social policy, privacy, and economics.

1.2 Why Do We Need to be Concerned About Epigenetics?

When the impetus for epigenetic changes is caused by stressors, a period of starvation for example, epigenetics provides a powerful evolutionary advantage: it deploys a tool that records the challenge and can prime genes to efficiently respond to similar threats in the future. The issue for public health is that the challenges met at one point of life, or in one generation, might not be appropriately answered when epigenetic programming meets different circumstances in the future. In other words, a metabolic program installed to provide support during times of scarcity might instead, during times of plenty, predispose to obesity, diabetes, and heart disease. The news is not all bad. We are not necessarily slated to be the victims of our ancestral metabolic and environmental histories: many of the factors that influence your epigenetic status are controllable, and some are even reversible. A non-comprehensive list includes: quantity and quality of diet; socioeconomic status; social environment; quality of parental care; educational experience; prescribed and illegal drug use; geographical/environmental exposure to contaminants; and stress.

Epigenetics presents a challenge when the stimulus for change is the product of inadvertent manipulation by medical procedures and prescriptions, contamination by pollutants, or adverse and transient social or geographical conditions. Since individuals control neither the origin and behaviour of their parents, the care that they receive as children, nor the quality of the environment into which they are born, epigenetic health will inevitably become entangled with issues of social and environmental justice - these will require intervention or regulation at the political level.

Clearly most of the items on our list of epigenetic influences carry a social, ethical, legal, economic and political dimension. The costs of ignoring epigenetic threats are likely to be long-lived and expensive: who should bear them? Individuals, society, or future generations? Who should be responsible for a person's epigenetic state? Solely the individual? If we each inherit

the baggage of our parents' metabolic and social crimes and misdemeanors, is it fair that we be responsible for the attendant costs? How should we be empowered to rectify deficits - should we be granted privileged access to the medical histories of our progenitors? Should our children have access to our own? Should we impose sanctions for overfeeding children if the consequences of their weight and inactivity might last generations? And what about the environmental, social, and economic actors over which we hold no sway? Where do the boundaries for freedoms and obligations lie? If an industry inadvertently creates a product or waste that affects our epigenetic state, but this is undiscovered for decades, who is liable, and who should make redress? Can a balance be found that will protect exposed populations without drastically stifling innovation? Establishing precisely where to place the onus of responsibility and costs among individuals, businesses, governments, and society will be thorny issues.

An intelligent response to both preventive and remedial epigenetic health is obtainable, but requires a sophisticated understanding of the influences and outcomes, as well as a comprehensively mounted and politically inclusive response. Key to this will be education. Epigenetics provides additional and firm data to support social investments that will address long term economic and productivity imperatives while simultaneously addressing issues of social and environmental justice.

1.3 The Challenges of Understanding and Studying Epigenetics

Unlike genes, an epigenomic mark can change from cell to cell. This makes it difficult to assess an individual's overall epigenetic status. For example, some epigenetic marks might be accessible via a blood sample, others only through a biopsy of specific tissues such as brain, or liver, or muscle. Moreover, the complexity and combinatorial nature of epigenetic modifications means that there are different patterns of packaging changes that

can produce the same end result, and there are also many shades of grey in between “on” and “off” insofar as gene activity is concerned. Epigenetic marks act a little like a complicated audio volume knob that can muffle or blast a signal from a gene, with every increment in between. Being dynamic, epigenetics also change with age, and can sometimes be enhanced or even reversed by multiple influences. Finally, in some cases, an imprint might be transmissible to offspring: this is not always the case – some epigenetic marks are pervasive, others are highly tissue-specific and exclude eggs, sperm, and future generations. The basic mechanics of epigenetics, the complexity of the combinatorial code that it embodies, and how it can be experimentally or diagnostically assessed are covered in Chapter 2. This chapter will be helpful to understand the diversity and nuances of the mechanics, but it will not be necessary for understanding the gist and implications of ensuing chapters. We also include some further information on specific diagnostic strategies for the adventuresome reader with a recent biology background – these latter technical sections of Chapter 2 are not necessary for understanding subsequent chapters.

There is a wealth of data that links specific epigenetic mechanisms revealed in animal models (mice and rats in particular) to environmental cues. The same trends are seen in human epidemiological studies, however many of the larger social conclusions have relied upon inferences drawn by comparing animal and human studies. Not all organisms necessarily respond to environmental and epigenetic challenges in the same way, and there is an inherent risk in applying the mechanisms discerned in rodents to trends revealed by retrospective epidemiological studies in humans. That said, persuasive data is emerging to link specific epigenetic changes in humans to particular dietary or other challenges. In short, the data is looking good and delivers the message that we need to address epigenetic effects and health in a serious, comprehensive, and sustained manner.

1.4 The Astonishing Range of Effects

Examples of how epigenetic architectural modifications can affect cellular activity, or even how they can offer avenues for treatment, are especially evident in emerging studies on cancer (outlined as a concrete example of how the mechanisms of epigenetics can operate in Chapter 3). Since cancer is a genetic disease caused by mutation, any mechanism that alters gene behaviour has the potential to exacerbate or to mitigate the damaging effects of DNA mutations. The profound influence of the epigenome in cancer biology is an area of intense research, and already diagnostic, prognostic, and treatment opportunities are being developed. For example, epigenetic changes can signal specific changes in cell behaviour that have diagnostic and prognostic value. Indeed some of these epigenetic changes are deleterious and are coming to be known as "epimutations." But wouldn't it be lovely to circumvent a genetic mutation by encouraging epigenetic behavior modifications that can rectify or compensate for a problem? As promising as these avenues might be, the question of just who will have access to the resources to diagnose and mitigate health via epigenetic tools will prove to be a socially, ethically, and economically challenging question.

We should not become obsessively nervous about this attribute of our biology: in all seriousness, stress itself can modify the epigenome. Rather, we will argue that a little foreknowledge and preventative maintenance can go a long way to improving quality of life through to our declining years and on through to our children. Demographically, however, there is an urgent need to make immediate, comprehensive, though relatively modest investments in nutrition, health, as well as legal, social, and educational structures: these investments could mitigate much larger long-term costs. Case in point, as we will see in Chapter 4, periods of starvation or over feeding can alter the epigenome to influence a person's health status decades later, and can even

transmit across generations: apparently *you* are what your *grandparents* ate! By the same token, contemporary parents need to exercise care regarding what and how much they feed their children - it could have an effect upon prospective grandchildren. Should parents be regulated or taxed regarding the burdens they might be placing upon the future? What defines child- and generational-abuse? Should individuals enjoy the freedom to indulge in risky behaviour even before they become parents when there may be long term costs? Alleviating nutritional challenges, and educating stakeholders becomes a matter not merely of social, but also of generational justice.

Factors that can influence our epigenome surround us – they are ubiquitous. This means that the compounded effects of these influences can be hard to separate and to distinguish from one another. We are coming to realize that xenobiotics, such as drugs and environmental toxins, affect how our DNA is packaged and how it behaves. The number of studies that have examined long-familiar drugs and toxins and found profound epigenetic effects is expanding in a startling way. Drugs and their excreted metabolites, pesticides transmitted via our food and water supply, manufacturing and production contaminants - all of these categories of chemical are known to elicit deleterious and long-term epigenetic damage. Providing environmental justice will demand that we pay attention to geographical, political, legal, regulatory, economic and even to cultural frameworks in a manner that is much more long-viewed and cautious. The trick will be to achieve these objectives without stifling creativity and innovation (Chapters 5, 8). For example, like nutritional challenges, manufactured products might elicit an effect that remains hidden for decades or even generations. The legal implications of this lag time in terms of product and manufacturing liability are profound. If epigenetic effects are not seen for decades, how can litigation successfully compensate affected individuals: statutory limits might have expired; businesses might have closed or sub-licensed

production and responsibility; the state of knowledge at the time of manufacture might not have been sufficient that a business could reasonably have been expected to appreciate the risk; or the epigenetic modifications that are produced could be hard to link causally due to the complex and combinatorial susceptibility of the epigenome to multiple influences (Chapter 8).

For example, as we are coming to realize, the burden of toxicants leaking into the environment can exert unforeseen consequences upon our reproductive health, and most predominantly through endocrine disruption. Fungicides, the excreted metabolites of prescribed hormones and drugs, and bisphenol A, are just a few of the compounds documented to affect us, but the recent dimension of epigenetic harms they cause should give us reason to pause (Chapters 5, 6).

The endocrinology of birth is complex, so in retrospect, it should have come as no surprise that factors affecting conception, and the manipulations attendant with fertility and birth, such as in vitro fertilization, hormonal priming, Caesarian section, induced labour, breast feeding decisions, also have discernable epigenetic effects upon human infants (Chapter 6). The relative novelty of technologies associated with assisted reproduction means that potential long-term effects upon the human epigenome are still unknown – the first generation of "test-tube" babies is only just now beginning to produce its own children. Will late life health changes and cross-generational effects be seen? It is too early to say. Preliminary manipulations of rodents suggests that care and caution need to be exercised, and close monitoring of procedures should be required of physicians in a field that, in many countries, deploy proprietary practices. At the very least, long term monitoring of the health of progeny derived by artificial means would seem to be prudent.

Are there some individuals who should be dissuaded from propagating? Should people be taxed according to the likely burden to society that they might transmit, and how would their

past behaviour be assessed and quantified in terms of risk? As pre-conception and prospective parents, what do we owe to our hypothetical offspring? What obligations does society owe to us and to them? How should multigenerational interests be balanced?

Not all influences are chemically direct: how we are socialized plays a role in epigenetic health. As will see in Chapter 7, the addictions, stress, and violence that often attend poverty can have devastating long-term consequences that ensure more of the same in subsequent generations. One mitigating influence – the number of years that you attended school! A reassuring note: if you are reading this, you might already be a member of an epigenetically privileged cohort – school really is good for your epigenetic health!

If we are willing to concede that epigenetics plays an important role in our lives, then we must also admit that information regarding the behaviour and health of our ancestors as well as ourselves is important both to our future health as well as to our children. Who should have access to that information? And even if medical, genetic, and epigenetic records are kept confidential, how much can be inferred about our epigenetic status from: surveys of our browser history; educational affiliations; parental data footprint; geographical location and history; marital status; dietary preferences; religious affiliation and practices (diet as well as communal comfort or stress); and hobby or sports activities? So your internet usage indicates that you play electronic games or stream videos for hours on end… should you be as insurable as your neighbor the yoga fitness instructor? Your telephone record suggests that you lived near an industrial park when you were a child – does your father's socioeconomic status and your exposure to heavy metals mean that you are more likely to suffer cancer, depression, or cardiovascular disease as an adult? Should prospective employers have the right to make use of this commonly available information to make inferences regarding the cost of your health benefits and the potential number of sick days

that you might be disposed to take? Privacy will be a much more complicated right to protect regarding epigenetic health and social data than perhaps any other feature of your medical history (Chapter 9).

1.5 An Agenda for Change

Each chapter concludes with a short list of provocative questions, as well as some solutions that should give you pause to either consider or worry. Neither list is intended to be comprehensive, but both should help to expand the horizons under consideration. In Chapter 10, we sum up by discussing some practical steps forward that will encourage stakeholders to become cognizant and engaged with the implications of their life styles, histories, and choices. While many of the issues that we will address (social, health, legal, and geographical justice) already have common currency in debate, nevertheless epigenetics will provide impetus to consider these matters more urgently – their ramifications do not reside merely in the present or near term futures – they are of generational consequence. Moreover, they are all linked by a biological mechanism, and this suggests a need for careful and comprehensive coordination to maximize benefit. Since epigenetics is evolving rapidly as a science, and the procedures for studying it are still somewhat young, our toolkit for understanding, tracking, and evaluating will no doubt continue to evolve.

Chapter 2

A Beginner's Guide to the Mechanisms and Assessment of Epigenetics

Natalie Gosselin and Curtis Foreman

Abstract

Epigenetics refers to the decoration and remodeling of chromosomal material to both package and regulate the behaviour of enfolded genes. This repackaging is dynamic, complex, and involves many different proteins and RNA molecules. Together these players interact with DNA to make architectural changes, but the changes alter gene behaviour without altering DNA sequences. The combinatorial possibilities of factors involved in this process exceed the informational complexity of the DNA that comprises our genomes. This chapter outlines the different interacting structural proteins and enzymes, as well as the diverse set of RNA molecules that help to modulate chromosomal form and function. It is helpful to get feeling for the diversity and complexity of epigenetic packaging options in order to understand some of the challenges that researchers and physicians face when trying either to define an individual's epigenetic status, or to clarify how their epigenetic status is affected by environmental stimuli. The technical challenges are several fold. First, different cells can carry different epigenetic imprints, and even in a single organ, similar cells can reach the same epigenetic effect by different paths. Second, epigenetic imprints are susceptible to many different

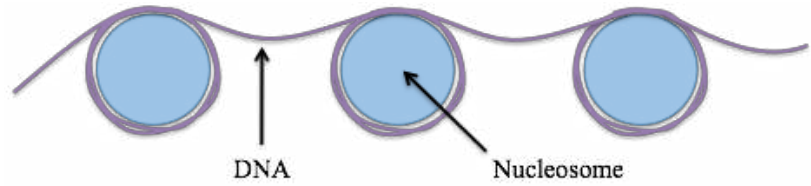
influences – it is hard to define specific causes and effects. Third, some epigenetic states are transient, others are less so. Fourth, depending upon which tissue experiences epigenetic re-programming, the effect may be heritable or not. Fifth, some epigenetic states might be reversible. Lastly, epigenetic states change with age. We conclude in section 2.6 with a list of assessment tools used in epigenetics. This last section provides technical details for the interested. If the technology does not interest you, relax… it is not necessary to understand how these tools function to understand the rest of the book.

2.1 Introduction to Epigenetic Mechanisms.

Deoxyribonucleic acid (DNA) is the hereditary material of all non-viral organisms on earth. It is found in every living cell, and each living thing has a unique DNA sequence that distinguishes it from every other. DNA sequences are the major focus of studies ranging from biology, to medicine, pharmacology, and heredity. Recently, this perspective has been challenged by the discovery of epigenetics. Epigenetics is the study of stable changes in the packaging of the genome that are independent of any changes to the genome sequence itself (Berger et al. 2009). A huge variety of epigenetic modifications can occur, and these changes engender a new informational "code" that augments and modulates the activity and information carried by DNA **nucleotide** sequences. Epigenetic changes, like DNA itself, can also convey information in a heritable manner. By regulating the packaging of DNA, epigenetic mechanisms regulate gene activity, and in a real sense, mediate and inscribe a record of the genome's interaction with its experience and environment.

The structure of DNA is one that is familiar to many, even those uninvolved in the study of biology: two strands of nucleotides are coiled into a double-helix. While this is the basic structure of DNA at the simplest level, it does not fully reflect the

way that DNA is packaged in a cell. The DNA double-helix is itself coiled around complexes of cellular proteins known as



nucleosomes (Figure 2.1). Each nucleosome is composed of eight **histones** comprised of four pairs of: H2A, H2B, H3 and H4 (Figure 2.2).

Figure 2.1: *The structure of DNA wrapped around histones. DNA (shown in purple) wraps two times around each nucleosomal protein complex in a reiterative pattern reminiscent of pearls on a string.*

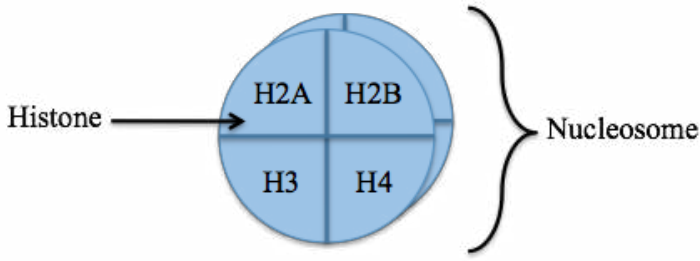


Figure 2.2: *Histone proteins compose the nucleosome. Each nucleosome contains two each of histones H2A, H2B, H3, and H4.*

Afterwards, an even higher order of architectural complexity is established by interactions with multiple accessory proteins and RNA molecules that serve as a scaffold to further coil and organize DNA/nucleosomes.

The genes encoded by DNA are functional in the sense that

they code for either protein or RNA molecules that will themselves carry out a function in the cell. The ordered complex of DNA, RNA, and protein that resides in the nucleus of the cell and that constitutes a chromosome is called chromatin. Loosely packed chromatin is known as euchromatin, whereas tightly packed chromatin is called heterochromatin. The degree to which DNA is wrapped around nucleosomes and other proteins/RNA largely determines the extent to which the embedded genes can be exposed, active, and expressed. When DNA is wrapped around nucleosomes loosely, it is easier to activate the genes that are located in that region. Conversely, DNA that is tightly wound around nucleosomes can form a more densely packed architecture that inhibits the activation of encompassed and even neighboring genes. This packaging can prevent gene activation by excluding factors, such as activating proteins called transcription factors, that are necessary for genes to turn on and to serve as the template to transcribe RNA. These alternatively wound conformations of chromatin constitute opposite ends of the spectrum of activity. Chromatin can, however, exist on a spectrum of varying levels of compaction, not only the aforementioned polar-opposite states (on versus off). In other words, chromatin packaging can vary gene activity like a volume knob: on, off, and all levels in between.

We will briefly outline some of the players in this differential packaging of genes. If you have the interest and fortitude to get more technical, the attributes of each are expanded in Sections 2.2, 2.3 and 2.4. The nucleotide bases that comprise DNA can be the site of a modification known as **methylation**. In particular, one of the four nucleotide bases, known as cytosine, is a base that may be decorated by addition of a small methyl group in vetebrates such as ourselves. Methylation of DNA is typically associated with a repressed chromatin state, however the effect depends on the location of the methylation (Bird and Wolffe 1999). This will be discussed further in section 2.2.

DNA decoration by methyl groups can alter the suite of

proteins that are recruited to chromatin, and moreover, some of these proteins can themselves also be modified. For example, each histone in the nucleosome has sites on it that can also be modified: histones can be methylated, acetylated, phosphorylated, ubiquitinated, glycosylated, and/or SUMOylated. It is not necessary to know what all these different modifications are to understand that there is quite a repertoire of possibilities given than several of these features can be altered at multiple sites, in combination, over the span of a single histone molecule. Methylation of histones, like methylation of DNA, is associated with tight packaging and repressed gene activity, whereas **acetylation** is associated with activation of the region of associated DNA. However, the large variety of histone modifications that occur can induce different effects when combined in various ways. Section 2.3 will expand further upon histone modifications and their epigenetic effects on gene expression, however it is safe to say that the complexity of histone modifications in combination may comprise a code more complex and subtle than that of DNA itself.

Other actors can play a role in modifying gene packaging – we will briefly list some of them here, and then expand upon them later in Section 2.4 for the truly curious. Long noncoding RNA (**lncRNA**) molecules are those RNA molecules that can have either a repressive or expressive effect on regions of the genome. A famous example of a lncRNA is involved in X-chromosome inactivation in the cells of females. The human genome is optimized to work best with a certain number of active genes: males have only one X chromosome and a very much smaller Y. The X carries many more genes than the Y chromosome, so a type of gene dosage must be achieved to compensate for the gender difference. Since human females are born with two X-chromosomes, one must be inactivated by compaction (into what is called a Barr body). The lncRNA involved in this case is called XIST. Where this RNA molecule attaches and decorates a

chromosome, that chromosome is packaged to within an inch of its life and it remains, to all intents and purposes, quiescent. lncRNA molecules provide gene regulation in ways that proteins cannot (Lee 2012).

MicroRNA (**miRNA**) molecules are smaller RNA molecules that associate with a protein and RNA complex to either cleave an RNA transcript or halt protein synthesis (Schwarz et al. 2003). They tend to bind to targets with a degree of promiscuity, so one individual miRNA can regulate the products of multiple genes (Lim et al. 2005).

miRNAs are themselves regulated by circular RNA (**circRNA**) molecules. These RNAs are resistant to miRNA-mediated degradation due to their circular shapes. They have the ability to bind miRNA molecules and to sequester them, thereby preventing them from performing their full function (Franco-Zorrilla et al. 2007). RNA species and their effects on epigenetics will be discussed further in section 2.4, but as you might perceive, the combinatorial complexity of these gene packaging components (proteins, lncRNA, miRNA, and circRNA) is vast.

Since the epigenetic changes discussed in this chapter alter the packaging, behavior, and responsiveness of genes to challenges and environmental cues, they have many implications for human health. In order to use epigenetic information to diagnose and characterize diseases, there must be a way of assessing the state of these modifications. Assessment of epigenetic states is described in section 2.5.

2.2 DNA Methylation.

2.2.1 Methylation and CpG dinucleotides.

The four nucleotides that make up DNA are: cytosine, guanine, adenine, and thymine (C, G, A, and T). In a DNA strand, nucleotides can be joined in any linear sequence, but when DNA assembles into a double stranded duplex, each nucleotide in one strand binds a complementary nucleotide on the other (A to T and

C to G; Figure 2.3).

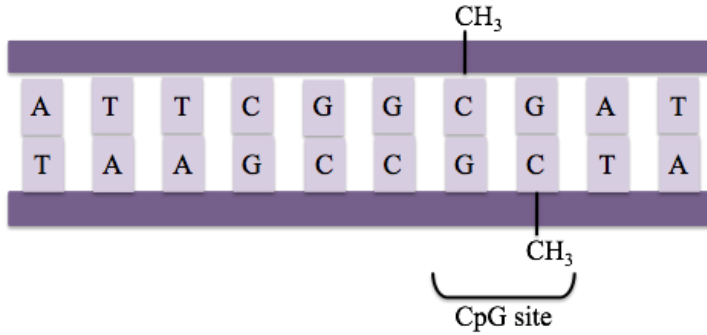


Figure 2.3: *Cytosine and guanine form bonds across double helices. Cytosine followed by guanine along the length of a DNA strand constitutes a CpG site.*

DNA methylation involves the addition of a methyl group. A methyl group is simply a carbon atom bound to three hydrogen atoms denoted CH_3. DNA methylation occurs only on the cytosines (C's; Figure 2.4), and specifically when the cytosines reside beside a guanine (G). The methylation sites are known as CpG dinucleotides or islands. In double stranded DNA, the complementarity of base pairing (C-G, and T-A) means that on the opposite strand, a CpG sequence will sit across from a CpG sequence oriented in the opposite direction. CpG sites occur throughout the genome, and the majority of them are methylated. The methylation of these CpG dinucleotides is important because they provide stability to DNA and silence regions of the DNA that must remain stable and inactive. An example of silenced DNA are retrotransposons: these are remnants of viral DNA that inserted into our ancestors' genomes. These CG-rich regions have been kept from harming us by methylation-mediated gene silencing.

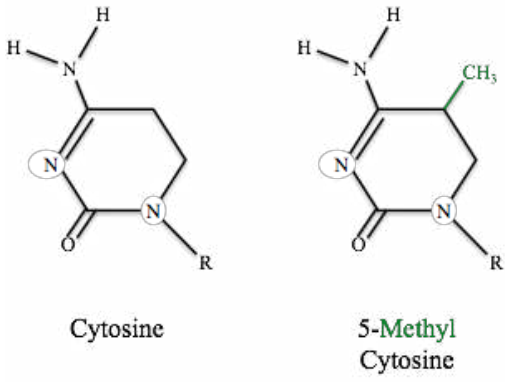


Figure 2.4: *The structure of cytosine and 5-methylcytosine. The methyl group is shown in green as an addition attached to carbon five of cytosine. CpG dinucleotides are typically not methylated when they occur in regions of the genome that contain active genes or gene regulatory regions called enhancers and promoters. Repression of genes by DNA methylation is a common cause of diseases such as cancer, but also occurs as a natural part of the mechanism to turn genes off when they are not needed.*

2.2.2 DNA Methyltransferases (DNMTs).

DNA methylation is carried out by proteins known as **DNA methyltransferases (DNMTs)**. These proteins are enzymes that catalyze the addition of methyl groups to cytosines. There are two main varieties of DNMTs: *de novo* methylases and maintenance methylases. *De novo* methylases are those that add methyl groups onto previously unmethylated DNA. Maintenance methylases are DNMTs that maintain the methyl pattern of DNA as it is replicating. DNA is replicated in a semi-conservative manner: each of the two strands of DNA provides a template for the synthesis of a new, complimentary strand of DNA during replication. While the template strands will continue to carry the original methylation pattern, the newly-synthesized strands will be free of methylation until acted on by maintenance methylases

(Klose and Bird 2006).

As mentioned previously, DNA methylation typically has a repressive effect (Bird and Wolffe 1999). If methylation occurs on a gene, it usually acts to recruit the suites of protein necessary to compact that region, and this in turn represses expression of the gene. This effect can vary. Not all DNA regulatory regions activate genes – some repress. If methylation occurs in a repressive regulatory region of DNA, it would inhibit the repression and thus the gene would be activated. Moreover, it is often the case that genes are regulated by the interactions of several different proteins with different DNA regulatory sites. Methylation could preferentially inhibit one interaction while facilitating another. For these reasons, it cannot be assumed that methylated DNA is necessarily repressed.

2.2.3 Methyl Binding Proteins (MBPs).

Methyl groups are relatively small when considering the size, scope, and complexity of a DNA molecule, or indeed a DNA sequence that might encompass tens of thousands or even a million nucleotides. While methyl groups have the ability to directly block some proteins (such as transcription factors) from binding to DNA, their main action occurs through the recruitment of accessory and metabolic proteins to form a complex. Proteins that recognize methylation marks specifically are methyl-binding proteins (MBPs). MBPs recognize methylated DNA, and recruit other protein and RNA factors to this region to assist in remodeling and repression. Some proteins that may be recruited by MBPs are histone-modifying proteins, which will modify neighboring histones, and thereby further propagate and amplify the change caused by DNA methylation. MBPs provide a link between DNA methylation and histone modifications, as well as overall chromatin state changes (Jones et al. 1998).

2.2.4 Demethylation.

DNA demethylation is a controversial topic. Some investigators believe that it plays a critical role in balancing DNA methylation states, whereas others deny it. One mechanism that is proposed for demethylation is the conversion of 5-methylcytosine to 5-hydroxymethylcytosine (Figure 2.5). This is carried out through the action of a group of enzymes known as Ten-Eleven Translocation proteins (TETs) (Tahiliani et al. 2009). Other mechanisms of active demethylation have been proposed but most are not widely accepted. A mechanism of passive demethylation has received more approval. Passive demethylation means that demethylation is occurring simply due to lack of maintenance of the methylation status. This means that DNA is replicated without the methyl marks being replaced as the new strands of DNA are synthesized during cellular proliferation. This task is normally carried out by maintenance methylases. Without maintenance methylases, demethylation occurs (Klose and Bird 2006).

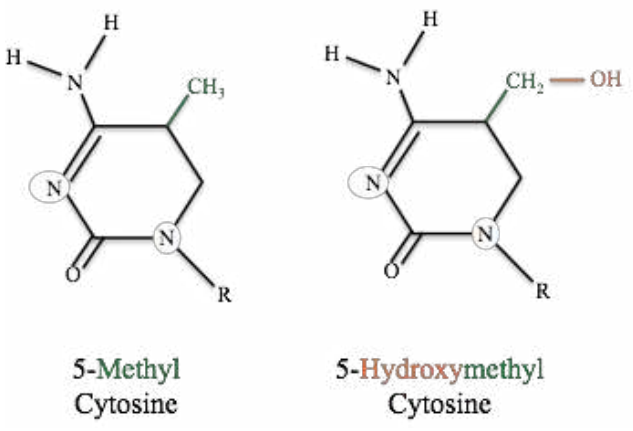


Figure 2.5: *Demethylation can occur through a 5-hydroxy-methylcytosine intermediate.*

2.3 Histone Modifications.

2.3.1 Nucleosome and histone structure.

As was previously discussed, DNA wraps around assemblies of histone proteins to form nucleosomes (Figure 2.1). Approximately 147 base pairs of DNA wrap around each nucleosome, with up to 80 base pairs separating one nucleosome from the next. Histones each have an amino terminal tail that is pointed outwards away from the nucleosome and is available to be modified.

2.3.2 Histone Acetylation.

Acetylation involves the addition of an acetyl group. The addition of an acetyl group to histones neutralizes the positive charge on the lysine amino acid residues of the histone. This positive charge is usually a driving force for interaction of histones with negatively-charged DNA. Without the positive charge, the attraction between the histones and DNA relaxes, altering the intimacy of their interaction, and thereby also increasing the availability of DNA for interaction with other factors – in essence, genes are now available to be activated.

Addition of acetyl groups is mediated by **histone acetyltransferases (HAT)** (Roth et al. 2001). Removal of acetyl groups is typically performed by **histone deacetylases (HDACs)** (Grozinger and Schreiber 2002). Both HATs and HDACs are key enzymes in regulating chromatin structure through modification of histones. HDACs have been shown to combine with MBPs to form a larger complex in response to DNA methylation. Association between HDACs and methyl binding proteins would stimulate histone deacetylation in regions of DNA that have been methylated. This is consistent with the idea that acetylation causes a more relaxed, activated chromatin state, whereas deacetylation is associated with a more repressed and inactivated state.

2.3.3 Histone Methylation.

Histone methylation is similar to DNA methylation in that

methyl groups are added. They are different in terms of what proteins mediate these processes. Recall that DNA methylation occurs through the action of DNMTs (DNA methyl transferases). Histone methylation is carried out by two different types of enzymes depending upon the site of the methylation. Histone methylation occurs on either arginine or lysine amino acid residues. The addition of methyl groups onto arginines is done by protein arginine methyl transferases (PRMTs) (Lee et al. 2005). Lysines can be mono-, di-, or tri- methylated (Figure 2.6, 2.7) (Lachner et al. 2004). This process is mediated by histone lysine methyltransferases (HKMTs) (Lachner et al. 2003)). Conversely, demethylation can also occur from both arginines and lysines, through multiple different mechanisms. Arginine demethylation occurs through deiminases which convert methylated arginine to another amino acid called citruline (Bannister, 2005). Lysine demethylation occurs through the action of an enzyme called lysine-specific demethylase 1 (LSD1) (Shi et al. 2004). The methylation state of histones can have varying effects on the resultant gene expression depending upon the site of methylation and other modifications that may be present.

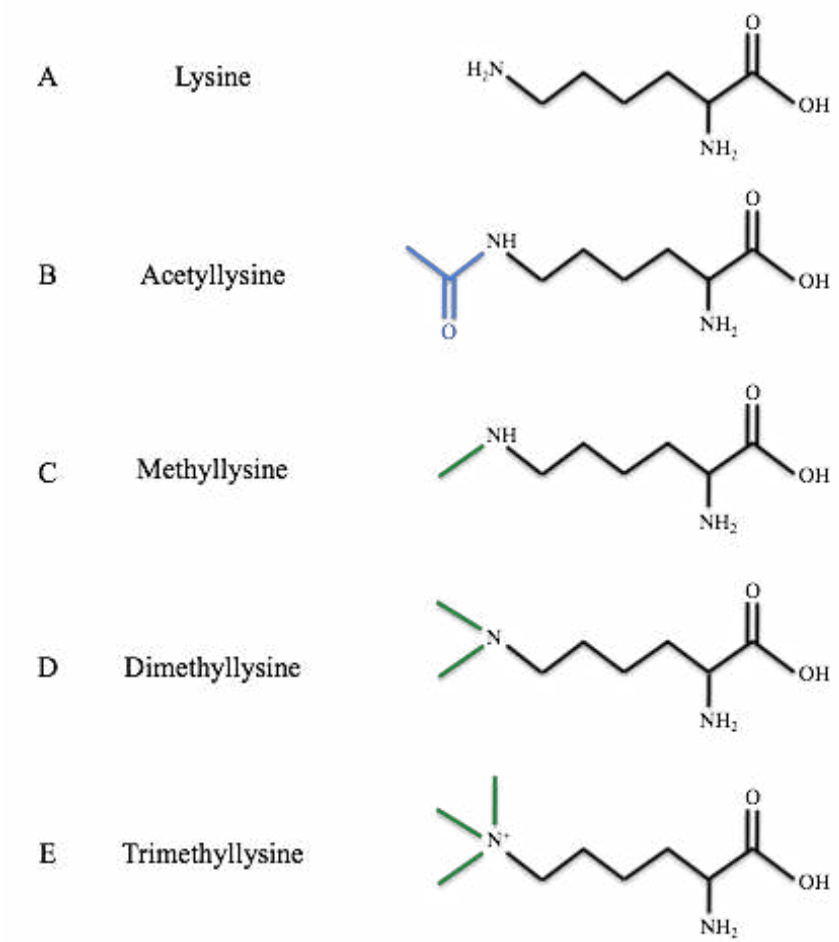


Figure 2.6: *Modifications that occur on lysine amino acids.* ***A*** *Lysine in its native state.* ***B*** *Acetylated lysine. The acetyl group is shown in blue.* ***C-E*** *Mono-, di-, and tri-methylated lysines. The methyl groups are shown in green.*

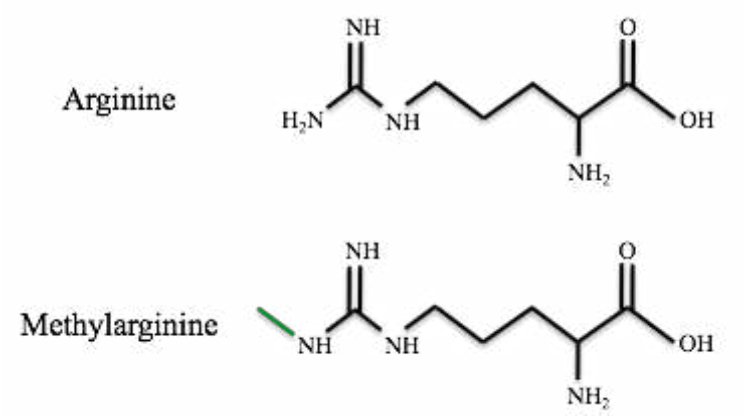


Figure 2.7: *Methylation can occur on arginine amino acids. Methyl group is shown in green.*

2.3.4 Phosphorylation.

Phosphorylation is probably the most commonly discussed protein modification. It is mediated by histone kinases which add a phosphate group onto proteins. Phosphorylation occurs on serine (Figure 2.8), threonine, and tyrosine amino acids. Dephosphorylation is carried out by protein phosphatases. Histone phosphorylation typically causes an increase in gene expression or a general loosening of chromatin in the region.

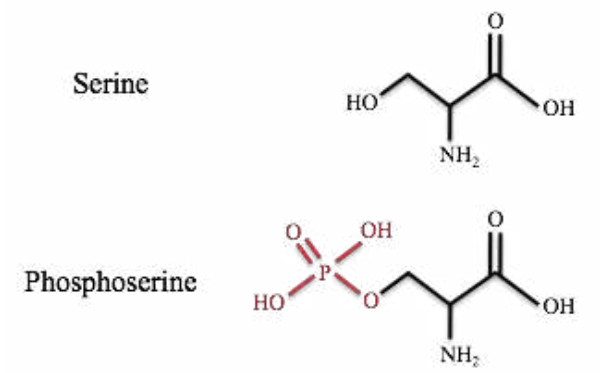


Figure 2.8: *The structure of serine before and after phosphorylation. Phosphate group is shown in red.*

2.3.5 Ubiquitination.

Ubiquitination is the addition of ubiquitin groups onto a protein. Ubiquitin is unlike any of the previously mentioned histone modifications in that it is not a small molecule. Rather, it is a large peptide (protein sub-fragment) that represents a significant addition to a histone protein if added. Ubiquitin groups highlight or mark a protein for degradation and removal. The outcome of ubiquitination can be either positive or negative in terms of overall gene expression in that region (Muratani and Tansey 2003).

2.3.6 SUMOylation.

SUMOylation is the addition of SUMO groups to the histones. SUMO groups are, like ubiquitin, peptides. These have an exclusively repressive outcome. They achieve this by either blocking acetylation from occurring at the site of SUMOylation or by recruiting HDACs to remove acetyl groups (Yang et al. 2004). In some contexts, SUMO groups compete with ubiquitin.

2.3.7 Combinatorial aspect.

The above-described modifications can occur in combinations. The outcome with respect to DNA packaging, conformation, and activity depends upon the various modifications that are present. Detection of an acetyl group on a histone does not necessarily mean that gene expression is occurring in that region. All other modifications that are also present in that same region must be taken into consideration. Histone modifications are referred to as combinatorial for that reason. Indeed, the “histone code” is liable to be more varied and complex than the sequence of DNA itself..

2.4 RNA Species.

2.4.1 Overview of ncRNA.

There are many species of RNA that can exert functions themselves without being translated into protein. RNA molecules known as messenger RNA (**mRNA**) are those that are translated into protein (Figure 2.9). However, there are other types of RNAs that do not get translated into protein such as transfer, ribosomal, and telomeric RNA (**tRNA, rRNA**, and **telRNA**), to name a few. These latter are involved in the machinery of protein synthesis and consolidation of the structural ends of chromosomes without themselves encoding a protein. These functional RNA molecules are broadly classified as noncoding RNA (**ncRNA**). This is because they are RNA molecules that do not encode a protein, but perform a different function in cellular life. Finally, there is a constellation of new RNA species that are now being discovered and that play an important role in epigenetics.

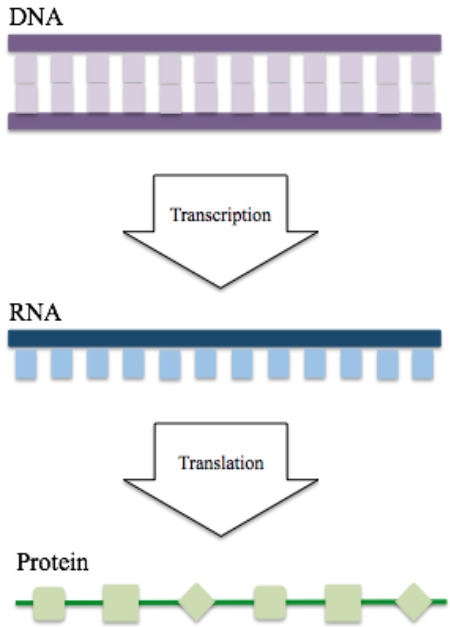


Figure 2.9: *The Central Dogma of molecular biology. Illustrated is the transcription of DNA into transient RNA molecules that act as messengers for the translational coding of protein molecules.*

While proteins are very important in cellular processes, noncoding RNA molecules are not to be overlooked. Interestingly, only around 1% of the genome transcribes mRNA that encodes proteins, whereas somewhere between 70% and 90% of the genome is transcribed into non-coding RNA at some point (Lee 2012). RNA molecules can be transcribed from almost anywhere in the genome, including areas that overlap with known protein-encoding genes. The speculation is that there are around ten noncoding RNA molecules transcribed from within the span of any "regularly" coding gene (Lee 2012). This means there is a huge volume of DNA that is being transcribed, the product of which has yet to be understood and characterized for the most part. Some lesser-understood ncRNA molecules are those that are implicated in epigenetic mechanisms. These include long noncoding RNA (lncRNA), microRNA (miRNA), and circular RNA (circRNA), which will all be discussed for the remainder of this chapter.

2.4.2 lncRNA.

Long noncoding RNA molecules are RNA molecules that are not translated into protein, and are not associated with either regular cellular processes (such as tRNA and rRNA) nor are they characterized by specific cleavage and folding patterns (as is miRNA) (Ernst and Morton 2013). They can be thousands of nucleotides long. Long-noncoding RNA molecules are known for being allelic in nature (Lee 2009). This means that regulation by lncRNA can be specific to one of either the maternally inherited or paternally inherited chromosome. This can be illustrated by the example of X-inactivation in females. We know that females carry two X chromosomes, as opposed to the X and Y carried by males. The Y chromosome is very small compared to the X. In order to compensate for having double the genetic complement of men (and thereby preserve a correct "dosage" of functional genes), women turn off one X chromosome via a process known

as X-chromosome inactivation. The mechanism by which this occurs involves a very important lncRNA known as *Xist* (Brown et al. 1992). The key in X chromosome inactivation is that one chromosome must be fully compressed and shut down whereas the other is fully active; there cannot be any variability in the expression from either one. This is known as allelic specificity (Lee 2009).

The way that RNA molecules are able to achieve such a high level of specificity is because of the way they are produced, their stability, and the way they are compartmentalized and stored in specific regions. RNA molecules are nucleic acids, just like DNA. In this sense they have a sequence, and this can bind to complementary sequences of DNA to interact with only specific target sequences, in this case DNA. There are many proteins that bind to DNA: numerous examples can be found among the transcription factors (DNA activating and repressing proteins). The difference between proteins and RNA is that proteins typically recognize short DNA sequences to bind, whereas RNA molecules are able to bind to long sequences, giving them a specificity that proteins may lack (Lee 2012).

DNA is an extremely stable molecule. It needs to be, since it will direct cellular activities throughout the life of the cell. RNA on the other hand, is a labile molecule. It is synthesized to pass a message along, either to encode proteins or to fulfill one of many other functions. Once it has done this, it often needs to be removed immediately from circulation by degradation. This is an aspect of lncRNA that is manipulated in certain biological processes, ie: those that require brief signaling or signaling specific to the site of RNA transcription. This is how lncRNA is able to act in an allelic manor. In the case of X-activation and other cases, lncRNA is degraded almost as soon as it is made. This means that it doesn't have an opportunity to diffuse away from the site of synthesis; the lncRNA is likely to act near the X chromosome from which it is transcribed, not upon a neighboring

chromosome (Sun, 2006). Furthermore, even if small amounts of lncRNA are actually able to diffuse from their site of synthesis, the rates of degradation by cellular machinery ensures that concentrations would likely become insufficient for remote activity (Lee 2012).

lncRNA molecules can also restricted to a distinct region of action via the binding of tethering proteins. Tethering proteins will bind to RNA molecules during synthesis and anchor them to that region thereby preventing diffusion away from the target DNA sequence (Lee 2012).

In the example of the lncRNA involved in X chromosome inactivation, the Xist lncRNA specifically recruits proteins involved in chromatin repression. This is what causes the inactivation of the chromosome (Wutz 2011). However, it is not always true that lncRNA will silence any given region. In fact, there are lncRNA molecules that are involved in turning on expression of DNA (Lee 2012).

While there has been a lot of research focusing on various lncRNA molecules in recent years, it is still unclear what their importance is to the cell. Most of the hypotheses derive from experiments where certain lncRNA are turned off. Many of these experiments do not result in a noticeable change in cell physiology leading many to speculate that lncRNA molecules have redundant functions (Lee 2012). More research is needed to clarify the functional importance of most lncRNA molecules.

2.4.3 miRNA.

MicroRNA (miRNA) molecules are much shorter than lncRNA molecules, and once processed, they average roughly 23 nucleotides per molecule (Bartel 2004). Their action is specific and targeted to repress genes. The way that miRNA molecules function is through interaction with a complex known as an RNA-induced silencing complex (RISC) (Schwarz et al. 2003).

miRNA molecules are formed through the folding of an initial

RNA transcript and then its subsequent cleavage to produce a double-stranded RNA molecule, one strand of which will be recognized by, and pair with, the RISC. Together, RISC and the miRNA bind to a specific protein-encoding mRNA . The miRNA provides the sequence specificity to target specific mRNA molecules (Schwarz et al. 2003). While there is some specificity in gene targeting, there are miRNA molecules that can recognize up to hundreds of different gene products – in other words, related families of mRNA sequences (Bartel 2009; Shukla et al. 2011). Most miRNA molecules bind to the untranslated region (UTR) of an mRNA. If multiple mRNA sequences share a conserved UTR **motif**, then one miRNA can recognize any one of these mRNA molecules (Lim et al. 2005).

When the RISC/miRNA complex binds to a target mRNA molecule, there are different fates that can arise for the mRNA. Binding can stimulate the RISC complex to cleave the protein-encoding mRNA molecule, effectively precluding production of the protein. This typically occurs when the miRNA and mRNA sequences are very similar so that binding is strong. Alternatively, when binding is weaker, the RISC and miRNA complex will bind to the mRNA and then either: halt translation of the mRNA through the ribosomes (protein factories); allow more miRNA to bind; or send the mRNA for processing and eventual degradation (Baulcombe 2004). Regardless, the fate of the mRNA is to be either destroyed or at least not translated, meaning the product of that gene is effectively turned off.

2.4.4 circRNA.

As we saw above, miRNA molecules regulate genes at a post-transcriptional level. The genes that encode miRNA are themselves subject to regulation like any other gene, including those that encode proteins. However, there is another layer of activity that regulates the levels of miRNA. This level requires circular RNA (circRNA) molecules. circRNAs have the ability to

bind miRNAs and to inhibit them. Their circular structure allows them to bind miRNA without being rendered susceptible to degradation via the mechanism that miRNA uses to induce mRNA cleavage (Franco-Zorrilla et al. 2007). Currently, our understanding of circRNA molecules is limited, but it appears to promise another layer of complexity to be considered when analyzing gene regulation and epigenetics.

Through this chapter we've seen various RNA species that can interact and regulate one another, as well as various genes. The interactions are summarized in Figure 2.10.

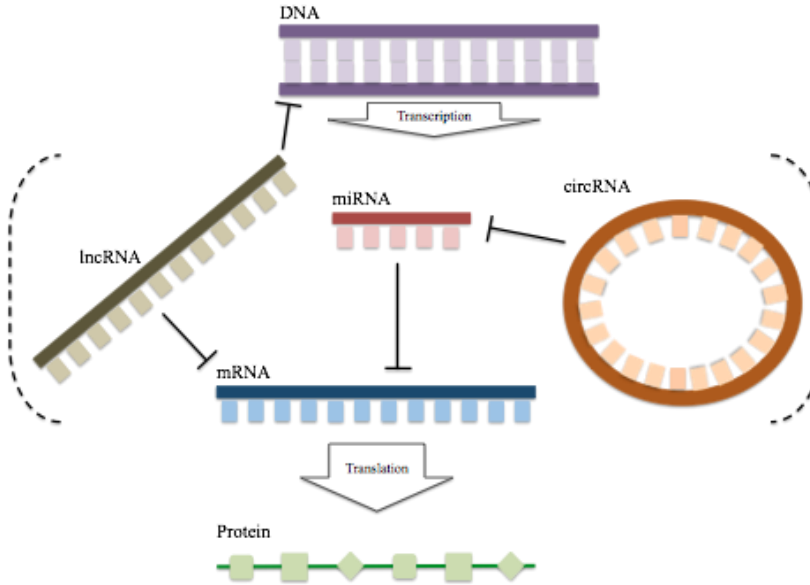


Figure 2.10: *The Central Dogma of molecular biology with feedback and interactions between RNA species. DNA is transcribed into a pool of different RNA species. The traditional mRNA is still translated into protein. miRNA and lncRNA inhibit protein-encoding mRNAs. circRNA potentially reverses this by inhibiting the miRNAs. lncRNA can also effect the DNA directly, either by affecting transcription of new RNA molecules, or by physically covering DNA or altering its shape sufficient to preclude interaction with other protein or RNA factors.*

While DNA and proteins are typically the most discussed components contributing to chromatin structure, this section describing the impact of RNA on epigenetics has hopefully provided some enlightenment about why RNA is an equally important player in the regulation of gene expression and chromatin structure.

2.5 Introduction to the Assessment of Epigenetic States.

Understanding the molecular subtleties of epigenetics is one thing, assessing these epigenetic marks in a way that advances the field is quite another. Before diving into the gory details of epigenetic assessment techniques there are some preliminary difficulties with epigenetic assessment as a whole, especially when it comes to human subjects. Firstly, epigenetic states are dynamic and can vary substantially between different tissue types. For example, epigenetic patterns change with age, and at different rates in different tissues. This means that a mouth swab of oral epithelial cells is not representative of the epigenetic status of the whole body - epigenetic marks might affect only a single gene in a single cell type. If that cell type happens to reside in the brain, then a blood test will tell you nothing. From a human patient's point of view, this might mean uncomfortable prospect of biopsies of sensitive tissues like liver, brain, or gonad. Imagine having to request a brain biopsy from a living colleague to test for epigenetic effects (although there may be some colleagues in whom the lost material passes unnoticed!). Furthermore, if you do not know in advance which gene or suite of genes are liable to be affected by epigenetic changes, it can be hard to design a test to screen for effects. That said, if epigenetic changes are detected in blood samples, they do not necessarily covey information concerning organs, nor whether the imprint will have been transmitted to the germ line for propagation to future generations. Finally, as will become clear in the ensuing chapters, many

different influences act upon the epigenome, so establishing a discrete causal link can be very difficult – there are simply too many confounding factors at play.

Even presupposing the availability of a tissue sample, what is to be epigenetically assessed? DNA methylation, histone modification, regulating RNA species, etcetera? In most cases, there is far too little scientific literature to systematically narrow down which epigenetic path to take. It is also not feasible to collect sufficient tissue sample and have the financial resources to perform all tests. Furthermore, one should also consider how much of an effect the biopsy itself may have on the epigenome of the tissues implicated.

The second issue is that the definition of epigenetics is not static, but rather is ever evolving, much like the definition of a gene has over the last few decades. Some believe that epigenetics must be considered heritable (Berger et al. 2009). Others believe that only mechanisms that change chromatin structure should be considered to be epigenetic (Bird 2007). Perhaps the safest definition is one that conveys the notion that absolutely any mechanism that changes the expression of a gene, and that that doesn't involve change in nucleotide primary sequence, is epigenetic. Adopting the latter definition, the possibilities of "what is epigenetics?" becomes nearly endless.

Finally, given the number and complexity of potential DNA methylation sites, as well as the innumerable ways in which histones and other chromatin proteins may be modified, it may often be hard to assess the meaning of the state of a DNA sequence or its associated histones. For example, a single gene might have a half dozen or more CpG islands available for methylation, and different numbers and arrays of them might be methylated (or not) in different cells even within the same tissue. Without even having approached the evaluation of histone modifications, the epigenetic code is already complex and subtle.

To make this section brief, only two putative epigenetic

modifications have been explored for assessment, methyl DNA and histone modification. These assessment techniques may be used on all tissues using very small sample (biopsy) quantities. One exception is the strategies that employ bisulfite treatment to identify methyl DNA: this used to require a substantial amount of sample tissue, but the technique is now very much more efficient.

The importance of epigenetic assessment is apparent in the correlation of epigenetic marks with predispositions to, and involvement in, disease. Indeed, a better understanding of the role of epigenetics in disease may facilitate the discovery of preventative measures, accurate prognostic assays, treatments, or even cures. Epigenetic assessment is facilitated by specialized biomolecular techniques that are capable of identifying methylated DNA regions, genes under the control of modified histone proteins, and epigenetically active RNA species. These techniques include DNA amplification by **polymerase chain reaction (PCR)**, **DNA sequencing**, and **immunoprecipitation (IP).** Often, these techniques are used in combination. This chapter will cover the basics of today's popular epigenetic assessment techniques, and identify their limitations and some ramifications from the consumers' point of view.

2.6 Methylated DNA (MeDNA) Assessment

DNA methylation (MeDNA) is one of the best understood epigenetic markers with a plethora of assessment techniques, many involving the bisulfite treatment of DNA. A simple approach is to use restriction enzymes to cut the genome into manageable sizes. One variation of this approach employs restriction enzymes that have a differential ability to cut at methylated CpG sites. A sample can be digested, and depending upon where it is methylated, differently sized fragments will be cut. The strategy is then either to visualize these differences on a gel, cut them out, and then clone/sequence them, or, to use polymerase chain reaction (PCR) to amplify the fragments

following the addition of adapter sequences to their ends. In this latter instance, the PCR sequences can be cloned or sequenced (Esteller 2005).

Another strategy requires that methylated C's be chemically converted to make them stand out. Bisulfite converts any unmethylated cytosine residues in DNA to uracil (Frommer et al. 1992). These changes can be then be detected by conventional DNA sequencing or PCR (polymerase chain reaction) approaches – comparisons are made to DNA samples that have not been bisulfite treated, and this highlights the nucleotides that must have been methylated (and protected from bisulfite conversion). Treating DNA with bisulfite to find methylated C's is analogous to using a black light used to find bodily fluids on surfaces and clothes. Since the base pair complement of uracil is converted to adenine instead of guanine, sequencing techniques are ideal for accurately locating unmethylated cytosines within DNA. Unfortunately, bisulfite treatment also degrades a large portion of the initial DNA template, and this reduces its effectiveness with scarce DNA samples (Grunau et al. 2001). Another limitation is that 5-hydroxymethylcytosine, like 5-methylcytosine, is also resistant to bisulfite treatment. This is a problem since the consequence of 5-hydroxymethylcytosine on gene transcription is different from 5-methylcytosine as described earlier. The Tet family of enzymes (Section 2.2) may mitigate this problem by converting 5-hydroxymethylcytosine to 5-carboxylcytosine that is read as a thymine when sequencing bisulfite treated DNA.

An alternative to sequencing bisulfite treated samples, is to use polymerase chain reaction (PCR) conditions that will discriminate between untreated and bisulfite-converted sequences. Depending upon the DNA primers used to amplify a target sequence, bisulfite treated sequences can alter whether or not the sequence can amplify – a comparison of PCR products on a gel will tell you if the target sequence was originally methylated or not (Figure 2.11).

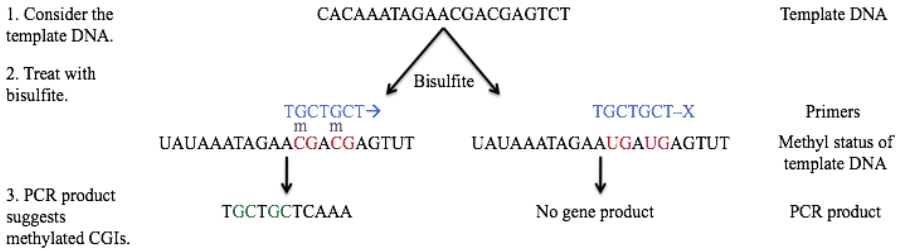
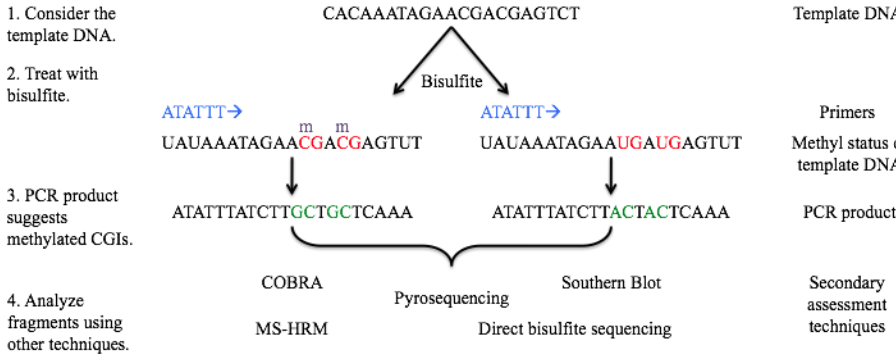


Figure 2.11: *Flow chart of methyl DNA (MeDNA) assessment by MSP. Methyl specific PCR takes advantage of a PCR technique using methylated CpG island specific primers for the rapid assessment of DNA methylation status.*

Bisulfite treated DNA opens many doors to PCR and sequencing techniques. The difference between PCR and sequencing is that PCR amplifies targeted DNA sequences thousands of times until they are abundant enough to be visually detectable, while sequencing is the systematic identification of each nucleotide base in the sequence. Bisulfite sequencing benefits from being more in the way of a brute-force approach – you do not necessarily need to know anything about the DNA sequences to begin with – you can capture sequence data in an unbiased manner. DNA enrichment was initially required for this treatment due to severe degradation of DNA from bisulfite treatment and these clones had to be amplified for any direct sequencing sensitivity. Any kind of cloning is labor intensive and expensive, thus other sequencing techniques are now preferred (Tost and Gut 2007). Unlike the old method of bisulfite sequencing (dideoxy chain termination), pyrosequencing does not require enrichment of DNA and it is therefore not quite as labor intensive (Tost and Gut 2007). Nucleotide incorporation is identified and reported by a light signal as a complementary sequence of DNA is polymerized by sequencing enzyme. The

light signal intensity may then be quantified. The trade-off is that the technology itself is expensive and the sequence read length is short – 300-500 base pairs instead of the 1000 base pairs formerly achievable.

Figure 2.12: *Flow chart of MeDNA assessment by BSP.*

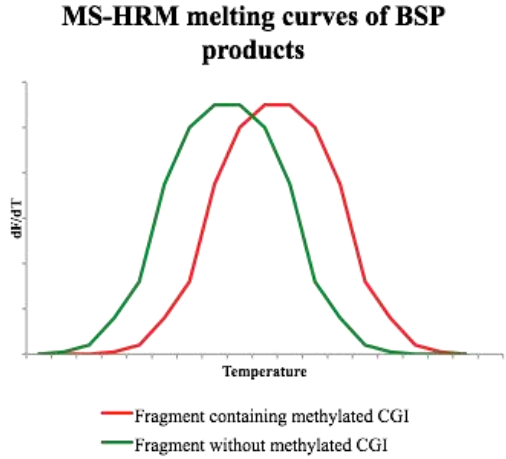


Bisulfite specific PCR, unlike MSP, amplifies upstream regions of DNA with primers not containing possible methylated CpG islands. The products produced are then assessed by using a secondary rapid technique.

Essentially two PCR techniques are commonly used: methyl specific PCR (MSP) (Figure 2.11) and bisulfite specific PCR (BSP) (Figure 2.12). The key difference between the two is primer composition. MSP includes CpG sites in their primers and BSP does not. Due to the nature of MSP primers, a PCR product is observed when a CpG site is methylated (Herman, 1996). If the template is unmethylated then the MSP primer would not hybridize to the template DNA resulting in an absent PCR product as shown in Figure 2.11. PCR products are DNA fragments resolved on agarose or acrylamide gels into singular bands. This is a cheap and fast 5-methylcytosine assessment of a single or small collection of genes. The limitation of MSP is that the template DNA sequence must be known in order to design primers (Herman et al. 1996). Other problems arising from any PCR

technique is non-specific primer hybridization which requires careful annealing temperature optimization for each primer set.

Figure 2.13: *The figure represents a melting curve using methylation specific high resolution melting (MS-HRM) of two BSP PCR products. The red melt curve is representative of the*



PCR product that contained a methylated cytosine and therefore requires a higher temperature to dissociate the double stranded DNA to single strands. The red product lacked one or more methylated cytosines and acquired the weaker uracil bases resulting in a lower temperature melt curve.

The power of BSP is that it does not require detailed knowledge of a gene or genes of interest. Primers may be designed for highly repetitive DNA sequences that do not include CpG islands. Excellent examples are the Alu elements. **Alu sequences** are often found upstream from many genes' non-transcribed regions including the promoter, repressor, and enhancer domains that may be littered with CpG islands (Yang, 2004). After PCR, the DNA fragments can be subjected to methyl DNA (MeDNA) assessment using a variety of techniques including: sequencing, Southern blot analysis, combined bisulfite

restriction analysis (COBRA), and methylation specific high resolution melting (MS-HRM).

- Southern blots allow a MeDNA specific DNA primer conjugated to a fluorescent or radioactive reporter to probe a collection of DNA fragments that have been previously immobilized on a membrane.
- COBRA uses a CpG island-specific restriction enzyme approach (Xiong and Laird 1997). Restriction enzymes reduce PCR products to smaller fragments by double stranded breaks. The number and size of these fragments of bisulfite treated DNA containing methylated CpG islands will differ from those without methylation. The restriction enzyme approach is easy, accurate, fast and cheap.
- Finally, another technique involves methyl sensitive high resolution melting (MS-HRM). This techniques takes advantage of the differential melting properties of DNA based on nucleotide length and composition (Wojdacz and Dobrovic 2007). A higher cytosine-guanine composition present in bisulfite-treated methylated CpG island DNA fragments will result in a higher melting temperature than unmethylated CpG island fragments. Figure 2.13 illustrates this phenomenon as a melt curve with the y axis, dF/dT, representing the change in fluorescence over change in temperature. In this instance, the fluorescent molecules are designed to fluoresce only when bound to double stranded DNA: as the temperature increases the DNA melts into single stranded DNA, and fluorescence declines in proportion. The increased cytosine and guanine content results in a higher melting temperature due to the triple bond between them, resulting in more energy required to break compared to double bonds formed by thymine or uracil and adenine. Like COBRA, MS-HRM is relatively fast, easy, cheap and very accurate

at determining methylated CpG islands at a locus (Wojdacz, 2007).

The previously discussed techniques are often combined for confirmation and accuracy of results. As an example, MSP amplified fragments may be subjected to pyrosequencing for methylated CpG island confirmation (Shaw et al. 2006). Combined techniques may also elucidate additional information of a DNA sample such as methylation heterogeneity when combining pyrosequencing with MS-HRM (Candiloro et al. 2011). Most importantly, combining BSP and sequencing techniques results in **global methylation** assessment (Yebra and Bhagwat 1995). Global methylation has great significance in medical diagnostics since cancers often exhibit a particular methylation signature that is detectable in blood samples (Delpru, 2013).

Other MeDNA global assessment techniques that do not require bisulfite treatment include methyl DNA immunoprecipitation (MeDIP) and methyl binding domains (MBD). The two use the similar concept of isolating digested methylated DNA by binding 5-methylcytosine and immobilizing it and the attached DNA sequence – this can later be processed and analyzed (Jacinto et al. 2008) (Fig. 2.14).

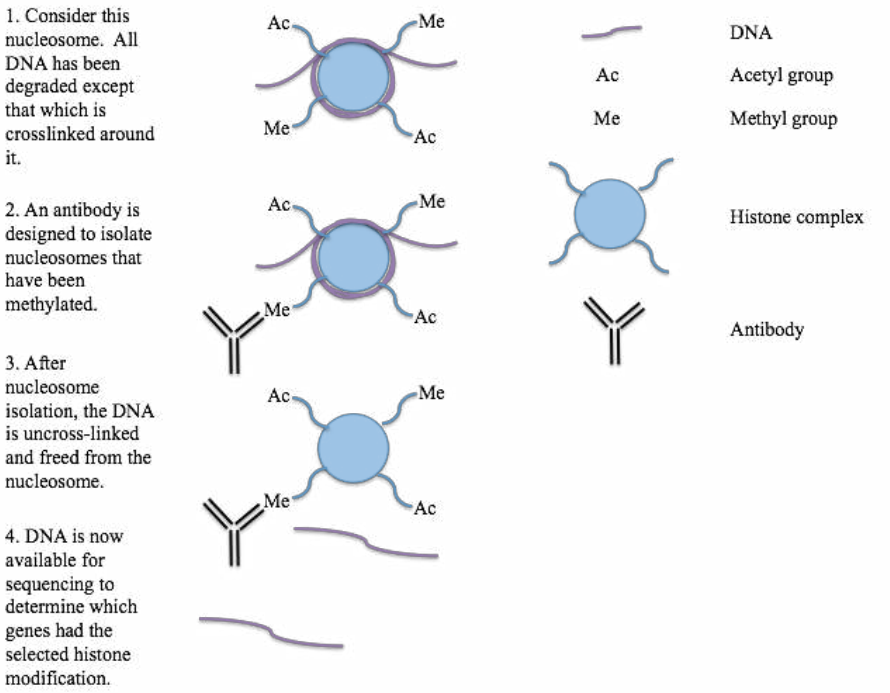
- MeDIP uses an antibody approach and MBD utilizes proteins that bind preferentially to 5-methylcytosine. To help conceptualize immunoprecipitation imagine the following: When fishing, a fisherman wants to catch a particular fish (5-methylcytosine) and to do this he will use a specialized bait (antibody). Once the fish is caught the fisherman will use rod and line to isolate the fish (immunoprecipitation). Isolated DNA may then be subject to sequencing or a microarray (Palmke et al. 2011). Microarrays use a myriad of microscopic complementary DNA spotted and dried in arrays on a glass – each spot

might potentially bind specific sequences present in your sample. There may be hundreds to thousands of distinct DNA sequences spotted on a slide. Your DNA sample of interest can be PCR amplified, fluorescently labeled, and then incubated upon the microscopic dots. Complementary strands stick to each other: since your sample DNA is labeled, a fluorescent signal can be detected and linked to a known sequence upon the array. These experiments provide a massive amount of information. Microarrays are often used in medical diagnostics since they provide comparative information between two cell types, for example, those deriving from cancer and normal tissue (Palmke et al. 2011). The drawbacks of microarrays are not inconsiderable: they are expensive; results may be inconsistent; the massive amount of information they provide is not necessarily useful; and validation and analysis of results can take a long time (Chagovetz and Blair 2009).

There are not many methods outside of chromatin immunoprecipitation (ChIP) for assessing the epigenetic states of histones (Huebert et al. 2006; Pillai and Chellappan 2009). ChIP uses the same isolation strategy as MeDIP, but with a different target using a different bait. In this technique, a DNA sample prepared so that multiple species that are 146bp long can be cross-linked to nucleosomes: the exposed DNA between nucleosomes is then degraded. Once the cross-linked DNA is immobilized on the nucleosomes, antibodies are used to specifically recognize and bind to particular histone modifications; some antibodies will recognize acetyled histones, other will bind methylated, SUMOylated, ubiquitinated, glycosylated, or phosphorylated histone. The repertoire of antibodies available for this approach is quite large and specific. For example, there are reagents available that are sufficient to discriminate between histone H3 methylation

at the 4th, 9th, and 27th amino acid position on the protein, and even between different methylation sites at each of those positions etc.. This might give you a glimmering of how varied and complex the combinations of modifications can be.

Figure 2.14: *Flow chart of histone modification assessment by immunoprecipitation. Antibodies are designed to target a specific histone modification like in this example, histone methylation.*



Epigenetic assessment technique	Mechanism	Information provided by technique
Direct bisulfite sequencing	DNA sequencing of clonal vectors following bisulfite treatment	Methylome in its entirety, global MeDNA, and single gene MeDNA assessment
MSP	DNA amplification of template DNA via CpG containing primers after bisulfite treatment	Single gene MeDNA assessment
BSP	DNA amplification of template DNA via non-CGI containing primers after bisulfite treatment	Global MeDNA and single gene MeDNA assessment
Pyrosequencing	Short read sequencing technique involving a luminescent reporter	Global MeDNA, single gene MeDNA, and global gene identification under a histone modification assessment
MeDIP/MBD	Immunoprecipitation of DNA containing methyl cytosine which is then probed or sequenced	Global MeDNA and single gene MeDNA assessment
ChIP	Immunoprecipitation of targeted histones with desired mark followed by sequencing of cross-linked DNA	Global gene identification under a histone modification

Table 2.1: *Summary of epigenetic assessment techniques*

Whatever DNA/histone combination is then bound and precipitated by antibody is then cleaned up and the DNA is un-cross-linked and made available for analysis by sequencing or microarrays (Huebert et al. 2006; Pillai and Chellappan 2009). 146bp sequences are more than enough to identify which gene or genes are associated with the targeted histone modification. Much work to be done with regard to assessing histone modifications. In yeast alone there are 51 conformations of modifications identified by **mass spectrometry** (Jiang et al. 2007). Different combinations also have different implications on gene expression (Jiang et al. 2007). Linking histone modification assessment data with genetic expression will be quite the undertaking, but it will greatly expand our understanding of the role of combinatorial histone modifications in gene expression.

2.7 Conclusions

Epigenome analysis is liable to be fraught with many of the same challenges and concerns that were elicited by genome sequencing. For example, who should have access to data, and under what circumstances? How will privacy be protected, and how long will samples and data be stored? If direct-to-consumer epigenome testing kits become available, how will they be regulated, and how will individuals be taught to employ and interpret them with intelligence, caution, and integrity? Will it be possible to quantify an individual's epigenome given that all tissues and cells within that person are liable to carry a slightly different imprint? Or will generalized and averaged assays be sufficient to indicate general health trends? If epigenomes can change with time and experience, will this immensely complex fingerprint ever find utility to identify and characterize people?

Epigenetics is a quickly growing field of biology, but there is still much more to be explored. Individuals interested in getting their methylome assessed will not only have to consider which tissues to assess but also when, since methylation patterns differ

between tissues and over time. For example, 5-methylcytosine deamination is a product of aging and accumulates over time. Perhaps at some stage some people will advocate that we assess the global methylation status of an individual's gametes before they manifest the intent to reproduce? This practice would ensure that epigenetically healthy material is inherited by the offspring, and this would help to indicate corrective dietary measures, or to minimize the transmission of possibly deleterious trans-generational effects (Chapter 5). However, after assessment should individuals commit to life changing decisions based on what is, after all, relatively dynamic information? There is also the issue of financially disadvantaged populations: should money determine who can afford the expense of a methylome assessment? Who should pay to mitigate epigenetic harms? What should be done for the equal opportunity of epigenetic assessment? These ethical issues are of the realm of eugenics and are discussed in Chapter 8.

In summary, epigenetic tools are abundant for global MeDNA, single gene MeDNA and global histone modification assessment. However, the analysis of the massive amounts of data they provide has not yet matured into something suitable for solving today's ethical, legal, and scientific complications.

Provocative Questions

- How can an epigenome be characterized if the marks that differentiate cell are so complex and varied?
- Since epigenetic imprints vary from cell to cell, and tissue to tissue, will biopsies be required from every tissue type?
- If an epigenome is to be modified by drugs or diet, given the aforementioned constraints, how will success be monitored?
- Who will bear the cost of assessing an individual's epigenome, and will simple blood tests become sufficiently sophisticated to provide generalized prognostic

information?

- Given the rapidly changing and complex methods of assessment, how will non-specialists, such as physicians ever manage to keep abreast?
- Given that there are many different epigenetic routes for cells to reach a particular endpoint, is there value in trying to assess an epigenomic imprint? Will a result that comprises averaged status be informative?
- Will it be possible to predict whether or not a person's epigenetic status will convey to the next generation? Should such an attempt be made?
- How will the huge datasets derived be housed and secured from intrusion?
- Given that an individual's epigenomic imprint changes with time, how often should their imprints be re-assessed?
- How will the cost to benefit balance of epigenetic testing be assessed?

Possible Solutions

- Given the epidemiological scale, longevity, and trans-generational effects of epigenetics, resources should be directed to developing comprehensive, accessible, and reliable methods to assess epigenetic imprints.
- Multiple and representative samples should be assessed globally to define variation between individuals, genders, ages, economic strata, geographical areas, and genetic backgrounds.
- Education of policy makers, health officials, medical practitioners, as well as science and health teachers should be updated to include consideration of epigenetic effects and mechanisms.
- Economists, medical practitioners, ethicists, and social scientists should be engaged to evaluate the relative costs and benefits of epigenetic assessments and interventions for both individuals and for society at large.

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Chapter 3

Cancer: An Example of Epigenetic Peril and Promise

By Sandy Zakaria, Marisa Market, Jessica Hebert

Abstract

Cancer epigenetics offers a good introduction to how both the mechanisms and tools of epigenetics are utilized by clinicians and researchers to unravel new possibilities for diagnosis, prognosis, and manipulation (treatment). For example, hyper-methylation appears to play a role in silencing tumor suppressor genes, while the overexpression of some miRNA genes exacerbates this condition. We discuss how epigenetic markers can serve as indicators of tumor aggression and progression. By screening for these epigenetic markers (like lowered global methylation status and a loss of acetylation and methylation on histone proteins), a more accurate diagnosis and prognostication of tumor character and stage can be made. Finally, by manipulating epigenetic states via either global or target-specific means, prospective therapies for cancer treatments have potential to offer effective solutions to formerly intractable problems.

3.1 Introduction

Over the past decade, research into cancer epigenetics has begun to reveal the mechanisms by which normal cells adopt cancer phenotypes. In 2000, a consistent relationship was shown

between DNA hypermethylation (many methyl groups attached) in tumor suppressor genes and cancer phenotypes (Esteller et al. 2000; Bygren et al. 2001): aberrant methylation and acetylation patterns are now known to contribute to cancer. Epigenetic analysis permits researchers to examine cancer profiles by comparing normal to cancer cells (Hedenfalk et al. 2001). More recently, global hypomethylation (few methyl groups attached), was identified in all cancer cells (Kaneda et al. 2004). We now know that carcinogenesis is governed in part by epigenetic mechanisms in which the degree of hypermethylation of tumor suppressor genes, the level of global hypomethylation, and aberrant histone modifications are indicative of cancer development and progression. The importance of epigenetics as a causative agent in cancer will be assessed in this chapter, as well as its importance for prognosis and treatment.

3.2 How Epigenetic Mechanisms Induce Cancer Development and Progression

Epigenetic mechanisms of **methylation** and **acetylation** modify the structure of DNA, histones, and **miRNA** (microRNA) and can, on occasion, lead to cellular malfunction (Esteller 2007; Suzuki et al. 2012). The same epigenetic mechanisms that control normal functions can be deregulated to induce uncontrollable cell growth and rapid proliferation. If this happens, the newly attained cellular functions can produce cancer cells that will compromise the health of the individual. Examples of this can be seen in Figure 3.1.

An individual's DNA is susceptible to epigenetic modification at cysteine residues as mentioned in Chapter 2. When cysteine residues become modified with methyl groups, this will lead to a new epigenetic signature (Esteller 2007). In general, hypermethylation will decrease gene expression and downstream gene targets, whereas hypomethylation will increase gene expression and downstream gene targets.

3.2.1 DNA Hypermethylation of Tumor Suppressor Genes

Cancer cells display elevated methylation at the **CpG sites** of tumor suppressor genes. Tumor suppressor genes encode proteins that maintain the integrity of numerous cellular processes, but especially those that are involved in cell division or programmed cell death (**apoptosis**). The gating of entry into either division or death is critical to normal cellular function.

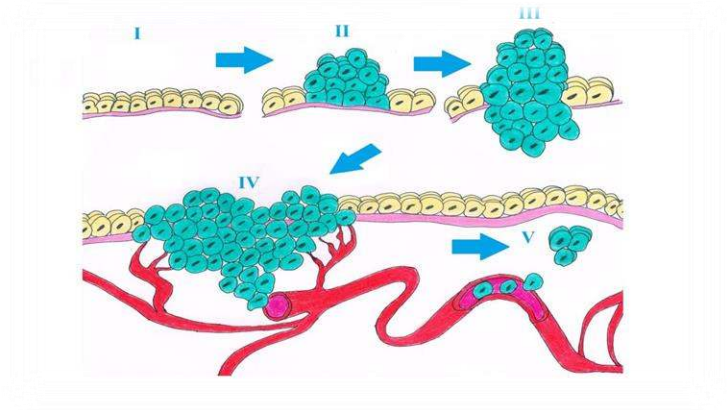

Figure 3.1 *Stages of carcinogenesis I-V.*

Stage I: *the epigenome is unaltered allowing for normal function of cells.* ***Stage II:*** *epigenetic modifications occur to encourage benign cell proliferation.* ***Stage III:*** *genetic and epigenetic modifications begin to accumulate and cells will adopt a more aggressive and invasive phenotype.* ***Stage IV:*** *cancer cells are transformed into malignant cells due to an increasing accumulation of genetic and epigenetic modifications and now have the potential to migrate through circulatory system.* ***Stage V:*** *cancer cells exit bloodstream to establish a secondary tumor site and begin to proliferate. Accumulations of epigenetic modifications such as hypermethylation of tumor suppressor genes and miRNA, global DNA hypomethylation, and aberrant histone modification will compound and amplify and the genome will in turn become more unstable. Cancer cells will attain a malignant phenotype that will adopt invasive and aggressive cellular properties.*

When tumor suppressor genes become hypermethylated, their activity is down-regulated and they are often silenced. As a result, aberrant cellular processes develop, such as premature cell cycle entry, avoidance of apoptosis, defects in DNA repair, and

impaired cell adhesion. All of these processes will contribute to cancer formation and progression (De Carvalho et al. 2012).

Tumor suppressor genes are not merely essential for regulating the cell cycle, but they are consequently vital for maintaining genome stability. A well-studied tumor suppressor gene known as *TP53* is needed to ensure proper cellular genetic composition before cellular division. The role of this gene's encoded protein, p53, ensures genetic integrity and stability of cells thereby minimizing the possibility that gross genetic and chromosomal anomalies will be passed down to daughter cells. An *in vivo* study has shown that p53 function is essential for tumor prevention and cellular senescence (Agarwal et al., 1995; Qian and Chen 2013). The antitumor activity of p53 is essential. Cells that have suppressed p53 activity via hypermethylation will lose genetic integrity and stability. Other tumor suppressor genes that operate to regulate the cell cycle such as retinoblastoma (RB) and p16 can also be subjected to methylation of their regulatory regions to induce gene silencing (Murao et al. 2006). For example, in cutaneous squamous cell carcinoma, tumorigenesis is associated with hypermethylation of RB/p16 pathways (Murao et al. 2006).

Tumor suppressor genes such as *CDH1* and *CDH13* are needed for cellular adhesion to maintain the complex architecture of cells as they form organized tissues (Graff et al. 1995; Toyooka et al. 2001). Several types of cancer can arise when *CDH1* and *CDH13* are hypermethylated. The silencing of *CDH1* and *CDH13* affects downstream genes in their respective signal cascades, and this causes cells to lose adherence to their neighbours. Subsequently, cells acquire the ability to migrate and metastasize to establish secondary sites of growth elsewhere via the circulatory system. According to the National Cancer Institute, an individual with ovarian cancer that has metastasized has a 5 year survival rate of 44% whereas an individual with ovarian cancer that has not metastasized has a 92% chance of survival after a 5 year period. The consequence of hypermethylating *CDH1* and *CDH13* is that

cancer cells become more aggressive and invasive, thereby dramatically decreasing survival rates. Hypermethylation of CDH1 is associated with metastasis 74% of the time in these tumors (Yeucheng et al., 2006)

DNA repair mechanisms ensure the integrity of the genome. Tumor suppressor genes that are responsible for DNA repair mechanisms can fail to work properly when they are hypermethylated at CpG sites. Without proper repair functionality, cells cannot overcome mutations that arise within their genome, and they become even more susceptible to mutations and this contributes to carcinogenesis. For example, hypermethylation is apparent in the *BRCA1* gene that is associated with breast and ovarian tumors, and the DNA repair gene *hMLH1* is hypermethylated in colorectal, endometrial, and gastric tumors (Esteller et al. 2001; Esteller 2005).

3.2.2 Global DNA Hypomethylation of Epigenomes

Tumor suppressor genes aside, most regions of the tumor epigenome have a trend of global DNA hypomethylation. Epigenomes of cancer cells undergo a decrease of DNA methylation of approximately 20-30% compared to normal cells (Esteller 2005). Epigenetic hypomethylation is also a critical factor for maintenance of genome integrity. For example, normal methylation of the epigenome is needed to silence repetitive sequences left by ancestral viral invasions such as transposable elements (Esteller 2005). These viral remnants constitute almost 42% of our genome, and are normally kept stable and inactive by methylation. When hypomethylation occurs, these ancient viral sequences can become mobile and take chunks of chromosome with them as they translocate, leading to the chromosomal translocations that are a hallmark of mutation and cancer (Esteller et al. 2001; Esteller 2005). Mobilized sequences can also have dire effects upon gene expression when they re-insert into a random location that might include a formerly intact and vital gene: this

can either disrupt the "host" gene's regulation or its function (Walsh et al. 1998). Chromosomes require special organization of their middles and ends (centromeres and telomeres). Hypomethylation of centromeric regions of DNA can enhance susceptibility to the formation and progression of tumor cells by disrupting chromosomal segregation during mitosis – daughter cells receive abnormal numbers or parts of chromosomes, and this also enhances the descent to gross mutational disruption (Esteller 2005).

In addition, the loss of methylation of other genes, such as those encoding growth factors or cell cycle regulators, will induce aberrant cell growth and division leading to carcinogenesis. For example, global DNA hypomethylation can reduce the methylation status of imprinted genes *(IGF2* and *H19*) to result in their overexpression whereby they contribute to carcinogenesis (Esteller 2005).

Even though the degree of global DNA hypomethylation is an indicator of tumorigenesis and progression, global hypomethylation initially exerts its effect through only a minority of affected genes (Esteller 2005; Fraga et al. 2005). Later, the hypomethylation of many genes and transposable elements have consequences that are, ultimately, reflected in increased rates of mutation, chromosomal degradation, and tumor progression.

The assessment of an individual's **global methylation** status should be interpreted very carefully as there is a tendency to lose methylation with age (Dunn 2003). This makes it difficult to calibrate relationships when considering hypomethylation as a driver of carcinogenesis. For example, two mouse lines that were deficient in two different genes were crossed and then a tumor assessment was made. The first mouse line possessed a disrupted gene for the enzyme DNMT1: it could not attach methyl groups to cysteine residues and this resulted in hypomethylation of several gene regions (Esteller 2005). The second mouse line had a genetic mutation in the tumor suppressor gene *adenomatous polyposis coli*

or *APC*. This mutation predisposed mice to develop colon tumors (Esteller 2005). Data was collected that showed two separate outcomes: decrease in colon tumor formation and increased risk for the formation of lymphomas (Laird et al. 1995; Gaudet et al. 2003).

Correlation studies prove an existing relationship between hypomethylation and carcinogenesis. Whether hypomethylation is a consequence or causative factor of cancer likely depends upon the initial genes targeted by the disruption, and the genetic context within which these disruptions occur.

3.2.3 Histone Modifications

The packaging of DNA around histones into **nucleosomes** is particularly important for the expression of genes (Chapter 2). To review briefly, histones comprise a family of five proteins: H1, H2A, H2B, H3, and H4. These histone proteins can be subjected to posttranslational modifications to affect the intimacy of their interaction with DNA sequences and thereby regulate expression of multiple genes at once (Herranz and Esteller 2007; Davie, 1998). Most commonly, histone tails extending from H3 and H4 proteins are epigenetically manipulated by methylation and acetylation. As explained in Chapter 2, methylation of histone terminal tails causes DNA configurations around histone proteins to inhibit gene expression whereas acetylation of histone terminal tails relaxes nucleosomal configuration to promote gene expression (Herranz and Esteller 2007). The combination of histone modifications present in our epigenome, by virtue of the number of ways that histones are modified, is quite complex. These modifications are sometimes referred to as the histone code, and the combinations and information carrying capacity they embody exceeds the informational capacity of the genome's DNA sequence!

A common feature of cancer cells is an epigenetic modification of H4 histones. H4 histones can be subjected to

numerous modifications at the N-terminus tail. Specifically, many cancer cells have a loss of trimethylation at lysine 20 and a loss of acetylation at lysine 16, predominantly where the histone associates with repetitive DNA sequences (Fraga et al. 2005). The degree of loss of acetylation and methylation at histone H4 dictates tumor progression: larger decreases in acetylation and methylation correspond to higher a degree of tumor aggressiveness (Fraga et al. 2005). These histone modifications can therefore provide a predictive indicator for aggressive cancers. Generally, aberrant histone modification appears to be responsible for aberrant epigenetic status in the cancer epigenome (Fraga et al. 2005). For example, acetyltransferase recruitment to H4 histone was decreased near the tumor suppressor *TP53* gene, thereby silencing the tumor suppressor activity of p53. Abnormal functions of methyltransferases, such as *MLL,* have also been linked to malignant cancer phenotypes (Fraga et al. 2005).

In hematological cancers, chromosomal translocation can arise from dysfunction of histone acetyltransferases such as EP300, CREBBP, NCOA2, MYST3 and MYST4 (Rodriguez-Paredes and Esteller 2011). Also, histone modification enzymes can interact with other proteins to induce hematological and solid cancers. For example, EP300 and CREBBP can associate with oncoprotein E1A and SV40 large T antigen to cause hypoacetylation of histone 3 at lysine 18 (Rodriguez-Paredes and Esteller 2011). This change to the epigenome results in the activation of genes that allow for cellular growth and proliferation (Rodríguez-Paredes et al. 2001). Furthermore, genetic mutation of EP300 is correlated with increases in malignant transformation phenotypes in colorectal, gastric, breast, and pancreatic tumors (Gayther et al. 2000). The overexpression of **histone deacetylases** such as HDAC1, HDAC2, and HDAC6 also play a role in tumorigenesis (Rodriguez-Paredes and Esteller 2011).

Histone methyl modification enzymes have many downstream gene targets that encourage cell transformations. It is

estimated that 80% of infant leukemia cases and 5-10% cases of all other lymphocyte leukemia cases result from a methyltransferase atypically interacting with 50 gene targets (Rodriguez-Paredes and Esteller 2011). These leukemia promoting genes will elicit abnormal recruitment of enzymes to histone 3 – lysine 79 (Krivtsov and Armstrong 2007; Rodriguez-Paredes and Esteller 2011). The overexpression of histone methyltransferase is also seen in other cancer profiles. For example, extra recruitment of enzymes to histone 3 – lysine 27 effectively produces an overexpression of the methyltransferase EZH2 in prostate and breast cancer. Also, studies show SMYD3, a histone 3 – lysine 4 methyltransferase, is overexpressed in colorectal cancer (Rodriguez-Paredes and Esteller 2011).

Recently, a relationship between the extent of aberrant histone modifications and DNA methylation has been elucidated. For instance, the loss of **histone acetylation** and the loss of histone methylation on histone 3 – lysine 9 will result in the recruitment of DNA methyltransferase 1 to the regulatory regions of genes and increase DNA methylation (Herranz and Esteller 2007). We must infer that not only does methylation of DNA allow for histone modifiers to be recruited to the neighbourhood, but that the reverse is true as well -histone modifiers also help to regulate methylation of DNA.

3.2.4 Hypermethylation of miRNA Genes

miRNA are small non-coding RNA molecules. They constitute approximately 22 **nucleotides** and have the ability to affect gene expression post-translationally (Suzuki et al. 2012). As we saw in Chapter 2, miRNA have the ability to bind to **mRNA** molecules via complementarity to either inhibit activity or to promote degradation of the targeted mRNA (Suzuki et al. 2012). miRNA genes can be subjected to epigenetic modifications such as hypermethylation which will silence them, and indirectly permit the expression of the targeted mRNA. Evidence suggests that

miRNA silencing is involved in cancer formation and progression. For example, the down regulation of expression of *miRNA-15*, *miRNA-16*, and miRNA *let-7* by hypermethylation are linked to **chronic lymphocytic leukemia** and *Ras* oncogene expression. miRNA expression patterns are also linked to histone modification. miRNA molecules were assessed in control and acute lymphoblastic leukemia patients. **Acute lymphoblastic leukemia** patients showed: hypermethylation at CpG islands of thirteen miRNA; high prevalence of histone marks that reduce gene expression (dimethylation of histone 3 – lysine 9); and low prevalence of histone marks that allow for gene expression (trimethylation of histone 3 – lysine 4) compared to control patients (Roman-Gomez et al. 2009).

The *miRNA-124* family has tumor suppressor properties since *miRNA-124* regulates the cell cycle. Aberrant function of *miRNA-124* leads to activation of cyclin-dependent kinase 6, inducing cancer cells to enter the cell cycle and allowing for inactivation of the tumor suppressor Rb gene by **phosphorylation** (Lujambio and Esteller 2007; Lujambio et al. 2007; Agirre et al. 2009). Epigenetic silencing via hypermethylation of *miRNA-124* is apparent in 70% of colorectal cancers (Suzuki et al. 2012). *miRNA-124* are also hypermethylated in acute lymphoblastic leukemia where *miRNA-124* methylation status is indicative of higher mortality rates (Agirre et al. 2009).

An essential role of the *miRNA-34* family is to target p53 pathways. *miRNA-34* can be subjected hypermethylation at its CpG sites: this causes cells to grow uncontrollably and permits avoidance of apoptosis (Suzuki et al. 2012). Silencing of *miRNA-34* is prevalent in many types of cancers; oral, esophageal, gastric, colorectal, pancreatic, breast, lung and renal cancer (Suzuki et al. 2012). The *miRNA-34* family has two main classes: *miRNA-34b* and *miRNA-34c*. When they become methylated, metastasis as well as higher reoccurrence and mortality rates can result (Wang et al. 2011).

The *miRNA-9* family also plays a role as a tumor suppressor: methylation within CpG sites of its promoter is prevalent in breast and pancreatic cancers (Suzuki et al. 2012). In gastric cancer, methylation of *miRNA-9* is correlated with increased metastasis, proliferation, and invasion (Lujambio et al. 2008). Other mi-RNA molecules that act as tumor suppressors are *miRNA-1*, *miRNA-125b*, *miRNA-129-2, miRNA-137,* and *miRNA-145*. All are found to be methylated at CpG sites and are associated with several cancers (Suzuki et al. 2012).

3.3 Clinical Applications of Epigenetics in the Field of Oncology

DNA methylation and histone modification patterns provide indicators for carcinogenesis - they are signposts of tumor stage, and they can identify tumour sub-type and provide prognostic measures for treatment. An individual's susceptibility to cancer is dependent upon various factors including; the degree of DNA hypermethylation of tumor suppressor genes, the global hypomethylation of genes, or the extent of aberrant histone modifications. Since cancer cells have specific epigenetic marks associated with development and progression, these marks will have great utility in the field of clinical oncology for cancer detection, and ultimately, for pharmacoepigenetics (Rodriguez-Paredes and Esteller 2011).

3.3.1 Cancer Cell Detection

Sample DNA and histones can be assessed for epigenetic marks to characterize an individual's epigenetic susceptibility to cancer, or for the presence of abnormal cells. Technological advancements have provided the methods to assess the DNA methylation status of normal versus cancer cells (Rodriguez-Paredes and Esteller 2011). In some cases, non-invasive samples can be obtained to assess epigenetic status (Laird 2003). For example, feces can be collected from colorectal patients, urine can

be collected for bladder cancer assessment, and sputum can be collected for lung cancer assessment (Rodriguez-Paredes and Esteller 2011). Early detection methods are based upon the principle that hypermethylation of select CpG sites occurs early in tumorigenesis. Hypermethylation of CpG sites has been detected early in tumor suppressors $p16^{INK4a}$, $p14^{ARF}$ and *MGMT*, which are involved in colorectal cancer development (Rodriguez-Paredes and Esteller 2011). Increased DNA methylation of tumor suppressor genes may be an indicator for cancer progression.

Early detection methods are also very useful for individuals who inherit genetic risks for cancers such as mutations in *BRCA1* and *BRCA2* (Rodriguez-Paredes and Esteller 2011). Early screening could improve timely detection and increase survival rates. Also, aberrant histone modifications are present in low grade prostate tumors (Esteller 2007). For example, the most promising early detection biomarker for prostate cancer is the hypermethylation of the *GSTP1* gene. The specificity of the *GSTP1* gene to prostate cancer as compared to tumor tissues such as breast, liver, and kidneys makes *GSTP1* ideal (Rodriguez-Paredes and Esteller 2011). Approximately 80-90% of CpG sites are hypermethylated in *GSTP1* in prostate cancer and obtaining biological specimens such as urine or serum is relatively simple (Brooks et al. 1998; Rodriguez-Paredes and Esteller 2011). Finally, *GSTP1* hypermethylation is not found in benign proliferating cells but only in pre-malignant and malignant cells making *GSTP1* a specific marker for malignant prostate cancer. Presumably the repertoire of markers will expand to the extent that physicians will also be able to differentiate between the prostate tumours that need immediate attention, and those that can be left in situ

3.3.2 Tumor Prognosis

Tumor prognosis is a measurement taken to assess the characteristics and severity of a tumor. A tumor prognosis evaluation is based upon levels of DNA methylation and histone

modification. For example, DNA hypermethylation of tumor suppressors *DAPK, p16^{INK4a}*, and *EMP3* are correlated with tumor invasiveness in lung, colorectal and brain cancer (Esteller 2007). Technological development in the field of epigenetics has allowed for the development of prognostic dendrograms where levels of global DNA can be assessed using CpG arrays (Rodriguez-Paredes and Esteller 2011). Prognostic dendrograms have identified unique and useful markers of aberrant methylation patterns in lung cancer reoccurrence and metastasis in colorectal patients (Rodriguez-Paredes and Esteller 2011). In breast cancer, the degree of histone modification is an important factor in tumor prognosis. Individuals who have low to moderate levels acetylation at histone 3 – lysine 18, acetylation at histone 4 – lysine 12, dimethylation at histone 3 – lysine 4, trimethylation at histone 4 – lysine 20, and dimethylation at histone 4 – arginine are classified with a poor prognosis (Chervona and Costa 2012). It was also discovered that histone modifying enzymes are responsible for certain tumor characteristics. The over-expression of the methyltransferase EZH2, which methylates histone 3 – lysine 27, results in tumor aggressiveness and poor prognosis in breast cancer patients (Chervona and Costa 2012). Several other specific histone modifications are seen in lung, prostate, leukemia, esophageal, gastric, kidney, liver and pancreatic cancers that are reflected in poor patient prognosis (Chervona and Costa 2012).

3.3.3 Pharmacoepigenetics

The field of pharmacoepigenetics uses an assessment of an individual's epigenetic architecture to evaluate the best method for cancer treatment options. For example, hypermethylation of the *MGMT* gene is associated with chemoresistance. In normal cells, the DNA repair enzyme MGMT repairs the damage caused by chemotherapeutic drugs, however, in cancer cells with epigenetically silenced *MGMT*, the protein is unavailable to repair the mutations. Therefore, a strategy that epigenetically silences

MGMT might make tumors more susceptible to chemotherapy drugs (Baylin and Jones 2011). Other hypermethylated DNA repair genes can be used to predict or modify chemotherapy response. These include the MLH1 response to cisplatin treatment in lung cancer, and WRN response to irinotecan treatment in colorectal cancer (Rodriguez-Paredes and Esteller 2011). In short, epigenetic mechanisms can be used both to assign the most effective chemotherapy to individuals, as well as to bend a tumor's epigenetic characteristics to make it more susceptible to treatment.

3.3.4 Epigenetic Marks for Therapeutic Drugs

Therapeutic drugs can be used to target aberrant DNA methylation and **histone acetylation** (Figure 3.2). This is accomplished by inhibiting enzymes responsible for DNA hypermethylation to re-activate tumor suppressor genes and prevent global histone acetylation in cancer cells. Demethylating drugs that inhibit **DNA methyltransferase** activity such as 5-aza-cytidine or 5-aza-2-deoxycytidine (Decitabine) are promising in this regard. Success has been seen in treating acute promyelocytic leukemia, however, the lack of specificity of the drug target is an issue (Herranz and Esteller 2007). In large doses, demethylating drugs can non-specifically demethylate genes in normal cells and contribute to overall global hypomethylation: the consequence of these "off target" effects are unknown.

Hypoacetylation can be reversed to the original epigenetic state via inhibition of histone deacetylases. Histone deacetylase drugs fall into four classes: hydroxamates (TSA and SAHA), short chain fatty acids (valproic acid), cyclic peptides (desipeptide) and benzamides (MS-275) (Herranz and Esteller 2007). These drugs have antitumour activity by causing cellular differentiation, cell cycle arrest in G1/G2, and apoptosis. They have been successful in treating hematological cancer and solid tumors (Herranz and Esteller 2007).

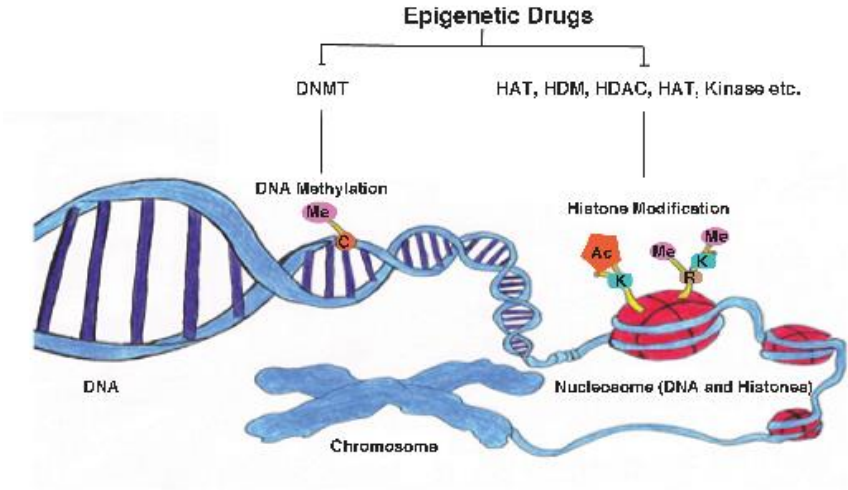

Figure 3.2 *Mechanisms of epigenetic drug treatment.*
Epigenetic drugs are used for cancer therapy where inhibition of enzymes can induce epigenetic changes. Inhibiting **DNMT (DNA methytransferase)** through epigenetic drugs will prevent the recruitment of methyl groups to DNA that can be useful for preventing hypermethylation of tumor suppressor genes. Inhibiting HMT and HDM (Histone methylation modifiers); HDAC and HAT (histone acetylation modifers) can prevent aberrant histone modifications that contribute to carcinogenesis. For example, loss of trimethylation at lysine (K) 20 and a loss of acetylation at lysine 16 on H4 histones, a hallmark of cancer cells, can be manipulated by epigenetic drugs to restore original epigenetic architecture.

The most promising demethylating drug to date is Zebularine. Zebularine, an oral drug, is chemically stable and has low toxicity in normal cells (Yoo et al, 2004; Cheng et al., 2004a; Cheng et al., 2004b). Using 5-aza-2-deoxycytidine, there is an impressive 40-54% response rate in **myelodysplastic syndrome** patients. The high response rate to 5-aza-2-deoxycytidine has encouraged the FDA to approve it for patients with myelodysplatic syndrome. Clinical trials of histone deacetylases are also showing great promise. Phase one trials with depsipeptide show a partial response with minimal side effects in patients unresponsive to conventional chemotherapy treatments (Herranz and Esteller 2007). Also, clinical trials of valproic acid and SAHA are in the final phase:

SAHA has been found to have antitumour activity in solid and hematological cancers (Herranz and Esteller 2007).

3.4 Conclusions

Presently, there is evidence for several correlations that confirm environmental toxins as a causative factor for the development and progression of cancer. Environmental toxins such as bisphenol A, RDX, contaminated drinking water, air pollution, benzene, chromium, aluminum, mercury, lead and pesticides have the ability to alter epigenetic mechanisms (Hou et al. 2012). Statistics suggest that thirteen million people die annually from environmental toxins, where 24% of these deaths are preventable (Hou et al. 2012). DNA methylation and/or histone modification are providing a causative link between environmental exposure and cancer. Eliminating all possible environmental toxins would be difficult: society is supported by commerce which is fueled by the products that utilize many of these toxic chemicals. Nevertheless, it is unethical to knowingly expose individuals to chemicals with a proven causative link to cancer. Therefore, government subsidies and grants should be directed to epigenetic research in order to elucidate epigenetic mechanisms and to develop new therapies. Epigenetics has proven to be an early biomarker for cancer development, an indicator of tumor progression, and a predictor of pharmacological efficacy. Governments and policy makers should recognize that epigenetics has huge potential to become a powerful detection method and eventually, to be used in treatments for cancer. Health care systems will ultimately have to provide epigenetic testing to the public to permit early detection and improved treatment outcomes at reduced costs to the health system and the affected individual. Funding can also provide opportunities to improve current DNA demethylation and histone deacetylase drugs, which have shown great success in clinical trials. Overall, epigenetic mechanisms must be regarded as the mechanism that links the environment and cancer. As such, epigenetics offers a

unique and useful tool for diagnosis, prognosis, and treatment.

Provocative Questions

- Will epigenetics facilitate a personalized assessment and treatment of cancer?
- What will the cost of epigenetic screening be? Who would have access? When would indviduals be screened? Given that an epigenome changes over life, how often will testing be necessary?
- Where would the information be stored? How would it be protected?
- So much potential… So little information – Are epigenetic marks causative or coincidental?

Possible Solutions

- Research needs to be directed to differentiating causative versus merely associational relationships between epigenetics and cancer
- We need to develop faster, cheaper, and more efficient ways to assess an epigenome.
- All stakeholders need to be consulted on how data will be stored, analyzed, and shared. Confidentiality protocols need to be ensured.
- The field is fast moving – physicians, patients, and policy makers need to enjoy a mechanism to provide timely and pertinent education, intervention and treatment.
- There should be more clarity regarding intellectual property rights – should they be restricted to drug products, or will confusion reign with respect to ownership of mechanisms elucidated (much like patent law vacillated over the status of newly discovered genes)

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Chapter 4

Dietary Epigenetics: Are You What Your Parents Ate?

Lina Chaker, Roni Hetzel, Kaela Scott, and Marisa Market

Abstract

The epigenome can be modified by many different factors, one of which is diet. This chapter focuses on both acquired and inherited nutrition-dependent epigenetic modifications that occur within or across generations. We begin by differentiating between acquired and inherited epigenetic modifications. Intra-generational and inter-generational epigenetic effects are considered acquired, while trans-generational epigenetic effects are considered inherited. Intra-generational refers to effects seen in the same generation as the environmental change; inter-generational refers to effects seen in their children, likely exposed either in utero or as precursor gametes; trans-generational refers to effects seen in future generations that were not exposed, even as gametes, to the environmental change. This chapter proceeds by exploring the multivariate diseases, such as obesity, diabetes, eating disorders, Crohn's, and Coeliac disease, that can be associated with nutrition-dependent epigenetic modifications. Specifically, potential trans-generational epigenetic effects are examined through two retrospective studies: the Dutch Famine and the Överkalix cycles of overfeeding and malnutrition. These studies are compared to established epigenetic data available from rodent studies. By examining the pattern of disease development in people exposed to

different nutritional conditions during different periods of development, a link can be established between nutrition and the epigenome. The inherent weaknesses of retrospective studies in humans are then discussed. Finally, we address some of the ethical and social implications that dietary and lifestyle decisions might convey to our progeny and to society at large.

4.1 Introduction

It is common sense that what you eat today will affect your health tomorrow. What may not be commonly acknowledged, however, is that nutritional challenges experienced by your parents or grandparents could lead to health consequences for you as an adult. The method by which this is likely to occur is through modification of your parents' or grandparents' epigenome (via certain mechanisms that are discussed in Chapter 2), which leads to alterations in your own gene expression. Therefore, it is epigenetics or other non-genetic mechanism that provides a likely explanation for how nutritional challenges or surpluses can exert effects upon individuals much later in life, as well as between parents and offspring, and onwards through to subsequent generations.

Acquired vs. Inherited Epigenetic Changes

Broadly speaking, epigenetic changes can either be acquired or inherited. In order for an epigenetic change to be considered acquired, the epigenome must be directly affected by an environmental event. Thus, if an adult, child, fetus, or even the gametes that eventually give rise to an individual are exposed to an insult that results in epigenomic modification, this would be considered an acquired epigenetic change. This last point might be a bit confusing, and understanding the process and timing of **gametogenesis** will make it easier to understand how the gametes of a fetus may acquire an epigenetic change. Gametogenesis is the process by which haploid gametes (egg or sperm) are produced from diploid germ cells. Although the purpose of oogenesis

(development of eggs) and spermatogenesis (development of sperm) is the same, these processes differ in many ways, including developmental timing. In females, oogenesis begins in the fetus and egg development is soon arrested in Meiosis I – a sort of suspended animation that stores the egg in nearly mature form until it is needed. Thus, females have gametes already present while they are growing as fetuses in utero. While the bulk of eggs are stored this way, recent research has also discovered oogonial stem cells are responsible for the development of some eggs into adulthood as well (White et al. 2012). By contrast, males initiate spermatogenesis when the primordial cells go into meiosis during their slow growth period, just before puberty (Pembrey et al. 2006). Thus, sperm are not present in the male fetus during gestation. Therefore, it is possible for a female to be exposed to an environmental change at any time throughout her life, from fetus through to adulthood, and for this change to affect her gametes through epigenetics. This change to the epigenome of her gametes will then lead to an altered epigenome being expressed in her children (Figure 4.1). Hence, even though her children were not present during the environmental change, the *prospective* effects on their epigenome are still considered to be acquired because the gametes that led to the creation of her children were present during the environmental challenge. In males, the environmental challenge is most likely to exert a long term effect in progeny if it occurs during the pre-pubertal slow growth phase of development, or to a lesser extent, afterwards into adulthood. This concept is examined in more detail in Section 4.4 under Trans-generational Mechanisms.

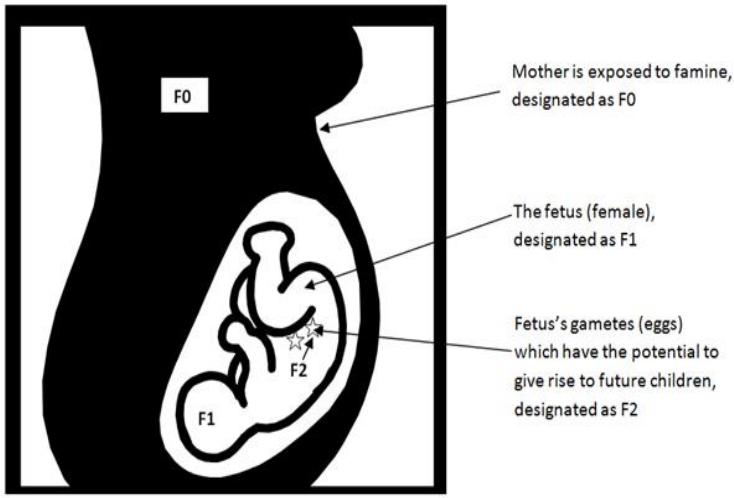

Figure 4.1: *The environment has a direct effect on all three generations.*

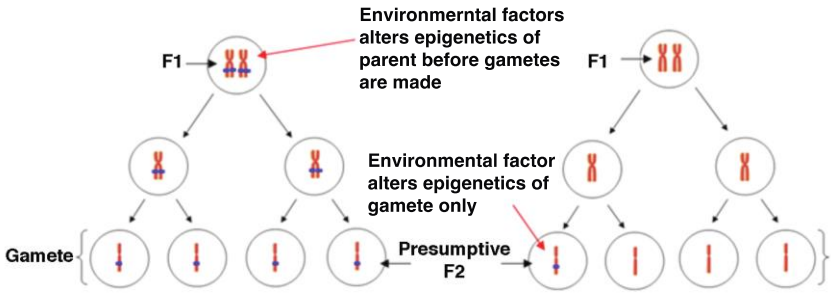

Figure: 4.2*: Environmental changes during specific times in gametogenesis, the meiotic formation of the gametes (sex cells or germ line cells), could theoretically modify sex chromosome epigenetic imprints (Pembrey et al. 2006). The left image depicts a change transmitted through a parent to the first generation F1; thus the change is inherited since it occurs first in the F1 cell precursor to gametes. The F1 cell that gives rise to the gametes may have been exposed at any point in its development. The right image depicts a change after gametogenesis, where the mature gamete is directly affected by the environmental stimulus and transmits the effect to the next generation, the F2s.*

Inherited epigenetic changes, however, refer to changes that occur

in one generation and are passed on to future generations via germ cells not yet present at the time of challenge. Therefore, inherited epigenetic effects are passed on to offspring due to epigenetic changes that occurred in pre-gametic cells, which then become gametes (Burdge et al. 2011). Figure 4.2 highlights the differences between epigenetic changes that occur in pre-gametic cells and those that occur in gametes.

Intra-, Inter-, and Trans-generational Epigenetic Effects

The differences between acquired and inherited epigenetic modifications help us to distinguish between intra-generational, inter-generational, and trans-generational epigenetic effects (Figure 4.3). Intra-generational and inter-generational epigenetic effects are both considered to be acquired, despite the different way that they develop. In either case, the changes to the epigenome are induced *de novo* (spontaneously) within a single lifespan, thus affecting only the exposed individual. However, it is the type of environment that induces these changes that separates the meaning of these two terms. Intra-generational effects occur within the exposed individual's lifespan, and result in later life changes. For example, the food you eat or the pollution to which you are exposed could lead to effects some years later. Contrastingly, inter-generational effects are due to changes that are imposed during the gamete or fetal stage of life (ie; direct exposure but before birth): the environmental challenges are transmitted to the gametes or fetus via the parental environment. Parental environmental stresses upon the sperm or egg that eventually contribute to a new person define one sub-category. Another sub-category arises when the change is installed in embryos in utero. This latter is also known as developmental or **fetal programming** (Gabory et al. 2011). An example of these effects would be a situation in which the food a mother ate while she was pregnant elicited an epigenetic change in the fetus that resulted in her child having an altered phenotype. In either instance, epigenetic changes would not necessarily be passed

to future generations and therefore could not be considered trans-generational.

Finally, trans-generational epigenetic effects are inherited and therefore they occur in one generation and are passed down to the offspring, and onward through successive generations (Skinner and Guerrero-Bosagna 2009). The mechanisms by which epigenetic modifications lead to intra-, inter-, and trans-generational effects will be discussed in the following sections.

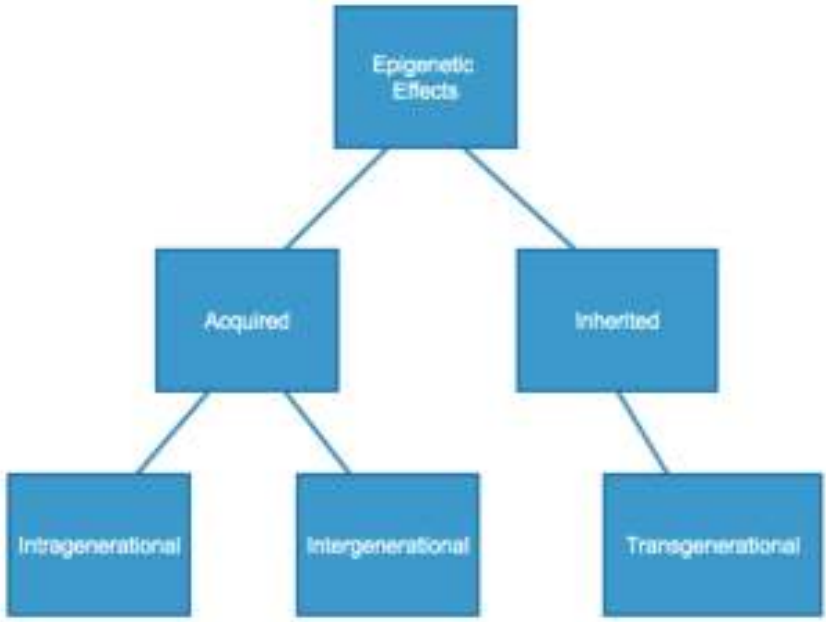

Figure 4.3: *A change occurring in the mother's somatic cells will not affect the fetus; it is an acquired change and called intra-generational. Likewise, a change occurring in the fetal somatic cells will not affect its gametes; it is an acquired change and called intergenerational.*

With respect to nutritional-dependent epigenetic modifications, a period of malnutrition or surplus food would be considered to be the epigenetic "event" or "challenge" that we have been referring to. This chapter focuses on these changing nutritional circumstances and the role that diet plays in the

modification of the epigenome and consequently, upon gene expression. How changes to the epigenome can contribute to the development of **multivariate** diseases, including obesity, diabetes, eating disorders, Crohn's disease, and Coeliac disease, is also examined. This is done primarily through the analysis of retrospective studies.

Simply put, our nutritional status alters the packaging of our DNA on a continuous basis, and this in turn alters the behaviour of genes. Especially when the conditions during the time of challenge do not reflect conditions later in life, our near-, mid- and long-term predisposition to disease is altered as well. Although some experimental information (molecular characterization of epigenetic states) is available in mammals regarding the influence of environmental shifts on the phenotypes of successive generations (Burdge et al. 2011), the majority of our knowledge comes from retrospective studies. In this field, two historical cohorts are of particular interest, the Dutch Famine cohort and the Swedish Överkalix cohort. Finally, we will conclude by outlining possible consequences and ethical concerns that epigenetic data may pose for society.

4.2 Intra-generational Epigenetic Effects

Intra-generational epigenetic effects are an important contributing factor to the development of certain multivariate diseases over the lifetime of a person. Obesity and its related condition, diabetes, are both considered global epidemics and major public health concerns by the World Health Organization (WHO 2014a; WHO 2014b). While it is true that unhealthy eating habits and a sedentary lifestyle are contributing factors, there are multiple additional influences that contribute to the pathology of these diseases, some of which may include epigenetic changes. (Melzner et al. 2002; Lefterova et al. 2008; Nielsen et al. 2008; Vucetic et al. 2012).

4.2.1 Obesity

In the U.S., more than one third of adults are obese (have a body mass index (BMI) above 30 kg/m^2). In addition, another third are classified as overweight (have BMI of 25-30 kg/m^2) (CDC 2014a; CDC 2014b). Worldwide, obesity has nearly doubled since 1980 and more than 10% of the world's adult population is currently obese (WHO 2014a; WHO 2014b). Being overweight presents as a major risk factor for the development of heart disease, hypertension, stroke, certain types of cancer, and diabetes (specifically type 2 diabetes mellitus), which encompass some of the leading causes of preventable death (WHO 2014a; WHO 2014b).

Although obesity arises from biophysiological, psychological, social, and economic factors, epigenetic factors appear to modify physiological processes and to contribute to obesity at the cellular level. Adipogenesis is the key physiological process in fat tissue production by which lipocyte precursors become mature adipocytes (fat cells). A transcription factor called peroxisome proliferator-activated receptor gamma (PPARγ) is considered to be a master regulator of adipogenesis and it activates a large suite of other genes to produce a mature adipocyte (Lefterova et al. 2008; Nielsen et al. 2008). This master regulator of fat cell growth seems to be regulated by and causes some of its effects through epigenetic mechanisms. The promoter of PPARγ is demethylated during adipogenesis, and as a consequence, it activates itself and other targets by increasing the transcription of a histone monomethyl transferase (Fujiki et al. 2009; Wakabayashi et al. 2009).

The dysfunction of epigenetically regulated appetite and reward pathways in the brain may also play a role in obesity. Dopamine is a key player in the reward pathway, and is synthesized in response to a pleasure-invoking stimulus. A chronic high fat diet reduces the activity of dopaminergic pathways in the

brain, which means that an individual needs to eat more in order to receive a sufficient feeling of pleasure and reward (Vucetic et al. 2012). An epigenetic explanation is offered by a study in which rats were fed a high fat diet. They displayed, along with decreased reward circuitry, DNA methylation associated repression of the promoters of genes involved in dopamine synthesis and transport (Vucetic et al. 2012). A high fat diet in rats has also been shown to decrease the expression of the transmembrane opioid receptor (μOR), decreasing the pleasure stimulus that results from eating and thus requiring that a person eat more to enjoy a certain reward level (Vucetic et al. 2011). This decreased expression is correlated with repressive features such as increased DNA methylation, increased H3K9 methylation and decreased H3 acetylation (Vucetic et al. 2011).

Leptin, the hormonal product of the *Ob(Lep)* gene, is produced in adipose tissue and plays a significant role in monitoring the "appetite center" of the brain's hypothalamus (Figure 4.4) (Cripps et al. 2005). The leptin gene has CpG islands in its promoter that can control its expression epigenetically (Melzner et al. 2002). In pre-adipocytes, there is a high degree of DNA methylation and no expression of leptin, whereas mature adipocytes express leptin, correlating with promoter demethylation (Melzner et al. 2002). Constantly high levels of leptin may impair its function in inhibiting appetite via hypothalalmic receptors, however, more research is necessary to fully understand the connection between obesity and the epigenetic regulation of leptin (Ling and Groop 2009).

Our final example of a possible physiological mechanism by which epigenetics may promote obesity through acquired intra-generational modifications may be through maternal "flavour preference" (Mennella et al. 2004). This phenomenon is thought to occur during the period of breastfeeding where flavours/fats ingested by the mother are carried through to the milk (Mennella et al., 2001). Early exposure to specific flavours may alter certain

neurological processes that then lead to a distinctive pleasure reinforcement response when the food is consumed later in life (Delport and Pollard 2010). Whether or not epigenetics plays a role in this development of dietary preference remains to be seen.

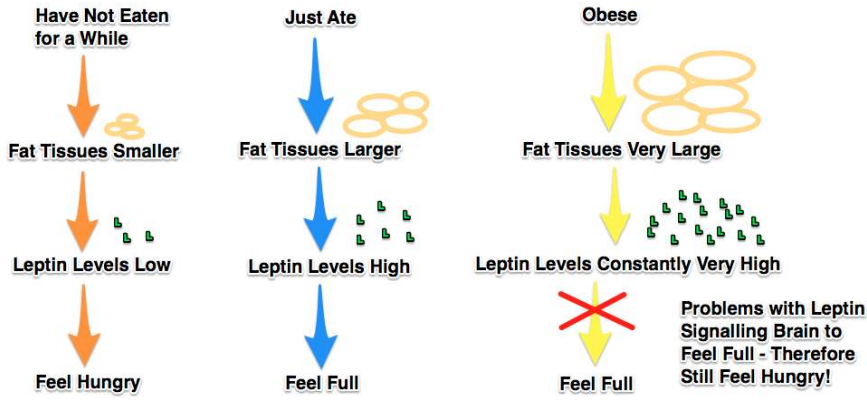

***Figure 4.4**: When you have not eaten in a while and your fat stores are low, you will have low levels of leptin and feel hungry. After you have eaten and your fat stores increase, your leptin levels will increase. Leptin will then signal to your brain to make you feel full. If you are obese and have a constant amount of large fat stores, your body will constantly have high amounts of leptin. However, the constant high amount impairs regulation and your brain does not receive the "fullness" signal. Thus, you feel hungry even when you have plenty of fat stores.*

4.2.2 Diabetes

Diabetes is a chronic disease that occurs either when the pancreas does not produce enough insulin, as in type 1 diabetes, or when the body cannot effectively use the insulin it produces, as in type 2 diabetes mellitus or gestational diabetes mellitus (WHO, 2014b). This can lead to hyperglycemia, or raised blood sugar, which over time leads to serious damage of many of the body's systems, especially the nerves and blood vessels (WHO, 2014b). As of 2011, approximately 347 million people worldwide have diabetes, and the World Health Organization projects it will be the

seventh leading cause of death by 2030 (Danaei et al. 2011; WHO 2014b).

Many studies have supported the idea that epigenetic mechanisms are involved in insulin regulation and diabetes. In pancreatic beta cells, the insulin gene displays active epigenetic marks including hyperacetylation of H4 and hypermethylation of H3 at lysine 4. Importantly, these epigenetic marks are not seen near the insulin gene in other cell types (Mutskov et al. 2007). Furthermore, the transcriptional co-activator PPARGC1A (peroxisome proliferator activated receptor gamma coactivator-1 alpha) gene is involved in mitochondrial oxidative metabolism, and appears to be expressed differently in diabetics versus non-diabetics (Ling et al. 2008). DNA methylation of the PPARGC1A promoter is elevated in pancreatic islets (groups of cells that make the insulin) among patients with type 2 diabetes mellitus compared with healthy subjects (Ling et al. 2008). When PPARGC1A is methylated, its expression is reduced in diabetic islets (Ling et al. 2008). Importantly, PPARGC1A expression is positively correlated with glucose-stimulated insulin secretion in human pancreatic islets, suggesting that this epigenetic axis of regulation may ultimately have an effect on insulin levels (Ling et al. 2008).

4.2.3 Eating Disorders

Like obesity and diabetes, eating disorders also show multivariate origins that are not based solely upon genetic or social anomalies. Epigenetic disturbances may contribute to anorexia nervosa and bulimia nervosa symptoms by affecting neuronal dopamine signaling. This is crucial because the dopaminergic system is involved in the regulation of body weight, eating behaviour, and the reward system (Frieling et al. 2010). In one study, patients showed an elevated expression of dopamine transporter gene activity when compared with control patients, and this was correlated with hypermethylation of the dopamine gene's promoter (Frieling et al. 2010). This means that individuals

exhibiting anorexia nervosa or bulimia nervosa symptoms show altered dopamine function compared with individuals without a disorder. Although down-regulation of the dopamine receptor 2 gene was seen in both nervosa groups, it was accompanied by hypermethylation of the gene's promoter only in patients with anorexia nervosa (Frieling et al. 2010).

Atrial natriuretic peptide (ANP), a hormone that has anti anxiety effects, may also have implications in the pathophysiology of eating disorders via epigenetic regulation (Strohle et al. 2001; Frieling et al. 2008). Patients suffering from both anorexia nervosa and bulimia nervosa showed decreased activity of the ANP gene, correlated with hypermethylation of the ANP gene's regulatory region (Frieling et al. 2008). However, whether these epigenetic changes are causes or effects of eating disorders are questions that require further research (Frieling et al. 2010).

4.3 Inter-Generational Epigenetic Effects - Overfeeding and Supplementation

In addition to intra-generational effects contributing to the increased incidence and prevalence of multivariate diseases, inter-generational effects probably also play a role in the development of these diseases. This first part of this section focuses on inter-generational effects that arise from overfeeding, specifically with respect to the development of obesity and diabetes. Over the last few decades, the prevalence of obesity and diabetes among children and adolescents has increased dramatically (Pinhas-Hamiel and Zeitler 2005; CDC 2014b). An explanation for these trends suggests that both adult and childhood obesity and diabetes rates may be due to a cyclical propagation of these diseases through generations. This propagation could be mediated by epigenetic modifications in utero, also known as **developmental programming** (Gabory et al. 2011). Furthermore, the mechanisms by which epigenetics plays a role in predisposing these conditions are likely similar to those mentioned in Section 4.2.

4.3.1 Obesity

Since inter-generational epigenetic effects are often due to changes that are imposed during the fetal stage of life, these environmental challenges are thus transmitted to the fetus via the maternal environment. This means that examining the nutritional state of the mother during pregnancy could provide insight into the development of obesity and other diseases in the offspring.

Numerous studies have reported that maternal pre-pregnancy body mass index (BMI) and gestational weight gain are positively associated with the BMI of offspring at birth as well as during infancy, childhood and early adulthood (Gale et al. 2007; Mamun et al. 2009; Rooney et al. 2011). At the time of birth, infants born to obese women tend towards increased adiposity (fat tissue), insulin resistance, and cord blood leptin levels (Battista et al. 2011). Another study suggests that children born to obese mothers are twice as likely to be obese by two years of age (Whitaker 2004). Furthermore, large cohort studies point to an increase in adolescent and adult obesity for at least 25 years if individuals weigh more at birth (Curhan et al. 1996a; Curhan et al. 1996b). A study in rats has supported the idea that maternal obesity is a risk factor for later obesity in offspring. First, obesity was induced in rat dams (mothers) through an obesogenic liquid diet before mating and throughout pregnancy (Shankar et al. 2008). After birth, the pups were cross-fostered to lean dams. Compared to those born from lean dams, the offspring from obese dams exhibited increased adiposity and insulin resistance (Figure 4.5) (Shankar et al. 2008).

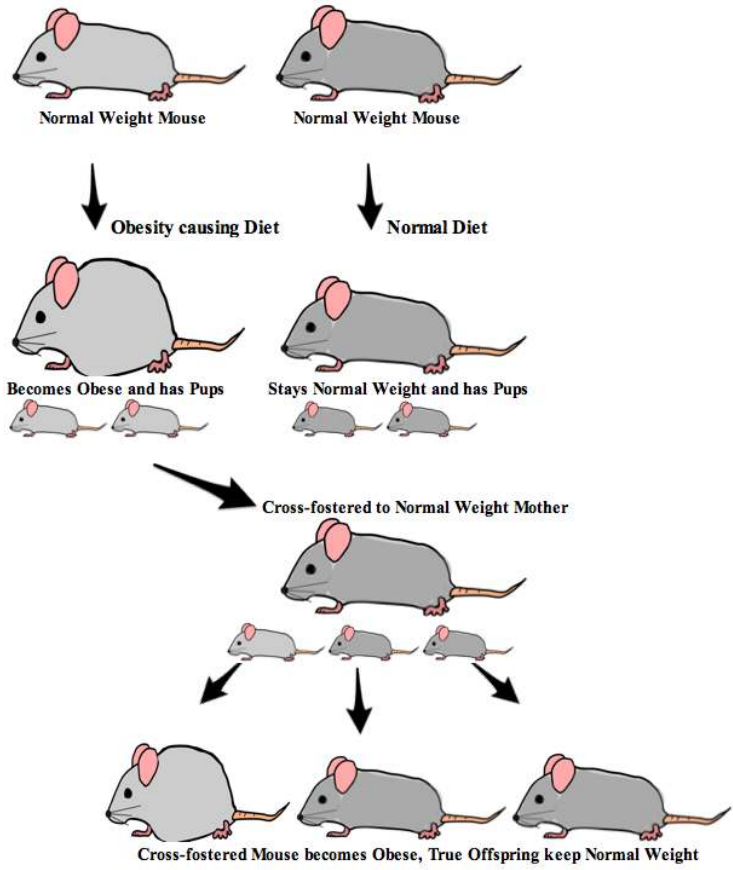

***Figure 4.5**: Obesity was induced in a normal weight mother (therefore, the propensity for obesity was not due to genetics). The pups from the obese mother were then cross-fostered - that is, raised and fed by a normal weight mother. These pups that were cross-fostered ended up with increased adiposity compared to the true offspring of the cross-foster dam.*

4.3.2 Diabetes

Similar to the development of obesity, looking at the nutritional state of the mother may provide important clues as to the mechanism by which diabetes is propagated. Population based studies have demonstrated that the offspring of diabetic mothers have an increased risk for glucose intolerance and type 2 diabetes mellitus (Silverman et al. 1995). A study of the Pima Indians of

Arizona, who have the world's highest incidence and prevalence of diabetes, showed a genetic transmission component (Knowler et al. 1978). The study also revealed that the cycle of inter-generational diabetes can be explained by environmental factors, since the strongest single risk factor for type 2 diabetes mellitus was exposure to maternal diabetes in utero (Dabelea et al. 1998). Children born to diabetic mothers showed a 10-fold increase in the prevalence of diabetes by early adulthood compared with children whose mothers did not develop diabetes until after delivery (Heerwagen et al. 2010). This inheritance could be in part due to exposure to a diabetic intrauterine environment (Dabelea et al. 2000).

In another study, individuals whose cord blood or amniotic fluid insulin levels were elevated had a three to fourfold risk of developing glucose intolerance and type 2 diabetes mellitus during childhood (Pinney and Simmons 2012). This suggests that persistent fetal exposure to hyperinsulinaemia in the uterine environment contributes to the development of diabetes through epigenetic mechanisms. This notion is supported by studies showing that impaired glucose tolerance of adolescent offspring is associated with maternal hyperglycemia and not with the specific type of diabetes of the mother (Silverman et al. 1995). A study in rats also showed that although an intrauterine environment that provides normal glucose concentration cannot overcome a genetic predisposition to diabetes, a hyperglycaemic intrauterine environment significantly increases the risk of diabetes in the adult life of the offspring (Gill-Randall et al. 2004).

4.3.3 Maternal Diabetes and Offspring Obesity

Obesity and type 2 diabetes mellitus are closely related; consequently, there are many studies where maternal diabetes seems to predispose offspring to obesity. One study showed that newborn infants of women with gestational diabetes have 20% more body fat than infants of women with normal glucose

tolerance, regardless of birth weight (Catalano et al. 2003). The aforementioned Pima Indian study also looked at the differing BMIs of siblings born before their mother was diagnosed with diabetes compared to those born after the diagnosis. The results showed that the mean BMI was 2.6kg/m^2 higher in offspring of diabetic pregnancies than in offspring of non-diabetic pregnancies (Dabelea et al. 2000). In contrast, there were no significant differences in risk of diabetes or BMI between offspring born before and after the father was diagnosed with diabetes (Dabelea et al. 2000). This is similar to what was discussed in 4.3.2 and therefore supports the idea that this difference might also be based on exposure to a hyperglycemic uterine environment.

Researchers have also studied the effect of maternal diabetes on offspring using a mouse model of insulin deficiency created via injections of streptozotocin, a chemical that induces a diabetic-like state (Steculorum and Bouret 2011b; Steculorum and Bouret 2011a). Insulin deficient dams had offspring with significantly higher pre and post-weaning body weight compared to the offspring of control mice. Moreover, they had increased fasting glucose levels, increased insulin levels, and displayed increased food intake during their adult life (Steculorum and Bouret 2011b; Steculorum and Bouret 2011a).

Insulin deficiency and hyperglycemia during pregnancy might also exert inter-generational effects in offspring metabolic regulation via leptin resistance. In the study previously mentioned, streptozotocin injections caused the offspring to develop abnormally organized feeding pathways in the hypothalamus. As adults, these offspring showed increased food intake and leptin resistance (Steculorum and Bouret 2011b; Steculorum and Bouret 2011a). This reduced ability of leptin to activate neurons within the hypothalamic feeding pathway may contribute to the mechanism by which uterine hyperglycemia leads to obesity. Further study is required to discover any epigenetic mediator of this long lasting environmental effect.

The *maternally imprinted mesoderm-specific transcript* (MEST) gene is involved in growth of the embryo and placenta, and is also implicated in a mechanism by which a hyperglycaemic uterine environment could predispose offspring to obesity (Lefebvre et al. 1998). MEST showed decreased methylation levels in the umbilical cord blood and placentas from mothers with gestational diabetes compared to samples from mothers without the condition (El Hajj et al. 2013). In other studies, an enlargement of adipocytes and fat mass expansion occurs where MEST is overexpressed (Kamei et al. 2007). Likewise, decreased blood MEST methylation levels were observed in adults with morbid obesity when compared with normal control animals (El Hajj et al. 2013). In sum, these observations suggest that decreased methylation of the MEST gene, possibly due to a diabetic in utero environment, could cause its over-activity leading to increased fat cell growth in the offspring later in life.

4.3.4 Maternal Supplementation

A change in gene activity due to diet-related epigenetics changes is associated with the development of other diseases, including schizophrenia and cardiovascular diseases, among others (Kirkbride et al. 2012a; Kirkbride et al. 2012b). Methylation of these gene regulatory regions can sometimes be restored to their original state using dietary supplements administered during pregnancy. Deficiencies with respect to methyl donors – the metabolic substrates that provide the methyl groups for epigenetic modifications– such as vitamin B12 and folate, are responsible for decreased methylation within genes and regulatory regions (Kirkbride et al. 2012a). Numerous studies have revealed that neural tube defects can be significantly reduced by vitamin B12 or folic acid supplementation during early pregnancy (Brown et al. 1996; Susser et al. 1996; Kirkbride et al. 2012a). In fact, studies show that the offspring may develop increased cognitive function and a reduced risk of autism by supplementation with these methyl

donors (Schmidt et al, 2011; Kirkbride et al., 2012a). In addition to vitamin B12 and folic acid, a methyl donor proven to have long-term epigenetic effects is a protein called genistein, a soy-derived isoflavone and phytoestrogen. This nutrient has been shown to shift coat color distribution in mice via methylation of sequences near the agouti gene (Dolinoy et al. 2007). In addition to changing the coat colour phenotype, genistein also protects offspring from obesity (Dolinoy et al. 2006). Another study concluded that methyl donors can reverse demethylation caused by the estrogen mimic bisphenol A (BPA). BPA was identified as a common contaminant that leached from food packaging and plastics. In the study, BPA perturbed coat colour, obesity, and diabetes in mice (Dolinoy et al., 2007). To conclude, these studies support the hypothesis that maternal diet may play a role in modulating the epigenome. Thus, not only does the type and quality of food have an effect, but also the quantity.

4.4 Potential Inter-generational Epigenetic Effects - Dietary Restrictions and Starvation

As previously explained at the beginning of this section, inter-generational epigenetic effects arise due to maternal exposure to an environmental event while the offspring is still in utero. While the first half of Section 4.3 focused on the effects of overfeeding and supplementation on the epigenome and thus offspring development, this half will focus on the effects of dietary restrictions and starvation. In sum, the environmental event or challenge here is a decreased level in the quality or quantity of the diet.

CASE STUDY: Nutrition Supplements in Guatemala

A nutritional supplement study was conducted between 1969 and 1977 in El Progreso, Guatemala. The results of this study may provide information about the relationship between nutrition and the epigenome (Behrman et al., 2009). This study differs slightly from the above studies in that the supplements were given while the mother was still in her youth, before pregnancy and even prior to puberty. Two villages were given a high calorie protein supplement drink called *atole* while two similar villages were given *fresco*, which was low in calories and did not contain protein. Records of supplement consumption were kept for children aged 0 – 7. Subsequently, during a follow-up, a comparison of the offspring of the women who received these different supplements as children were evaluated on measures of birth weight, height for age scores, and weight for age scores (from ages 0-12). Children, particularly sons, of mother's who received *atole* had a significantly higher birth weight, height, and weight (Behrman et al., 2009). Therefore, the results of this study support the relationship between supplements and an altered offspring phenotype, perhaps through altered methylation as the epigenetic mechanism.

While the first half of Section 4.3 focused on the effects of overfeeding and supplementation on the epigenome and thus offspring development, this half will focus on the effects of dietary restrictions and starvation. In sum, the environmental event or challenge here is a decreased level in the quality or quantity of the

food consumed.

4.4.1 Dietary Restrictions

The term "dietary restrictions" may imply restricting caloric intake to some drastic level, much like extreme dieting. However, for the present instance, "dietary restrictions" will mean total caloric intake, while still providing essential nutrients. In terms of epigenetics, dieting in this sense is considered to be one of the most effective methods of achieving a maximum lifespan (Tollefsbol 2014). Reducing total caloric intake by 25–60% while still providing essential nutrients led to a 50% increase in lifespan in rhesus monkeys. This is due to DNA methylation affecting the activity of histone deacetylases (enzymes introduced in Chapter 2) (Tollefsbol 2014).

Another form of dietary restriction involves limiting glucose intake. However, the epigenetic results of glucose restriction differ depending on cell type. In most cells, glucose restriction causes an increase in *hTERT* (human telomerase reverse transcriptase) and a decrease in *p16*, a gene that slows cellular replication. Both components have the potential to enhance life expectancy. Possibly, one way that they might achieve this is via their effects upon pre-cancer cells: *hTERT* and *p16* can induce **apoptosis** (programmed cell death) of the cells (Figure 4.6) (Li et al. 2010). Thus, dietary restrictions can have a profound impact on gene expression in different cell types.

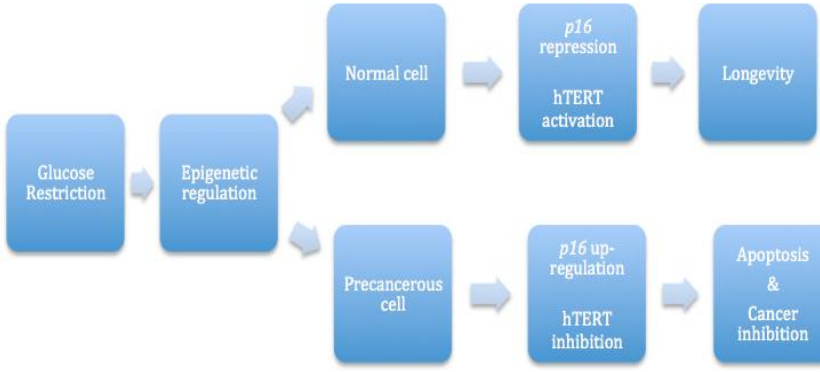

***Figure 4.6**: This flowchart describes the differing impact of glucose restriction on precancerous and noncancerous cells.*

Malnutrition, however, is the dietary restriction of all foods and therefore has negative consequences for the body. There is evidence to indicate that **telomere** shortening, a sign of cellular senescence, is accelerated by malnutrition: a protein, TORC1, that is deployed in the maintenance of telomere length in organisms as diverse as yeast and humans mediates this effect (Ungar et al., 2011). In humans, telomere shortening is cumulative and irreversible: acute fetal malnutrition results in shortened telomeres even if an adequate diet is restored (de Rooij and Roseboom, 2013). Therefore, as was concluded at the end of the Maternal Supplementation subsection, not only does the quality of the diet affect the epigenome, as is seen with the development of diseases such as obesity and diabetes (discussed earlier in this chapter), but the quantity of the diet in terms of restricting both calorie and glucose consumption has also been proven to affect a person's lifespan.

4.4.2 The Dutch Famine

Near the end of World War II, German forces imposed restrictions on the transportation of supplies to the west

Netherlands between October 1944 and May 1945 (Lumey 1992; Roseboom et al. 2011). It is estimated that the food intake dropped to below 1000 calories a day per person (Burger, 1948 as cited in Lumey, 1992; Roseboom et al., 2011). This defined time period, location, and level of malnutrition provides a level of control not always possible with retrospective studies, and it represents an exceptional opportunity to examine the epigenetic effects of nutrition in people who lived through this event. The long term consequences of the Dutch Famine on the health of prenatally affected individuals was marked. A broad range of adult onset symptoms and diseases appeared in time-sensitive (exposure/onset) (Ravelli et al. 1999; Roseboom et al. 2006) and gender-specific ways (Ravelli et al. 1999; Tobi et al. 2009).

Timing-specific effects

Studies show that regardless of the fetus' gender, early gestation is the most vulnerable time, since this is the busiest trimester in terms of establishing the body plan of an embryo. Famine exposure was shown to increase risk of obesity and coronary heart disease much later in life, and to raise prognostic indicators such as lipid levels and blood clotting dynamics (Roseboom et al. 2006). Some of the later onset metabolic and cardiovascular diseases may be due to changes in the hypothalamic-pituitary-adrenal response to stress, which was also higher in these individuals.

During mid gestation, organs continue to develop and the fetus grows in size and weight (Roseboom et al. 2006). Thus, a fetus affected during this stage would experience organ and growth-specific impairments. Late-onset consequences include renal dysfunctions related to diabetes, as well as obstructive airway diseases (Roseboom et al. 2006).

Fetuses exposed to famine during the third trimester exhibited the common trend of decreased glucose tolerance (Roseboom et al. 2006), independent of birth size and body

proportions. Although fetuses exposed at this time point had the lowest birth weights, overall they were the least affected by the famine (Stein et al. 2004). Furthermore, an increase in hypertension and high blood pressure was seen in both men and women who were exposed during gestation, compared to their same sex siblings who gestated under normal nutritional circumstances (Stein et al. 2006).

Recent studies suggest that both men and women conceived at the height of the famine had a two-fold increased risk of developing schizophrenia (Susser et al. 1996). Schizophrenia can be caused by the reduced expression of the *Reelin* gene. Since this gene's regulation is dependent upon DNA methylation, an epigenetic mechanism may be implicated (Kirkbride et al. 2012b).

Finally, fetuses exposed to famine during any point in gestation were more likely to have decreased glucose tolerance and increased rates of type 2 diabetes mellitus.

Gender-specific effects

Many studies provide evidence to support the idea of gender-specific effects due to famine exposure. One study found modifications in methylation levels of two genes involved in cardiovascular disease that were restricted to men (Tobi et al. 2009). In terms of obesity, both male and females who were prenatally exposed to the famine displayed abnormal fat distribution in adulthood (Ravelli et al. 1999). The gender specificity of obesity is determined by the timing of fetal exposure. Men exposed to the famine during early gestation had an increased chance of becoming obese in adulthood, while those exposed during late gestation had a decreased chance of obesity later on in life. For females, there was an increase in obesity only if they were affected by the famine in early gestation, and were properly nourished throughout the rest of the pregnancy (Ravelli et al. 1999). The suggested mechanism is a modified central endocrine regulatory mechanism. Table 4.1 provides a summary of the

differences between men and women who were exposed to the famine at different periods during gestation.

Gender	Early gestation	Mid gestation	Late gestation
Women & Men	Glucose intolerance	Glucose intolerance	Glucose intolerance
	Altered blood coagulation	Renal dysfunction	
	Stress sensitivity		
	Coronary heart disease		
	Increased breast cancer risk		
	Changes in fat distribution		
	Increased chance of obesity		
Women	Higher cholesterol levels	Higher cholesterol levels	Higher cholesterol levels
			Increased fat depositions at age 59
			Higher BMI at age 50
Men	Changes in methylation at 1 gene related to cardiovascular health	Changes in methylation at 1 gene related to cardiovascular health	Changes in methylation at 3 genes related to cardiovascular health
			Decreased chance of obesity

Table 4.1: *Famine impacts on fetuses are specific to the timing of famine exposure and fetal gender.*

In summary, those conceived during the famine had: an increased risk of schizophrenia and depression; a more severe atherogenic plasma lipid profile; adverse responsiveness to stress; and twice the rate of coronary heart disease. They also performed worse in cognitive tasks, which is a sign of accelerated aging. Moreover, a higher incidence of type 2 diabetes mellitus and increased glucose intolerance were observed in both genders.

A possible mechanism by which exposure to an environmental challenge, such as the Dutch Famine, in utero could result in long-term health consequences for the adult offspring is provided by the fetal origins hypothesis (Roseboom et al. 2006). This hypothesis implies that adaptations for the survival of the fetus occur at the potential cost of long-term consequences

(Roseboom et al. 2006). Stress in utero may "program" changes that enable the child to cope with an immediately challenging environment while coincidentally leading to the development of disease later in life, perhaps via an altered hypothalamic-pituitary-adrenal response to stress (Gluckman et al. 2005a; Gluckman et al. 2005b; Roseboom et al. 2006; Bogdarina et al. 2007). Possibly, if the environment experienced in utero fits the actual environment after birth, the fetus will thrive; however, if mismatched, the individual may suffer a pre-disposition to disease later in life.

Numerous Dutch Famine studies provide mechanistic evidence to support the fetal origins hypothesis (Painter et al. 2005; Roseboom et al. 2006). For example, the *insulin-like growth factor 2* gene *(IGF2)* is central to many of the results in the Dutch Famine studies. This gene plays a crucial role in early development, and plays a role in brain growth and development. Certain epigenetic modifications to this gene and adjacent sequences may later result in the development of schizophrenia (Pidsley et al., 2012: (Kirkbride et al. 2012b). Early data supporting the idea that prenatal exposures have long lasting epigenetic effects was based on the methylation levels tested at the *IGF2* locus. At the age of sixty, the women who experienced the famine in utero showed hypomethylation of the *IGF2* locus compared to women who were not exposed in utero (Heijmans et al., 2008). Although the level of methylation on this gene was not associated with birth weight, the level of methylation increased with age (Heijmans et al. 2008). A separate study monitored methylation levels of the *IGF2* gene in relation to a methyl donor, namely folic acid (Hoyo et al. 2011). It demonstrated that low methylation of these regions deregulated the *IGF2* gene, but that this could be reversed with folic acid intake. This was also demonstrated in a rat study, where folic acid had a permanent restorative effect upon methylation levels on the *IGF2* gene, even after the nutrient was removed from the rats diet (Waterland et al. 2006).

These studies indicate a potential for epigenetic

intervention via dietary rectification of methylation levels. The significance of the timing of fetal exposure in determining the gender specificity of many of the **epiphenotypes** described above suggests an interaction between sex hormones and DNA methyl transferases (DNMTs), enzymes that play a role in methylation, and this will be important to study in the future (Tobi et al. 2009).

4.4.3 Crohn's and Coeliac Disease

If epigenetic changes can be induced in fetuses of starving mothers, then it is reasonable to assume that diseases that disturb adequate nutritional absorption could also cause epigenetic modifications. Examples of these diseases include **Crohn's disease** and **coeliac disease**. In Crohn's disease, the preponderance of female patients may be related to the female-to-female specific imprinting of a predisposition to the disease (Zelinkova et al. 2012). This is supported by a speculative study that showed no sex-specific pattern for Crohn's disease when the disease was transmitted through the father, but showed significantly higher rates of daughters being affected when the disease was inherited maternally (Zelinkova et al. 2012). These gender specific effects are reminiscent of the inter-generational effects of the Dutch Famine, but no mechanistic evidence of epigenetic phenomena have yet been identified in the evolution of these diseases.

The relationship between epigenetics and coeliac disease has also been examined. Parental coeliac disease may contribute to the etiology of non-syndromic cleft lip and palate through epigenetic mechanisms. Unlike Crohn's disease, both maternal and paternal coeliac disease appears to perturb offspring equally, and the effect is postulated to be mediated by genomic imprinting (Arakeri et al. 2010). Again, since no mechanistic evidence is presented that links the phenomena to epigenetics, the hypothesis is interesting, but speculative.

4.5 Potential Trans-generational Epigenetic Effects

An epigenetic effect cannot be said to be truly trans-generational unless an exposure in one generation produces a measurable outcome in a following generation that was never exposed to the altering factor (Pembrey et al. 2013). Few studies on the trans-generational effects of nutrition have been conducted, so retrospective studies on human cohorts have provided an alterative approach. As was explained in Section 4.3, the defined duration, location, and level of malnutrition during the Dutch Famine has provided an important cohort from which to study the effects of nutrition upon the health of future generations. There is also another cohort, the Överkalix cohort, which has been used to infer the effects of nutrition cycles upon the epigenome and the predisposition to disease in future generations. Unlike the Dutch cohort, the Overkalix data relies solely upon demographic measures: investigators have yet to examine the epigenetic biochemistry of potentially affected genes. Both of these cohorts will be examined in this section in order to illustrate effects that are good candidates for epigenetic modifications can be passed down trans-generationally.

4.5.1 The Dutch Famine

Nutritional deficits during fetal development not only result in intra-generational epigenetic effects, transferred from mother to fetus, but also promote defects through subsequent generations (Skinner and Guerrero-Bosagna 2009). Pregnant women and their fetuses experienced this starvation. Although fetal death due to malnutrition was high, many of those that did survive nevertheless developed normally and went on to have children of their own (deJong et al., 1981 as cited in Lumey, 1992). The study of these children constitutes the bulk of the trans-generational studies done on humans. As with the inter-generational studies, the relationship between the timing of in utero famine exposure of fetuses that went on to become mothers, and its effect upon the birth weight of this generations' firstborn offspring was examined (Figure 4.7).

Studies conducted using these children looked at both timing- and gender-specific effects, similar to what was described in Section 4.3.

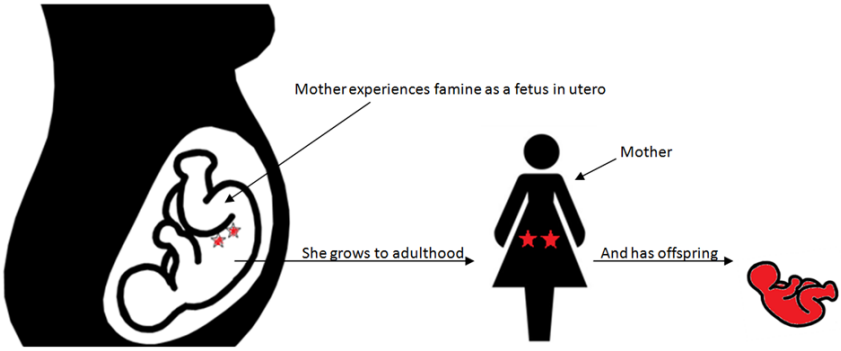

Figure 4.7: *Generational terminology as a pictogram. The term mother in the following section refers to the female fetus grown up, and the term offspring refers to her children (red).*

Timing-specific effects

The fetuses that were exposed to famine in the first trimester (early gestation) later as adults delivered firstborn babies that were statistically lower in birth weight, even if the mothers' own birth weight was not depressed (Lumey 1992). A lack of similar results in the parents exposed during their second and third trimester suggests that epigenetic modifications are more likely to occur during early gestation. Contrarily, a second study of a different cohort found that mothers exposed to the famine in the first trimester had babies within the normal weight range, however instead of delivering subsequent babies with progressively heavier birth weights (the normal trend), the firstborn was the heaviest, and subsequent offspring were progressively lighter (Lumey et al. 1995). Although the results are not consistent, they reveal that the mother's nutrition in the womb may have an effect on the offspring's birth weight. Why is this important? It is important because low birth weight is associated with increased susceptibility to cardiovascular disease, obesity, and type 2 diabetes during adult life (Jimenez-Chillaron et al. 2009).

Gender-specific effects

Subsequent studies looked at both men and women exposed to malnutrition at an early stage of their fetal life and its effect on their offspring (Painter et al. 2008). The children of undernourished female fetuses were found on average to be shorter, have a higher ponderal index (adiposity) at birth, and have higher rates of autoimmune disease later in life (Painter et al. 2008; Roseboom et al. 2011). The children of men exposed in utero had a higher BMI and weight, while women's children showed no difference in either characteristic (Veenendaal et al. 2013). Gender specific trans-generational effects of in utero exposure are still being studied, as is the effect of the timing of famine exposure. Thus far, an effect of lower overall health can be seen: there is an association between the in utero nutrition of your mother and father, your birth weight, and health later in life.

Supporting Mammalian Studies

Prenatal underfeeding

Experiments on rodents and other mammals also demonstrate both timing-specific and gender-specific results, providing support to the retrospective Dutch Famine studies. One study revealed the effects of a parental (F0) protein restricted diet during pregnancy and/or lactation upon the grand-offspring (F2) generation in rats (Figure 4.8) (Zambrano et al. 2005). It should be noted that the rodent lactation period is developmentally equivalent to the third trimester in humans, and it is usually folded into the accounting as such. First generation females (F1) received a low protein diet in utero or during initial feeding. This affected the glucose and insulin metabolism of their offspring (F2). Female F2 offspring maintained higher glucose and lower insulin concentrations than F2 males. These results depended on the timing of the protein restriction of their parents, whether it was only during pregnancy, only during lactation or during both pregnancy and lactation. Both male (if restricted during lactation)

and female (if restricted during pregnancy) F2 showed decreased insulin sensitivity (Zambrano et al. 2005). This indicates that maternal protein restriction timing affects male and female offspring differently.

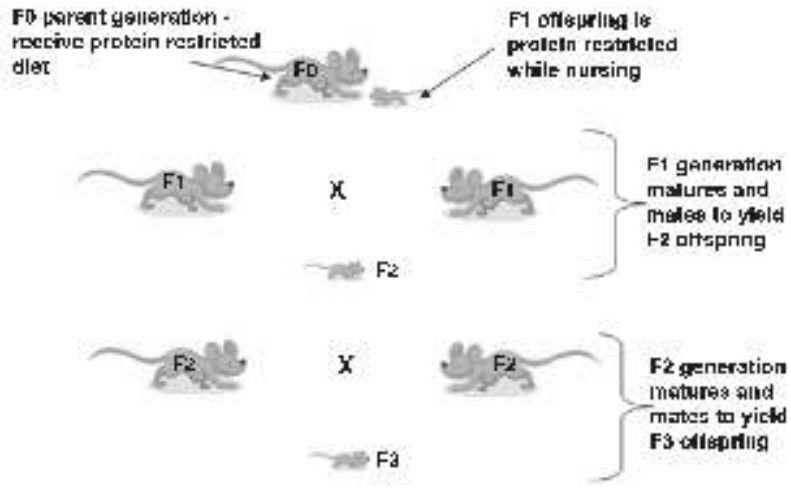

Figure 4.8: *Generational designations (F0, F1, F2, and F3) for rat studies.*

In another study, the female F1 generation was exposed to either an adequate or protein-deficient diet in utero and during early development (Benyshek et al. 2006). The F2 generation was found to be insulin resistant and the great-grandchildren (F3) showed altered glucose homeostasis. The F3 generation showed gender specific effects. F3 females exhibited higher glucose levels and F3 males exhibited lower glucose levels than controls, though a trend towards normalization could be seen in both cases (Benyshek et al. 2006). Even though the magnitude of the epigenetic effects lessened, they persisted as far as the great-grandchildren!

In Jimenez-Chillaron et al.'s (2009) study, F0 females received either a normal diet or a calorie restricted diet (50%) throughout pregnancy. Therefore both male and female F1

offspring were malnourished in utero. This resulted in impaired glucose tolerance and insulin resistance in both male and female F2 offspring. This was found to be passed through both maternal and paternal lines. F2 males and females had increased adiposity, which progressed through the maternal line. However, in contrast to retrospective human studies, low birth weight in mouse F2 offspring was affected by their fathers, the F1 malnourished males (Jimenez-Chillaron et al. 2009). Human studies initially revealed only a maternal effect Lumey (1992), however the Överkalix study has since revealed the potential for transmission through both parents. Therefore, these studies support the idea that protein restriction has an effect on inherited glucose tolerance and insulin resistance, but that the gender-specific effects may vary according to species or the nature of the dietary challenge.

Prenatal Overfeeding

In contrast to studies where rats were placed on a restricted diet, Burdge et al. (2011) put rats on a high protein diet. Three generations of mothers (F0, F1, and F2) were given this increased protein diet during pregnancy and lactation. It was found that the high protein diet improved the ability of offspring to regulate glucose and fatty acid metabolism. Here the F1, F2, and F3 generations are the analyzed offspring. The high protein diet led to an increased ability to maintain glucose and lipid levels and to regulate body weight by the F2 generation. Such changes were also inherited by the F3 generation. This suggests that, with regard to over-feeding, both the in utero environment (intergenerational epigenetic changes) as well as the modified maternal epigenome (trans-generational epigenetic changes) contribute to affect traits in offspring (Burdge et al. 2011). What does this all mean for humans? Glucose regulation and metabolic alterations can be passed through generations. This is important, since insulin resistance is known to be related to heart disease, high blood pressure, obesity, and diabetes, which are all related to a poor

health prognosis later in life.

4.5.2 Överkalix: Cycles of Overfeeding and Malnutrition

Överkalix, Sweden, experienced periods of low food production interspersed by periods of high food availability throughout the 1800's. During high food availability periods, residents overfed as compensation for the periods of malnutrition (Kaati et al. 2007). The Överkalix cohorts provide us with distinct periods of varying food availability and defined groups of individuals for study, much like the Dutch Famine cohort. Before we delve into the findings, some background information is necessary to fully understand the studies. A male's slow growth period (SGP) occurs between the ages of 9-12 and a female's between the ages of 8-10 (Bygren et al. 2001; Pembrey et al. 2006; Kaati et al. 2007). This slow growth period is the period just before puberty, leading up to the pre-pubertal growth spurt (Bygren et al. 2001).

Kaati et al. (2002) looked into the effects of overfeeding and famine exposure during the slow growth period of grandparents and parents and tied them to health changes in the grandchildren. They found that nutritional changes had trans-generational effects with respect to the risk of diabetes and cardiovascular disease, mainly through the male line (see figure 4.9). If the paternal grandfather was overfed during his slow growth period, his grandchildren were likely to have a shorter lifespan and a four-fold risk increase of death due to type 2 diabetes mellitus (Kaati et al., 2002). By contrast, if his exposure was to famine, he passed on a resistance to diabetic death among his grandchildren (Kaati et al., 2002; Bygren et al., 2001). Therefore, paternal grandfather overfeeding in his slow growth period increases health risks in grandchildren and underfeeding decreases them. Paradoxically, overfeeding during the slow growth period of the father offers protection from diabetes as the cause of death (Kaati et al., 2002). Paternal famine exposure and maternal

overfeeding during the SGP decreased the risk of cardiovascular disease of the offspring (Kaati et al., 2002; Pembrey et al., 2010). The paternal grandmother's exposure to famine also decreased the risk of cardiovascular disease in grandchildren (Kaati et al., 2002). These complex results demonstrate how your parents' and grandparents' eating patterns may have an effect on you (Table 4.2 and Figure 4.9).

Supporting Mammalian Studies

Mammalian studies support a male-mediated pattern of results evident in humans. In contrast to epigenetic modifications that may result from in utero experiences that are dependent solely upon the mother, paternal transmission must involve epigenetic modifications before or during spermatogenesis. Ng et al. (2010) tested whether the diet of male rats had an effect on offspring health using males fed a high fat diet or control diet throughout life. The paternal high fat diet was linked with decreased glucose tolerance and insulin secretion in female offspring, which is similar to the finding in humans that paternal obesity is linked to increased risk of diabetes in children (Ng et al. 2010). Specifically, the pancreatic β -cells that produce insulin were found to have altered expression of 642 genes. These expression differences are thought to be caused by epigenetic mechanisms.

How risk of death due to diabetes is affected by nutrition

	Overfed	Exposed to famine
Father	Increased	----
Paternal grandfather	Increased	Decreased

How risk of cardiovascular disease is affected by nutrition

	Overfed	Exposed to famine
Father	------	Decreased
Mother	Decreased	------
Paternal grandmother	------	Decreased

Table 4.2: *The effects of overfeeding or famine exposure during the SGP as found by Kaati et al. (2002). The dashed lines indicate that the results were not mentioned for these particular conditions.*

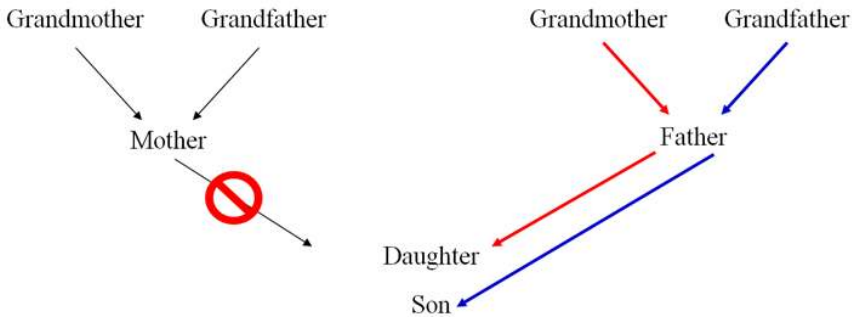

Figure 4.9: *Flow chart depicting male line pattern inheritance. It is through transmission by the grandchild's father that grandparental effects occur. Furthermore, gender effects were found to occur: Kaati (2007) found overfeeding in the father's slow growth period increased mortality rate in sons. However, these results were not significant in Bygren et al.'s 2001 study. Only the paternal grandfather's access to surplus food that showed a negative effect on lifespan (Bygren et al., 2001). Furthermore they found that both parents' food intake affects the daughter's mortality, where good parental food conditions increase mortality risk and poor conditions decrease it. They also determined that paternal grandfather's food supply during his slow growth period affects the mortality risk only of grandsons (high food intake means higher risk of mortality and vice versa) and that the paternal grandmother's food supply in fetal, early life, and slow growth period only affects granddaughters (Pembrey et al., 2006; Kaati et al., 2007). This suggests that the father's sperm may carry information about the ancestral nutrition from either his mother or his father (Kaati, 2007).*

For instance, hypomethylation was seen in the Ill3ra2 gene, the gene that showed the highest difference in expression (Ng et al. 2010). In another study, male mice born to overfed mothers passed features of metabolic syndrome (insulin resistance and impaired glucose tolerance) onto their male offspring and grandchildren, though there was a progressive weakening of the phenotype (Pentinat et al. 2010). This demonstrates male-line inheritance, supporting Kaati et al.'s (2007) human studies. In summary, these studies support the idea that environmental effects causing obesity in males may have long-term health implications such as obesity and diabetes in their offspring and grandchildren, possibly transmitted via epigenetic modifications.

Trans-generational Mechanisms

The last question you may be wondering is: "if these trans-generational effects are due to epigenetics, then how are these epigenetic changes passed through the generations?" Epigenetic inheritance is a strong candidate for three reasons; some epigenetic states can be transmitted to the next generation(s); specific nutritional exposures can alter the epigenetic state; and trans-generational methylation responses to specific exposures have been shown in experimental animals (Kaati et al. 2007). This type of research is barely underway, and technology has only recently been developed to examine epigenetic modifications. So the short answer is we do not know – but we have some ideas, and a few hypotheses have been advanced.

1. The Feed-forward Control Loop

Similar to the fetal programming hypothesis for inter-generational epigenetic mechanisms, a **feed-forward control loop** has been proposed as the link between grandparents' nutrition and the grandchild's growth (Pembrey 1996; Bygren et al. 2001; Kaati et al. 2002). The mechanism could be a specific response to the

parents' nutritional state, which directly modifies the setting of the gametic (sex cell) imprint on one or more genes determining growth in the offspring (Pembrey, 1996). For example, a change may occur in an individual's epigenome or the epigenetic imprint of their gametes by starvation during their slow growth period. This imprint may facilitate the efficient conservation of calories and may be passed to successive generations. If starvation conditions continue, future generations will already be epigenetically prepared for them.

2. Sex Cell and Sex Chromosome Involvement

The involvement of the sex chromosomes in trans-generational effects was suggested by Pembrey et al. (2006). The role of the Y chromosome might explain paternal grandfather to grandson effects as these could be mediated by an imprint carried on the Y chromosome. Perhaps something about the unmatched Y and X chromosome in fathers permits a different erasure and re-establishment of an epigenetic imprint? The X chromosome, along with any epigenetic modifications, would therefore be passed from grandmother to granddaughter via an intermediary father only, explaining paternal grandmother to granddaughter effects (Pembrey et al., 2006) (Figure 4.10). Alternatively, the trans-generational transmission of imprinted states could have nothing to do with the sex chromosome *per se*, but instead reflect the differential capacity of sperm to carry miRNA that are packaged in response to environmental challenges: these miRNAs would presumably impart cues for subsequent epigenetic modification (Dias et al., 2013; Marczylo et al., 2012).

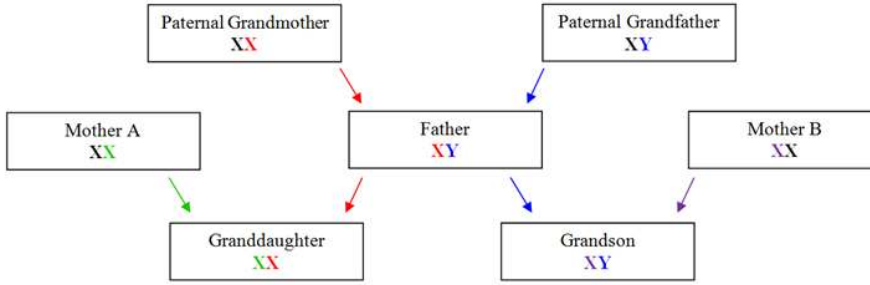

Figure 4.10: *The blue pathway indicates paternal grandfather to grandson effects. The red pathway indicated paternal grandmother to granddaughter effects.*

The sex cell hypothesis has several attractive attributes. For example, environmental changes during specific times in gametogenesis could modify sex chromosome epigenetic imprints (Pembrey et al., 2006). Since trans-generational effects must involve the germ line, this process would not be expected to have the same timing in males and females. As was explained in the Introduction, gametogenesis occurs later in males (Pembrey, 2013). Spermatogenesis, the formation of mature sperm cells, occurs just before puberty, during a male's slow growth period . This is when trans-generational effects of starvation in males are their most profound. Oogenesis, the formation of primary oocytes or ovum, occurs mainly during fetal life. However, recently it has been suggested from mice studies that oogenesis continues beyond the fetal period, presenting a reason why a female's slow growth period could also be associated with multigenerational and gender specific effects (Pembrey et al., 2006).

Research done by Anway et al. (2008) gives support to this hypothesis. They found that the critical period to influence the germ line epigenome through methylation is during the epigenetic programming of the germ cells when they migrate to the gonads during sex determination. Inducing permanent alterations in the germ line DNA methylation pattern would therefore allow the

phenotype to become trans-generational (Anway et al. 2008; Anway and Skinner 2008; Burdge et al. 2011). In Anway et al.'s (2008) study, rats were exposed to vinclozolin, a compound previously shown to cause changes to hormonal systems during the embryonic period (Gore and Dickerson 2012). The toxin altered the expression of 196 genes in the testes in the F1, F2, and F3 generations. Most of these genes showed decreased expression. Since the F2 germ line (presumptive sperm) is also exposed directly to the vinclozolin, it is the F3 generation that manifests trans-generational effects (Anway et al. 2008). This study also showed that effects are mediated through the paternal line. Pembrey et al. (2010) coined this the "Y dipstick hypothesis": sex-specific segregation of diet influencing information transmits via the Y chromosome. He put forward the idea that DNA sequences within the non-recombining part of the Y chromosome may accumulate epigenetic marks in response to early experience. Thus, it is the Y chromosome imprint that is more significant (Pembrey 2010). Moreover, the X chromosome is inherited relatively unmodified since in the father the X and the Y are unmatched - there is no other chromosome to act as an imprinting template. The consequence could be that the X imprint is transmitted with less degeneration. Thus epigenetic gametic inheritance is one possible mechanism for an environment's ability to affect multiple generations. The major failing, however, is that even fourth generation offspring (F3), sometimes of both genders, have exhibited effects in rodent diet/epigenetic studies.

To conclude, epigenetic changes may reach the F3 generation by a combination of *de novo* imprinting and the retention of some epigenetic memory. Studies have subsequently shown that metabolic syndrome effects fade by the third generation; this reinforces the idea that trans-generational phenotypic changes may occur through imprinting (Benyshek et al. 2006; Pentinat et al. 2010). Epigenetic modification can occur gradually over a few generations, but genetic changes due to

selection or genetic drift require a longer period of change. This supports epigenetics as a potential answer to inherited effects due to nutritional changes. Moreover, genetic defects tend to be irreversible over the span of a couple of generations, and yet we have seen that folic acid can reverse some of the effects discussed in rodent studies. Thus, the role of epigenetic mechanisms in trans-generational nutritional effects is gaining more support through these studies.

4.6 Inherent Weaknesses of Retrospective Studies

Despite the usefulness of retrospective studies, they have their drawbacks. For example, the reliability of reported years and the severity of starvation and overfeeding in Sweden can be questioned. Although these challenges spanned well-defined years, different individuals would have had varying access to resources: the reported caloric intake values during these periods and specific lengths of starvation may not be accurate. The fact that these retrospective studies relied upon telephone interviews, data based on participant responses, and food estimates based on municipal boundaries adds variability by the distancing effects of time and memory. This in turn leads to the over- or underestimation of trans-generational effects. Furthermore, both the Dutch Famine cohort and the Överkalix cohorts resided in specific geographical regions. There could be confounding environmental effects experienced by these cohorts that would not occur in a randomized sample. Moreover, although both cohorts are relatively homogenous Northern European populations, given the historical patterns of settlement, marriage, and propagation, they might have represented a narrow genetic background, and have responded idiosyncratically to potential epigenetic modifications. One must then ask, how generalizable are these results?

Despite possible drawbacks, these historical alterations to human nutrition offer unique opportunities to study trans-

generational nutritional effects in humans. It is neither possible nor ethical to collect a randomized group of individuals, place them in an environment without confounding variables, and starve or overfeed them to observe the epigenetic effects.

4.7 Implications for Society

Epigenetics allows us to understand the ways in which environmental cues, such as diet, impact the expression of our genes and our prospective descendants. However, the knowledge of this relationship also raises new medical, legal, and ethical concerns that have implications for both political leaders and the average citizen alike.

4.7.1 Obesity as a Disease

Discovering more about the uncontrollable factors in disease development could lead to patients refusing individual responsibility for their condition. For example, as of June 2013, the American Medical Association has labeled obesity as a 'disease' (AMA 2013), although it can be combatted through diet and lifestyle changes.

Although framing obesity in this way could promote greater social acceptance of this condition, researchers say that this could also perpetuate the perception that weight is unchangeable or fated and thereby render self-regulatory efforts seem futile (Hoyt et al. 2014). In this latter study, an online survey revealed that participants who read articles about obesity being a disease placed less importance on healthy eating and weight loss, and expressed less concern over weight when compared to obese people who read control articles. These findings suggest that the messages people hear about the nature of obesity - like conceptualizing it as a disease, passed on by ancestors through genetics or epigenetics – could take the onus off individuals and lead them to point an accusing finger at their genome or epigenome (Hoyt et al. 2014). This would not only have consequences for the way certain

diseases are perceived, but it would also have consequences for healthcare costs and resources as a whole.

CASE STUDY: The Chinese Famine

The 1959-1961 Chinese Great Leap Forward famine will likely provide a new cohort that can help to test the generalizability of the previous human studies, and whether or not these effects are solely specific to Northern European populations. In the 1959-1961 famine cohort, both intra- and inter-generational impacts have been reported. Preliminary inter-generational studies reveal that children born to mothers who had experienced the famine in utero have a higher risk of mortality in infancy (Song 2013). Gender effects are also seen, with maternal exposure more significantly affecting the sons and paternal exposure more significantly affecting the daughters (Fung and Ha 2010). Surviving offspring only just reaching the age at which adult diabetes or cardiovascular disease risk usually emerges. Will this cohort, which is genetically different from the Dutch famine or Överkalix cohorts, and which also presents a much larger demographic, exhibit similar health risk factors and predispositions? And what kind of trans-generational effects might be seen?

4.7.2 Obesity in Children: A Case for Child Abuse?

If a parent makes poor choices before, during or after pregnancy, could the negative epigenetic impact upon the prospective child be considered child abuse? Child neglect is typically defined as placing a child at risk for serious harm through the failure of caregivers to seek or provide necessary care, medical or otherwise (Varness et al. 2009). Thus, childhood obesity could

be considered neglect by contributing to the development of conditions such as severe obstructive sleep apnea, uncontrolled type 2 diabetes mellitus, and advanced fatty liver disease. Not only does childhood obesity increase the risk for serious harm during childhood, it also increases the risk for the development of diseases as an adult (Varness et al. 2009). While the law states that parents have a duty to protect their children from harm, the law also supports parents raising their children according to their own values and beliefs (CYH.COM 2012). Hence, this issue can be approached from numerous perspectives, making it difficult to discern whether or not childhood obesity is, in fact, child abuse.

It is clear that parents are responsible for their children, however, parental responsibility to unborn children in terms of inherited epigenetic propensities to certain diseases is not as clear. First and foremost, Canadian law does not grant person status to a fetus. Similar debates have met with mixed results in the United States. Where a pregnant woman has the right to refuse medical treatment that is in the best interest of the fetus and abortion is legal, maternal autonomy often rules (ACOG 2005). However, in the past, criminal charges have been brought against women for their abuse of alcohol during pregnancy (Lisko 2006). Could it then be argued that a mother who abuses food during pregnancy, and therefore exposes her unborn child to epigenetic consequences, should also be charged? The extent of maternal responsibility is a grey area in many jurisdictions. Opponents of criminalization of certain maternal behaviors during pregnancy argue that it violates the mother's constitutional rights, and that this type of regulation is a slippery slope (Lisko 2006). Thus, the dissemination and deployment of epigenetic knowledge may add fuel to these arguments.

Epigenetic risks incurred during pregnancy could place an unjust burden of responsibility on women (Chadwick and O'Connor 2013). While the majority of human fetal effects discussed were effects of in utero exposure, recent evidence also

points to epigenetic paternal contributions to obesity and diabetes. Responsibility of the parents could then extend to regulating their activities to prevent epigenetic harm to the gametes that may create children in the future – where does the line get drawn? Preconception? Chadwick and O'Connor (2013), argue that "trans-generational justice demands not that those alive today live their lives slavishly devoted to the yet- to-be-born, rather, that the interests of current and future generations be balanced".

4.7.3 Governmental Control of Nutrition

Ideally, a government enacts laws in order to protect the citizens of that country. A current topic of discussion is how to regulate the kinds of foods that are available to the public, potentially providing them with better nutritional choices: but how will this be accomplished without infringing upon the rights and freedoms of the public? For example, from 2002 to 2013 the mayor of New York City, Michael Bloomberg, proposed a ban on large sugary drinks. This policy would have prohibited restaurants and other eateries from serving soda pop or other sugary beverages larger than 16 ounces. This was thought to be an important first step in fighting rising obesity rates. It was unlikely that the proponents of the move were conscious of the potential for epigenetic harms - these arguments were not advanced. Opponents, however, argued about government encroachment upon freedom in decision-making. The New York City's board of health unanimously passed this law, but it was invalidated by a New York Supreme Court Judge six months later (Grynbaum 2014). Although policies such as these may have the potential to increase public health and decrease health care spending, will personal freedom be the cost? How willing are we to live in a country where the government has control over our nutritional, exercise, and health choices? Should the needs and health of the *prospective* many trump the freedoms of those currently living their lives? Given the demographics of obesity, diabetes, and health, how are

future health costs to be controlled?

4.7.4 Correlational Conclusions

Links between the nutritional intake of one generation and its effects on subsequent generations are *correlational* in nature. Since the multiple mechanisms of action and influence upon the epigenome cannot be definitively separated, quantified, and defined, causation cannot be explicitly characterized. This affects the ability of government and institutions to enact laws or policies regulating environmental conditions that may exert adverse epigenetic effects. It also reduces the ability to assign responsibility to current generations for the negative consequences that they may impose upon their descendants.

4.7.5 Privacy

The epigenome and epigenetic effects will eventually embody a substantial dataset that patients will want to keep safe-guarded. **Privacy**, as we will see in Chapter 9, is important with respect to any type of health information. Equipped with the knowledge of trans-generational effects, we now know that your parents' and even grandparents' health can have far reaching effects on your current and future health. Therefore, your parents' and grandparents' information could become a privacy issue for you, as it could affect job prospects or insurance coverage if information is leaked. In sum, how can protective policies be created in a non-discriminatory way? Conversely, descendants might have a legitimate claim upon the health records of their antecedents – how do we balance their respective interests?

4.8 Conclusion

The food we eat can affect us long after our meal has ended. Furthermore, our dietary and lifestyle choices can impact our children and grandchildren, through acquired and inherited

epigenetic modifications that give rise to intra-, inter-, and trans-generational effects. All of these factors and modes of transmission can affect our future society's health, productivity, and economy. Understanding how the epigenome is modified and how diet can lead to epigenetic changes will allow for more effective medical strategies to deal with this category of health effects. For example, knowing that obese and diabetic mothers transmit epigenetic modifications to their child in utero, thereby contributing to disease predisposition, could facilitate strategies, education, and interventions to reduce the prevalence of obesity, diabetes, and related disorders both in the mother and her offspring via epigenetic-based medicine (Lehnen et al. 2013). It is crucial to understand the effects and mechanisms that overfeeding and malnutrition have on the transmission of generational diseases, and retrospective studies have been an important part of the discovery process. Could 21st century North America be the newest cohort for the study of overfeeding, and trans-generational effects, the likes of which have yet to be seen? If we do not change our path, the answer may be grim.

Provocative Questions

- **Retrospective studies**

  Is the information researchers receive from patients about reported years and the severity of periods of starvation or overfeeding reliable? Are we over- or under-estimating inter-generational or trans-generational effects?

- **"Not my fault"**

  Could deeming multivariate conditions, such as obesity, a "disease" induce patients to minimize their sense of individual responsibility for their condition?

- **Epigenetics and disease in children- child abuse?**

  Mothers who abuse alcohol during pregnancy have been

convicted. Could a mother who abuses food causing lasting epigenetic harm to her child, also be tried?

- **Governmental control of nutrition**

Should governments enact laws and dietary regulations? Where should the line between public good and personal freedoms be drawn? Since studies have indicated gender and cultural differences in epigenetic effects and disease risk, how can protective policies be made non-discriminatory?

- **Correlational conclusions**

Current retrospective studies and experiments on model organisms provide a strong case for the link between nutrition and epigenetics. However, the evidence still remains correlational. Should governments enact nutritional policies based strictly on correlational data?

- **Privacy**

How can we protect privileged information, such as where your parents and grandparents grew up and what they ate, so that it does not affect job prospects or insurance coverage? Should we all have the right of access to the health records of our parents and grandparents?

Possible Solutions

- Education is currently our only tool to mitigate epigenetic harms and diseases that are due to diet and nutrition. Perhaps people might change their behaviours if they knew that their actions would affect their children and grandchildren as well.
- Education could be specially targeted to expectant-mothers to help improve the epigenetic prospects in utero.

- Feeding, nutrition, and educational food preparation programs could be implemented to teach schoolchildren the importance of nutrition with respect to the epigenome. Starting younger is better.
- Finally, the possibility of government-imposed restrictions or taxation regimens on dietary choices could prove to be beneficial for the public, depending on their willingness to accept the intervention.

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Chapter 5

Chemical Epigenetics: Prescriptions, Pollutants, and Picking your Poison

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Abstract

We interact with chemicals every day – they arrive in the form of recreational drugs, prescriptions or by environmental exposure. All can have an effect on our epigenome. They are externally derived substances that we ingest, and we call them xenobiotics. The xenobiotic chemicals discussed are thought to have a basis in epigenetic modification and may cause changes over both the near and long term. Pharmaceutical drugs can both negatively (DES) and positively (valproic acid and ritalin) affect the epigenome; alterations caused by alcohol, amphetamines, cocaine, nicotine and morphine have been linked to addiction. Cautionary measures as well as the legal and ethical responsibilities of pharmaceutical companies are also discussed. Environmental factors show variable effects upon the epigenome: environmental toxicants are most often associated with human diseases, but chemo-preventative receptors are involved in preventing and correcting harmful epigenetic variations. For the most part though, air pollutants and dietary xenobiotics have been correlated with disease and adverse health effects. Liability, insurance, governmental action, chemical safety protocols, and personal awareness are

needed to deal with drugs, environmental toxins, and their population-wide epigenetic effects.

5.1 Introduction

When most people hear the word "chemical", they think of strange liquids bubbling in vats or test tubes. In fact, everything you can see or touch is some kind of chemical, and life itself can be reduced to a series of chemical reactions. Humans are quite good at harnessing chemical properties to manipulate the complex reactions in our body with drugs to produce desired effects or to suppress disease. Understanding chemical reactions allows us to protect crops, build homes, and create everyday objects. Just as drugs can exert powerful effects on pathogens, environmental chemicals can also invade and interfere with processes of the body, and some of these effects are now known to be epigenetic in nature. They can be elicited both intentionally and incidentally. Chemicals not normally manufactured within our bodies, but that find their way there by one means or another are called **xenobiotics**, and they will be the focus of this chapter. Recreational and medicinal drugs, as well as chemicals in our environment can leave marks and alterations upon our epigenome that science is only recently revealing. We will examine these interactions, beginning with drugs and followed by the environmental chemical context within which we live.

5.2 Epigenetics and Drugs

In 2003, the Human Genome Project reached completion, affording us the information and tools to study our own biological blueprint. While the Human Genome Project will doubtless lead to advances in research practices and safer drugs, the promise of easy and safe genetic manipulation remains largely unfulfilled (Wrobel et al. 2009; Verma et al. 2011; Brunschweiger and Hall 2012). This is because of the unexpected complexity of the genes themselves, and in part, because the technology is still too blunt to finesse

without causing collateral damage. Epigenetics is an example of unexpected complexity, but it may also offer a tool to manipulate gene activity. Epigenetics is now understood to play a role in regulating gene activity in most animals and plants

Gene therapy has delivered promising results in clinical trials, but is showing limited success in practice (Tani et al. 2011). There is another way to alter gene expression without physically changing the genetic code. As outlined in Chapter 2, in the case of certain genes, epigenetic changes can provide a regulatory function. By modulating the intimacy of interaction between histones and DNA, problematic genes that cause certain diseases can be "turned off" without having to alter a single **nucleotide**. While drugs that change epigenetic factors circumvent the challenges of traditional gene therapy, they come with their own risks and problems. Research is revealing that many of the adverse effects of addictive drugs can be attributed to epigenetic factors and pharmaceutical drugs previously considered safe may have extensive epigenetic consequences, including cancers and birth defects. In this section, we will discuss the unintended epigenetic side effects of pharmaceutical drugs that are presently in widespread use, the beneficial effects of marketed drugs and the epigenetic changes that contribute to making drug addiction heritable.

5.2.1 Epigenetic Side Effects of Prescribed Drugs

Clinical and/or experimental evidence has demonstrated that a number of drugs already on the market have persistent epigenetic side effects that need to be addressed. While the extent or severity of these risks is unclear, it might be prudent to assume that any one of the drugs presently on the market has the potential to affect the epigenome.

Negative Drug Effects

On September 19th, 1941, **diethylstillbestrol** (**DES**) was approved by the United States Food and Drug Administration. The drug was prescribed to pregnant women to prevent the risk of pregnancy complications and miscarriage. Not only was the drug ineffective at preventing pregnancy complications, but we now know that it had epigenetic consequences for the children exposed to the drug in utero. Women born to mothers who had taken DES experience impaired fertility and are predisposed to cervical cancer, breast cancer, and **clear cell adenocarcinoma** (Palmer et al. 2001; Troisi et al. 2007; Hoover et al. 2011). Men who had been exposed prenatally to DES suffer a higher incidence of genital malformations, most commonly **epididymal cysts** and **hypoplastic testes** (Wilcox et al. 1995). It is now known that DES causes measurable epigenetic changes: the methylation patterns in genes of the estrogen pathway are altered (Li et al., 2003). It is unknown how these changes affect development – do they alter cell responsiveness and behaviour, or do some cell types form abnormally or die out altogether?

There are concerns that the effects of the drug could propagate even further to subsequent generations. For example, preliminary reports suggest that DES causes increased risk of ovarian cancer in the granddaughters of woman who had taken DES while pregnant (Titus-Ernstoff et al. 2008). There are not enough third generation DES victims old enough to mount a comprehensive study, so understanding the multi-generational effects of DES will take some time. However, the emerging appearance of these symptoms is consistent with the knowledge that epigenetic alterations can persist through multiple generations. For more information on DES and the associated legal debate, see Chapter 8.

While the case of DES highlights how ignorance of epigenetic consequences can lead to disasterous health issues, epigenetic knowledge can be utilized to discover novel treatments for disease. By regulating the genome, it is possible to choose which genes are expressed and which are inhibited, creating a new

strategy that can be used for medical applications. Drugs that act directly on the epigenome, for example valproic acid, can functionally accomplish the goal of changing how the genome is expressed without the technical challenges and risks inherent in gene therapy.

Positive Drug Effects

Valproic acid is a short branched fatty acid currently used in the treatment of epilepsy, although the mechanism of how it mitigates seizures is unknown. Recent research shows that valproic acid acts on the epigenome by up-regulating 726 genes and down-regulating 577 others in rat cortical neurons, including a gene that is implicated in epilepsy (Fukuchi et al. 2009). The drug works as a **histone deacetylase (HDAC)** inhibitor, which means that it acts on the epigenome directly to regulate DNA – histone interactions. Valproic acid's epigenetic effects have become the focus of many research studies since it is a relatively cheap and safe compound and has a myriad of unexpected benefits. The drug has been shown to render multiple types of cancer cells more susceptible to radiation therapy: it arrests their cell cycle. Valproate affects multiple cancers from human **erythroleukemic cells** to breast cancer cells - this diversity of effect, despite the varied and heterogeneous nature of cancer, is impressive (Karagiannis et al. 2006; Travaglini et al. 2009). Valproic acid also has the benefit of targeting cancer cells specifically - it affects normal cells much less (Travaglini et al. 2009). The benefits of valproic acid do not stop at cancer and epilepsy; it has neuroprotective properties that promise possibilities for treating multiple neurodegenerative diseases (Monti et al. 2009). One study even showed that valproic acid's epigenetic effects facilitate the development of perfect pitch learning in humans and mice – a skill which is normally impossible to acquire after early development (Gervain et al. 2013).

We Need to Employ Caution

Valproic has been approved safe by the FDA, but DES shows that caution must be exercised with any drug that affects the epigenome, especially one like valproic acid that acts directly upon epigenetic machinery. Valproic acid was found to increase **neural tube** defects in mice by 1-2% because of its epigenetic effects, although it is still prescribed as an anti-epileptic medicine (Tung and Winn 2011).

In fact, many currently prescribed drugs are thought to display epigenetic side effects. Methylphenidate (MPH) is often prescribed to children with attention deficit hyperactivity disorder, and is most commonly known by the trade name Ritalin. Methylphenidate alters the population of lncRNA present in the brain (Wu et al., 2015). Rats treated with MPH through adolescence exhibit different responses to emotional stimuli at adulthood than untreated rats. The MPH treated rats displayed decreased responsiveness to rewarding stimuli such as sugar and sex, but increased sensitivity to aversive stimuli such as **swim stress** (Bolanos et al. 2003). MPH causes up-regulation of 5 neurotransmitter genes, including the genes *Grik2* and *Htr7*, which can remain up-regulated even into adulthood (Adriani et al. 2006). *Grik2* is linked to changes in reward perceptions, and *Htr7* may play a role in depression (Tang et al. 2004; Guscott et al. 2005). This suggests that the epigenetic regulation of these genes could underlie the permanent effects of MPH exposure.

Antidepressants, contraceptives, chemotherapeutics, and general anaesthetics are just a few more of the currently available pharmaceuticals that show signs of epigenetic side effects (Csoka and Szyf 2009). For example, fluoxetine, found in antidepressants like Prozac, alters **DNA methylation** and **chromatin** structure leading to altered cerebral gene expression and hormone levels, and this can result in sexual dysfunction and infertility. Oral contraceptive pills affect gene expression immediately and long-term, and are linked to an increased incidence of breast cancer in

women who took the pills prior to their first pregnancy. General anaesthetics, may cause neuronal injuries, brain protein misfolding, as well as postoperative cognitive dysfunction that persists, via epigenetic changes, long after the surgery (Csoka and Szyf 2009). These side effects in some cases sound minor; a 1-2% increase in neural tube defects caused by valproic acid could be judged as inconsequential when compared to its positive effects. However, epigenetic side effects are distinct from most other side effects because they *may* be heritable. Heritability adds a new dimension when judging the cost and benefit ratio of medications: the patient's long term epigenetic health and prospective descendants should be considered.

5.2.2 Addictive substances

Alcohol

Alcoholism is a serious addiction that can lead to **cirrhosis** and increased risk of throat, mouth, and liver cancer (Thun et al. 1997). There are two types of alcoholism: Type I and Type II. **Alcoholism Type I** is acquired in adulthood; Type 1 alcoholics often feel guilt associated with their addiction. In contrast, **Alcoholism Type II** begins as early as adolescence, and is more likely to cause violent behaviour (McGue 1999). An analysis of 577 Swedish men found that Type I alcoholism is associated with genetic and environmental factors in males, but Type II alcoholism is associated with a family history of abuse, where the postnatal environment plays little if any role. In reference to this latter etiology, men whose biological mothers were alcoholics had higher incidence of Type II alcoholism, even if they were raised by non-alcoholic foster mothers (McGue 1999). Not only does alcoholism appear to be heritable, but the negative effects of alcoholism upon cognitive function can be inherited as well. Both prenatal alcohol exposure and maternal alcoholism history play a significant role in the propagation of alcohol related disorders (Cottencin et al. 2009). The reason for heritability of alcoholism is

a mystery, but the interplay between environmental and genetic effects in the development of alcoholism suggests that addictions operate through epigenetic mechanisms. Indeed, the questions of how fetal alcohol exposure leads to alcoholism, and how alcoholism can be passed down through family histories, may only have their answers in epigenetic behaviour.

Rat fathers that had been exposed to alcohol sire offspring with problems including congenital malformations and cancers, and these effects last for generations (Stockard 1913). This effect isn't genetic in the classical sense - alcohol is not a mutagen that can install mutations inherited via the germ cells (Phillips and Jenkinson 2001). The offspring were never directly exposed to alcohol, and yet paternal exposure to alcohol had lasting consequences. This study adds to a growing, somewhat controversial body of evidence suggesting that paternal exposure to drugs may affect children for generations, even when the drugs do not mutate genes (Friedler 1996). To investigate the possibility that addiction heritability is epigenetic, investigators looked to see whether alcohol has effects on the epigenome.

The gene *TLR4* encodes a lipid sensory receptor called *toll-like receptor 4*. *TLR4* activation contributes to binge drinking and epigenetic mechanisms are suggested to be involved (Liu et al. 2011). A surplus of product from this gene was shown to cause damage to the liver and brain in mice (Pascual et al. 2011). *TLR4* is normally important for recognizing microbes in the body and for signaling an immune response. Alcohol decreases the **acetylation** of the H4 histone and the activity of **histone acetyltransferases (HATs)** in the frontal cortex, **hippocampus**, and **striatum** of mice that carry a normal *TLR4* gene. In mice with *TLR4* receptors, ethanol exposure normally causes inflammatory damage to the brain, memory loss, liver damage and behavioural disorders, while mice engineered to be mutant deficient for this receptor gene are protected from such damage (Pascual et al. 2011). The biochemical basis for this difference was tested: mice lacking

TLR4 receptors could absorb alcohol, but would not experience any decrease in the net acetylation of H4 histone measured in biopsied material. Other genes now known to be epigenetically affected in human alcoholics include: alpha synuclein which is hypermethylated in alcoholic patients and that is important for neuronal synapse function (Bonsch et al., 2005); *monoamine oxidase A,* a neurotransmission modulator that is methylated in female alcoholics who are co-dependent upon nicotine (Philibert et al., 2008); *vasopressin* and *atrial naturetic peptide* are hyper- and hypomethylated respectively, and this correlates with degree of craving (Hillemacher et al., 2009); the *H19* and *insulin like growth factor regulatory domain* is abnormally methylated in the sperm of alcoholic males, suggesting a possible route for transmission to offspring (Ouko et al., 2009). Finally, alcohol appears to alter the epigenetic status of a gene important for neuronal function in mice, *brain derived neurotrophic factor* (*Bdnf*; Stragier et al, 2015). Whether or not these changes are of trans-generational importance remains to be seen.

What could alcohol change via epigenetic modification of histone H4 that would result in an increased chance of alcoholism? Evidence suggests that alcohol affects chemosensory systems in the brain to make the substance taste more palatable (Youngentob & Glendinning, 2008). In this latter study, rats were inferred to have inherited epigenetically altered gene expression in the taste and olfactory systems - changes that predisposed them to develop a preference for ethanol compared to their non-exposed peers. This conclusion must be regarded with caution, however, as no genes were examined directly. Fetal alcohol exposure could increase the chance of alcoholism by simply making it taste less bitter. Nevertheless, olfaction and taste provide a plausible epigenetic mechanism that would help explain the heritability of alcoholism (Youngentob and Glendinning 2009).

Other Addictive Substances

The epigenetic activity of addictive drugs doesn't stop at alcohol. Amphetamines can, through epigenetic pathways, subvert their stimulation pathway to become less effective over time. For example, amphetamines change the imprint status of a gene called *DeltaFosB* in brain – this a gene has carry-on effects in the regulation of other genes such as *c-Fos*, *histone H3 methyltransferase*, and *KMT1A* , a lysine methytransferase. Note that these last two genes are likely to alter the epigenetic status of additional genes. The end result is an addict needing more amphetamines to achieve the same high (Renthal et al. 2008). Zhou et al. (2011) measured the epigenetic effects of alcohol and cocaine in the post-mortem brains of human cocaine abusers and alcoholics. Cocaine has some of the same epigenetic effects as alcohol in the hippocampus, the area of the brain affecting memory, which suggests a common mechanism that both links and escalates addictions (Zhou et al. 2011). Although these substances cause several adverse effects, the following study describes a resistance to addictive behaviour across generations. Male rat offspring of **sires** that had self-administered cocaine showed a resistance to cocaine abuse behaviours (Vassoler et al. 2013). This difference in behaviour was not observed in female offspring or in male offspring of sires who self-administered saline (i.e. controls). Neither was it observed in the male offspring's self administration of sucrose and, therefore, cannot be attributed to operant learning deficits. Thus a decreased reinforcing effectiveness is seen among the male sire offspring (Vassoler et al. 2013). Can this be deemed a 'positive effect'?

Nicotine is argued to be a gateway drug. Levine et al. (2011) developed an animal model using mice to determine if previous exposure to nicotine primed their response to cocaine. By measuring addiction-related behaviours and the synaptic plasticity of the striatum, an increase in cocaine-associated responses was seen only when nicotine was administered first. Cocaine had no influence on nicotine effects when the order was reversed.

Nicotine was found to inhibit HDAC (histone deacetylase) in the striatum, thereby enhancing cocaine's ability to induce expression of the *FosB* gene, a key player in cocaine addiction (Levine et al. 2011).

Addiction is not only caused by ancestral and prenatal factors: how one is raised in early childhood also has an epigenetic effect on the predisposition to addiction (Schwarz et al. 2011). When rats are introduced to morphine, their **glia** increase the expression of a subset of **cytokines**. These particular cytokines are predictive of whether the rats will become addicted to morphine. Rats that were raised by nurturing and loving mothers had significantly more of a competitor cytokine, IL-10, which inhibits the activation of addiction-related ones in the offspring. Well nurtured mouse pups are less likely to become addicted to morphine upon exposure. Presumably, poor parenting decreases IL-10 expression, and this renders offspring more vulnerable to additive behaviour. It was found that the IL-10 gene expression is modulated by differential methylation patterns, where nurtured rats show nearly a 5-fold decrease in methylation compared to rats that were poorly nurtured (Schwarz et al. 2011). This explains, in part, some of the social influences upon epigenetics and substance abuse. A greater understanding of these mechanisms could facilitate cures and countermeasures to combat addictive drugs.

5.2.3 Future of Pharmacology in an Epigenetic World

Epigenetic pharmacology is an emerging field that should be treated with equal measures of caution and enthusiasm. Since it is now obvious that epigenetics constitute an inextricable part of the blueprint of life, epigenetic treatments present challenges, especially when it comes to safety testing and ensuring the health of future generations. The ability to fully understand complex conditions like addiction, and the prospect of creating drugs that present novel solutions to medical conundrums should be weighed against those challenges.

Epigenetic testing is imperfect. Pharmaceutical companies, armed with epigenetic knowledge, should be responsible to test their drugs for epigenetic changes. The tragic case of DES stands as an example of the consequences of ignorance when it comes to epigenetic side effects; however, the cost of epigenetic testing is unclear. Should the epigenetic effects of every drug taken to market be studied, including those already being sold? How many post-patient generations should be analyzed? Much of the time, rodents reveal epigenetic effects that are similar to those found in humans: we share a near-identical assembly of genes, regulatory pathways, and activities. But the gene regulatory pathways are complex and can vary in subtle but important ways between species. Moreover, no single epigenome map exists since, unlike DNA, an epigenetic map would need to take into consideration each and every tissue and cell type, parental legacy, sociological, and environmental factor in order to provide a useful reference point. Some steps are being taken to assemble this information (The Encyclopedia of DNA Elements, called the ENCODE project for short), but given the combinations and variables that will need to be considered, the project will be mammoth indeed.. Moreover, given the interplay of all of the aforementioned factors, cause and effect relationships would be difficult to determine. Finally, methylation patterns change naturally with age, so longitudinal studies showing changes in methylation, acetylation, miRNA, lncRNA etc., will need very strong controls to establish causation. For true confidence, multiple generations of humans would have to be monitored, and this is impractical since it would effectively slow drug development to a halt, and raise development costs to prohibitive levels. The current program of epigenetic testing undertaken by pharmaceutical companies has been examined and deemed reasonable, given the current understanding of epigenetics (Alyea et al. 2012). As understanding grows, though, will current testing methods continue to be adequate? It is clear that some responsibility must be taken by drug developers and purveyors for

the epigenetic effects of their products. The trick will be to balance the expense of testing before it risks becoming burdensome both to developer as well as to society at large. Look at the stalled pipeline of antibiotic development: similar cost disincentives have dangerously impaired our ability to control infection. Clearly, care will have to be exercised when introducing policies, because both economic as well as epigenetics effects can last for generations.

The pharmaceutical industry is no stranger to regulation, and for good reason. Modulating the chemical balance of any system as complex as a body is a dangerous and difficult task. Epigenetic research will not only change how companies approach drug safety and testing, but also how they consider waste management and their consideration of patient metabolites. By extension, patients will also have to assume some responsibility for safe and compliant practices. For example, it is not uncommon on Canadian television to see public service announcements encouraging safe disposal practices for expired pharmaceuticals: patients have to acknowledge that they are also consumers with a responsibility for proper disposal. The reason for this is clear: our water supply is becoming measurably contaminated. If substances that affect epigenetics are released into the environment, they expose humans and other animals to potentially harmful trans-generational effects. In fact, as you'll read in the next section, such toxicants already have polluted the ecosystem, due largely to ignorance.

5.3 Environmental Epigenetics

According to the World Health Organization, more than 13 million deaths occur annually due to environmental causes. As much as 24% of disease is caused by exposures that can be averted: pollutants are among the list of environmental threats to human health (Prüss-Ustü and Corvalán 2006). Compounds that are of natural and artificial origin, chemicals that can have harmful, positive, or neutral effects, and molecules that contaminate the

environment for numerous years, all encompass our everyday exposure. Increasingly, scientific evidence is shedding light on the changes that environmental chemicals exert upon the epigenome.

5.3.1 Toxicants & Chemopreventative Reversers

The environmental chemicals we are exposed to can be grouped broadly into two categories: **environmental toxicants** or **chemopreventative reversers** (Kim et al., 2012). These variations induce opposite effects on the epigenome in relation to their primary effects upon methylation (Kim et al., 2012). Environmental toxicants are strongly emphasized throughout the literature since they present the most obvious impact on human disease. Various human diseases have been attributed to environmental toxicant effects on the epigenome, a prime example being cancer (Goodman & Watson, 2002; Chapter 3). Current evidence suggests a second category of epigenetic alteration effects: the "chemopreventative reversers." These potentially prevent and correct harmful epigenetic perturbations.

Environmental epigenetics encompasses how environmental exposure to nutritional, dietary, physical, and chemical factors can alter gene expression and modify individual genetic susceptibility through changes in the epigenome (Kim et al. 2012). In this field of study, DNA methylation is investigated as a product as well as a diagnostic signpost of exposure to environmental toxicants. (Yang and Park 2012). Mixed exposure to environmental toxicants and chemopreventative reversers simultaneously makes interpretation and understanding of the etiology of environmental effects complicated. However, given the complexity of environments, it is perhaps inevitable that many studies have focused upon mixed exposures of multiple environmental toxicants, the combinatorial nature of which leads to outcomes that are more deleterious than the additive effects of separate chemicals individually (Christiansen et al. 2009). By

exposing multiple environmental toxicants simultaneously a "1+1=3" effect occurs: the synergisms are complex.

5.3.2 Pathways of Exposure & Health Implications

Air Pollution

Respiratory diseases and epigenetic imprinting imparted by environmental exposure appear to be linked. Animal studies have shown that chronic exposure to air pollutants can lead to noticeable changes to the methylation patterns of inflammatory genes, in addition to changes in **miRNA** expression patterns (Jardim 2011). Recall, miRNA may act as an upstream epigenetic modifier that causes later biochemical alterations to genome packaging and the behaviour (Bartel 2004; Sevignani et al. 2006) (Chapter 2). Even a 2 hour exposure to diesel fumes is sufficient to induce measurable epigenetic change (Jiang et al. 2014).

Epidemiological studies suggest that exposure of a mother to tobacco smoke may transmit the risk of asthma to her children (Alati et al. 2006) or even grandchildren (Li et al. 2005). Therefore, it is important to consider air pollution as an issue not just of environmental but also of generational justice. Other common air pollutants with epigenetic implications include: ground-level ozone, diisocyanate, endotoxins, allergens, and pesticides (Miller and Ho 2008) (Table 5.1).

Many individuals are exposed to workplace air pollution. For example, gas station attendants and traffic police experience low-dose benzene exposure that is measurable in blood samples (Bollati et al. 2007). This exposure is linked to global DNA hypomethylation and the specific hypermethylation of p15, a tumour suppressor gene. p15 is important because of its role in cell cycle regulation. These characteristics are often seen individuals with **acute myelogenous leukemia** (Bollati et al. 2007). High-level benzene exposure has also been associated with an increased risk of this disease (Snyder 2002). Working conditions vary quite broadly; some individuals are much more likely to be exposed to

hazardous substances than others (see case study on Heavy Metals).

Dietary considerations can work synergistically with air

CASE STUDY: Heavy Metals in and Around the Workplace

A significant concern relates to the exposure of workers and individuals to heavy metals in and around the workplace. Factories may use heavy metals during production; both the workers and people living in the area surrounding the factory become exposed to heavy metals by air, but also by contact. Numerous papers have outlined the effects of heavy metal exposure on the epigenome. These heavy metals include arsenic, cadmium, chromium, lead, mercury, tungsten, and nickel. They all exhibit an epigenetic component, resulting in various cancers, cardiovascular disease, and trans- generational effects (Martinez-Zamudio and Ha 2011). One can only speculate at the long term effects upon people who garden, play, or inhabit structures built on land that is reclaimed from industrial use.

Steel plant workers are exposed to inhalable particulate matter which is associated with an increased risk of lung cancer (Cantone et al. 2011). Particulate matter contains carcinogenic and toxic metals such as arsenic and nickel. It was found that human exposure is associated with an increased amount of histone 3, lysine 4 methylation and histone 3, lysine 9 acetylation in blood leukocyte samples, especially in individuals with more years of employment. These regional changes are associated with open chromatin states also present in lung carcinoma cells (Cantone et al. 2011).

Animal models have been used to show that the DNA of sperm in mice exposed to air from a steel plant exhibit hypermethylation compared with control animals. The change persists even after the environmental stimulus is removed. This raises flags regarding trans-generational effects (Yauk et al. 2008).

pollution to lead to diseased phenotypes. Dietary supplements to provide methyl donors during pregnancy can increase the risk of respiratory disease and these effects are compounded with air pollution exposure (Hollingsworth et al. 2008). Table 5.1 outlines various research which associates the epigenetic changes of asthma and atopy (allergic hypersensitivity) linked genes with environmental stimuli.

Table 5.1 *Representative Research on Gene-By-Environment Interactions In Asthma or Atopy (*Adapted from Miller & Ho, 2008)

Dietary Exposures

Pesticides

The typical North American diet is contaminated with various xenobiotic chemicals. Extensive research has been done on the epigenetic effects of vinclozolin, a commonly used agricultural fungicide, which is also an **endocrine** disrupting chemical. Vinclozolin is very difficult to wash off with water. Increasingly, this chemical is shown to lead to specific and adverse epigenetic effects such as altered methylation patterns of genes encoding a protein phosphatase, and another encoding a serine threonine kinase (Anway et al., 2005). It is anti-androgenic and induces spermatogenic defects, male infertility, breast cancer, kidney disease, prostate disease, and immune abnormalities (Anway et al. 2006).

An additional pesticide, methoxychlor, is estrogeneic and also has been found to exert trans-generational effects including changes to sperm number and motility. These chemicals, among others, produce measurable epigenetic methylation changes within the germline that have been linked to impaired embryonic testis cord formation and increased spermatogenic cell **apoptosis** in the adult testis (Skinner and Anway 2005). These changes are also

correlated with adult onset male infertility (Guerrero-Bosagna et al. 2013).

Vinclozolin and methoxychlor are just a couple of the many pesticides of concern. Other examples include: DDT, paraquat, dieldrin, and propoxur (Collotta et al. 2013). A common feature of many of these contaminants is that they are endocrine disruptors. Herbicides, pesticides, fungicides, and heavy metals, all have been shown in various models to have adverse health effects and further research is being done to elucidate the epigenetic effects (Collotta et al. 2013).

Plastics

Food and beverage products are often packaged in plastics that leach chemicals with harmful effects. **Bisphenol-A** (**BPA**) is one of these chemicals. BPA acts as an **estrogen** mimic and is used in conjunction with polycarbonate plastics. The classical example of BPA contamination is via disposable water bottles. Studies have revealed that BPA induces hypomethylation of various genes and is associated with higher body weight and higher risks of breast and prostate cancer (Dolinoy et al. 2007). Early stem cell development is sensitive to BPA. However, it has been shown that maternal supplementation of diet with methyl donors, such as folic acid or the phytoestrogen genistein, can reverse the effects of such hypomethylation in early development (Dolinoy et al. 2007). This is an example of an environmental toxicant and a chemopreventative reverser, this time a normal component of diet, working antagonistically.

It is crucial that we are aware and understand where our food sources come from and what chemicals they contain.

Chemopreventative Reversers

A few supplements have been identified with the potential to reverse changes caused by chemicals like alcohol, BPA or even heavy metals (Kim et al. 2012). Methyl donors, such as folate,

methionine, betaine, choline and vitamin B12, affect methylation. Downing et al. (2011) compared the *Igf2* gene methylation patterns in the embryos of dams given alcohol during pregnancy. They found that the embryos of mice not given methyl donors were hypomethylated at the *Igf2* locus while those receiving supplementation had increased methylation of the *Igf2* gene. Thus the effect of alcohol hypomethylation was reversed (Downing et al. 2011). Evidence also suggests methyl donors can reverse metal and BPA effects (Kim et al. 2012). Unfortunately, in contradistinction to these remediating effects, research also indicates that in utero exposure to a diet rich in methyl donors may enhance the severity of allergic airway disease in mice and that this predisposition has trans-generational effects (Hollingsworth et al. 2008). Dietary polyphenols, such as genistein derived from soy, are among other things, inhibitors of DNMTs (the genes encoding DNA methyltransferases – epigenome modifiers) and are implicated in reversing methylation of tumour suppressor genes involved in multiple types of cancers (Kim et al. 2012). Other chemopreventative reversers include vitamin D3, the tomato carotenoid lycopene, and selenium (Kim et al. 2012).

5.3.3 Environmental Justice

As research progresses in this burgeoning field, more health effects will undoubtedly be revealed. It is important to evaluate environmental conditions consistently, to perform chemical and biological risk analyses to prevent subsequent health complications, and to fulfill our obligations to future generations who will inherit both our physical environment as well as our epigenome. Some epigenetically associated disease examples are listed in Table 5.2. Research continues to reveal a growing list of diseases that can be attributed to epigenetic modifications.

The environmental domain requires regulation and legislation to keep individuals safe from exposure and subsequent health effects, but responsibility should be a two way street.

Personal chemoprevention as well as government regulation of environmental toxicants has been recently emphasized for individual and societal protection from environmental toxicants including cancer (Kim et al., 2012).

5.4 Considerations

5.4.1 Regulation and Liability

Insurance

Currently in the U.S., laws exist to protect individuals from insurance discrimination under the Genetic Information Nondiscrimination Act (GINA) (Hudson et al. 2008). By contrast, in Canada, a person who has their DNA tested and does not disclose a defect to their insurer could lose coverage. There is no legislation in either jurisdiction that explicitly covers epigenetic features. Medication and residential histories may indicate increased risk of epigenetic changes and consequently also predisposition to diseases. Should this permit insurance companies to charge more for health insurance based upon your geographical proximity to pollutants? This is an important question as there are economic and social inequalities that affect levels of environmental exposure, the affordability of health care, and access to insurance coverage. Who will protect economically disadvantaged individuals from under-representation or discrimination on the basis of parental legacy, upbringing, or other socio-economic factors that they could not reasonably be expected to control? What if effects are not manifest for a generation or more? Will the burden of proof to establish causal links be too onerous to permit successful remedy? How short or long should statutory limits be?

Toxicant	Biomarker	Related Disease	First author, year

Alcohol	MTHFR	Lung Cancer	Vaissiere, 2009
Benzene	PARP-1	Leukemia	Gao, 2010
BPA	Hoxa10	Abnormal uteri development	Bromer, 2010
DES	Lactoferrin	Uterine cancer	Li, 2003
Dioxins	Foxp3	Colitis	Singh, 2011
Vinclozolin	H19, Gtl2	Abnormal male reproductive system	Stouder, 2010
Arsenic	p53	Various cancers	Hou, 2012
Cadmium	LINE-1	Various cancers	Hossain, 2012
Chromium	45s rRNA	Trans-generational cancer	Cheng, 2004
Lead	COL1A2	Preterm birth	Hanna, 2012
Mercury	Rnd2	Neurotoxicity	Arai, 2011
UV-B	p16, RASSF1A	Skin cancer	Nandakumar, 2011

Table 5.2 *A Selection of Epigenetically Associated Diseases. (Adapted from Kim et al. 2012)*

Liability

Corporate and personal liability already presents a problem that needs attention as epidemiological studies reveal associations between epigenetic signatures and disease states (Chapter 8). If epigenetically induced diseases arise as a consequence of pharmaceutical or environmental exposures, questions will arise regarding who is liable, especially since many of the effects might not become perceivable within normal **statutory limits**. If reparative actions are pursued, there will need to be a framework for enforcement and determination of the extent of the damages. For example, if workers who are employed in factories that utilize hazardous chemicals later show epigenetically-based diseases, should the company be held liable if all possible precautions were not taken to limit exposure? What if exposure risks were unknown at the time? The same questions might also be asked in cases pertaining to prescription drugs.

Government

Taxation models could be developed to reserve funds and provide reparations when pharmaceutical or environmental toxicant harms are later identified. Such reserves could ensure that affected citizens will have recourse to aid in future generations when the offending company might no longer exist. Models of similar funding approaches to facilitate public health solutions already exist, such as vaccination development programs. In the United Kingdom, the government has allocated money to incentivize vaccine development by manufacturers. This allows for vaccines to be more affordable because they have established a competitive context and market that may otherwise have been non-existent. This is referred to as a "pull" approach (Surowiecki 2004). Perhaps similar approaches can be taken when considering corporations and the health of citizens – models could be developed to reward positive epigenomic maintenance throughout the corporate world, without placing inordinately heavy encumbrances upon businesses.

As previously mentioned, some people, - those in certain workplaces and socioeconomic classes - are likely to be disproportionately affected. The government must represent all stakeholders. This includes those most severely affected and those who are most impoverished and lacking a voice (Mansfield 2012). It also includes those that are most likely inflicting harmful epigenetic modifications such as heavy polluting corporations and producers of various xenobiotic chemicals. Clearly, the government has an important role to play when in mediating and developing policies and practices that will promote and ensure both a healthy and productive population, as well as a vibrant and sustainable economy.

Chemical Safety Analysis Protocols

It is of utmost importance to have standardized procedures to effectively measure the epigenetic implications of new and

existing chemical compounds. Techniques must be perfected to measure changes and to publish the epigenetic effects of toxicants so that risks can be understood by all (LeBaron et al. 2010). Government agencies, industrial stakeholders, and researchers could compile information as well as generate new and centralized databases concerning the chemical hazards in relation to epigenetics. Testing agencies such as the American Food and Drug Administration, the Canadian Health Agency, and other bodies should implement requirements for manufacturers to test, to examine potential effects, as well as to mandate policies for corporations to follow when performing clinical trials. As recent experience demonstrates, the present model for cataloguing, reporting, and making information from trials accessible could benefit from greater transparency: even negative results could be useful to future development and safety. When balanced against the potential risks of liability and litigation, corporations might find it cheaper to make data public.

5.5 Conclusion

Chemical exposure, whether by intention via drug use or inadvertently via environmental exposure, is an important field of study because of the complex mix and high volume we are exposed to each and every day. There are various questions and concerns that have arisen from epigenetic studies: for example, which commonly used but heretofore untested chemicals and drugs are harmful? What effects might their use have upon my children's children? Research may lead to better prevention and treatment options even if currently employed but unsustainable practices are upset. This moves us into the frame of thinking of the environment and our place in it as a larger and more complex interactome (Guthman and Mansfield 2012).

How do we create healthy environments to promote positive epigenomic reinforcement and to limit human disease? This remains a question of importance that will be addressed

through policy initiatives, government intervention, corporate accountability and transparency, changing societal dynamics, and education. It is important that all stakeholders are aware of the potential consequences of chemical exposure both at a genetic and an epigenetic level. This means that personal education is likely to be as important as actions by larger bodies.

Many chemicals that we take for granted may suddenly be revealed as environmental toxicants upon further inquiry. Exposures can have adverse health effects, whether they are from pharmaceutical drugs, addictive substances, air pollution, dietary components, or by any other route that xenobiotics can invade our everyday lives. Often, if a substance is not immediately or visibly harmful, we do not think of the effects of long term exposure to these chemicals. Epigenetics is increasingly likely to indicate that it is no longer enough to understand how our genetic code is written; we must understand how it is read, stored, and accessed by the body. In a practical sense, in terms of immediate effect, any chemical that affects how our DNA is expressed deserves the same respect and caution that we reserve for mutagens.

Provocative Questions

Chemical and pharmaceutical development.

- How should we balance the benefits of development against potential long term and inherently unknown epigenetic risks and costs?
- How much can developers be encumbered before development ceases? Is there a middle ground between responsible testing and advancement?

How will new products be tested? How will liability be assessed?

- Different tissue and cell types can have different epigenetic marks - how would one determine which tissues to test?

Can comprehensive yet simple tests be developed? Will they be cost-accessible?

- How could one determine causation – how do we distinguish between the single compound being tested versus the other myriad of factors (inheritance, socioeconomic status, diet, aging, drugs etc.)
- How many generations should be tested? Will this slow development, increase liability?
- Who should determine the procedures and policies that pertain to epigenetics-oriented interventions in medicine law, ethics, or society?
- Where should we draw the line between corporate and personal liability?

Remediation

- If a process or treatment has generational consequences, how will a fair cost-benefit analysis weigh competing interests?
- Environmental toxicants are often distributed within geographically finite boundaries. Who's responsible to clean up the environment? What onus falls to society, business, and individuals to mitigate geographical risks?
- What is to prevent agencies and companies from discriminating against individuals on the basis of their geographical and employment histories (ie; their possible exposure, epigenetic status, and possible health pre-dispositions)?

Possible Solutions

Chemical and pharmaceutical development will be determined with time and experience.

- Laws and regulations mandating the testing for epigenetic effects should not only be in place, but should be reviewed and refined often.
- At the very least, if testing every drug for epigenetic side-effects is found to be impractical, the fact that the drug has not been tested should be included in the list of side effects: this creates awareness and a market preference for drugs that are tested.

Genetic testing was imprecise in its infancy, so it is likely that epigenetic testing methods will improve with time.

- Tissues that are the target of, say, a drug, may be a testing priority; subsequently, tissues often involved in high risk diseases, like stem cells in which changes are often linked to rapidly developing diseases, are important to test.
- All drugs containing substances that are known to have epigenetic effects should be subjected to multi-generational testing or monitoring, even drugs that have no previously discovered mechanism.
- Privacy policies/non-discrimination laws need to be expanded to cover epigenetic risks and histories.

Remediation is a difficult problem to solve; past and current issues with known negative consequences, such as industrial pollution, highlight this point.

- It will take time and effort by researchers and agencies to push companies and government to solve these issues – communication, education, and consultation among all stakeholders will be critical.

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Chapter 6

Developmental Epigenetics and Reproductive Medicine:
Apparently Always a Parent!

Marisa Market and Jessica Hebert

Abstract

If diet during pregnancy can epigenetically affect the fetus, then it follows that those environmental and social experiences that have the potential to alter maternal physiology might also produce fetal modifications. It is also likely that such changes might be mediated by the placenta. A common theme in epigenetic studies revolves around the role of hormones in effecting long term changes to patterns of DNA packaging and behaviour. It is not surprising then, that hormonally assisted deliveries, birth control, patterns of nursing, as well as the manipulations that underlie ART (Assisted Reproductive Technology), especially IVF, are linked to imprinting changes. In vitro fertilization (IVF) involves both hormonal priming of the mother, as well as direct manipulation of gametes under highly artificial conditions. The demonstration of specific DNA methylation changes outlined in the previous chapters, as well as the generational persistence of imprinting defects in other studies (such as diet-induced changes), make careful assessment of such

procedures all the more compelling. Medical interventions (such as Caesarean section or the administration of epidural analgesia and hormonal labour-inducing agent Pitocin) that have been trademarks of assisted labour and delivery are being questioned again in the light of their potential to modify the epigenome, as are some of the practices that follow immediately after birth, such as breast or bottle feeding. Epigeneomics might also give us pause to interrogate the surprising ways in which surrogate or adoptive mothers might influence the development of an individual despite their different genetics.

6.1 Introduction

Your autobiography might often start with the date you were born. That is the beginning of your story. However, the epigenetic mechanisms that influence the construction of who you are and who you will become begin much earlier. As we have learned in previous chapters, it is possible to inherit epigenetic traits that span at least two generations. Apparently you cannot be too careful in your choice of parents: all joking aside, the epigenetic bonuses or deficits that come packaged along with your inheritance of DNA matter. In this chapter, we will discuss epigenetic mechanisms associated with: pregnancy environment, Assisted Reproduction Technologies (ART), oral contraception, birth, and parental behaviours.

6.2 Epigenetic Interactions of Maternal Environment and Fetus

Maternal taste preference for high-energy foods consumed during gestation can be transmitted to offspring, thus reproducing an affinity for this diet in future generations (Gicquel et al. 2003; Delport and Pollard 2010). In this way, attributes of the broader social environment can be transmitted via the intimate molecular connection of the mother to the fetus, and via parental social interactions with the delivered child. The mechanics of this

relationship remain somewhat hazy, however a picture is emerging in which epigenetics plays a prominent role. If one's choice of food can be attributed to influences such as epigenetics, prenatal programming, and learned cultural norms, it becomes clear that culture and biology work in an interactive and reinforcing fashion: diet culminates in biological alterations, and it is these changes that further influence dietary choices and culture. As we saw in Chapter 4, under-eating, over-eating, or certain nutrients can all elicit epigenetic changes, sometimes even pre-conception, and such changes are heritable. If dietary experience can affect the fetus, then it stands to reason that social exposures and psychological experiences that alter a mother's physiology during pregnancy could also affect the fetus and newborn (Bale et al. 2010).

6.2.1 Depression during Pregnancy

Women who were depressed during pregnancy bore infants of lower than average birth weights (Gicquel et al. 2003; Laprise 2009; Liu et al. 2012). These latter authors contend that depression disrupts the proper functioning of the hypothalamic/pituitary hormonal axis (consisting of interactions between the hypothalamus, pituitary, and adrenal glands), but only in the African American women within the study cohort. Results indicated a 2.4% higher methylation level compared to those women that identified as non-depressed. Moreover, in samples of umbilical cord blood, DNA methylation patterns differed for 10 genes compared to infants born to unstressed mothers within the cohort. This increased methylation could contribute to an increased vulnerability to neurodevelopmental disorders (Liu et al. 2012). Additionally, African Americans exhibit lower levels of DNA methylation in umbilical cord blood samples compared to Caucasian participants (Adkins et al. 2011). Finally, African American women experience a higher rate of pre-term delivery, and lower birth weights, compared to Caucasians, even when financial incomes are comparable (Collins et al., 2007). What these

studies may truly indicate is that the maternal environment affects offspring (as has been asserted before). However, these studies neglect to account for the possibility that correlations made with race will be confounded by external cultural and economic features that are unrelated to genetics (see Chapter 7).

6.2.2 Smoking during Pregnancy

Maternal habits of cigarette smoking harm the fetus. Head circumference and low birth weight (LBW) are common side effects in babies born to mothers who smoke cigarettes throughout pregnancy. The consequences of cigarette smoke inhalation during gestation is at least somewhat mediated by methylation (Breton et al. 2009). In samples of umbilical cord blood, tests reveal methylation shifts in response to smoking, potentially explaining part of the correlation of smoking and LBW (Murphy et al. 2012). A major challenge of this study is that changes detected in the methylation of umbilical cord blood samples might not reflect changes in the body of the delivered baby, however, the results certainly raise cause for concern. Women who quit smoking for the duration of pregnancy were compared to a smoking and a non-smoking group: their newborns compared favorably to non-smokers relative to those who smoked. Another study of smokers showed down-regulation of four different micro RNAs in the placenta (miRNAs, Chapter 2). Recall that miRNAs are assistants that control essential cell activity (Maccani et al. 2010; Maccani et al. 2013). Since the placenta mediates the transfer of nutrients to a fetus, it seems likely that these changes could indicate a significant mechanism by which maternal smoking alters fetal health. If a specific miRNA is down-regulated, whatever target it is supposed to interact with is up-regulated. The researchers suggested that the down-regulation of these four miRNAs miR-16, -21, -146, and -182), and corresponding up-regulation of their target genes disturbed processes of cell cycle regulation, development, immune system adjustment, and growth in the placenta. These alterations

could also confer changes in fetal metabolic programming, including development of neurological strucutres (Maccani et al. 2010; Maccani et al. 2013).

6.2.3 The Placenta

During the first trimester, the placenta develops from **trophoblastic** tissue and serves to nurture the fetus until birth. The human placenta is the first embryonic organ to develop, and it provides the resource for the mother and fetus to exchange gas, nutrients, waste, and metabolites. It is structured to permit this exchange between maternal and fetal blood without direct mixing. Nevertheless, potentially harmful substances and pathogens can pass across the placental barrier. Some examples include medical drugs (the morning sickness medication thalidomide), illicit drugs (heroin), alcohol, chemicals from cigarette smoke, and even certain pathogens (HIV) can cross the placental barrier.

Changes in the epigenome of placental tissue might result in low birth weight and pre-term birth. Changes in placental imprinting, however, may be responsible for many more diseases. This is due to the fact that aberrant epigenetic changes that are readily repaired in embryonic tissue appear to persist in placental tissue (Mann et al. 2004). This is not to say that imprinting modifications in embryonic tissue do not result in imprinting diseases, it highlights the major role that placenta plays in fetal development through epigenetic regulation. Diseases caused by changes in the placental epigenome include gestational diseases, such as Gestational Trophoblastic Disease (GTD) and **pre-eclampsia** (CASE STUDY 6.1, 6.2), as well as diseases that impact the rest of the child's life. Pre-eclampsia is an umbrella term used describe a variety of deficiencies that result in dangerously high maternal blood pressure. There are likely multiple different causes, some of which might be epigenetic in origin.

CASE STUDY 6.1: Gestational Trophoblastic Disease

Gestational trophoblastic disease describes a group of rare tumors that are caused by derailed **trophoblast** growth in a woman's uterus. The cells that make up the trophoblast layer that should eventually become the placenta begin to grow abnormally and can cause various tumors. The most common type of tumor is a **hydatidiform mole**, which has the potential to develop into a carcinoma. Studies have found that the cause of this abnormal growth is improper epigenetic marks within embryonic stem cells (such as aberrant tumor suppressor gene methylation and the hypermethylation of transcription factors that play an important role in maintaining embryonic stem cell pluripotency) (Nelissen et al. 2011).

CASE STUDY 6.2: Pre-eclampsia

Pre-eclampsia is characterized by high blood pressure and excessive amounts of protein in urine. From this, **eclampsia** can develop, which is characterized by the onset of seizures, usually in the last half of pregnancy.

There are many factors that can lead to pre-eclampsia, but an important mechanism involves the fetal **hypoxia/**reoxygenation response pathway. The reduced methylation of genes encoding hypoxia-inducible factors leads to increased transcription of proteins that prevent proper trophoblast differentiation. This then leads to a failure of placental trophoblast cells to effectively invade and remodel maternal spiral arteries, ultimately leading to abnormal placental blood circulation. Due to hypoxia and re-oxygenation stress, pre-eclampsia can develop. The mechanism that senses maternal vascular pressure is disrupted and this leads to a generally broadcast cue to constrict arteries - high blood pressure ensues. This can then lead to eclampsia. Pre-eclampsia is a common cause of fetal growth retardation in developed countries, causing both pre-term birth (PTB) and low body weight (LBW). Intrauterine growth retardation (IUGR) and LBW are negative health predictors for newborns and children, as they are associated with the development of chronic diseases expressed later in life (such as cardiovascular disease, hypertension and type 2 diabetes). Finally, improper placental development due to aberrant methylation can also cause harm to the mother where poor placental development is associated with maternal hypertension. Therefore, aberrant DNA methylation in the placenta impacts fetal growth, the development of diseases later in life, and could affect maternal morbidity (Blair et al. 2013).

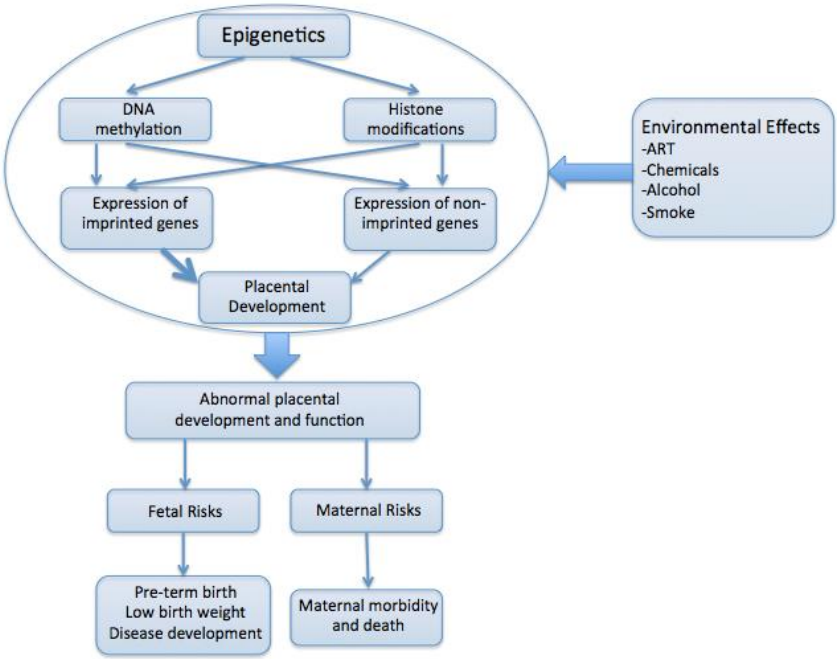

Figure 6.1 *Factors affecting placental development. (Nelissen et al. 2011) Placental development is determined by many factors including DNA methylation and histone modifications that regulate gene expression, as well as environmental factors such as medical procedures (ART), and exposure to toxic substances (chemicals, alcohol, smoke), among others. Abnormal placental development can result in consequences for both the fetus and the mother. The fetus may be born pre-term, have low birth weight or could be more at risk of developing diseases later in life. Maternal risks include morbidity and death.*

Epigenetic changes arise in both the embryo and the placenta: aberrant DNA methylation and histone modification of many genes have been observed under the circumstances discussed in CASE STUDY 6.2 Such genes include *H19* and *IGF-2* (Li et al. 2005). However, epigenetic alterations in the placenta appear to be more common, especially in the placentas of babies that were conceived using assisted reproduction technologies (ART). While the precise

cause of this is unknown, some theories suggest that the placenta is more affected since it derives from outer trophectoderm cells, which by virtue of being the most external cells of the early stage embryo, are in closer contact with the Petri dish culture medium in which maternal and paternal contributions are combined. They are also the first cells to differentiate and therefore undergo slightly longer exposure as differentiated cells. They might simply also be more strongly affected by the *in vitro* environment. The trophectoderm cells have different DNA replication patterns and might consequently have less capacity to maintain imprints (Mann et al. 2004). Furthermore, increased prevalence of diseases resulting from placental imprinting modifications could reflect the diminished capacity of presumptive placental cells to deal with epigenetic challenges (for example hypoxia, nutrition, disease, or drugs) compared to cells that go on to form the body of the fetus itself (Mann et al. 2004). Aberrant epigenetic marks in placental tissue could lead to altered placental function, which may in turn lead to diseases such as pre-eclampsia or **placental previa**. Either outcome results in disrupted growth and development of the fetus, ultimately leading to LBW or PTB (de Waal et al. 2014).

It is important to note that aberrant methylation and histone modification can arise in non-ART children as well. Studies have shown that the environment under which the placenta develops (maternal diet, smoke, alcohol, chemicals, etc.) can also alter epigenetic marks (Nelissen et al. 2011). A disturbance in placental development or function through changes to the epigenome could alter fetal development and increase in disease susceptibility later in life. Not to be forgotten are the effects that anomalous fetal growth could have upon maternal health: difficult pregnancies and births can have long lasting effects. However, more research still needs to be done in order to fully understand the relationship between the placenta and long term epigenetic sequelae.

6.3 Assisted Reproductive Technologies

The causes of male or female infertility are numerous and broad. There are many genes and mechanisms involved in the fertilization of an egg by sperm and the progression from a single-celled zygote through to a human baby; the cause of any one person's infertility is complex and unique. Assisted reproductive technology (ART) has revolutionized the field of reproductive medicine and solved the problem of infertility for millions of people around the world. The first "test tube baby", Louise Brown, was born in 1978 (Laprise 2009), and by 2008, an estimated five million babies were born that were conceived through *in vitro* fertilization (IVF) and other reproductive techniques (ESHRE 2012).

ART is considered to be any medical technique or procedure that aids in the conception of a child. The extent to which reproductive assistance is required depends on the cause of infertility and can vary from helping with the production of eggs to injecting sperm nuclei directly into an egg. Some examples of ART include:

- *Ovulation induction/ Superovulation stimulation:* Gonadotropin releasing hormone analogues are used to control the timing of ovulation and the number of mature eggs released from the ovary (Elder and Dale 2000).
- *Egg retrieval:* Eggs are harvested from the ovary to be used for fertilization. They are then: cultured in an incubator; fertilized; cultured some more; and then transferred back to the female reproductive tract or to cryopreservation vessels.
- *Fertilization:* Fertilization is the process of sperm and egg fusing to form a zygote and can occur *in vitro* or in vivo via artificial insemination.

- *Embryo transfer:* Following *in vitro* fertilization and culture, the embryo is inserted into the uterus to grow and develop further.
- *Cryopreservation:* Extra eggs and sperm can be frozen in order to preserve them for future use, avoiding the need for multiple egg and sperm retrievals. Embryos can also be cryopreserved if too many were fertilized *in vitro* (Kaariainen et al. 2005; Iliadou et al. 2011).
- *Artificial/ Intrauterine insemination:* The process of inserting sperm into a woman's uterus in order to facilitate in vivo fertilization.
- *Third-party reproduction:* This form of ART includes egg donation, sperm donation, and the use of a surrogate or a gestational carrier. A gestational carrier is a woman who carries and delivers a baby that is not biologically hers, while a surrogate donates her eggs for fertilization and gestation. In a recent innovation, a donated egg can be stripped of its own chromosomal DNA to be replaced by DNA from the prospective mother. Since the mitochondrial genes come from the donor egg, and the genomic chromosomes from the father and mother, these embryos have three genetic parents.

6.3.1 In Vitro Fertilization

In vitro fertilization (IVF) is the process that involves removing mature eggs from the uterus, fertilizing them outside of the body, and implanting one or more embryos back into the uterus. This is done in order to facilitate fertilization and early embryonic development for people who would otherwise be unable to conceive. A typical IVF cycle includes:

1. Ovarian stimulation/ Superovulation
2. Egg retrieval

3. Fertilization: This step may vary depending upon the ability of the sperm to fertilize the egg. If the sperm are unhealthy or lack the ability to fuse with the egg, intracytoplasmic sperm injection (ICSI) may be used. ICSI involves injecting a single sperm nucleus into a single egg using a micropipette in order to increase the odds of successful fertilization (Iliadou et al. 2011).
4. Culturing.
5. Fertilization.
6. Culturing again for a while to assess embryo viability and health.
7. Embryo transfer to a receptive womb.

ART, especially IVF, has been linked to the development of imprinting disorders in children. These disorders are generally thought to be caused by aberrant DNA methylation and/or histone modifications that lead to the improper expression of certain genes, and the consequent development of a disease. The imprinting disorders that have been most strongly correlated with ART include Beckwith-Wiedemann syndrome (Gicquel et al. 2003; Maher et al. 2003; Laprise 2009; Vermeiden and Bernardus 2013), Angelman syndrome (Gicquel et al. 2003; Maher et al. 2003; Laprise 2009) and Silver-Russell syndrome (Kagami et al. 2007; Laprise 2009; Vermeiden and Bernardus 2013) (CASE STUDY 6.3). It is important to note, however, that some studies refute a link between these imprinting diseases and ART. It is an ongoing and controversial topic of research (Zheng et al. 2011; Vermeiden and Bernardus 2013).

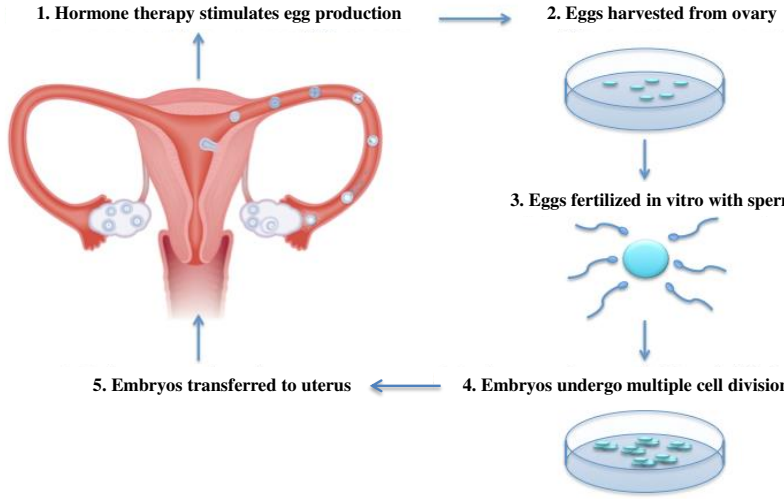

Figure 6.2 *Summary of the IVF procedures.*
IVF consists of numerous procedures. The exact procedures may vary, and this is based on the person(s) undergoing IVF. Generally, hormones are used to induce superovulation, eggs are harvested from the woman's ovaries, these eggs are fertilized by sperm in vitro, the embryos are cultured on media and allowed to undergo multiple cell divisions before finally being transferred back to either the mother's or donor's uterus.

In order to understand the reasons why ART might cause imprinting disorders, it is necessary to understand the process by which the epigenome is established during gametogenesis (production of the egg or sperm) and embryo development. Prior to implantation of an embryo into the uterine wall, there are two major phases of epigenetic reprogramming that occur: 1) at **gametogenesis**, when the sperm and egg develop, and 2) shortly after fertilization.

1) During gametogenesis, the epigenetic marks that were present in the pre-gamete cells are largely, though not completely, erased. Then, new sex-specific imprints are established; a

paternally directed pattern is imposed in sperm, and a maternally directed pattern is imposed in eggs. This erasure and re-imprint is necessary because the chromosomes in the gametes will have derived from and carry a partial imprint from a mixture inherited from each gender of grandparent. This is known as genomic imprinting (Johnson 2005; Iliadou et al. 2011; Market Velker et al. 2012).

2) During fertilization and early embryo development, generalized DNA methylation patterns and histone modifications take place along with specialized epigenetic processes that include the maintenance of genomic imprints, silencing of retroviral elements, and **X chromosome inactivation**.

Genomic imprinting allows for the expression or silencing of each copy of the alleles (pairs of genes) inherited from each parent via sperm and egg. The imprint is dependent upon whether the mother or the father provided that allele. These imprints remain intact, despite other DNA modifications that occur in early development, in order to protect the regulated expression of specific alleles from each parental chromosome.

Retroviral elements are ancestral remnants of viral genes that are present in our genome (Market Velker et al. 2012). They can be mobile and pose a disruptive threat to genome integrity. In order to protect against the deleterious effects of these elements, they are silenced during this period by epigenetic modification.

CASE STUDY 6.3: Imprinting Disorders

Beckwith-Wiedemann syndrome (**BWS**) Beckwith-Wiedemann syndrome is an overgrowth syndrome causing macrosomia (excessive birth weight). Half of BWS cases are caused by epigenetic changes at one of two genes on chromosome 11: H19/IGF-2 and KCNQ1OT1 (Gicquel et al. 2003; Laprise 2009). Normally, H19 is expressed only from the maternal alleles while IGF-2 is expressed only from the paternal alleles. The KCNQ1OT1 gene is normally methylated on the maternal alleles, therefore being expressed solely by the paternal alleles (Gicquel et al. 2003). Loss of imprinting at the imprinting centers of these genes leads to aberrant transcriptional regulation, resulting in BWS (Laprise 2009).

Angelman syndrome (**AS**) **/ Prader-Willi syndrome (PW)** Angelman syndrome primarily affects the nervous system and can lead to delayed development. Normally, AS is caused by the loss of function of certain genes on the maternal chromosome 15, due to gene deletions through paternal uniparental disomy (Laprise 2009) (inheriting two alleles from the father and none from the mother). Less than 5% of AS/PW cases are due to imprinting errors in the gene SNRPN DMR, however, all cases associated with ART are due to loss of methylation on the maternal allele of this gene (Laprise 2009). This suggests that there is something about ART procedures that is increasing the incidence of AS/PW caused by epigenetic alterations.

Silver-Russell syndrome (**SRS**) Silver-Russell syndrome is a slow growth disease characterized by intra-uterine and postnatal growth retardation (Rossignol et al. 2008), resulting in low birth weight and failure to thrive (Laprise 2009). SRS is a "sister" disease to BWS, as it is also due to epigenetic errors in genes on chromosome 11. Two-thirds of cases are due to hypomethylation of H19/IGF-2, while the remaining cases are due to epigenetic mutations on other chromosomes (Laprise 2009).

Finally, **X chromosome inactivation** is crucial during

development of females in order to compensate for the second set of genes that they receive through their XX pair. This adjustment of gene dosage is necessary because a male's Y chromosome is smaller and carries fewer genes compared to the X chromosome. X chromosome inactivation is carried out by condensing it 90% into inactive, tightly packaged **heterochromatin**. Epigenetic marks aid in the process of DNA condensation that mediates X-inactivation (Market Velker et al. 2012).

Given the timing of *in* vitro fertilization procedures relative to epigenetic remodeling, there is a possibility that procedures will disrupt imprinting erasure/re-imprinting and alter the epigenome (Laprise 2009; Feng et al. 2011).

6.3.2 Contributing to the Development of Imprinting Diseases: The In Vitro Environment

Despite efforts to simulate the female reproductive environment, a Petri dish cannot replace a uterus and fallopian tubes. The natural environment is extremely complex and cannot be completely recapitulated by the type of media and incubators used for embryo culture. Variables introduced by this artificial culture environment include composition of the medium, the addition of serum albumin, temperature, and the oxygen/CO_2 concentration at which the gametes and embryos are incubated.

Studies on *in* vitro culture media have concluded that embryo culture affects global DNA methylation patterns, **chromatin** remodeling and gene expression (Market Velker et al. 2012; Feng et al. 2011). Early embryo development is programed to be flexible enough to respond adaptively to the environmental changes that occur as the embryo moves from the fallopian tubes to the uterus, and then implants (Johnson 2005). However, when subjected to the vaguaries of artificial culture conditions, researchers suspect this adaptive quality is subverted. Studies have also examined the effects of adding serum albumin to culture media (Bavister 1995). Serum albumen is an additive that is frequently used to supplement

cultures and to enhance cell viability and growth. Mouse studies showed that the addition of serum resulted in changes in the methylation and expression of numerous imprinted genes, including *H19* and *IGF-2* (Khosla et al. 2001; Market Velker et al. 2012). It is hard to predict the epigenetic consequences of media supplementation upon human embryos, however the addition of both serum and serum substitutes has been used for several years to improve human embryo viability and implantation success (and we are not even discussing the subtle and proprietary changes that for-profit clinics might deploy).

The temperature at which embryo culture occurs is not as homeostatic as the temperature an embryo would experience *in vivo*, and this may also introduce epigenetic effects. The eggs/embryos in their Petri dishes are removed from the incubator for observation under microscopes. The transition from one venue to another, or from the incubator to the transfer pipette, and then into a womb introduces temperature swings and physical stress.

Finally, oxygen/CO_2 levels in the female reproductive environment are dynamic from pre-implantation through to post-implantation and are crucial to the development of the embryo. However, embryos are often cultured *in vitro* at a static concentration of 20% or 5% oxygen, and removal from the culture chamber to the microscope introduces new variability. Therefore, it is possible that inappropriate gas concentrations (and attendant shifts in pH) could impair the development of the embryo, genetically or epigenetically (Rinaudo et al. 2006; Nelissen et al. 2011; de Waal et al. 2014). In summary, the abnormal environment in which gametes are stored and fertilized, and where early embryo development takes place could affect the embryonic epigenome (Laprise 2009).

Additional factors could also impinge upon success and the epigenetic health of the embryo. First, the physical and chemical manipulations that gametes and embryos undergo during the *in vitro* process could not only introduce DNA damage, but could

also have an effect on the epigenome. Second, superovulation and use of immature sperm could alter the genomic imprints that are formed during gametogenesis or introduce incompletely imprinted sperm to the egg. Imprint marks are still being established in oocytes during meiosis. Therefore, the use of gonadotropins during superovulation procedures may have an epigenetic impact, perhaps by restricting the amount of time available for imprint acquisition. Although imprint marks are established before meiosis in sperm, immature sperm might not be fully developed or the use of immature sperm may cause imprint changes in the resulting embryo (Laprise 2009).

Finally, the Intra-Cytoplasmic Sperm Injection (ICSI) procedure could lead to media or acrosome pieces (pieces lost during normal fertilization) being introduced into the egg, or to a physical disruption in the egg via the micropipette (Maher et al. 2003; Kalra et al. 2011). Both of these situations could affect the embryo's epigenome.

6.3.3 In Vitro Fertilization and Low Birth Weight

Low birth weight and pre-term birth have been associated with assisted conception and reproduction (Schieve et al. 2002; MacDonald et al. 2009; Kalra et al. 2011). It is hypothesized that low birth weight and pre-term birth could be due to epigenetic alterations that arise during IVF procedures. Potential factors that could induce low birth weight or pre-term birth include ovarian stimulation, oxygen tension, culture media type, serum addition, and multiple embryo implantation.

There may be a link between the use of exogenous hormones to induce superovulation, and birth weight. By comparing live births from fresh embryo transfers (where the maternal environment had just experienced ovarian stimulation) with cryopreserved or frozen-thawed embryo transfers, researchers were able to compare the effects of these stimulatory hormones (Kalra et al. 2011). Interestingly, there was a higher incidence of low birth weight in

children conceived after fresh embryo transfer. Might freezing have mitigated otherwise damaging hormone concentrations and effects? Could subtle damage induced by freezing have elicited impromptu repairs? While the mechanism by which low birth weight occurs is currently unknown, one possibility is that the reproductive environment (altered by exogenous hormones) affects epigenetic marks either withing the placenta or the embryo that acquired during early development (Kalra et al. 2011).

As previously discussed, oxygen tension during embryo culture has been found to affect epigenetics: these changes could lead to low birth weight or pre-term birth (de Waal et al. 2014). As previously mentioned, the type of culture media used during IVF has epigenetic consequences (Johnson and Krasnow 1990; Market Velker et al. 2012). Some studies have also concluded that embryo culture can affect the birth weight, suggesting that this is the reason behind the higher risk for low birth weigh in IVF singletons (Dumoulin et al. 2010). Other studies, however, suggest that there is no significant relationship (Carrasco et al. 2013; Lin et al. 2013). Could these disparities be due to undisclosed and proprietary differences in embryo culture and transfer practices? Changes in the methylation and expression of imprinted genes such as *H19* and *IGF-2* have been reported (Market Velker et al. 2012). This is important because these genes are involved in growth development and the development of imprinting disorders. In fact, the use of serum for in procedures used to propagate domestic livestock has been associated with Large Offspring Syndrome (Walker et al. 1996; Market Velker et al. 2012) and Beckwith-Wiedemann syndrome in humans.

During embryo transfer, often more than one embryo is implanted into a woman's uterus in order to maximize the chance of pregnancy. Sometimes multiple embryos implant and begin to grow, with some terminating over the course of gestation – generally between 5 and 10 weeks; this is known as a "vanishing twin" (De Sutter et al. 2006). Singletons that result from a double

embryo transfer (due to a vanishing twin) imply a suboptimal implantation environment. This environment could contribute to a higher miscarriage rate and less-than-ideal placental development and fetal growth leading to lower birth weight (De Sutter et al. 2006). It is not known if this environment affects the epigenetic health of the remaining fetus. Alternatively, another simple explanation might be that in the first place, procedures are being deployed to aid embryos that are in some way already sub-optimal (see section 6.3.4 below). In summary, the loss of embryos over the course of harvest, culture, transfer, and implantation may be the result of technical deficits, procedural insults to the embryos, or merely to poor egg or embryo quality. and Distinguishing between the possibilities will not be trivial.

Assisted reproductive technologies have provided many people with the welcome option to reproduce. Given the potential for imprinting diseases, low birth weight, pre-term birth and intra-uterine diseases, it is crucial to confirm or refute the relationship between ART and disease development. Moreover, studies based on animal models must be extrapolated with caution to the human reproductive process since significant difference exist between species with regard to both fertilization and development (Iliadou et al. 2011). Furthermore, there are no long term studies that track the development of IVF babies into late adulthood: IVF is a relatively recent procedure, so ART children are not old enough to reveal late-onset diseases (Johnson 2005). Moreover, they are only just beginning to have children of their own, and trans/multi-generational effects need to be assessed. Finally, the epigenetic effects of superovulation upon the mother or egg donor are still not clear. So, in reality, many of the epigenetic consequences of ART and IVF remain unknown and some people have suggested that a discussion is required regarding whether it is ethical to continue ART under these circumstances (Amor & Halliday, 2008; Rothstein et al., 2009; von Wolf et al., 2015).

6.3.4 The Condition of Infertility

An alternative hypothesis as to why IVF may result in imprinting disorders is that the condition of infertility or subfertility is what is responsible for the epigenetic alterations, not ART procedures. If a reproductive system is not able to: produce gametes; undergo sperm-egg fusion; permit the embryo hatch from blastocyst stage or to implant properly, there must be a nutritional or physiological deficit, or one or more mutations that are inhibiting these processes. Therefore, by artificially aiding the reproductive process, these traits are being passed along to the next generation despite the fact that they are suboptimal. These genetic mutations and/or epigenetic alterations may be inherited by the next generation and may unfortunately manifest as imprinting disorders.

An example of one IVF procedure where this might be the case is ICSI (Intra-Cellular Sperm Injection), which was originally thought to be the direct cause of ART-related imprinting diseases. ICSI compensates for unhealthy or weak sperm that are not able to travel to the egg or to fertilize it without assistance. Therefore, ICSI circumvents fertilization barriers that have been imposed by evolutionary pressures (Laprise 2009). Ergo, it is possible that these suboptimal sperm carry DNA damage that affects the epigenetic imprinting process, and that these deficits are being passed onto the offspring (Maher et al. 2003; Laprise 2009). Numerous studies have looked into this hypothesis and found that children were more likely to have imprinting disorders related to subfertility rather than ART (Vermeiden and Bernardus 2013). Thus, the quality of the gametes used should be taken into consideration and perhaps gametes with DNA damage or genomic imprinting defects should be avoided if possible (although it is by no means clear how this would be assessed – the ability to detect and exclude these gametes is not yet feasible). Also, in extreme cases, we knowingly use immature eggs or sperm if the male or female cannot produce fully matured gametes: this raises both

practical and ethical issues (Laprise 2009). Likely, it is a combination of both the condition of infertility *and* ART that play a role in the disruption of epigenetic marks and the development of relevant diseases.

Continued studies on ART- conceived children and adults, especially long-term studies, are necessary in order to better understand the relationship between epigenetics, ART, and disease. ART-conceived adults are beginning to have their own children now, and this could provide the opportunity to investigate the trans-generational effects (genetic or epigenetic) of these procedures.

We have learned that the pre-conception and conception conditions, intrauterine environment, and external experiences of mothers during pregnancy can alter methylation patterns in a fetus. From this arises the important ethical issue of responsibility. When a drug, environment, or personal decision has deleterious epigenetic effects for descendants two generations down the line, who bears responsibility for the pain and suffering that they endure? When a wrong has been done by people who actually abided by the laws of their time, can blame fairly be placed from the perspective and situation of more recent technological and ethical standards? It easy to say that it is a pregnant woman's ethical obligation to create a healthy environment for her fetus, and that this should include avoiding drugs and dangerous work environments. How practical is this though, when there are social, economic, and cultural influences that might subvert such care?

So, given that epigenetic harms can be absorbed even before conception, where should lines of obligation and responsibility be drawn? Methylation or **histone acetylation** modifications incurred before conception or during gestation can alter gene expression in life outside the womb, and in this sense, biological science could be used to further question the "starting point" of life. Currently in Canada for example, criminal law does not limit abortion, nor are fetuses deemed to be persons (section

223 of the Criminal Code of Canada), thus it can be inferred that women have no legal obligation to their fetuses. In the process of determining legal responsibility, epigenetic studies could reopen the legal "when-does-life-begin" debate. This will be tricky legal, ethical, and social terrain indeed.

6.4 Oral Contraceptives

Oral contraceptives can be used for a variety of reasons, but are generally used to treat menopausal disorders, or to control a woman's menstrual cycle, and prevent pregnancy (Hilakivi-Clarke et al. 2013). Oral contraceptives may also be referred to as "synthetic estrogens" despite the fact that they contain a mixture of steroid hormones including **estrogens** and **progesterones**.

Oral contraceptives prevent pregnancy by preventing the pituitary gland from producing the hormones (LH and FSH) necessary for the ovaries to release a mature egg. Furthermore, oral contraceptives thicken cervical mucus making it more difficult for sperm to travel toward the fallopian tubes. Finally, oral contraceptives thin the lining of the uterus so that implantation of a fertilized egg cannot occur. Despite the fact that combined hormone oral contraceptives work by preventing fertilization, many of the ethical questions surrounding the use of oral contraceptives stem from what some critics define as an essentially abortive function. This stance questions a woman's right to control her bodily and reproductive functions,. Tangentially, even though there may be medically pertinent reasons to prescribe the hormones to teenagers in order to regulate the menstrual cycle, some critics have voiced concerns that this will encourage early sexual behaviour.

6.4.1 Oral Contraceptives and Cancer Risk

Since oral contraceptives disrupt certain **endocrine** axes, their potential for epigenetic effects on various cells and tissues has been the focus of recent studies. Many epigenetic effects are being

investigated including an increased risk of developing breast cancer, disruption of macrophage function, and changes in mRNA expression patterns of a wide variety of genes.

The link between estrogens and breast cancer appears controversial: some studies have confirmed a causative relationship, while others not (Lodha et al.). An association between methylation on the estrogen receptor alpha **CpG island** (ERα) and breast tumor subtypes with bleak prognoses has recently been identified (Izadi et al. 2012). Izadi *et al.* (2014) suggest that never experiencing a full term pregnancy or waiting until after 30 years of age to do so could possibly present higher chances for circling estrogens, natural or synthetic, and/or environmental carcinogens to induce aberrant methylation in the breast tissue before it undergoes pregnancy-triggered differentiation. Furthermore, some results go so far as to suggest that exposure to synthetic estrogens may increase the risk of developing breast cancer not only in the women who used these pills, but in their daughters as well (Collaborative Group on hormonal factors in breast cancer, 1996; Hilakivi-Clarke et al. 2013). The potential epigenetic mechanisms of these trans-generational effects include epigenetic modifications in mammary glands and germ cells, thereby causing a heritable susceptibility to breast cancer development.

Although it has been well known and generally accepted for years now that oral contraceptives reduce the risk of ovarian cancer in women (Collaborative Group on Epidemiological Studies of Ovarian et al. 2008), only a few studies have examined the differences between high and low hormone dose regimens on cancer risk. For example, one study found associations between high-dose progesterone and reduced risk, leading the authors to postulate that progesterone is the central component in the protective effect of oral contraceptives against ovarian cancer (Rodriguez 2003). Another found that low-dose progesterone offers the stronger protection (Lurie et al. 2007). Clearly, studies

on the exact causes of the reduced risk of ovarian cancer in women with prolonged exposure to oral contraceptives have been ambiguous. However, one more recent study yielded interesting results that could possibly explain the ambiguous findings. Faber *et al.* (2013) illustrated that the estrogen potency in the oral contraceptive did not affect the level of reduced ovarian cancer risk. Progesterone alone also did not reduce risk. The authors suggest that the protective effect may be due to anovulation (an egg-free menstrual cycle), finding a 6% risk reduction for each year a woman took oral contraceptives, and this may be independent of estrogen and progesterone intake altogether (Faber et al. 2013).

6.4.2 Other Epigenetic Side Effects

Since oral contraceptives are ingested systemically, they disrupt the hormonal environment throughout a woman's body and this has the potential to affect many biological functions, including the disruption of phagocytic macrophage functions. Macrophages in the bloodstream rely heavily on cues provided by the "milieu" in which they function. A decrease in **global methylation** was observed in the white blood cells of women that were exposed to synthetic estrogens. This hypomethylation is in turn associated with a predisposition to atherosclerosis and other age-related chronic diseases. Furthermore, aberrant methylation of genes in white blood cells could impact immune function, potentially leading to very serious autoimmune diseases or failures to remove abnormal, pre-cancerous cells (Campesi et al. 2012).

Abnormal methylation of DNA could have a plethora of consequences depending on the gene that is affected. A specific example of this is the altered expression of DNMT3a (DNA cytosine-5-methyltransferase 3a; a methyltransferase responsible for controlling gene methylation and the epigenetic imprint) within the developing rat amygdala following exposure to estradiol and dihydrotestosterone (Kolodkin and Auger 2011). The DNMT3a

changes in turn affect multiple other genes, thereby perturbing amygdala function. As we will see in the chapter discussing the estrogen mimic diethylstilbestrol (DES, Chapter 8), there is a possibility that oral contraceptives may have many more lasting and subtle epigenetic effects than presently appreciated.

6.4.3 Oral Contraceptives and Risk to Progeny

From 1960 to today, the number of women using oral contraceptives in the United States has grown from about one to eleven million (Jones et al. 2012). In 1990, the annual FDA Consumer Report declared a general opinion (taken from government, medical, and public sources) in support of the safety and effectiveness of oral contraceptives. More recently, Strifert (2014) noted that the increased prevalence of **autism spectrum disorder** coincides with the increased use of oral contraceptives. He invokes the Knudson hypothesis that was developed to explain the development of cancer (Knudson 1971): an accumulation of "multiple hits" to the genome contributes to the mutations leading to cancer. In the case of autism spectrum disorder, oral contraceptive install the epigenetic changes necessary for Autism Spectrum Disorder to develop. The first "hit" is the damage done to the egg from continued use of the pill. The second "hit" could be epigenetic, environmental, or genetic and this compounding of damage is what yields an autistic phenotype in the progeny (Stifert 2014). There is no direct evidence to establish a causal link between hormonal contraceptives and the disorder, so the relationship could just be coincidence.

6.4.4 Estrogenicity of Drinking Water: Are Oral Contraceptives Part of the Problem?

In vivo fish experiments have demonstrated the potency of EE2 (Ethinyl Estradiol- an artificial estrogen found in oral contraceptives) to be much higher than that of natural **estrogens**

(E1, E2, E3) and the documented environmental effects of oral contraceptives are a growing concern (Thorpe et al. 2003). Estrogen contamination of drinking water is a rising concern: the increase in intersex fish and human reproductive issues raises some alarms (Massart et al. 2006; Wise et al. 2011a). Natural estrogens (E1, E2, and E3) as well as synthetic estrogens (EE2) enter the water supply from many different sources (Table 6.1) (Velicu and Suri 2009). EE2 is such a huge concern because of its higher potency compared to E1, -2, and -3. However, the compiled data depicted EE2 as playing a relatively small role in the total estrogenicity of drinking water. In The Netherlands, EE2 was found to account for about 1% of total human estrogen excretion, which is just one source of estrogens - in the United States, it is likely that the proportion of EE2 is less than 1% (Wise et al. 2011a).

Other noteworthy sources of estrogen include mimics in pesticides like: vinclozolin; organochlorine pesticides, a prime example being DDT; and atrazine, which is known to be extremely mobile in the environment (Thurman and Cromwell 2000; Solomon et al. 2008). Runoff of these pesticides are less potent than E2, however the quantities detected in surface waters are much higher and can produce deleterious effects on aquatic life at levels as low as 0.1 ppb (parts per billion) (Hayes et al. 2010). Although it has been roughly estimated that approximately 40% of the EE2 ingested reaches sewage as active EE2 (Johnson and Williams 2004), the amount of EE2 in relation to all the other estrogens contributing to the estrogenicity of drinking water poses a minimal risk to human health (Wise et al. 2011a).

Source	Waste Management	Where does it go?	Escapes Drinking water treatment?	Measured in drinking water?
Homes	Treated	Surface water	YES	YES
Livestock	Untreated	Surface water	YES	YES
Industry	Treated	Surface water	YES	YES
Agriculture	Untreated	Ground water	YES	YES
Landfills	Untreated	Ground water	YES	YES

Table 6.1 *Sources from which estrogen has the means to enter our water supply.* Adapted from Velicu et al., 2009.

Only water waste from homes and industrial sources is treated twice. If our goal is to reduce the estrogenicity of drinking water, then we are going to have to look at all of the different estrogen sources and act on them. Wise et al., (2010) propose some general ideas of ways to address each identified source: improvements to waste water treatment, detection methods/monitoring, and estrogenicity tests; frequent chemical policy reform, since testing protocols and chemical information need updating; reduction in overall use of EE2; and further research in this area.

Critics have strongly disagreed with the characterization that EE2 posed minimal health risk to humans (Grzybowski 2011). Performing his own calculations based on the Dutch population statistics that Wise et al., (2010) presented, he concluded that EE2 likely contributes 93% of estrogenicity in German drinking water. He appears to have factored in a value for potency that enormously increases results. Without a more detailed explanation of his methods, one must read with a critical eye.

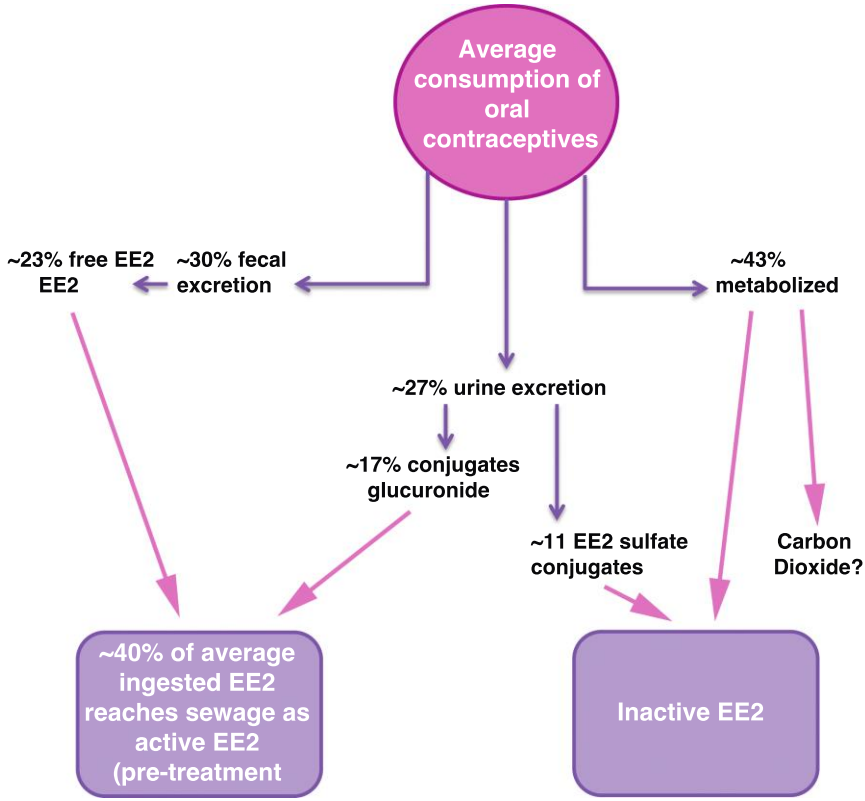

Figure 6.3 *Estimation of percentage of EE2 from oral contraceptives before water treatment. EE2 is a clear contributor to increased water estrogenicity. The amount of active EE2 excreted and available in the system, even considering this is only an estimate, is astonishing. However, taking into account the detection rates of between 0 and 1 per cent of EE2 in drinking water, we should have confidence in our water filtration and treatment plants and focus our attention on what we can do about other sources of estrogen coming into our drinking water. (Adapted from Johnson and Williams, 2004.)*

Wise and colleagues respond with a reiteration of two main points that perhaps they did not make clear in their original paper. The first point is that water treatment systems have become increasingly effective, so the amount of EE2 actually in the water that reaches our taps falls to virtually nothing (Wise et al. 2011b). But are the discussants talking about the same geographical areas,

the same populations, etc.? Perhaps not all water treatment facilities are equally sophisticated. Depending on the population and its practices, the amount of natural and synthetic estrogens coming into the system could differ. The second rebuttal point stressed by the authors is that there are many other overlooked sources of estrogenenic compounds that can be just as harmful as EE2 from oral contraceptives (Wise et al. 2011b). There are still natural estrogens from human waste and untreated agricultural runoff, estrogenic plant compounds, and even other synthetic estrogens from industrial waste. EE2 from oral contraceptives likely plays a role in the estrogenicity of drinking water, but it is not a big enough role to be solely responsible for this growing concern. We must look to all estrogen sources if we want to address the issue.

6.5 Birthing Method

Dahlen *et al.* (2014) invites those who argue for medical intervention during labour and delivery to take a moment and consider what is being given up. Physiological birth, normal vaginal delivery without medical intervention, could be the key to the health and stability of not only the baby, but the future children of that baby. Our birthing day could be a much more significant event in our epigenetic construction than we have given it credit (Dahlen et al. 2013; Dahlen et al. 2014). EPIIC (Epigenetic Impact of Childbirth) is a group that specifically studies the epigenetic effects of childbirth. The EPIIC hypothesis posits that the physiological birth process, from labour to delivery, is in place to exercise purposeful and positive stress on the fetus in preparation for life outside the uterus. The argument is advanced that birth epigenetically programs specific genes involved in calibrating immune response, controlling weight regulation, as well as suppressing tumors. These changes to the epigenome are heritable and could conceivably lead to lifelong, perhaps trans-generational, consequences to health (Dahlen et al. 2013)

During a vaginal delivery, contractions of the uterus and fetal hypoxia stimulate a significant perinatal stress response. There is an increase in stress hormones like cortisol and catecholamines, which serve to: mobilize fuel for the journey through the birth canal; trigger lung-liquid reabsorption to facilitate air-breathing after birth; and to activate the hypothalamic-pituitary-adrenal axis. These processes contribute to the maturation of certain organs, the nervous system, and the immune system (Schlinzig et al. 2009; Cho and Norman 2013). This increase in stress hormones is different in babies delivered by elective Cesarean section: concentrations rise rapidly and suddenly as opposed to the gradual increase that is experienced during vaginal birth. This altered perinatal stress pathway might have epigenetic consequences via modification of gene methylation (Schlinzig et al. 2009). Altered DNA methylation has been observed in glucocorticoid receptors in the **hippocampus**, which leads to higher stress sensitivity, and in genes involved in the pathway that regulates T-helper cell type populations, possibly contributing to the development of immune diseases (Cho and Norman 2013).

6.5.1 Medical Intervention During Birth - The Caesarean Section

During an elective Cesarean section, an incision is made in the lower abdomen in order to remove the baby without having it pass through the birth canal. Since Cesarean sections are surgical procedures, there are potential issues and complications that do not arise during a vaginal birth. This could cause the mother or baby more harm if the procedure is carried out inappropriately. On one hand, a physician must respect their patients' decisions, but also must not expose the mother or baby to unnecessary harm (Muula 2007).

Hematopoietic stem cells were extracted from the umbilical cord and analyzed for differential methylation between infants born by Caesarean section and infants born by vaginal

delivery (Almgren et al. 2014). Cesarean newborns showed more methylation than vaginally delivered newborns and these results were independent of maternal and infant characteristics such as age, weight, gender, and parity. Just over half of the differentially methylated positions were linked to known genes. Surprisingly, about three quarters of the differentially methylated positions in the Cesarean newborns were hypomethylated when compared to the vaginally delivered newborns. Affected genes were mainly those with responsibilities in the metabolism of sugar, and in regulating cell **apoptosis**. At least one gene related to the immune system was differently methylated, and this particular gene has strong associations with a genetic predisposition to type I diabetes (Lie et al. 1999; Almgren et al. 2014).

The clear evidence that the epigenetic profile of the hematopoietic stem cells in umbilical cord blood is altered by mode of delivery could become an issue for umbilical cord blood banks. Can this blood be used for transplants if it is not epigenetically altered? Almgren *et al.* (2014) theorize that these differentially methylated positions do not take immediate effect, but rather require a successive "hit" or "hits" to trigger disease. The next logical step is to compare adolescent and adult hematopoietic stem cells of Cesarean versus vaginally delivered individuals to see whether or not the differentially methylated positions retain their epigenetic marks over a lifespan. Cesarean sections lead to a higher incidence of certain diseases including: asthma, allergies, lower respiratory infections, type 1 diabetes, celiac disease, peripheral and central adiposy, obesity, and inflammatory bowel disease (Magnus et al. 2011; Azad and Kozyrskyj 2012; Bager et al. 2012; Cho and Norman 2013; Flemming et al. 2013; Mesquita et al. 2013). So, not only is our birth *day* a critical life event, but perhaps more importantly our birth *way* makes all the difference: unquestionably, emergency Caesarian sections can save lives, but how should physicians weigh the cost/benefits of the procedure, and how much should we

worry about elective procedures?

6.5.2 The Cesarean Section and the Microbiome

The human body is covered in mutualistic and commensal microorganisms that constitute our normal **microbiota** (Penders et al. 2006). In utero, however, humans are generally considered to be microbe-free (although this has recently come under scrutiny – see Aagaard et al., 2014; Romano-Keeler et al., 2015). It is during birth that normal microbial populations are established in the infant. These microbes are acquired primarily during the journey through the birth canal, and then later from the environment when breathing, breastfeeding, and contact with the parents begins. Babies delivered vaginally are colonized by bacteria from the mother's birth canal and perianal region, while those delivered via Cesarean section are colonized by bacteria from maternal and non-maternal skin, and from the hospital environment (Penders et al. 2006; Cho and Norman 2013). The "hygiene hypothesis" suggests that an altered neonatal gut microbiome leads to changes in the neonatal immune system, potentially predisposing babies to develop certain diseases such as asthma and allergies (Schlinzig et al. 2009). Gut bacteria prime the immune system (Penders et al. 2006) and change the proportion of T-helper cell types so that the immune system can mature. A disrupted balance of microbes in a neonate's gut could prolong this maturation period and impair functional efficiency. This in turn increases the risk for immune diseases later in life (Schlinzig et al. 2009). Moreover, it is also important to note that babies delivered by Cesarean section are more likely to receive antibiotics: the composition of the neonate's microbiota can be changed for years. An altered microbiome would result in unusual metabolite production. These metabolic products could alter the environment for developing cells, influence epigenetic marks and change the expression of certain genes. These are confounding factors when discussing the differences in the microbial flora of babies that were delivered

vaginally as opposed to by Cesarean (Schlinzig et al. 2009). Hence, the establishing human microbiome can both alter and be influenced by numerous factors and is an important aspect of many biological processes, including epigenetic regulation.

6.5.3 Medical Intervention During Birth - Epidural Analgesia and Pitocin

Epidural analgesia, more commonly referred to simply as an epidural, is a common and effective pain reliever during labour. An epidural injection contains a combination of low doses of local anaesthetic and opioid. Epidural analgesia desensitizes the mother and blocks the pain of uterine contractions. Another effect of an epidural is paralysis in the lower body. What does this mean for the physiological birth process if the woman is disconnected from sensation of her body? Data taken from a large cohort of women in Australia showed that one of these effects is a higher risk of Caesarean section after administration of an epidural (Bannister-Tyrrell et al. 2014). Fetal distress is listed as the reason for intervention in a small fraction of cases, so, the epidural seems to be putting more than just a block on the pain, it appears to be quite effectively slowing down labour to the point of having to perform a C-section (Bannister-Tyrrell et al. 2014). One tool to jump start a slow or stalled delivery is to administer oxytocin to the mother.

Pitocin, or synthetic oxytocin (synOT), is also a usual intrapartum (period of time between pregnancy and delivery) intervention. It is used to increase the frequency and intensity of contractions with and without epidurals (Bell et al. 2013). If Pitocin is administered before an epidural, the contraction pain becomes too intense to bear without anesthetic aid. Thus, most of the time, Pitocin and epidural analgesia go hand-in-hand. It culminates in an increased chance of ending by Caesarean section.

As with research on effects of epidural analgesia, studies on Pitocin outcomes are just beginning to surface. One of the first discovered effects that synthetic oxytocin can have on a

developing neonate brain is higher risk for Autism Spectrum Disorder (ASD). Studies have shown a solid predictive correlation between synthetic oxytocin administration and development of ASD later in childhood (Kurth and Haussmann 2011). Oxytocin administration correlates with childhood onset of ASD almost 2:1 compared to controls. Additionally, male infants exposed to Pitocin appeared to have higher risk of developing ASD, although the authors admit this could simply be because of the fact that ASD is generally more frequent in males (Kurth and Haussman, 2014).

Another consequence of synthetic oxytocin for the baby is less optimal levels of pre-feeding cues and breastfeeding behaviour (Olza Fernandez et al. 2012; Bell et al. 2013). This latter group saw medium-low levels of pre-feeding cues and organization in 75% of infants with mothers who had intrapartum oxytocin. In contrast, 100% of the infants with mothers who had no oxytocin had medium-high levels of pre-feeding cues and organization.

Education is the key in creating an informed birth plan and in helping to make tough choices during labour and delivery.

6.6 Breastfeeding

Whether or not to breastfeed is an important decision that must be made by every new mother. It is also the topic of an ongoing ethical debate. Breast feeding has been associated with many benefits, including the provision of antibodies and vital enzymes to protect the baby and aid in immune system development (Jackson and Nazar 2006), as well as providing an overall increase in neurocognitive function (Quinn et al. 2001). Breast milk contains secretory IgA antibodies that can protect the baby from harmful pathogens, and leukocytes, that are thought to influence immune system development. Furthermore, breast milk contains lysozyme (which destroys bacterial cell walls), lactoferrin (which removes excess iron to stop microbial growth), as well as **nucleotides** and complex sugars (which help fight off pathogens

and aid in immune system development) (Jackson and Nazar 2006). Breastfeeding has also been shown to: increase the size of the thymus gland, whose function is T lymphocyte development and maturation; cause an increased immune response to childhood vaccines; protect individuals from allergies and the development of autoimmune disorders (Azad and Kozyrskyj 2012); and to prevent the development of diabetes mellitus (Jackson and Nazar 2006). Breast milk also appears to aid in neurocognitive function, resulting in babies with higher IQ's (Quinn et al. 2001). Moreover, breast milk is free, does not allow for extreme over-feeding, and can help mothers to lose weight. However, noxious substances like polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and infectious diseases (HIV/AIDS) can also be passed from mother to baby via breast milk. Furthermore, breastfeeding may be painful and some people still consider it inappropriate or unacceptable to breastfeed in public.

6.6.1 Breast Milk and the Epigenome

Breast milk is a biologically active substance that includes genetic materials, such as microRNAs, stem cells, and organic substances that affect epigenetic marks and gene expression (Ozkan et al. 2012). Studies have also indicated a relationship between post-natal nutrition, the microbiome, and epigenetic regulations that influence development and the predisposition to certain diseases. MicroRNAs have been implicated in controlling the expression of epigenetic regulators like **DNA methyltransferase** and **histone deacetylase** (Chapter 2). Therefore, if transmitted through breast milk to a baby, this genetic material has the potential to alter epigenetic marks (Ozkan et al. 2012).

The presence of stem cells that are harbored in the baby, but that are derived from the mother or prior siblings in the birth order is known as microchimerism. These "foreign" stem cells might also play a role in development, particularly of the immune

system (Miech 2010; Chan et al. 2012). Breast milk is a significant source of maternal-fetal microchimerism (Dutta and Burlingham 2010), and the maternally derived cells may persist in the offspring for years: they could perform unknown epigenetic effects.

Furthermore, it has also been hypothesized that early post-natal nutrition (breast or formula feeding) epigenetically programs certain organs via the gut microbiome (Mischke and Plosch 2013). The epigenome of intestinal cells and first line immune cells would be most susceptible to these changes, but so would other tissues that come into contact with microbial metabolites, such as fat and liver cells. This is because foods cause adjustments to the composition of gut microbiota: changing the type microbes present alters the cocktail of metabolites released. Studies indicate that breast milk stimulates the development of a microbiome dominated by carbohydrate-fermenting bacteria like *bifidobacteria* and *ruminococci*, which produce large amounts of the secondary metabolite folate. Folate is an essential compound in one-carbon metabolism that provides methyl groups to regenerate S-adenosyl methionine (SAM). SAM in turn provides the methyl groups for DNA methylation. These bacteria may also affect epigenetic marks via increased histone modification since areas of DNA methylation act to attract histone-modifying enzymes. Thus, these bacteria play a role in DNA methylation (and subsequently histone modification), potentially altering epigenetic marks in certain tissues (Mischke and Plosch 2013). On the other hand, formula contains more proteins in order to compensate for low protein quality. More protein therefore travels to the colon and causes an increase in the growth of proteolytic bacteria such as the firmicutes *lactobacilli*, *clostridia*, and *streptococci* that produce butyrate.

Butyrate is the product of microbial fermentation of undigested carbohydrates and proteins. It serves as an energy substrate and therefore contributes to fat accumulation, but it also inhibits the actions of histone deacetylases. This means that in the presence of high levels of butyrate, histones could remain

hyperacetylated, resulting in the epigenetic changes that lead to up-regulation of certain genes. This mechanism of hyperacetylation has been implicated in various cancers, sickle cell anemia, as well as cholesterol, lipid metabolism and storage disorders (Mischke and Plosch 2013). Studies have linked these microbial mechanisms to the incidence of obesity in breast fed versus formula fed

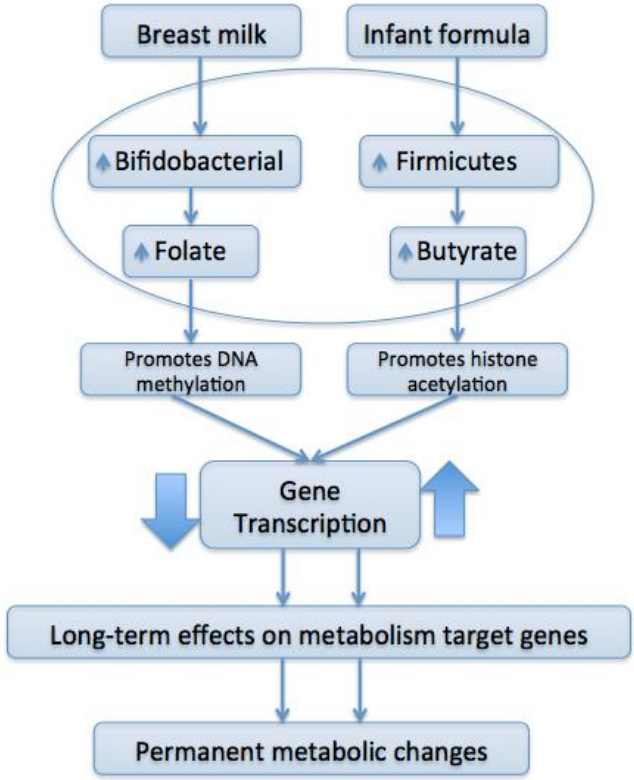

Figure 6.4 *Epigenetic pathways initiated by the reception of breast milk or formula respectively.* Adapted from Mischke, 2013. *The type of nutrients a baby receives is able to cause life-long metabolic changes. Breast milk has been shown to nourish bacteria that promote DNA methylation. This leads to a decrease in gene transcription and is associated with the development of certain characteristics (tend of be leaner as an adult, increased levels of*

intelligence, and increased immune response to vaccines). Formula-fed infants tend to have different gut bacteria that promote histone acetylation, which in turn promotes gene transcription. This leads to the development of other characteristics (predisposition to obesity, cancers, and cholesterol and lipid metabolism and storage disorders).

children: breastfed children are generally leaner well into adulthood (Mischke and Plosch 2013). None of the aforementioned precludes a role for the bacteria that are transferred from mother to child by the intimate contact of breastfeeding. More research is required to understand the role of epigenetics as a link between breast milk and offspring characteristics/ disease development.

6.7 Maternal Programming

We still struggle with questions of nature versus nurture: how much of who we are is genetic and how much is influenced by our experiences? Studies of twins, for example, provide a way of exposing those differences that are derived solely from environmental variables (Haque et al. 2009). Now, mechanisms of methylation and histone modifications offer an entrée to solving this puzzle: environment, genetics, epigenetics, and psychology have never been more entwined. Any infant's environment includes a caretaking individual(s) who shape(s) the infant's immediate world. Early nurturing or rearing deficits now appear to lead to epigenetically encoded mental stability or disorders respectively in later years (Sullivan and Lasley 2010).

One such disorder is chronic physical aggression (CPA). This behavior constitutes an aggression that usually diminishes before kindergarten but that instead persists through to adulthood with predictable consequences for criminality and family stress. Provencal *et al.* (2013) studied a group of males diagnosed with childhood physical aggression on a CPA trajectory and found they showed lowered **cytokine** activity, associated with epigenetic

modification, compared to the control group. The underpinning causes of the association between CPA trajectory and methylation remains a prominent question (Provencal et al. 2014; Provencal et al. 2015): which came first: the methylation or the childhood physical aggression? What events during fetal development or extremely early infant life could be contributing factors? For a more on this study, refer to Chapter 7.

6.7.1 Imprinted Maternal Care Behaviour

In rats, mothering behaviours can be inherited and some of this character may be transferred across generations when the mother fails to nurture sufficiently, and the pup is stressed. This has an impact upon its epigenetic imprint: if a female pup was cared for by a good mother, the pup grew up to be a good mother herself and treat her pups to the same high standard of licking/grooming behaviour to which she had been treated (Sapolsky 2004; Weaver 2007; Weaver 2009). Other studies corroborated these results, and demonstrated the epigenetic mechanisms that underpinned the perpetuation of this behaviour: a gene in the stress response pathway, glucocorticoid receptor, is marked by post-natal experience. Pups absorbed not only maternal caregiving attributes, but differential sensitivity to stress. The imprint is reversible by fostering. Subsequently, another gene in the stress response pathway, **brain-derived neurotrophic factor (BDNF)** was found to be affected by maternal care patterns (Blaze et al. 2013). Pups that were maltreated by their caregivers and pups that were pulled away from their biological mothers, even though they were well-nurtured by their foster caregivers, all had significant methylation on their BDNF gene. Finally, the effects of fear and stress upon the epigenome, during early and adult life, can be passed on to progeny (Sullivan and Lasley 2010; Dietz et al. 2011). These features are discussed in more fulsome manner in Chapter 7, but they raise concerns for the effects of parental care and skills upon epigenetic health in humans.

6.7.2 Depression and Suicidal Behaviour

Unfortunately, depression is a common mental condition that can open the gateway to even more serious conditions such as Major Depressive Disorder (MDD), otherwise known as clinical or unipolar depression. This condition is usually a crucial casual element of suicidal behaviour (Dwivedi 2009). We know that epigenetics is a factor in susceptibility to depression, MDD, suicidal behaviours, as well as other psychological disorders. Programmed cell death, or apoptosis, can be caused by *pan75* neurotrophin receptor impairment. Neurotrophins, are proteins that help to maintain the function, growth and survival of neurons. The methylation status of *BDNF* and *pan75* is linked: low activity of the *BDNF* gene impairs the availability of the pan75 receptor. Suicidal behaviour is also associated with lower expression of the *BDNF* gene and the *TrkB* receptor. The reduced expression of both of the genes is partly due to methylation changes that are detectable in autopsy specimens of suicide victims. Clearly, suicidal behaviour may have a more complex etiology than formerly supposed (Dwivedi 2009). The experience of depression and its consequent effects upon the epigenome are not tied solely to the individual who experiences them. Recall in the beginning of the chapter we explored the epigenetic effects of depressed mothers on fetuses during gestation. This kind of cyclical epigenetics is part of what makes causative relationships so extremely difficult to define.

CASE STUDY 6.4 – Post Traumatic Stress Disorder (PTSD)

Adverse social environments, in utero challenges to infant biology, a history of familial PTSD, and traumatic childhood experiences such as sexual or physical abuse are all ways in which epigenetic mechanisms play a role when it comes to vulnerability to PTSD (Yehuda and Bierer 2009). We, like rats, are all born with a pre-programed attachment system allowing us to identify, remember, and bond with our primary caregivers. In a healthy environment, the caregiver is strongly associated with safety, effectively turning off fear. On the other hand, in an abusive environment, a child can become desensitized to the difference between safety and fear. Instead of turning off fear, the attachment system turns off the ability to fear, because no matter what we always seek out attachment in early life (Sullivan and Lasley 2010). In rats, Sullivan and Lasley (2010) found that once the rats became older and started to venture from the nest, their ability to learn fear from outside the nest functioned properly and they demonstrated behaviour comparable to repressing and possibly snuffing out previous fear, avoidance, maybe even memories, associated with the nest. The same could be thought to occur in adults who experienced abuse as children (Sullivan and Lasley 2010). The likelihood of an underlying epigenetic connection is a topic for further research.

6.8 Conclusions

Evidently, some conditions might not necessarily be the consequences solely of our life choices – we may inherit predilections from our progenitors and environment. This raises the question of whether epigenetic mechanisms will be used as a scapegoat. Will we take responsibility for what we can control, or will we evade it claiming that ultimately our health and behaviour is the fault of various external drivers of our methylation status? Because methylation can change over a lifetime and generations, it

is extremely difficult to know which epigenetic markers are inherited, which are acquired through our own life choices, nor which are combinations of both and to what degree.

Remediation is not necessarily impossible: when two *Agouti* mice homozygous for the *Agouti* mutation mate and conceive pups, the pups will inherit the *Agouti* coat colour and obesity traits. However, if during pregnancy, the mother is fed a methyl-rich diet, it changes the activity of the *Agouti* gene. In this case, too, methylation patterns can be improved (Waterland and Jirtle, 2003). Epigenetic alterations can be large or small. They can affect any chromosomal region, and thereby alter patterns of gene expression. We have only begun exploring all of the epigenetic pathways connecting environment, gene expression, and behaviour.

So what is a responsible strategy to pursue if we want to be healthy and to provide the best for our prospective descendants? We should live in a way that promotes our own epigenetic health and be considerate of the epigenetic health of prospective future children. Unfortunately, the epigenetic disadvantages that we inherit, we must live with, however, if we are proactive with regard to diet, behaviour, and environment, then perhaps these disadvantages will not affect us as severely, or perhaps they can be reversed. The field of epigenetics provides a deeper understanding of human biology and psychology and it poses both difficult questions as well as promising possibilities.

Provocative Questions

- Who is at fault when it comes to epigenetic disadvantages, especially ones resulting in major deficiencies? Should there be a law outlining legal epigenetic responsibility?
- Infertility treatments – Are they worth the risk and cost?
- Birth control – do formulations of the pill cause cancer via epigenetic means? What are the long term effects of the

pill, both for the individual, her offspring, and the environment?

- How much should we worry about estrogen and mimics in the environment – how severe will their effects be upon our epigenetic health, reproduction, and development?
- There seems to be a strong case for breastfeeding and birth free of medical intervention. Will epigenetic research push women to decide against epidurals and scheduled C-sections? Who gets to decide?
- How do we deal with pre-conception damage and liability? Should we?
- Could targeted treatment reverse methylation changes and therefore cure psychological disorders like PTSD and MDD (massive depressive disorder)?
- Allocating legal epigenetic responsibility could potentially necessitate extremely intrusive laws and policies that overwrite parental freedoms to varying degrees. How far should we go?

Possible Solutions

- Many consequences of Assisted Reproductive Technologies are still unknown. A focus must be turned to educating parents on all potential deleterious effects of fertility treatment options so informed decisions can be made. Long term studies spanning generations should follow-up IVF/ART progeny.
- Birth control may provide the conditions in the body where vulnerability to breast cancer is higher and susceptibility to ovarian cancer is lower. However, we do not know exactly what effects birth control has on eggs in the ovary – more study is needed.
- A mother always has the right to have an epidural, or refuse it. Her right to her own birth experience, and her own

assessment of pain she can or cannot handle, should not be compromised. Pain is scary, and this makes birth scary. The more women learn about physiological birth and the disruption of natural epigenetic processes, the better. With the proper care and intrapartum support, women will be endowed with better tools to make informed decisions.

- Novel treatments for psychological ailments such as depression and PTSD as well as physical disorders will be a future avenue for epigenetic study. Ethical concerns will inevitably surround epigenetic treatment since behavioural modification has a history fraught with baggage. As in the case of human fetal subjects, determining the effectiveness of epigenetic treatment in humans will inevitably begin in animal models and move to human clinical trials.
- Education needs to be pursued in a comprehensive, continuous, and evolving way to enable *all* stakeholders to make informed decisions, and help to construct fair regulatory, social, and legal frameworks.

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Chapter 7

The Epigenetics of Opportunity: Culture and Socialization

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Abstract

This chapter will explore the effects that culture can have on an individual's epigenome, as well as how an individual's epigenome may affect her place in society. For example, obesity, addiction, and depression plague traditionally marginalized demographics, but their persistence throughout generations may have epigenetic roots. We have known for a long time that mothers who smoke or suffer from depression tend to give birth to babies with lower birth weights, but we now know that this effect is correlated with measureable epigenetic changes in the infant. Additionally, maternal care during early childhood can cause demethylation: poorly nurtured rats display poor parental care themselves and treat their own pups badly, thereby perpetuating the cycle. Moreover, stress, fear, and drug addiction can have trans-generational effects on the epigenome, at least some of which might be reversed through diet. Finally, surveys are now demonstrating that socioeconomic status and exposure to aggression exerts effects upon the epigenome. Given that the human genome is now known to hold lineage-dependent differences, some investigators have interrogated the role of race in epigenetic modification. We

discuss how these studies lack a fulsome analysis of non-biological differences: components of culture such as diet, geographic location, and social/religious practices are likely to be playing a confounding role. We conclude with a discussion of culture's place in the study of epigenetics, and outline some of the challenges that society will have to face when epigenetics is integrated into how we understand culture.

7.1 Introduction

Humans exist and act both within a biological sphere as well as within a well-established social arena. Humans shape and guide society to fit their needs, but epigenetics now provides a tool that permits us to see how the societies we build can in turn affect us on a biological level. Conceptually, this is not new: genetically similar links have been long understood – for example lactose tolerance. Lactose tolerance is the result of genetic mutations that arose and conferred an advantage as humans developed agrarian societies (Laland et al. 2001). This genetic change was the result of a random mutation that became fixed by a cultural shift towards the domestication of livestock. The use of milk as a food source, where lactose tolerance could accommodate it, provided a reliable dietary supplement that rendered populations more likely to survive and propagate the genetic trait (Laland et al. 2001). Natural selection usually takes centuries to change the sequence of DNA and to produce a beneficial phenotype, however, culture can move much more quickly, and, as it transpires, so does epigenetics. The epigenome can change within a single generation (Szyf 2007; Dupras et al. 2012). Moreover, it is sometimes reversible and provides a flexible framework that can keep up with frequent cultural or ecological shifts. In sum, the epigenome provides a direct biochemical mechanism that links the influence of constructed environmental attributes, both physical and social, with the long-term packaging and behavioural patterns of our genes.

Among the most significant components of existing social frameworks is culture. Culture informs patterns of child nurturing and rearing, as well as patterns of organized behaviour, diets, socioeconomic stratification, and education. Culture refuses simple definition, but at its simplest, it can be considered to be a constructed framework of interacting elements that include world views, beliefs, traditions, artifacts and behaviours. These concepts are transmitted between generations and other cultural groups through social learning (Laland et al. 2001). Thus, culture can be simplified as a fluid collection of learned traits, artifacts, and practices that help to express, and at times codify, an individual's behaviour. Considering that enculturation is achieved predominantly through social means, culture can be considered to be among the most potent mediums for addressing the effect of the social environment on the epigenome.

In order to facilitate research, the sometimes nebulous concept of culture is often broken into discrete components to permit quantitative measurement (Laland et al. 2001). Cultural focal points for investigation may include dietary choices, social and physical activities (examples include drinking, smoking, exercise), religious denomination, and even the geographical spread of migrating populations (Braam et al. 1998; Laland et al. 2001; Toyooka et al. 2003; Delport and Pollard 2010). The evidence of socioeconomic impact upon an epigenetic profile contributes to the larger conversation regarding the role that socioeconomic status can have upon human health: it adds an additional layer of complexity upon the interactions that are manifest in our experience of geographical location, food sources, disease, climates, and social interactions.

No robust discussion of culture and geography can ignore issues of race, so the question arises, to what degree are the hard-wired genetic underpinnings (haplotypes) that differentiate race separable from the effects promulgated by cultural practices? This is understandably one of the more controversial and technically

challenging elements to distinguish. Indeed, we might even ask if it is even possible to identify a biologically sensible notion of race in the context of epigenetic studies? Race and links to epigenetic health factors have been addressed in the literature, however we will argue that some researchers have been too quick to claim that the minute biological differences between races have can explain epigenetic differences between populations.

The influence that maternal care, cultural practices, socioeconomic status, geographic location, and race have in facilitating changes to the epigenome will be addressed sequentially while we will also highlight the intricacy of their interactions. We will discuss potential avenues through which continued research and greater education concerning cultural effects upon the epigenome could be broadcast to benefit the larger community.

7.2 Socioeconomic Status and the Epigenome

7.2.1 *Introduction to SES-methylation patterns and research methods*

Socioeconomic status (SES) is a term that attempts to quantify level of opportunity/life chances that an individual or family enjoys, and it takes into account their hierarchical position in society, occupation, education level, and affluence. Not surprisingly, SES is tied to health, with individuals in less privileged socioeconomic positions more likely to be afflicted with multiple chronic health disorders up to 15 years earlier than individuals who occupy higher socioeconomic strata. (Barnett et al. 2012). Certain illnesses, such as heart disease, diabetes, and mental illness, are known to affect those with low SES at greater frequency compared to the more advantaged members of society (Adkins et al. 2011). Recently, research has examined the epigenetic mechanisms by which socioeconomic factors influence human gene expression, and ultimately, health and morbidity.

SES is an important determinant of the quality of one's life –

it impinges on life opportunities: access to health care, to healthy food, to education, to security as well as to opportunity. Low SES is associated with lower educational attainments, and lowered educational achievements are a predictor of employment status and income – a self fulfilling cycle. Health will likely suffer if one is born into a poorer family with a history of lower educational success. Consider the following scenario. As far back as 1999, in an isured household in the U.S., if one was diagnosed with a serious health problem, the individual, on average, will lose about $17,000 in out-of-pocket healthcare expenses and employment over the next year, not to mention the years following (Smith, 1999). A decrease in the individual's ability to work, in combination with medical costs, results in the reduction of SES. Evidence of direct inheritance of poor health and opportunity is sparse, but social epidemiology at the statistical level provides support to these claims (Loi et al. 2013).

Factors that affect patterns of **DNA methylation** include diet, lifestyle, cigarette smoking, and insufficient maternal care or diet. Other epigenetic modifiers, such as exposures to harmful substances such as water pollutants, pesticides, and other environmental toxins, are linked to poor SES and related sub-optimal geographical location and substandard living conditions (Rothstein et al. 2009). It is becoming clear that these factors can have adverse effects on populations via stable epigenetic changes, increasing risk in individuals and to specific strata of socio-economic cohorts (Rothstein et al. 2009). To better understand the influence that epigenetics can have on socioeconomic status, and how researchers can correlate methylation and socioeconomic status, consider the case study of the pSoBid cohort in the Case Study below.

CASE STUDY: Epigenetics in the pSoBid Cohort

Glasgow experiences high levels and distribution of population morbidity and mortality that cannot be explained by conventional risk factors. McGuinness et al. (2012) found an association between the SES of an individual and global DNA methylation patterns. The test subjects were a subset of the pSoBid cohort, a cross-sectional population-based study established in 2005 by the Glasgow Centre for Population Health (GCPH). The study was designed to track and investigate the psychological, behavioural, and biological determinants of ill health. Researchers hypothesized that social deprivation would be associated with a reduced methylation in the genome (i.e. hypomethylation) and that this would consequently account for enhanced inflammation and increased risk for disease, specifically cardiovascular disease (CVD).

Participants in the study comprised a subset of 239 participants (from a cohort of 666) that provided DNA for analysis. Participants were ranked using multiple deprivation indicators to define most and least deprived areas in Glasgow. The criteria were based on the Scottish Index for Multiple Deprivation (SIMD). Participants were then stratified so as to maintain a comparable distribution as in the original cohort. Other lifestyle factors such as diet, cigarette smoking status, and alcohol consumption were added to the base model, which already consisted of age, gender, and deprivation group. Following the extraction of DNA from peripheral blood leukocytes, a qualitative and quantitative analysis of DNA was performed. Researchers measured total DNA methylation level in blood samples as a percentage of the total DNA present. This method of analysis, therefore, did not reveal specific patterns of methylation that are associated with particular traits, but served as a general indicator of methylation extent and of epigenetic health.

Researchers applied linear regression models to reveal associations between global DNA methylation and SES lifestyle factors. Results showed that the middle age group, aged 45-54 years were the least methylated of the three age categories, and 16% less methylated than the youngest age group, 35-44 years old, and 10% less methylated than the 55-64 year old age group. The most socioeconomically deprived group was found to be 17% hypomethylated relative to the more privileged. Manual workers showed 24% more hypomethylation than non-manual working counterparts. Each year of education attained resulted in as much as 2.4% more methylation relative to less-educated participants.

Researchers analyzed sixteen biomarkers in hopes of finding a particular biomarker to help link socioeconomic status to risk of cardiovascular disease (CVD). Interleukin-6 (IL-6) and fibrinogen were studied in depth because they showed inverse relationships with respect to an increase in methylation. IL-6 had its greatest decrease by 20% in the oldest age group, whereas fibrinogen had its largest decrease by 7% in the youngest age group. Neither IL-6 nor fibrinogens were affected by any of the other lifestyle factors; therefore, any variable besides the base model, consisting of age, gender, and deprivation, had no effect on the amount of IL-6 and fibrinogen present. Since IL-6 is a biomarker of heart disease, its expression suggests a relationship between global DNA methylation and systemic inflammation (McGuinness et al. 2012).

In summary, global DNA hypo-methylation was associated with the most deprived participants. DNA hypo-methylation found in the most deprived could be the result of diet over a lifetime, or

during in utero development, environmental exposures, or any combination of factors (McGuinness et al. 2012). Although these are important findings, global methylation levels help little when it comes to pinpointing causal chains between the factors measured – effects could be indirect or coincidental (Landecker and Panofsky 2013). Another challenge the study presents is the limitations presented by the sample size; participants used for the study only represented the bottom 5% and top 20% of Scottish Index for Multiple Deprivation score. Ethnicity was not accounted for, the only related criteria deployed was to select for English speakers. The assumption was that the Glasgow cohort was ethnically mixed to an extent comparable to Liverpool and Manchester, where morbidity was not as severe. This could probably not be argued to represent the full population spectrum. Precisely why Glasgow citizens experience such a prevalence of cardiovascular disease remains uncertain, however, further studies concerning the relationship between hypo-methylation and increased expression of biomarkers should contribute to this conversation.

Similar studies corroborate these findings. Borghol et al. (2012) chose to focus on the regulatory sequences of specific genes to see if associations between childhood socioeconomic status and DNA methylation in adults could be identified (Borghol et al. 2012). Participants for the study came from the British Birth Cohort Study, an ongoing, longitudinal study, tracking males born in the same week during 1958. Researchers collected samples from forty males, aged forty-five years old at the time, and analyzed data based on participants' SESs in both childhood and adulthood. They were able to identify 1252 gene promoters whose methylation levels are associated with childhood SES (666 in high SES and 586 in low SES) and 545 associated with adult SES. Intriguingly, there was little overlap in the differentially methylated promoters associated with childhood versus adult SES (Borghol et al. 2012). Moreover, they also found clustering in specific regions of the genome linked to specific functions; in low

SES individuals, there was evidence of higher methylation, and thus repression, of sensory perception of smell and taste. Since DNA was only available in adulthood, it is unknown whether the methylation occurred in childhood, or if findings reflect experiences delivered in the prenatal environment. Further investigations exploring the relationship between pre-natal, childhood, and adult socioeconomic status health warrants future attention.

7.2.2 *Socioeconomic Status and Diet*

In the United States, being socioeconomically disadvantaged correlates with a higher likelihood of obesity: a lack of healthy food stores, safety, and places to exercise were the main factors holding back the disadvantaged (Lovasi et al. 2009; Rundle et al. 2009). Dietary choices are guided by many factors: economics, geography and cultural practices to name a few. In recent history, changes to the human diet (particularly in North America) have corresponded with a cultural shift from a nomadic lifestyle towards extreme sedentarism. This transition was facilitated largely by agricultural surplus and high carbohydrate/sugar diets (Chapter 4). Delport et al. (2010) specifically identify obesity as a product of both biological and cultural forces. Obesity can perpetuate itself trans-generationally through epigenetics, which can in turn make it even harder to escape the socioeconomic factors that can contribute to obesity: a Swedish study of 700,000 found that obese males performed significantly worse in school even when adjusting for intelligence and socioeconomic position. This discrepancy is attributed to discrimination in the educational system and general society (Delport and Pollard 2010).

The trans-generational effect of maternal food choices is associated with circulating levels of the hormone leptin, a protein that originates from adipocytes (fat cells) and plays a vital role in determining an individual's appetite via the hypothalamus. Energy-dense and highly processed diets during the third trimester

of pregnancy can cause the up-regulation of lipid transport across placental membrane. This will cause the fetus to develop more fat cells within its adipose tissue. As a consequence of the high fat intake and the increased leptin levels, the child will likely be born heavier and with an increased appetite during post-natal development. In adulthood, the expression of numerous genes within adipose tissue has been implicated in an individual's body mass index (BMI) calculations (including the methylation of leptin promoters), so it appears that epigenetically-driven phenotypic changes may account for body weight changes (Stoger 2008).

Socioeconomic status and culture not only affect diet content, but also meal frequency. The reality for many of the socioeconomically disadvantaged is not lack of food but inconsistent access and poor quality (Oldewage-Theron et al. 2006). Could there be an epigenetic consequence to consistent short term fasting, even if an individual does not starve? Funato et al. (2011) conducted research on the effects of fasting on the expression of **histone deacetylase (HDAC)** within mice. You will recall from Chapter 2 that HDACs are responsible for **histone acetylation** at specific genomic sequences to either repress or enhance gene activity, a function that has been previously linked to behavioural changes (Funato et al. 2011). The latter results showed that dietary alterations have an impact on the expression of different HDAC transcripts (numbered 1-11) in the medial hypothalamus, an area of the brain responsible for regulating eating and body-weight (Funato et al., 2011). After 16 hours of fasting, there was an increase in HDAC3 and HDAC4 expression and a decrease in the normally high hypothalamic levels of HDAC10 and HDAC11.

Other effects of fasting include the decrease in HDAC8 expression in a portion of the hypothalamus previously shown by other studies to exhibit changes in the expression of proteins associated with feeding behaviour (such as TRH: thyrotropine-releasing hormone). Another area of the hypothalamus involved in

aggression and reproductive activity was shown in this study to exhibit lower histone acetylation levels. Cumulatively, these effects indicate the extensive impact that fasting can have on expression levels in the brain, with meal time influencing a variety of potential functions ranging from eating patterns and homeostasis to aggressive and reproductive behaviour (Funato et al. 2011).

Xu et al. (2012) addressed the impact of fasting on 3-day old chicks at both 6 and 24 hour fasting lengths. Like Funato et al. (2011), Xu et al.'s (2012) research showed changes in expression levels within the hypothalamus. The study focused on the methylation of histone 3 at a specific amino acid: lysine 27, here referred to as H3K27. Fasting resulted in the significant increase in di- and tri-methylation of H3K27. EZH2, the H3K27-specific histone methyltransferase (HMT) responsible for the transfer of methyl groups to lysine, showed an increase in concert with the increased methylation (Xu et al., 2012). This alteration occurs within the pre-optic/anterior region of the hypothalamus, an area associated with maintenance of body temperature and feeding. This is similar to the functions of the hypothalamic locations addressed in Funato et al.'s (2011) research (Xu et al. 2012). Alongside these methylation events, a significant increase in thyrotropin-releasing hormone, an integral component to the metabolism-regulating hypothalamic-pituitary-thyroid axis, occurred after 24 hours of fasting (Xu et al. 2012). Fasting appears, therefore, to have an influence on metabolic regulation, particularly during early chick development.

Although these mechanisms and results cannot necessarily be directly applied to humans, they still serve to display the profound effect of diet on the epigenome and indicate the potential for human fasting practices to likewise produce epigenetic effects. Short-term fasting as a religious activity, however, is common; it is a component to belief systems such as Christianity, Islam, Judaism, and others. Since Funato et al. (2011) and Xu et al. (2012) discovered alterations to the epigenome in mice and chicks after

fasting durations of less than twenty-four hours, there is potential for short-term and culturally motivated fasting in religiously observant humans to also impinge on the epigenome.

7.3 Epigenetic Factors, Culture and the Mental State

7.3.1 *Early childhood, stress, depression, and anxiety*

There is a strong, consistent correlation between mental illness and low socioeconomic status, and while this link is not necessarily causative, nevertheless the relationship suggests a connection (Hudson 2005). Certainly, those who are mentally ill and have a low SES will have worse health than people who have the same mental illness but have a higher SES (Barnett et al. 2012). Mental illness also presents a vicious cycle, since by its nature it can limit opportunities for social mobility, not to mention adding to burdens by attaching a cultural stigma. The study of mental health stands to benefit greatly from ongoing research into epigenetics. While personality and mental disorders have clear roots in genetics, there are still many environmental factors that shape who we are.

Maternal behaviour can be inherited by female offspring. Sapolsky *et al*. (2004) found that rat pups that experienced high levels of licking and grooming from their mothers grew up to be mothers who licked, groomed, and cared for their own pups well. Demethylation and **acetylation** responses were a direct consequence of maternal care and caused permanent and heritable changes to the epigenetic packaging of specific gene loci that influenced behaviour later in life (Sapolsky 2004). Specifically, pups that experience high quality grooming have increased serotonin levels and demethylation of the glucocorticoid receptor gene. This in turn results in more glucocorticoid receptors being expressed in the brain, and lower levels of circulating glucocorticoid, a stress-related hormone (see figure 7.1). Ultimately, high quality maternal grooming behaviour and a greater resilience to stress emerged (Sapolsky 2004). Weaver *et al*.

(2008) discovered that reciprocal fostering of pups by good and bad mothers reversed the imprints inherited by the pups. So, if they had a mother who maltreated them, despite whether she was a biological or foster mother, the rat grew up to be a bad mother and to have a lower capacity to cope with stress. If the infant had a mother who licked, groomed, and cared for them properly, despite whether she was a biological or foster mother, the rat grew up to be a good mother, and in turn passed this trait onto her young (Weaver et al. 2005).

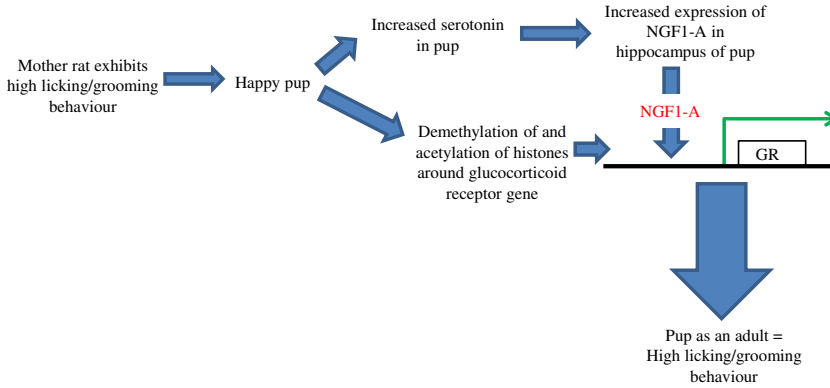


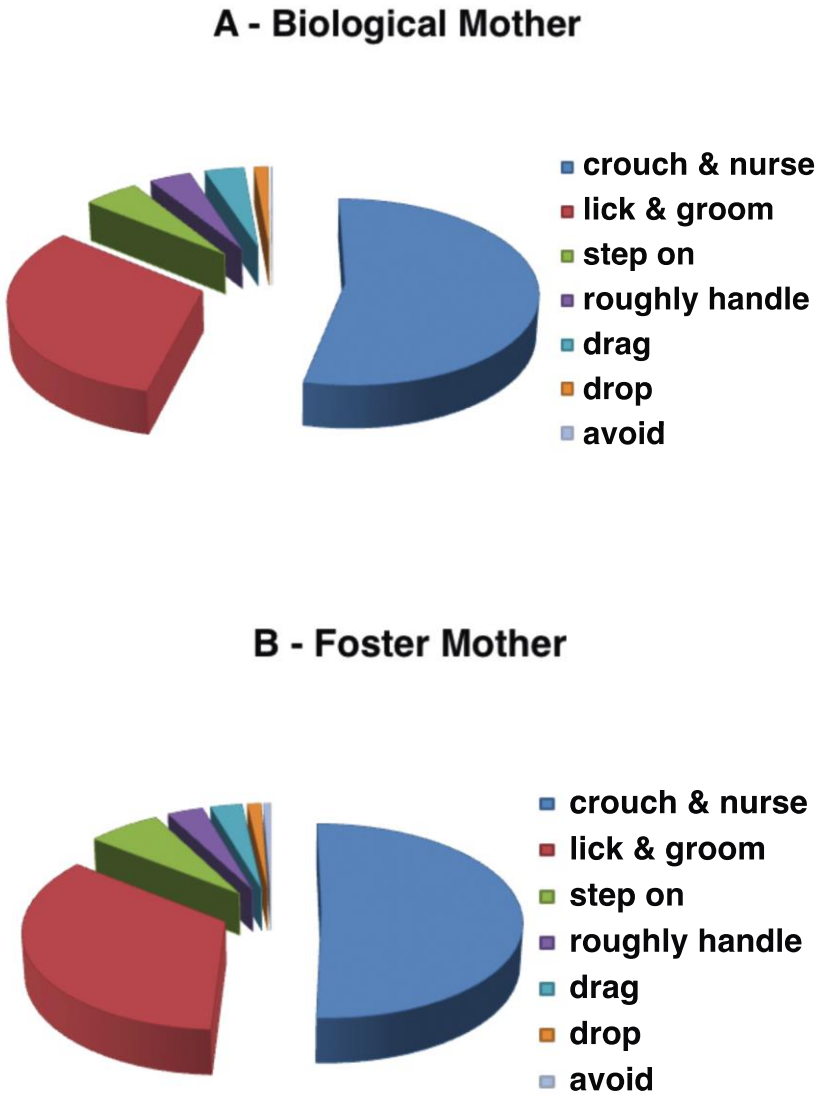
Figure 7.1 *Epigenetic mechanisms associated with imprinting of high licking/grooming behaviour. Adapted from Sapolsky, 2004. Rat pups who experienced high levels of licking/grooming from their caregivers demonstrated increased expression of NGF1-A in the hippocampus as well as a demethylation of and acetylation of the histones around the glucocorticoid receptor gene. These two conditions combined result in adult (previous pups) expression high hippocampal glucocorticoid receptors, low glucocorticoid levels, and ultimately high licking/grooming behaviour toward their own pups. Epigenetic imprints due to maternal care behaviour remain throughout the rats' lives and impact their own mothering style.*

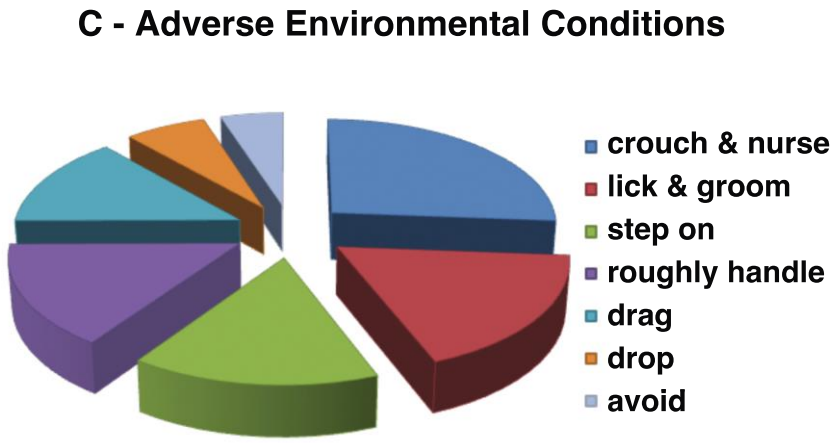
In another study, investigators studied infant rats reared by either a good or a negligent mother (Blaze et al. 2013). Like Weaver *et al.* (2008), they discovered that depending upon whether

the rats were reared by a biological parent or fostered, the female pups would be imprinted as good or bad mothers by the context of their conditions, and grow up to perpetuate that legacy (Figure 7.1). These behaviour differences, as well a sensitivity to stress, were linked to epigenetic imprinting of the stress-related glucocorticoid receptor gene (Weaver et al., 2008). Interestingly, Blaze *et al.* (2013) also found increases in methylation of the regulatory region of the gene for **brain-derived neurotrophic factor** (**BDNF**) in both males and females of the foster groups despite the nurturing environment. Finding increased methylation in the maltreated groups was expected. The BDNF gene encodes brain-derived neurotrophic factor, a member of the neurotrophin family of proteins that assist in nerve growth and maintenance. BDNF regulates patterns of neuronal sprouting and connectivity. Changes in methylation of BDNF are associated with high levels of stress-induced release of glucocorticoid. In this case, even the stress of being pulled away from their biological mother was enough to induce methylation of the *BDNF* gene (Blaze et al., 2013).

Abnormal packaging of the *BDNF* gene is a significant but small piece of evidence showing that early life trauma can have long-term consequences for brain wiring, and consequently also for behaviour (Roth and Sweatt 2011). Mice that are reared in a communal nest and that enjoy surplus maternal attention and care grow up to enjoy a predilection for adult social interactions, specifically ones that coincide with higher hippocampal BDNF protein levels (Branchi 2009; Branchi et al. 2009). By contrast, stress induces hippocampal atrophy, thought to be mediated by BDNF, and depression is the result (Warner-Schmidt and Duman 2006). The **hippocampus** is the brain region that forms and stores cue and context stimuli learned from fear conditioning, and that processes information from short-term to long-term memory.

Figure 7.2 *Maternal care behaviours exhibited in the three study group mothers.*





A *– caregiver was a good biological mother* ***B*** *– caregiver was a good foster mother to a pup from a poor one, and* ***C*** *– environment imposes maltreatment conditions.* Adapted from Blaze et al., 2013. *Biological relation to pups made no significant difference in caring behaviour in a normal environment. It could be hypothesized that the cross-foster rat pups still received the imprint of good mothering despite the stress-induced methylation from being pulled away from their biological mother.*

In mice, both male and female offspring of socially defeated fathers exhibit depression and anxiety-like behavior. Interestingly, these results were not usually observed when the offspring were created via IVF (Dietz et al. 2011). This could suggest that the epigenetic modification inherited by the offspring to induce the depressive or anxious behaviour is inherited when fertilization occurs in the fallopian tube and the natural process of implantation ensues. Alternatively, it could be hypothesized that the IVF process sometimes reverses or overwrites the epigenetic change inherited from the father.

Chronic and unpredictable maternal separation experienced by infant mice induces depressive-like behavior, and the male pups that experience maternal separation pass this behavior onto their offspring (Franklin et al. 2010). This is related to a phenomenon

called learned fear. If something repeatedly results in a negative or harmful outcome, an association is made between the event and the outcome, usually resulting in fear of that particular event. Normally, remembering danger is absolutely necessary for survival, so early memories based on fear cannot be overwritten and therefore will persist over the lifespan (Sullivan and Lasley 2010). Should this learning by fear give rise to any epigenetic modifications, they could be inherited by future offspring.

For example, the regulation of **chromatin** structure and DNA methylation is an important manager of gene transcription in the central nervous system: Zovkic and Sweatt (2013) found methylation changes in factors affecting the transcription of genes in the hippocampus. These changes also have the potential to be inherited. Therefore, if a parent learns a fear trigger and this causes alterations in chromatin structure and DNA methylation, the offspring could inherit these modifications and his or her hippocampus could develop so that it is more or less sensitive to the same stimulus as the parent (Zovkic and Sweatt 2013). This learned fear is also correlated with **post traumatic stress disorder** (**PTSD**) (Takei et al. 2011).

Early experiences are not the only way for epigenetic modifications to occur around DNA. Traumatic stress that occurs in adult rats can induce central nervous system gene methylation (Branchi 2009; Branchi et al. 2009; Roth et al. 2011). Hippocampal dysfunction in adults is also a response to traumatic stress that may be caused by epigenetic changes to the *BDNF* gene (Roth et al. 2011). It is reasonable to hypothesize that these brain and nervous system gene methylation patterns also happen in humans not only during childhood, but also in adulthood (Roth et al. 2011). Interestingly, susceptibility to PTSD in children of traumatized parents appears to reveal an epigenetic dimension that is trans-generational. For example adults whose parents survived the Holocaust were more susceptible to PTSD and recovered more quickly than their control counterparts (Yehuda et al., 2015; also

see chapter 6, specifically Case Study 6.4)

After Roth *et al.* (2011) found increased methylation in stressed rats compared to non-stressed rats, Roth and Sweatt (2010) hypothesized that a drug with the ability to reverse methylation may also reverse the abnormally low BDNF expression. After administration of a demethylating drug, BDNF was restored to normal in the stressed rats (Roth and Sweatt 2011). There is potential to reverse the behavioural consequence of this methylation, thus opening up novel treatment options for PTSD and other psychological conditions linked with epigenetic mechanisms. Weaver *et al.* (2005) also saw some success in methylation treatment in adult rats. In this case, they added dietary methyl donors to restore normal maternal parenting skills and stress responsiveness, thereby reversing the epigenetic effect of maternal behaviour on offspring DNA (Weaver et al. 2005). Further research needs to be done to determine if the reversion of normal expression in the adult brain directly results in reversion to normal maternal care behaviour as well.

These experiments all find epigenetic causes of traits linked with depression, anxiety and PTSD, providing scientific context for the common wisdom that early childhood nurturing is important for proper growth. Should these mechanisms prove persistent in the human epigenome, they would have implications for family structure decisions. This information would place more pressure on parents to stay home with their children, which for many is not an economically viable choice. The trans-generational effect of early nurturing would also help to contribute to a poverty loop. The solutions seem simple however (albeit difficult to implement depending on the political climate): either increased paid parental leave or increased access to high quality preschool.

7.3.2 *Aggression*

Another element of stress is the experience of aggression. Recent evidence suggests that DNA methylation of both **cytokine**

gene regulatory regions, and the transcription factor genes that regulate them, operate in response to environmental factors, such as social signals and parental behaviour (Provencal et al. 2013a). Cytokines are important signaling molecules for the immune system. Boys who maintain high levels of aggressive behaviour from kindergarten, through to adolescence, tend to be "impulsive, hyperactive, oppositional and rejected by their peers". Oftentimes, the domestic environment and familial relations of these individuals fail to encourage emotional regulation (Provencal et al. 2013a). The hypothesis was that stressful early life experiences are associated with methylation changes that could contribute to a **chronic physical aggressive (CPA)** trajectory. Recognizing the prevalence of physical aggression in this cohort of young boys, Provençal et al. (2013a) set out to test levels of cytokines in the blood with long-term regulation of physical aggression in them as young male adults (Provencal et al. 2013b).

Two groups were part of the study: one group of seven males on a CPA trajectory and one control group of twenty-five males on a normal trajectory. Aggressive behaviour was monitored by schoolteachers each year from ages six to fifteen. Provençal et al. (2013a) analyzed DNA methylation profiles of five cytokine genes that were known to show lower plasma levels in aggressive males. The methylation study employed blood samples taken from the males after they reached adulthood (Provencal et al. 2013b). Teacher-assessed hyperactivity and opposition disorder during childhood and adolescence predisposed the males in the CPA group to aggressive behaviour into early adulthood. 71% of the CPA group had a criminal record by age twenty-one, compared to 20% of the control group, and 57% of the CPA group reported acts of physical violence by the age of twenty-one, compared to 10% of the control group (Provencal et al. 2013a). Clearly early patterns of behaviour tend to predispose to similar patterns in adult life.

Tests for associations between DNA methylation and cytokine levels revealed 48 differentially methylated regions

associated with aggression (Provencal et al. 2013b; Provencal et al. 2014). Measured twice, at ages 26 and 28, for both the CPA and control group, the CPA group showed higher methylation and significantly lower concentrations of inflammatory cytokines when compared to the control group. The study found agreement with literature suggesting that associations exist between cytokines and CPA, suggesting that there was an inflammatory immune response elicited by conditions of aggression behavior and oppositional environments (Provencal et al. 2014) Their results also demonstrate the value of conducting gene-wide analysis of DNA methylation; restricting their observations solely to gene regulatory regions would not have allowed them to identify many of the differentially methylated areas implicated (Provençal et al., 2013b). This suggests that future research in the field should not limit analyses to known promoter regions.

Provençal et al. (2013a) only tested adult blood samples and therefore had no way of determining when aggressive behaviour becomes associated with methylation and cytokine levels in humans. It should also be noted that the methylation tests were made using blood samples, and are correlational – no assay could be made of brain tissues that would be more directly indicative of gene function in cells of the brain. This study and others highlighted within this chapter suggest that early experiences can have lifelong effects on individuals, but it is still unknown when methylation and demethylation begins and how patterns of methylation change throughout developmental growth, through infancy to adulthood. Finally, given that these studies were performed using a cohort that lived within a particular sub-district of Montreal, in the future it might be interesting to examine the role of habitat quality and physical environment as a contributing factor.

7.3.3 *Addiction*

Even when mental illnesses are not caused by epigenetics, epigenetic changes to the mental state can make life significantly harder for some people, especially those disadvantaged. Consider that increased aggression is one of the most significant mental factors in delinquency (Nagin and Tremblay 1999). Addiction to illegal substances physically compels people to commit illegal acts. Status as a criminal severely impacts one's position within their culture and social sphere, and can prevent already disadvantaged people from being able to find jobs or education opportunities. Many current prisons do not place an emphasis on rehabilitation, but how much blame can be placed on someone with the deck stacked against them biologically?

Misuse of drugs and alcohol is a common factor in criminal behaviour, especially since most "recreational" drugs are illegal. Now, studies demonstrate that drug and alcohol misuse could have an epigenetic dimension that provides a mechanism for transmission of addiction behavior across generations. Repeated abuse of drugs results in persistent epigenetic changes that could play a role in long-lasting alterations in behaviour. Byrnes et al. (2013) exposed rats to morphine during adolescence, before they began reproducing. Their eventual offspring had no fetal exposure to the drug, but nevertheless inherited weakened locomotor sensitization even after being treated with a drug to increase locomotion. The grandchildren of the original morphine-exposed rats, also inherited weakened locomotor sensitization. In humans, this could imply greater vulnerability to addiction (Byrnes et al. 2013).

Epigenetic changes might also provide targets for new treatment plans including reversal of addiction (McQuown and Wood 2010; Wong et al. 2011). There is potential for epigenetic reversal: Weaver et al. (2005) were able to reverse epigenetic changes to the stress hormone receptor in rats by feeding them a methyl-rich diet to reverse the demethylation of DNA. In this study, stress-induced behaviour was also reversed (Weaver et al.

2005).

Needless to say, epigenetic effects are not necessarily going to be confined to illegal and addictive drugs: prescribed medications also have effects - recall the example of the estrogen mimic DES (in Chapters 5 and 8). Presently though, the evidence for trans-generational effects has been more thoroughly elucidated with respect to addictions, and in rodent models.

More and more, we see a combination of the epigenetic advantages and disadvantages we make for ourselves, and those that we are born with and born into. Distinguishing between our choices, what we can control, and our circumstances, at least those that we cannot control, can be a convoluted puzzle. Let us look next at a circumstance over which we have no control: our ethnicity.

7.4 Epigenetics: Race and Ethnicity, or Culture?

7.4.1 *Introducing Race and Ethnicity*

The terms *race* and *ethnicity*, while similar, refer to specific aspects of the human experience. Race as a concept refers to broad sets of physical features (such as genetically encoded ranges of skin colour, facial bone structure) that are associated with the indigenous people that first flourished in different areas of the world. Ethnicity, while it is often associated with race, actually relates to a group of people who express cultural practices, and who hold beliefs, languages, and family history in common. While someone who identifies as Irish might have the same race as someone who identifies as Welsh, their ethnicities inform their respective cultures much more than their shared "race."

This distinction is crucial. Ethnicity is a social construction, it provides cultural and demographic context. Race is arguably also largely a social construct insofar as people will often self-identify, but it is only hypothetically a definition that is based upon physical characteristics – look hard enough at the genome, for example, and

you will find segregations of specific DNA sequence markers that correlate with ancestral patterns of dispersion and interbreeding. These markers, called haplotypes, mix over time, and over centuries of military, commercial, or social interaction, they have been exchanged by intermarriage etc. This mixing confuses the definition of race to the point that for the purposes of our discussion, it is almost useless. With regard to the mechanistic processes that contribute to the epigenome, "racial" differences have been very small and hard to find, and indeed, social characteristics carry more weight in the identification of factors that contribute to health predispostions (Kuzawa and Sweet 2009; Arbour et al. 2012). Ethnicities are useful when looking at a group, as they give insight into broad traditions or trends that allows for easier understanding of the actual cause of epigenetic differences. Racial and ethnic trends are only of interest when used statistically, almost always when study participants have self-identified

That being said, there is a controversial representation of racial disparities in the scientific literature as it pertains to epigenetics. Racial differences are suggested to correlate with variations in epigenetic profiles in a number of different health areas. Within a research setting, race is often employed to determine the etiology and risk factors of diseases. However, the variability of results that stem from the studies may indicate that the effectiveness of utilizing race is questionable. Our discussion will focus on an overview of studies on race that pertain to the epigenetics of cancer, atherosclerosis, cardiovascular disease, and methylation alterations made during gestation.

7.4.2 *Ethnicity and Epigenetic Studies*

One cancer study focused on the tendency for "Hispanic whites" to have improved survival over "non-Hispanic white" and "Black" sufferers of non-small cell lung cancer, a cancer that was addressed above in relation to smoking (Saeed et al. 2012). This embodied the concept of the "Hispanic paradox": despite

demographically existing within the lower socioeconomic tiers associated with less access to resources, health care, and wealth, Hispanic whites enjoy a higher survival likelihood. Saeed et al. (2012) list family structure and lower tendencies to smoke within this population group as factors in creating this paradox.

Studies with an ethnic dimension have also been conducted on health issues associated with atherosclerosis and cardiovascular disease (CVD). Analysis of leukocyte methylation in patients with atherosclerosis across numerous ethnicities indicates that "white" and socioeconomically advantaged participants exhibit higher Alu and lower LINE-1 methylation compared to "African-American" and "Hispanics" (Subramanyam et al. 2013). **Alu** and **LINE-1 elements** are ancestral remnants of retroviral invasion. They were once thought to be "junk" DNA, but are now known to play a role in chromosomal structure and gene regulation. They tend to be rich in **CpG sequences** and to undergo preferential methylation as a consequence. The number and distribution of these elements differs from one individual to the next, as well as between ethnic groups. Professed shortcomings of this investigation include the choice of Alu and LINE-1 as the focus of study. They are highly polymorphic or variable, and their functional significance as pertains to disease is unknown. As well, leukocytes in the bloodstream may not necessarily be the direct target of the social factors of interest in this study, and this could explain the disparities in results between this and other investigations of leukocyte methylation (Zhang et al. 2011; Subramanyam et al. 2013). Again, authors list social and cultural behaviours as factors in shaping the epigenetic differences found in this study (Subramanyam et al. 2013).

Most of the above studies show correlative relationships between race and epigenetic alteration patterns. However, an interesting study conducted on women provides a contrast to the other investigations addressed thus far. When women across different racial categories were subjected to controlled folate diets

(folate has previously been shown to be associated with methylation – it is part of the vitamin B complex that acts as a methyl donor), no significant variation in global leukocyte DNA methylation emerged – a simple dietary supplement superseded al else (Axume et al. 2007). Thus, race itself was not a significant factor in the actual mechanism of methylation in humans, rather, the trends that correlated with race and epigenetic effects more likely stem from cultural (eg; dietary) differences.

Liu et al. (2010) analyzed 196 individuals from five ethnic groups - Caucasian, Latino, Native American, African American, Asian, and mixed populations. They identified a total of four population specific factors that could differentiate between groups (Liu et al. 2010). Three of these factors are genetic **single nucleotide polymorphisms (SNPs)**; differential frequency of each SNP delineates a different racial category. The fourth was a population-specific methylation factor. In this case, differential methylation level was the population determinant: Caucasians had the highest methylation, followed by Latino and mixed participants, and lastly Native and African Americans. Despite this association between race and methylation levels, this factor only accounted for 0.2% of total methylation variance (Liu et al. 2010). This indicates that despite subtle genetic differences having an influence on methylation patterns, their role does not appear to be a large one relative to more substantial factors such as the environment.

Although this study provides an interesting perspective on methylation profiles and their connection to race, some aspects may affect the legitimacy of the results. Firstly, the sample size of 196 participants is small, especially considering the study's attempt to divide the individuals into 5-6 ethnic groups. Secondly, there appears to be a significant over and under-representation of different ethnicities across the groups: there are numerous Caucasian and Latino participants while there are few African American individuals. In the entirety of the study, there is only

one Asian participant (Liu et al. 2010). These discrepancies in sample size and quality may have compromised the results and conclusions of the study.

What can be conveyed by critical analysis of this research is that racial conclusions in scientific research need to be carefully evaluated. This does not imply that conclusions linking ethnic background and epigenetics should be dispelled. Rather, we need to be sensitive to that fact that multiple elements including historical background, geography, culture, and social environment unite to influence epigenetic modifications: race-associated genetic haplotypes appear to play a relatively minor role.

7.4.3 Race and Geography

Culture is often tied to the geographical location of a population. This is because cultural traits are learned and transmitted within both families and communities, and often in response to environmental cues. As a result, epigenetic alterations influenced by culture can be patterned in a global sense. The social and habitual activity of smoking illustrates this connection between methylation patterns and geography. When comparing the methylation levels of seven genes associated with non-small cell lung cancer in Toyooka et al.'s (2002) investigation, the studied countries were sorted into two groups. Group A consisted of USA and Australia, while group B encompassed Japan and Taiwan. Results showed that group A had significantly higher overall mean methylation index (MI) and higher methylation rates of the specific genes MGMT and GSTP1 (Toyooka et al. 2003). Toyooka et al. (2002) cited geography, culture, or race as potential contributors to the discrepancy. The data demonstrated methylation changes that coincide with the act and exposure to smoking. Cultural and social norms specific to different geographic regions may dictate the amount and variety of an individual's exposure. Examples of this could include: social stigmas on smoking near others; whether it is polite to accompany

someone to smoke; or whether smoking reflects a recreational or group activity. Relative to smoking health risks, these behaviours could potentially influence the methylation profile of an individual or a geographically delimited population.

Just as differences in epigenetics between countries can give context for the shifts in culture between large populations, epigenetic anomalies between areas within a country can be vital to study, especially when dealing with environmental consequences and xenobiotics (See Chapter 5). Mansfield's 2012 study of epigenetics and xenobiotics found that indigenous populations near bodies of fresh water were exposed to methyl mercury at a high rate, This toxin has epigenetic consequences and is a common contaminant of continental fish stocks. Indigenous people are more likely to be the most frequent consumers of fish as a result of their economic and cultural status: their proximity to the rivers and fish stocks that are exploited for subsistence or traditional fishing is an accident of economics, culture, and geography (Mansfield 2012). For these populations, fish is a vital and necessary food source. Government health advisories dispensed advice against fishing in the waters, but the information given out not only ignores the cultural dietary patterns of Native Americans, it places the burden of avoiding the xenobiotic on the people living by the water rather than the companies or processes that are polluting the lakes. Native Americans were given a warning that did not accurately reflect their needs. Moreover it was a warning that they could not act upon for economic and cultural reasons. In fact, the warning simply gave the polluters more support: someone ignorant of the cultural reality of the people affected could make the argument that the populations were warned against methyl mercury levels. The burdens of change should not be placed on those who are powerless to change them.

7.5 Conclusions

Policymakers and scientists must be incredibly careful that they understand the intricacies of ethnicity and race, especially as the world becomes more globalized and diverse. Research needs to focus on cultures instead of races, and include countries outside of North America so that we can collectively benefit by knowing the relationship between epigenetics and cultural factors such as diet, socioeconomic status, and even the way children are raised. You cannot change one aspect of society without causing a ripple effect, and all too often cultural factors reinforce themselves in feedback loops that are difficult to escape. There are no easy answers when dealing with the interaction between culture and our epigenome.

With its focus upon DNA alterations, biology of the epigenome has been necessarily reductionist in nature: anthropologist Jorg Niewohner argues that it will be necessary to broaden perspectives and to question assumptions if the full complexity and complexion of inputs is to be fully accommodated (Niewöhner 2011). Niewohner proposes the idea of the "embedded body"; an entity through which the cumulative contributions of the social, political, historical, environmental, evolutionary, and numerous other spheres can be adequately assessed. This method of redefining the body and the self as an entanglement of external contexts will guide scientists to expand their field of research beyond the restrictive focus on purely biomechanical machinery. He is not alone in his appeal – the genome scientists themselves are beginning to reference a new concept – the "interactome" (Li et al. 2014). It is difficult to design experiments that can take such a myriad of factors into account, but as the field of epigenetics grows, so too will our understanding of the interaction between the outside world and the individual's interior complement of packaged genes.

Provocative Questions:

- Will holding parents societally responsible for the epigenetic health of their children disproportionately burden marginalized or disadvantaged groups?
- Will epigenetic research be used to dispel racial biases, or reinforce them?
- Should we alter our epigenome to program a social or behavioral advantage?
- What policies should the government follow regarding data collection on the epigenetic trends of certain ethnicities? Should policies be developed to manipulate epigenetic trends?
- Should criminals be treated differently if they are epigenetically predisposed to crime?
- Stress causes epigenetic damage – should all bullying or intimidation be defined as a form of physical abuse?
- To what extent should governments, healthcare, social, legal, and educational systems intervene to educate or even moderate parenting skills and styles?
- If there is a family history of addictive behavior, would it be ethical to market or to administer medical interventions to treat offspring not yet addicted?

Possible Solutions

- Early education about epigenetics to parents and children
- Education for policy and law makers
- Epigenetic information freely available and tailored to specific cultures
- Research focusing on diverse populations across countries

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Chapter 8

Epigenetics and Law: The Quest For Justice

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Abstract

Epigenetic cases pose problems within the current legal system. When epigenetic harms occur, individuals should have recourse to legal action. Laws regarding who can take legal action, the time frames in which it can be taken, and the roles of various parties are examined using the challenges of diethylstilbestrol (DES) as a case study. We examine the ability of the legal system to encompass epigenetics within the context of statutory limits, dilution of liability, victim attenuation, and the challenges in identifying records and of proving direct causation. We then look at epigenetic harms with respect to testing policies and the directions in which they should be developed. These include establishing tests that look at not only epigenetic effects upon individuals, but also upon the reproductive and trans-generational effects a product might have. Finally, the chapter considers how privacy law and epigenetic cases intersect.

8.1 Introduction

Presently, epigenetics is not a term, mechanism, or process that is explicitly enshrined in law – the concepts and science are

simply too new, and the implications are too poorly understood. As we discussed in Chapters 1 and 2, epigenetics involves modifications to chromatin structure that alter the way that genes behave without altering the sequence of DNA itself. The modifications are to attached chemical groups and to associated proteins, and the informational complexity of the "histone code" exceeds that of DNA. Some epigenetic imprints are heritable, and they permit us to record experience of our environment upon the packaging of genes to alter/prime subsequent behaviour. Diet, drugs, environmental chemicals, traumatic experience, socio-economic status, parental care, even education, can affect your epigenome, and this has implications for your future health.

While *genetics* has been explicitly addressed in the United States under the Genetic Information Non-Discrimination act (GINA), there have been no cases to define whether or not *epigenetics* will fall into this same category and be protected. For example, epigenetic alterations do not change DNA sequence, so *genetic* non-discrimination may not apply to epigenetic cases. If the legal term *genetics* was expanded to include chromatin – the assemblage of DNA, RNA, and protein molecules that comprise chromosomes, then epigenetics would be covered under the act. A definition of *genetic* has not been fully explored by the courts. The few instances where epigenetics has been tested indirectly are in tort law.

The establishment of liability has specific requirements. A plaintiff must prove: that a harm has occurred; that the defendant was, through their inaction or negligent practice, the direct cause; that the defendant knew, should have known, or was negligent of the risks to which the plaintiff was exposed; that the complaint is made within **statutory limits**. Given the long lag time before epigenetic effects might become obvious, this class of case will present huge challenges for injured parties seeking remedy or compensation. In both the United States as well as in Canada, the principle of discoverability can introduce a loophole to strict

statutory limits. Instead, a suit must be brought within a reasonable timeframe following the first discovery of an effect, even if this occurs after normal statutory limits have expired. Epigenetic effects tested to date have become manifest well after statutory limits have expired, and since they are sometimes trans-generational in nature, the issue of dilution also comes into play. It can be hard to reconstruct who prescribed, manufactured, or dispensed a drug, and given the complex and combinatorial nature of epigenetic imprints, it is also hard to demonstrate direct and unadulterated cause and effect. The litigation surrounding diethylstilbestrol (DES) is illustrative of the complexities.

8.1.1 Diethylstilbestrol (DES): a case study in Tort Law

Beginning in 1938 **diethylstilbestrol (DES),** an unpatented, low cost synthetic estrogen was being distributed as a remedy drug for menopausal symptoms, senile and gonorrheal vaginitis, excessive menstrual bleeding and to suppress lactation (Downey and Gulley 1983; t Hoen and Dukes 2007). Many manufacturers that were producing DES eventually began promoting it for use in the prevention of miscarriage. The FDA requested that the manufacturers agree upon a standard chemical formula and uniform labeling, which in 1941 led these companies to form the "Small Committee" (Downey and Gulley 1983). DES was widely prescribed to pregnant women for the next three decades even though there were indications as early as 1947 that there was a threat of cancer to the user (Mascaro 1991). Women who had taken DES experienced a 30 – 40 % increased risk of breast cancer, preterm delivery, **pre-eclampsia** and miscarriage, while *in utero* daughters and sons suffered increased risk of genital defects and infertility. Between five and ten million individuals affected by the drug are prone to more than twelve medical conditions including a higher risk of cancers and infertility (NCI 2011).

The drug was withdrawn in 1971 after it was found to be

ineffective in preventing miscarriages and was found to be carcinogenic (t Hoen and Dukes 2007). However only recently, in the light of epigenetics, have studies revealed the mechanism that underlies the trans-generational impact of DES including, but not limited to, increased incidences of cancers and infertility (NCI 2011). DES is known to affect the methylation and therefore the regulation (but not the DNA sequence), of genes that encode proteins in the estrogen production pathway. Whether or not it is these changes that leads to later defects in function remains to be determined.

8.1.2 Legal Action Concerning DES Effects

One of the first examples of epigenetic harms being addressed by the legal system occurred in the United States when mothers and daughters exposed to diethylstilbestrol (DES) began suing manufacturers for damages due to reproductive problems and cancers in the exposed daughters. After successful first- and second-generation claims (mother and daughter exposed in utero respectively), third-generational claims were made by the granddaughters of DES-exposed mothers. However, the courts ruled that the manufacturers of DES were only responsible for the first and second generation effects of DES due to **victim attenuation**; the granddaughters were too far removed, or attenuated, from the initial offence (Rothstein et al. 2009b). Since the effects of DES were not evident until later in the daughters' lives, the statute of limitations – the time span in which legal claims are valid and pursuable – had to be extended in order for the individuals affected to be compensated. The courts also decided that the statute of limitations for the DES cases begins when the effects of exposure become evident or might reasonably be expected to cause notice, not when the actual exposure takes place (Rothstein et al. 2009b).

In 1990, a DES son filed a lawsuit against a drug company in the U.S. alleging that his *in utero* exposure to DES caused his

daughter's cancer (Mascaro 1991). This appears to be a precedent-setting lawsuit that claims DES caused injury to third generation victims. There have also been other third-generation DES cases for premature births where the babies were born with severe physical disabilities (Mascaro 1991). However, in his 2009 article, Rothstein notes that "claims brought by the granddaughters of the women who took DES have not been successful due primarily to the courts' unwillingness as a matter of tort theory… to extend liability…" (Rothstein et al. 2009b). For example, in New York, decisions flip flopped until the NY Supreme Court cited a precedent setting case where pre-conception injury was deemed ineligible as a basis for liability (Karen Enright, An Infant & C., Et Al., Respondents, V. Eli Lilly & Company, Et Al., Appellants.77 N.Y.2d 377, 570 N.E.2d 198, 568 N.Y.S.2d 550 (1991)). Unrecognized in the legal quagmire was that the infant did not solely receive injury indirectly because of physical harm done to the mother, but was likely to bear a significant and direct harm of an epigenetic nature herself. In other words, the harm was probably not caused by a physical deformity of the mother impinging that impinged upon fetal growth, but rather, was likely due to inherited patterns of altered gene activity.

8.2 Statute of Limitations

Statutory limits and repose present substantial constraints that prevent the system from dealing with epigenetic liability cases. Legally, the period for litigating claims is limited, and in most cases it runs out before any of the epigenetic harms manifest themselves (Khan 2010). Epigenetic harms can have a trans-generational impact, and the harms are sometimes unknown until after one or two generations have been born and displayed the effects of a harmful agent. For instance, it took years before the side effects of DES became known. Many of the women who took the drug are now deceased; yet many of the side effects are only

just now being encountered in their daughters and sons, as well as grand-daughters and grand-sons. Only time will tell if adverse effects will extend to further generations. It is this long latency period that makes it more difficult to accurately identify the product responsible for the harm, or even to assign blame for the negative epigenetic outcomes (Khan 2010). The imposition of strict time limits could eliminate future generations from filing claims. On the other hand, the absence of limitations could render the long-term nature of the economic environment too insecure to support vibrant business and product development.

Richard Abood discusses the pharmacist's Statute of Limitations in his book *Pharmacy Practice and the Law*. "The purpose of the statute of limitations is to prevent the litigation of stale claims years after the events that allegedly led to the harm, after memories have faded and witnesses have disappeared" (Abood 2010). While scientific tests might eventually confirm the effects of, say, a specific drug, the doses administered, the form they took, the identification of the specific manufacturing licensee, physician, pharmacist, could be difficult, and the degree of patient compliance might quite difficult to establish. Abood also reports that public policy favours the unencumbered practice of healing arts, "without fear of groundless and unjustified malpractice lawsuits, [which] has led to the enactment in many jurisdictions of reform laws that reduce the number of years a statute of limitations for medical malpractice" (Abood 2010). In many jurisdictions, there is a "discovery rule" which means the time limit does not start until the harm is discovered. For example in Ontario Canada, the Ontario Limitations Act 2002 provides for a two year period following discovery for litigation to be pursued. By contrast, some jurisdictions have a "statute of repose" which limits the discovery rule to prevent litigation decades after a drug's use (Abood 2010). This would prevent the claimant, especially second and third generation victims, from suing the individual doctor or pharmacist. Thus the chances of pursuing an epigenetics-related

claim against an pharmacy is almost impossible.

Some states have introduced changes to their legislation with regards to statute of limitations to make second and third generation claims more sustainable in courts (Rothstein et al. 2009a). Yet, even so, it is uncertain when statutory limits should expire, and for how many decades is it reasonable to hold a company liable. Perhaps, a potential defendant should not be exposed to the threat of indefinite liability, which is why statute of repose protects manufacturers (Khan, 2010). In instance of epigenetic effects, the statute of repose sometimes runs out even before the injury or harm itself has been noticed or identified, thus making it impossible to assign blame to anyone for the epigenetic harms (Khan 2010). The DES cases have proven an interesting testing ground in this instance – second generation claims have been permitted in some jurisdictions and under specific circumstances.

8.3 Tort Law

8.3.1 Liability

The next concern is how to hold parties legally responsible for epigenetic harms. Responsibility can belong to an individual, to an employer or state responsible for occupational or environmental exposure, or to a group of people working towards a common purpose, termed a collective (Hedlund 2012). Assigning legal responsibility to any of these types of groups requires causal responsibility – that the actions of the individual or collective involved directly caused the adverse outcome of interest (Hedlund 2012). While no laws currently exist regarding epigenetics and assigning responsibility for the causation of epigenetic insults, some believe that the tort system could be modified to address these issues.

Tort law is enforced to ensure public health and safety, thus it could potentially be used to address harms to the epigenome, but the requirements of tort law cases pose additional

problems for the victims of epigenetic insults (Khan 2010). In tort law, the victim is required to prove that the harm was a direct result of the defendant's actions by more than 50% probability (Khan 2008). Since individuals are exposed to thousands of different chemicals, and to the same chemicals from several different sources, it can be difficult to prove that exposure to a single product resulted in negative epigenetic effects (Khan 2010). Similarly, there has been little research on the epigenetic effects of the chemicals we are exposed to in everyday life, and the generation of such knowledge would be beyond the abilities of most plaintiffs (Khan 2010).

With current tort law, injury is only recognized when it affects an individual at or above the cellular level. Thus, epigenetic mutations introduced by chemical exposure would not be considered harmful unless they resulted in observable negative effects (Khan 2010). This poses a problem since epigenetic changes may not have noticeable effects for years or, in some cases, for generations. Can a company only be held liable if its actions result in epigenetic effects which are immediately observable? (Khan 2010; Rothstein et al. 2009b). Diseases are often viewed as binary, but epigenetic alterations can lead to a *predisposition* to developing a given disease: is this still grounds for a lawsuit (Khan 2010)? Finally, if the epigenetic effects a defendant has caused can be reversed by medication or other genomic intervention, does the defendant owe anything to the plaintiff? (Hedlund 2012). Should companies be held liable for causing epigenetic changes *only* when they have detrimental effects, or if *any* epigenetic alterations are proven? In addition, assuming that one is able to pinpoint and prove the fault of one particular manufacturer (defendant), how do you prove that the product is the sole cause of the outcomes? We are exposed to so many different chemicals and substances, thus making it difficult to establish one sole culprit (Khan 2010). Moreover, it is often difficult to pinpoint one product or actor responsible when there

are multiple manufacturers licensed to produce the same merchandise (Khan 2010).

It has been argued that it is unfair to put blame on current individuals for harms caused by past actors (Khan 2010). Since there can be a long latent period between exposure and effects, the makeup of individuals within a company producing an offensive chemical can completely change by the time the plaintiff makes a claim. Since the makeup of the collective could be entirely different, it becomes unclear if the collective can still be held legally responsible for the actions of its predecessors. Similarly, if a company that once produced harmful substances now only produces beneficial substances, it is uncertain whether the deadline of the statute of repose should be extended and the company held liable, as this could detract from the good the company is currently doing (Khan 2010).

Joint venture liability suits have also been filed for DES related cases. In these suits, all the companies who manufactured or sold DES are held jointly responsible and therefore proportionately liable for damages incurred irrespective of which manufacturer's doses were prescribed to a specific patient (Downey and Gulley 1983). In the Netherlands, collective settlements have been reached using a joint venture prosecution. A fund for victims was set up, however, it took 20 years to reach the agreement and the compensation has proven to be too low as the settlement has to be spread among multiplying claimants who subsequently discover side effects (t Hoen and Dukes 2007).

Another concern is whether **preconception torts** are legitimate claims for epigenetic cases. Preconception torts are claims made by offspring who are negatively affected by their parents' or physicians' actions before they were conceived, excluding existing genetic conditions or unintentional environmental exposures (Weiner 2010). These claims are similar to claims of wrongful life, in which parents are sued for knowingly conceiving and bringing a child into the world with a low quality

of life due to a predictable disability or other illness (Weiner 2010). Mothers have also been charged with child abuse when their actions during pregnancy, such as drug abuse, threaten the life and health of their child (Smith et al. 2013). Could cases such as that of DES be considered under preconception and pre-natal torts, thereby putting blame on the grandmothers and/or mothers for taking the drug while pregnant.

While these previous tort cases lend weight to the idea of preconception torts, there are many concerns with holding parents, physicians, or companies liable for their actions before conception. The parents could be held liable indefinitely due to their actions long before they ever thought of having children (Weiner 2010). As well, the legal system would need to clearly define what acts are considered harmful and punishable by tort law (Weiner 2010). Successful preconception torts would also raise concerns about the rights of the mother during pregnancy and at which point a developing fetus becomes recognized as a human being: this would have implications for abortion laws (Smith et al. 2013; Weiner 2010). Finally, given the latency of epigenetic effects and the lack of knowledge on the effects of substances on the epigenome, it is unclear whether *unforeseeable* negative effects could be punished by tort law (Weiner 2010).

Others have argued that pre-conception torts need not be dismissed out of hand if they argue a genetic basis for injury (Goldberg 2007). Moreover, genetic predisposition to be susceptible to an industrial toxin might help plaintiffs to meet the two-fold "more likely to have been affected than not" standard required for successful litigation in cases pertaining to toxins (Marchant 2006). By extension, epigenetic torts might also enjoy an opportunity to see their day in court since, like genes themselves, they can also embody both the measureable harm and the predisposition to injury.

8.3.2 Proving Causation and Obtaining Records

A further legal concern with respect to epigenetic cases has been the ability to prove that a drug caused the negative epigenetic effects. A plaintiff in any tort case should be able to identify the specific product that caused the harm and the causal chain of events that led to the injury must be unambiguous. This becomes difficult when the causal stimulus and the revealed effect are separated by 20 or more years, as is often the case for trans-generational epigenetic effects. How does one retrieve prescriptions that are that old, and demonstrate that a plaintiff was compliant with regard to the use of the drug?

Additionally, accessing medical records has proven to be another concern for epigenetic legal cases. The request to access personal health records requires that you know where the records are kept and in cases involving deceased progenitors, the executor of the estate must give permission. In Ontario, medical information is protected for 120 years after the record was created or 50 years after the person's death (IPC 2008; Personal Health Information Protection Act, 2004, S.O. 2004, c. 3, Sched. A). However, laws regarding how long medical professionals have to retain a patient's medical records vary greatly from 5-10 years depending where the person lived. In Ontario, the College of Physicians and Surgeons requires that records be retained for 10 years, but recommends 15. When you are making a second or third generation claim, which is the case in most epigenetic claims, it is difficult to have all the required information on hand to track the record, and sometimes a record might no longer even exist.

8.4 Models for Assigning Responsibility

It is a common idea that one should be held accountable for one's decisions, and up until today, our legal and regulatory framework has been concerned with assigning responsibility in a retrospective manner (i.e. blaming causally responsible stakeholders, when unfavourable outcomes are identified) (Gesche 2010; Hedlund 2012). However, when this model is applied to

epigenetic harms, identifying those to blame will prove to be a challenging task.

Some might argue that it is an individual's responsibility to make healthy choices with respect to diet and nutrition, exercising, or other major lifestyle choices. For that matter, where should the cost-benefit analysis place the threshold for advisable use of pharmaceuticals when the trans-generational consequences may be unknown? Thus, should there be any epigenetic side effects or potential health risks resulting from these choices, the individual should be held responsible to remedy these effects. Others argue that sometimes an individual may lack the knowledge required to make intelligent and healthy decisions, or may lack the socio-economic means to make better lifestyle choices to remedy their situation (Hedlund 2012). Moreover, the disadvantageous epigenetic side effects might be so profound or insidious that the cost of correction is unaffordable for the individual or his/her family. How is responsibility and blame assigned when more than one individual or group is involved? Although there are ways in which percentage blames can be assigned, the constantly changing and inherently complex nature of the epigenome likely means that the assignment of proportional responsibility will be difficult if not impossible to determine with exactitude. In addition, how does one deal with responsibility when there are structural conditions (social, economic and political) that affect the well-being of the individual and their life choices, and which contribute to epigenetic side effects? Finally, can it be regarded as fair to hold individuals responsible for the behaviour and habits of their ancestors – these too affect an epigenetic imprint? The complex combinatorial interplay of epigenetic influences also makes it difficult to assign a specific cause to an effect.

All of these factors shift the moral responsibility from the individual to society and the government, which will ultimately bear much of the cost associated with ill health and lowered productivity (Hedlund 2012). This raises the issue of **collective**

responsibility – how can this be managed?

One model of assigning blame is the **prospective responsibility** model. This model takes into account structural conditions and to some extent frees the disadvantaged from total responsibility (Hedlund 2012). An example of this type of program is the Finnish "Baby Box" program that was developed to entice and reward expectant mothers to attend early pregnancy consultations with medical professionals. The program was fundamental to a marked reduction in maternal and fetal mortality, and is discussed at greater length in Chapter 10.

The prospective model acknowledges that even if individuals are complicit in damaging their own interests, this creates costs to society in terms of health and productivity, not merely insofar as the individual is concerned, but also for successive generations who ought not to be punished. Prospective responsibility accounts for the multiple other inputs from the economy, geography, society that play a part in the epigenetic effects. The model also requires contributors to debate policy formulation: these may include individuals who have a wealth of knowledge, and researchers who study the field of epigenetics (Hedlund 2012).

In summary, because of the complexity of epigenetics and the number of interests and stakeholders that contribute to the system, it is difficult to determine the boundaries of responsibility between the individual and society, so it is economically and procedurally simpler to facilitate better health via societal support than by assigning individual blame.

8.5 Protection and Prevention: Adapting Testing Policies

In the U.S., Food and Drug Administration (FDA) rules and regulations are designed to prevent medical tragedies. Similar agencies perform a like function in other countries – in Canada for example, Health Canada performs this function. Many argue that it is time to bring new changes forward considering that our genome

is now known to respond to external signals in a diverse and complicated way (Szyf 2007). **Xenobiotics** affect us in many different ways, but their activity and pathways can also be different from individual to individual (Szyf 2007). Pre-market and license testing should therefore be required to address the epigenetic side effects of each drug, as well as to predict changes in the metabolic mechanisms whereby xenobiotics themselves could be modulated by varied epigenomes (Szyf 2007). Such testing should include both drugs that may have unintended epigenetic side effects, as well as the drugs that intend to cause an epigenetic modification as a pathway to treat a certain disease or condition. It is understandable why tests would be needed in the former case, where epigenetic side effects are unwanted and unexpected. Yet, testing the drugs that are intended to cause epigenetic modifications is necessary to ensure that such modifications are gene-specific, and not random and uncontrolled (Rothstein et al. 2009a).

Not only is testing a necessary measure, but there also is need for regulation regarding how and where the testing is done. Considering that epigenetic modifications are species specific, it is important to choose the right model organism for testing and if extrapolating to include humans, to do so cautiously (Rothstein et al. 2009a). Furthermore, testing drugs for epigenetic side effects should be considered with skepticism. Results from such tests may suggest epigenetic modifications that are not accompanied by symptoms, or that may not necessarily have a negative influence on an individual's health (Rothstein et al. 2009a). Since it has been previous shown that trans-generational epigenetic effects may not be immediately observable it is important to revisit tests in order to account for this. As such the results should not be taken as the final word, but rather, they should be seen as a guide, and further investigation should follow to determine possible long term effects.

Another precautionary regulation that might mitigate some

of the risks associated with epigenetics is trans-generational monitoring. Not only should a clinical trial assess the reproductive side effects of a specific substance, but it should also track the effects of the substance for at least one more generation to reveal, characterize, predict, and prevent further trans-generational harms. It is impractical to make human cross generational studies a part of the approval process - this could render the development of drugs a lengthy and impossibly expensive process – clearly cell and animal models will have to be developed to prognosticate potential pit falls, while simultaneously, longitudinal studies on humans should be required even after the drugs come to market.

8.6 Law and Taxation Models

Clearly, epigenetic testing, diagnosis, treatment, and remedy will come at a cost – how will the new expenses be borne ? With regards to taxation, three justice models have been suggested to correct for epigenetic disadvantages – the **luck-egalitarian model**, the **luck-prioritarian model**, and the **Rawlsian-egalitarian model** (Loi et al. 2013). These models would serve as a guide for taxation and redistribution of funds towards health care and other assets.

Engaging the luck-egalitarian model would serve as a social and economic leveler of sorts. The model operates to reduce inequalities to those that arise solely due to the choices an individual makes. Any other inequalities, for example due to genetics, parental legacy, or social status are corrected and subsidized. This includes the use of subsidized therapies to equalize epigenetic advantages when such therapies become available (Loi et al. 2013). This model would equalize social and genetic disadvantages so that similarly inclined individuals would have the same opportunities for success regardless of their starting position in society or genetic predispositions.

Luck-prioritarianism is similar to luck-egalitarianism as it requires genetic and social intervention to equalize opportunity.

However, luck-prioritarianism requires that helping individuals in the worst genetic and/or social state takes precedence over helping all other individuals. Thus, those in worse condition would receive a greater degree of aid and/or treatment than individuals who are slightly better off genetically, epigenetically, or socially (Loi et al. 2013).

The third justice model is Rawlsian-egalitarianism, or moderate egalitarianism. Unlike luck-egalitarianism and luck-prioritarianism, Rawlsian-egalitarianism would aid those in need only via social tools – for example, through tax cuts or subsidized education. Genetic and epigenetic inequalities would be viewed as the loss of a "natural lottery" and would be equalized, at most, by the redistribution of monetary assets and social aid in the favour of those less fortunate genetically (Loi et al. 2013).

Regardless of the justice model invoked, it has been suggested that a tax be levied on prospective parents to subsidize the genetic and/or social interventions for disadvantaged offspring. The tax would be collected for each offspring and the pooled money would be distributed either by a luck-egalitarian, luck-prioritarian, or Rawlsian-egalitarian system of social justice. Such a system would remove the burden of equalizing opportunities from society as a whole and place it on those who opt to have children (Weiner 2010). The practicality of taxing families when they are just starting out, and when family resources are relatively small seems dubious. Moreover, society has an interest in propagating new generations to replace and support the older ones, so why build in reproduction disincentives? The tax would discourage financially limited families from having children - a concern due to low birth rates already threatening many developed countries. The taxation of parents would also privilege wealthy people: only they might be able to afford to have children. Finally, such a tax would fuel the debate regarding abortion since financially unstable couples could find themselves in the unenviable position of having to choose between terminating the

pregnancy and of digging themselves into a financial hole.

Since one of the major concerns with imposing epigenetic liability is the lack of knowledge regarding the epigenetic effects of products on the market, some theorists have suggested placing a hefty "epigenetic tax" on products. The tax would increase the costs of producing such chemicals, and this additional expense would no doubt be passed on to consumers. So, taxes would undoubtedly increase the costs of production and decrease sales. How could companies be given incentive to ensure epigenetic safety? Taxation rates could be inversely tied to investment in research and prevention. This would benefit companies by decreasing both the taxes levied, as well as the potential liability costs of their product. Not incidentally, the program could be designed to expand knowledge of epigenetic risks and benefits (Khan 2008).

The pros and cons of shifting the burden of care from society to the individual, or vice versa, must be considered, as well as taxes levied to encourage epigenetic research on exposure to seemingly innocuous products. Eventually both stakeholders should be encouraged to promote environmental and social responsibility

8.7 Privacy Law

Aside from the legal concerns which arise in epigenetic cases, privacy law also has importance where epigenetic effects are concerned. Current law does not include any policies or acts which directly encompass epigenetics. The Canadian *Privacy Act* includes "information about an identifiable individual that is recorded in any form including…information relating to the education or the medical, criminal or employment history…" under its definition of personal information (Privacy Act of Canada, 2014). Loosely one could consider epigenetic information to be included under this if that epigenetic information is recorded in a form considered to be medical history.

With the significant role epigenetics can have on individuals and groups of people, assuming it can be categorized under the act is not enough. This is especially the case when many individuals are affected by unexpected negative epigenetic effects such as those resultant from taking DES. Inclusion of epigenetics within laws for genetics may be an option. In the United States, for example, the definitions of 'genetics' used in the existing genetic privacy laws presently might not encompasses epigenetics (Rothstein et al. 2009a). With the growing prominence of the field, and with legal cases of epigenetic harms presenting themselves, the inclusion of epigenetics into privacy law is crucial for the protection of individuals including for prevention of epigenetic-based discrimination. Privacy laws should be adapted and clearly define how epigenetics is encompassed, and how the privacy of individuals should be protected. Those who bring forth epigenetic legal cases should not fear breaches of their personal privacy with respect to their epigenetic information, especially when legal battles ensue and media involvement occurs, and even more so when information released concerning one individual can be used to make inferences either about either progenitors or descendants. We will discuss privacy more in Chapter 9.

8.8 Conclusion

With cases of epigenetic harms presenting themselves in court, the explicit inclusion of epigenetics into law will become necessary, especially because it affects so many realms of human health and opportunity. The uncertainty and challenges that have arisen in the DES litigations indicate that current law is inadequate to handle epigenetics. Moreover, those who present and adjudicate cases will need education to understand the new concepts and implications. Individuals who are affected by epigenetic side effects should have a right to obtain justice and to present an epigenetic case within a legal system which is suited to handle it. In order to provide justice and ensure that those who are liable are

held responsible, indeed to prevent future harms from occurring, laws that acknowledge the implications and effects of epigenetics must be discussed and formed.

Provocative Questions

- If the current legal system is not suited to deal with epigenetic cases, how can individuals obtain justice, and those responsible for them be held to account?
- How quickly can the legal system adapt to fit the needs of epigenetic cases? Will laws and policies concerning genetics be adapted or will new epigenetic-specific laws be formed?
- What are the implications of required epigenetic testing, for organizations, such as pharmaceutical companies? How will liability and costs be balanced to avoid impeding progress?
- Will laws for epigenetic harms due to medications or environmental effects be expanded to encompass epigenetic harms due to nutrition and lifestyle choices?
- Should those without the socio-economic means to change their lifestyles be offered assistance to present legal cases of epigenetic harm against the government?
- Will lack of epigenetic educational resources be a feasible argument for a legal case of epigenetic harm?
- How will the cross-jurisdictional nature of globally distributed pollutants with epigenetic effects be dealt with?
- How will the privacy of individuals and families affected by harmful epigenetic influences be protected when legal battles ensue?

Solutions

- Education in the field of epigenetics, and in previous legal cases of negative epigenetic effects.

- Acceptance of epigenetics requiring its own place within law
- Formation and discussion of epigenetic laws – locally, provincially, federally, and internationally – there are a lot of cross-jurisdictional issues that will require a comprehensive approach.

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Chapter 9

Epigenetic Privacy: Hacking Your Health?

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Abstract

Our improved understanding of epigenetics and how it can lead to ill health raises questions regarding how to acquire, store, manipulate, and access epigenetic health data. Like other types of medical and personal records, we need to consider how to handle information associated with a person's epigenetic status, however, this field of privacy and privilege is peculiarly vulnerable to exposure in additional ways that we will enumerate. Although pre-existing experimental standards have been adapted to ensure privacy for both patients and epigenetic study participants, vulnerabilities have been identified in the management of epigenetic data, especially with the growing affiliation of digital platforms and the use of internet tools to distribute, store, analyze and present data. The rapidly evolving, loosely regulated online environment could threaten confidentiality in ways unforeseen by both researchers and participants. Moreover, diverse online sources can be mined for additional information that could permit unscrupulous surveyors to infer your epigenetic status. Large data sets, thus assembled from diverse sources, can provide additional insights into an individual's epigenetic and prospective health status. Innocent online interactions could lead to exploitation of epigenetic data for manipulation and gain. The threats of unwarranted access by opportunistic enterprises, limited research, medical, and computational resources, and violation of privacy afforded by reverse association, emphasizes the need for epigenetic data

protection. Discussion of the implications of the current social and legal environments, and the increasing use of digital networks in epigenetic study and medicine, has made privacy a pressing issue.

9.1 Introduction

Patients have the reasonable expectation that their data - from medical visits, prescriptions, participation in health surveys, or simply providing background health information - will be handled with care and discretion. Despite the best of intentions, however, there have been some recent and spectacular breaches of this confidentiality. For example, in the United Kingdom, the National Health Service (NHS) was revealed to be selling highly classified information to many of the nation's insurance companies (Donnelly 2014). Although the data had been anonymized, apparently none of the civil servants involved had realized that insurance companies could compare their own records with those of the NHS to connect heath records to specific patients. Moreover, the sale of such information had no effect on improving patient care, but quite the reverse: compromised patients could risk being denied insurance coverage. The hazards of the aggregation of large medical data sets have already been articulated (Anderson 1995).

With epigenetics becoming a more prominent field, and the number of studies increasing, it will only be a matter of time before the detailed characterization an individual's epigenetic status becomes commonplace. Epigenetics is distinct from genetic data: it does not involve changes to DNA sequences *per se*, but to how those sequences are packaged and consequently behave. As we saw in Chapters 1 and 2, epigenetic changes to chromosomal architecture can take many forms, and the informational complexity, the differential distribution of these characteristics in different cell types, tissues, and individuals, and the constantly changing nature of an epigenome (with age, exposure and experience of different diets, environments, social activities etc), mean that epigenomic data sets will be much larger than those

describing genome sequences. They will also need periodic updating for each individual.

The absence of epigenetics as an explicitly defined term in regulation and law, the characteristics that make it both mechanistically and conceptually different from genetics, and its novelty in health practices means that few of the privacy issues and challenges that attend this new field have been identified, let alone discussed. We will attempt to remedy these deficits, and will show how epigenetic data not only embodies many of the same challenges as other large medical and genetic data files, but manifests other vulnerabilities as well. We will start with the issues of informed consent and epigenetic data collection, handling, storage, access and processing. We will conclude by outlining the accessory and somewhat novel means by which unscrupulous operators might develop a relatively accurate assessment of your epigenetic status and prospective health predisposition.

9.2 Data Collection

9.2.1 Collection and Data Housing Challenges in Epigenetic Studies

As with all large sets of biological data, privacy is important at all stages - from the assessment of the epigenome, to the storage of data, transfer and sharing, through to its computation, analysis, and presentation. Who is allowed access, to what data sets, and what security and protective measures should be in place, are all-important questions concerning participant privacy in epigenetic medicine and research.

Ethical practices are a constant concern in scientific research. Practice guidelines developed and enforced by research ethics and regulatory bodies ensure compliance. Defining ethics in human-related research requires attention to the participant’s contribution, and an understanding of its possible implications for the participant's life (Ludwin and Frideres 2014). It is the

responsibility of the researcher to conduct research in an ethical manner that is not at the expense of the individual studied (Ludwin and Frideres 2014). As interest in epigenetics continues to grow, an adjustment of ethical standards will be necessary in order to maintain data integrity. Privacy in epigenetic research is a matter of individual well-being that may be grouped with other, even physical, threats (Ludwin and Frideres 2014).

Prior to analysis, data collection is the driving force of any scientific study. Collection concerns and data handling are not unique to epigenetics (Kalapesi 2013), however, like genetic data repositories, epigenetic research often contains incomprehensibly large data sets or **big data** (Lohr 2013). Maintaining security throughout the screening process can be challenging as a result. Indeed, privacy assurance has been debated and proposed as a possible hindrance to scientific advancement (Kalapesi 2013). Still, the exact nature of personal risk in epigenetic studies has yet to be elucidated, as the implications have yet to be characterized.

A good researcher will understand why participant permission is not to be assumed, nor should it derived through manipulation or coercion (Ludwin and Frideres 2014). It is a matter of obtaining **informed consent** (Jallo et al., 2013) built upon the principle of respect for the participant while understanding the gravity of their commitment to the study (Ludwin and Frideres 2014). The challenges inherent in accumulating, storing, and making sense of big data, as well as the globalization of data curation, analysis, and research, present new issues in regulating data collection (Lohr 2013). Globally, there is a trend toward societal and digital decentralization: this is important to note when formulating privacy legislation (Kalapesi 2013). In such geographically and politically distributed structures, reliance upon a single governing body is difficult, especially when the objective is to regulate information sharing among diverse international entities. In these collaborations, sharing often involves third party tools that introduce additional risks since third party motives and intentions

are not always identical to those of the persons invested in the study.

Although it is simple to regulate those directly involved in epigenetic study under scientific standards, it becomes difficult to hold third parties to the same standards as their goals are different (Kalapesi 2013). Third-party regulation is further complicated when the services cross jurisdictional boundaries. For example, the lack of international policies presents an impediment to regulation and enforcement: for example, privacy laws in Canada will carry no weight in France and vice versa. If some part of your data is stored, processed, or analysed in a different jurisdiction, there is little that either you or your physician/researcher can do in the event of a breach of privacy. While accepting the inherently different goals of third parties and the tools they provide, investigators seeking to take advantage of these resources must account for these differences and limitations.

Data is now created and collected in new and distributed ways via connected and social communities (Kalapesi 2013). The traditional need for formal study approval to collect data is easily circumvented by harvesting from, for example, social media. Participants may not be aware that their personal data is being used, nor what it might divulge. Participant consent and privacy are lost in the implicit consent engendered by online posts or browser histories (Kalapesi 2013). A post to social media could provide a means for investigators or agencies to infer epigenetic status without the user's knowledge or consent.

9.2.2 Privacy and Informed Consent of Participants in Epigenetic Studies

Before privacy in storage and sharing of epigenomic data can be considered, it is important to understand how data is obtained. One method is collection through traditional participant-based studies. Due to the confidential nature of an individual's epigenome, researchers will have to gain informed consent. As for

any other medical or social science study, participants should understand the ways that their data will be stored, shared, and used: this is critical to building trust and public support for epigenetic studies. What constitutes informed consent? During the recruitment process, consent is usually defined as having two components. The first component is the participant-signed consent form that provides participants with written information on the study and the protocol that will be followed. The second component requires the research team to provide information and answer questions (Jallo et al. 2013).

Though the foundations of informed consent can be outlined for the researchers, there are still many obstacles to obtaining it from the participants. Some of these obstacles can include a lack of knowledge of the study or field of epigenetics, mistrust of the purpose of the study, language and literacy barriers, and health and socioeconomic status. These obstacles can limit the diversity of study participants (Jallo et al., 2013). Another issue that arises is related to the consent of individuals who are below the defined age of consent, as well as individuals who are unable to provide consent for themselves due to health problems or disabilities. Who has the right to consent for these participants? If inferences about an individual can be made from one individual's data do all relatives need to provide consent in order for data to be obtained for a study? One need only look to the clumsy publication of the HeLa cell genome to see how hurtful inferences can be made: relations of the person from whom the cell line was obtained were neither consulted nor informed (Hudson and Collins, 2013). Additionally, with the increasing use of open-access and wide ranging data sharing, will these individuals have the option to withdraw their epigenome data if they wish to in the future?

Informed consent can be promoted through different methods. It will be critical to provide study information in language stripped of scientific jargon, and with metaphors deployed to help clarify various epigenetic concepts. A FAQ sheet

can help participants to feel it is normal to have concerns and to ask questions pertaining to the study itself as well as to their privacy and anonymity concerns. Researchers should focus on allowing the participants to attain a better understanding, and should encourage questions. These methods have been shown to improve study enrollment rates to 89% compared to the wide range of 15-84% common to efforts that fail to promote informed consent (Jallo et al. 2013).

9.2.3 Access and Release of Epigenetic Data

Effectively safeguarding sensitive personal information goes beyond maintaining respect for the individual providing it. The definition of protection must have the plasticity and resilience to survive the challenge of increasingly integrated digital societies where respect for privacy has become more of an artifice then an actual practice (Kalapesi 2013). The traditionally accepted ethical model of data collection involves obtaining explicit consent directly from the owner (Ludwin and Frideres 2014): this involves the education necessary to make the consent informed, and it reaffirms the intentions of the researcher to expand current knowledge for the benefit of others and society alike. Online scientific resources for data could formerly be considered to enjoy informed consent provisions as one could reasonably expect that the participant was informed of all potential uses (Kalapesi 2013). Yet, in contemporary societies, data banks are no longer just collections of curated information associated with traditional **bioinformatic tools**. Our extended connections have created a new source of readily accessible and personal "big data". With the evolution of **data mining** of nontraditional sources, informed consent is often assumed to be unnecessary, however the uses to which the data might be put is often not transparent to the "donor" (Kalapesi 2013).

Since data may now be derived in many different ways, the various methods of its acquisition must be evaluated separately to

determine appropriate use (Kalapesi, 2013). For example, a recent initiative by Kaiser Permanente to retrospectively survey patient records and make associations regarding the health of offspring serves to indicate where the rules of ethical consent may be subject to intrusive abuse. The data had not been collected for this purpose, but had potential to reveal insights that might otherwise be unavailable. Informed consent was assumed irrelevant as the **inferred data** was obtained derivatively through family members (Kalapesi 2013). The study, however, could be considered a violation of personal privacy in the traditional sense. Does the benign purpose of the study justify the questionable means of its data use/collection? The aforementioned example of the NHS privacy breach serves to indicate how informed consent can fail, and the incident should warn us why strict restrictions on data use must be upheld.

Good intentions should not be assumed of any private entity as the pursuit of profit may incentivize circumvention of the intent of permission – transparency regarding the uses that data will be put to needs work (Kalapesi, 2013). Data privacy provisions should be adaptable, while still adhering to good ethics of collection: models that govern data use will have to adapt to accommodate the potential of constant access rather than attenuate future collection in order to protect privacy (Kalapesi 2013).

The United Nations recently attempted to suggest regulation to prevent privacy violations that are facilitated by interpolation of a person's everyday digital activity, and to account for the new methods by which data may be collected (UN 2013). While promising, this report acknowledges that it will be difficult to globally enforce privacy resolutions – the inter-jurisdictional nature of the problem means that the regulations could only serve as a category of moral request rather than act with the binding power of law. Voluntarily, UN-associated states may agree in principle to abide by such regulations, this agreement does not necessarily ensure compliance. Regional interests may supersede

global statements of principle regardless of consequence, rendering such resolutions somewhat ineffective.

Although some regional governments have developed privacy legislation intended to extend borders, such as European Union agreements (Klein 2008), Canada's Personal Information Protection and Electronic Documents Act (PIPEDA) is an example of legislation that recognizes a need for transferability (OPCC 2014a). The PIPEDA, building upon the principles of Canada's Privacy Act has been continuously expanded to protect health privacy. Yet, recent case law and government statements have made it clear that there is a preference for maintaining and growing international relations over ensuring cross-jurisdictional protection (Klein, 2008). This creates an avenue for international businesses to exploit loopholes and thereby bypass privacy provisions. We risk unnecessarily jeopardizing individuals' privacy, liberty, and equality of opportunity by privileging economic growth. Gaining a global consensus on privacy will take time to realize: advancements in our understanding of epigenetics will no doubt ensue over the interim, and protections will inevitably lag.

9.3 Data Handling

9.3.1 Online Networking and Epigenetics

The internet, as envisioned by Tim Berners-Lee, the man credited with its creation, was intended to provide a path to open, long distance information sharing, specifically of research materials (Owen 2014). More than 25 years later, it has evolved with other networks to provide fast connections and easy access to large stores of data. Initially, privacy was not considered an important factor. Nonetheless, privacy has become a main concern (Owen 2014). While the internet provides a convenient way to drive global collaboration between researchers, it is not without a degree of risk when it comes to fielding sensitive information.

Aside from the sharing of epigenetic data from online storage databases, there are many opportunities for data sharing through

online networking, social media, and mobile apps. For researchers and physicians, online networking is an increasingly important communication tool. Social media and online networks allow for these professionals to communicate with the public and each other with greater ease without the limitations of time-based appointments, and irrespective of geographic distances. There is even a 'Facebook of Science', a social network site called ***ResearchGate*** which allows data sharing among scientists to facilitate collaboration and the exchange of information (Gujarathi and Costa 2014). The use of online communication for non-scientists is also becoming a common way for individuals to obtain health information, share similar experiences through forums, and to participate in pharmaceutical clinical studies. The difficulty with this increased use of online networking is that many people are naïve or do not understand the relatively unregulated nature of privacy regulations and practices. Governing the flow of epigenetic data or information throughout all areas of online networking - social media, discussion forums, and through mobile apps, and patterns of digital device use - seems a near impossible task. A 2014 study on the issue summarized this by stating: "Privacy issues have been the prime disadvantage for the use of the internet and online data storage" (Gujarathi and Costa 2014)

Research boards and ethics committees can often review specific projects but when data becomes widely shared and accessible online, the intervention and regulation by these groups becomes difficult. Consideration as to how these governing bodies can be adapted to regulate and deal with online networking privacy issues is urgent. If adaptation of these agencies is not immediate or palatable, then new models for regulation should be considered. The individual responsibility of participating researchers and health companies must also be enforced. "Medical professionals who use social networking sites have unique responsibilities, since information posting and sharing could violate patient privacy. Life sciences companies should also consider that communication on

social networks is highly public, which allows small issues to escalate into public incidents" (Gujarathi and Costa 2014).

Additionally, the individuals who share their own epigenetic information or that of family members online must also understand the implications that this distribution of data might have. They need to keep in mind that there are privacy implications for relatives, and that breaches may also last for generations if trans-generational epigenetic effects are involved.

9.3.2 Epigenetic Privacy vs. Genetic Privacy

Since epigenetics has yet to reach a level of public awareness equivalent to genetics, many papers and studies are currently examining privacy concerns solely with respect to genetics and genomic data but not epigenetics. It is not clear yet what direction should be taken for epigenetics and privacy - whether it should be considered in conjunction with genetics or as a discrete challenge and issue. Certainly, epigenetics does not fit into the current "DNA sequence" definition of some genetic privacy legislation (see below). Since epigenetics encompasses a wide range of factors and topics, it seems that the subject should have its own discussion and legislative treatment. In this regard it might be beneficial to look at how genetics is being handled, and to use genetic legislation as a model to build upon for formation of epigenetic-specific regulation.

In the U.S., GINA (Genetic Information Nondiscrimination Act), and various other policies could be critiqued, improved upon, and adjusted to include elements that pertain to epigenetic privacy concerns. This would require broad consultation with diverse stakeholders and experts. The structure the GINA and the distinguishing reversibility/plasticity of epigenetic changes means that existing policy will not provide enough protection. GINA's disregard for phenotype (Rothstein, 2008) will have greater bearing in epigenetic protection considering the wider range of variation and of timing that can result from epigenetic alterations.

Policy-makers that seek to use GINA or any genetic privacy legislation must recognize this pitfall.

9.4 Storage and Access

9.4.1 Storage, Sharing, and Use of Epigenome Data

Once informed consent has been established, and the relevant epigenomic data of the individual is quantified and characterized, the data obtained will be stored, shared and analyzed. It is more common to store big data sets such as epigenomic data using cloud-based systems and databases. Privacy concerns play a major role at this stage due to the wide availability, distribution, and open-access nature of these systems, which, although constructed to promote data sharing and collaboration among researchers and physicians, they also engender a new dimension of perils.

Before online data storage, obtaining health records and study findings was time-consuming, limited geographically, and expensive in terms of housing physical copies of files. The degree of ease and speed with which online data can be accessed has changed how frequently data is shared and transferred. "Big data" sets have presented a profound storage issue. "Although both computers and the internet have become faster, there is a lack of computational infrastructure that is needed to generate, maintain, transfer, and analyze large-scale information securely..." (Costa 2013). **Cloud computing** has been suggested as the only solution which is viable to accommodate the scale of data collected over the course of epigenetic studies (Costa 2013). While some initiatives aim to embed privacy protection into the function of cloud computers (Lohr 2013), there are presently no formal industry standards. This is not the only cloud-based challenge. Data analysis, presentation, and delivery might also use distributed servers, agencies, and third-party tools. Moreover, given the complexity and specialization of analysis and presentation software, it might be the case that service providers are few in

number and geographically remote. Without even considering the risks that attend file transfers to remote locations, what regulations pertain to the privacy of data that is collected in Canada, housed via cloud computing in the United States or Europe, and analysed using software provided by a company in Iceland?

Does the concept of privacy need to be revised? Can an individual's data really be confidential and can its use in other studies be regulated? Online data storage and the subsequent global sharing of data has eliminated the idea of data being used for a single study, or by a single group of researchers. Will consent always be obtained for each use of the data once it is present in the open-access database? A recent study has asked some of these questions. "One approach is for the researcher to move from guaranteeing (and so protecting) privacy to practicing veracity, explaining to potential participants that their data will be accessed by, and shared with, others; those consenting to research have to be open to this." (Clarke et al. 2012). If data sharing is occurring on a global level, these privacy considerations need to not only be legislatively addressed in each area, but the regulation of these laws must also acknowledge and encompass cross-jurisdictional considerations.

One method currently in place to protect the privacy of an individual's data in online storage is **de-identification**. This process separates identifiable markers from an individual's data to prevent the data from being traced back to them specifically. Studies of genetic data have shown that **re-identification** is possible: researchers were recently able to identify about 50 participants who donated to the 1000 Genomes Project of "anonymized" genetic information (Hayden 2013). It has been proposed that re-identification methods be used as tests for the privacy protecting ability of various data storage systems (Malin and Sweeney 2004). Industry leaders have nevertheless identified the reality of digital storage vulnerabilities and instead support the call for research **transparency**: both researchers and subjects will

likely need to recognize the impossibility of complete privacy in research that utilizes online tools (Hayden 2013). Online data storage will force the re-evaluation of what is possible and plausible when assurances are given regarding online privacy. It can no longer be assumed that anonymized information is safe.

9.4.2 The Social Media Problem

The environment in which the data originates will dictate its handling, storage and access, and should be the focus of regulation, especially since many of the tools, mechanisms, and uses to which the data will be put are far from transparent to the provider. We are more connected than ever, and information sharing is no longer necessarily an intimate occurrence: blanket privacy protection at the point of collection is no longer practical (Kalapesi 2013).

Many retailers and large corporations use data mining to drive statistical research on current or potential clients (Duhigg 2012). **Predictive analytics** models consumer behaviour and personal events in order to understand future needs. While much of this information is collected by one's transactions with these companies, some may be bought from data banks that also mine a variety of sources including online social activity (Duhigg 2012). Surveys are one of the easiest sources from which to gather information as they tend to reveal a variety of lifestyle elements.

This sort of strategic analysis could be extended to reveal epigenetic status and prospective health. Slight decreases in genomic methylation, indicative of epigenetic status and health, have been linked to groups with low socioeconomic status (McGuinness et al. 2012). Even the nature of one's job correlates to **methylation** patterns and presumably, to disease risk (McGuinness et al. 2012). Combined social and commercial predictive data analysis can be cross-referencing with social, geographical, and employment data. Governments and commercial interests could conceivably derive a highly accurate picture of your epigenetic status and future health predispositions. Imagine how much

information could be gleaned and accurately predicted if the websites that were mined included blogs or chat rooms where people compared notes on depression, smoking, wine tasting, etc. Imagine how much more might be inferred about your general level of activity and fitness if your patterns of web surfing, movie streaming, or online gaming were assessed. Finally, inaccuracies within this mined data set are inevitable. How are researchers to go about validating mined information when no controls for variances are set in place, and how might citizens correct errors in the data that will affect their algorithmic quantification (Kalapesi 2013)?

9.4.3 Privacy Protection and Epigenetic Discrimination

Data-mining is valuable in surveillance and investigations, yet also provides a means for private **dataveillance** (collection and analysis of a person's digitally encoded actions) (Rubenstein et al. 2008). Loose online regulation provides for the essentially unregulated, if unethical, means to collect and store large data sets (Rubenstein et al. 2008). Without globally enforceable online privacy provisions, data-mining companies could take advantage of disingenuous end-users and threaten their privacy. The issue of privacy being subordinated to cross-jurisdictional interests is highlighted by a recent ruling of the European Court of Justice in which Austrian Max Schrems took issue with Facebook transferring his digital information to U.S. based servers (European Court of Justice, 6 Oct. 2015). The additional problems of online sharing in conjunction with re-identification threats further violates epigenetic privacy.

Privacy regulations must also take into account the potential for one's epigenetic data to be used as a means of discrimination. Whether third parties such as employers or insurance companies should have access to an individual's epigenomic data is an important question to address. While researchers and physicians utilize epigenetic data to better understand the factors that can aid in medical care, the intentions of third parties may not be as clear.

What questions or data should employers or insurance companies have a right to ask or access, if any? How can the use of such information by these companies be regulated, and monitored to ensure no discrimination is taking place? Should they be permitted to make inferences and discriminate based upon your educational record, your geographical location, your cultural habits and practices, or the socioeconomic status of your parents? For example, in Arizona, the Pima County Board of Supervisors recently voted to rule job applicants who were smokers ineligible to fill job vacancies… The potential for insurance services or job opportunities to be declined to individuals, or even their relatives, if their epigenetic data shows predispositions to certain health complications or diseases, should create a sense of unease.

The NHS scandal exemplifies a particularly egregious lapse of judgment that had the potential to harm the personal privacy anD opportunities of clients, but it is not the only "anonymized" data set in circulation. Indeed, releases of information like this are increasingly common. Newborn screening (NBS) programs, for example, are viewed as integral to effective postnatal care in the U.S. (Roberts et al. 2014). However, as with many other U.S. federal programs, screening results are publicly accessible. The original purpose was to provide early treatment for detected diseases: the health and societal benefits of this information as a predictive tool are obvious.

With the development of more advanced screening tools and the potential incorporation epigenetic information, complete epigenetic profiling may not be subject to privacy protection in the near future. Although informed consent and consent manipulation are topics hotly debated, the public health benefits of large scale surveys may have inadvertently provided an avenue for illicit exploitation (Roberts et al. 2014). Furthermore, **direct-to-consumer genetic testing** may not only facilitate the misinterpretation of one's genetic identity, and eventually epigenetic identity, but it also provides an additional resource for

third parties to link genetic to epigenetic information (Roberts et al. 2014). A prime example of this is the consumer DNA profiling company 23andMe that has already entered into agreements with pharmaceutical giants such as Pfizer to grant access to troll through their databases. Germany has recognized this as a threat and banned direct-to-consumer genetic testing services based on the public health implications. Still, these services remain widely available elsewhere. Open accessibility to these data stores has enabled the identification of individuals who made a point to be stay anonymous in certain aspects of their lives (Hayden 2013)

Additionally, if insurance companies provide extra services for epigenetic related issues there is a possibility for falsification of identity in order to obtain these services. If an individual's data is publicly accessible online or not stored securely, others who are unable to afford the insurance coverage may utilize the data in order to get the services for themselves. Aside from the financial implications this can have on the individual whose epigenetic identity is stolen in malicious computer hacker operations. It has been proposed that "…identity breaches can deleteriously affect the quality of care. Incorrect information can infiltrate the beneficiary's medical record and corrupt later medical decision making." (Taitsman et al. 2013). The idea that failure to protect an individual's privacy can also affect the quality of healthcare the individual may receive is disquieting and shows the significance of privacy in epigenetic research.

9.4.4 Epigenetics and Advertisements

The use of third-party tools in the form of networks, servers, and processing and storage systems is inevitable when it comes to epigenetic data. This raises the concern that all epigenetic data transactions have the potential to be monitored. **Packet-sniffing** (Ansari et al. 2002) is a powerful administrative tool to evaluate network function, however this tool also provides a method to analyze all shared information across a particular network,

sensitive or otherwise (Ansari et al. 2002). One category of business that utilizes packet-sniffing that could present a threat to epigenetic privacy is embodied by advertising agencies and marketing corporations. How would you feel if your health information was reflected by targeted ads over every web page you visited? What if billboards and TV advertisements were geared towards your personal health status as determined by analysis of your browser history or epigenomic data, or if your mailbox was flooded with ads and promotions related to your personal medical conditions? If third-party companies phoned you or came to your residence with offers for products or services all based on medical data you would consider private, how would you feel? This level of intrusion already occurs.

Google, and other search engines, monitor search inquiries as of way customizing advertising to each user. However, it is a part Google's privacy policy to refrain from ads targeted toward health and other sensitive identifiers (OPCC 2014b). Nevertheless, a man alleged that Google collected his private health information as a means to advertise to him after he had left webpages that he had visited (Lawton, 2014). In response to an admonition levied by the Canadian Federal Privacy Commissioner, Google asserted that the ad targeting was the result of **remarketing campaigns** (OPCC 2014b) and non-compliant corporate users of its services. The case raises questions about the shortfalls of online privacy regulation. Despite legislation that affords health privacy protection in countries like Canada where the aforementioned man was from, U.S. based companies are not held to the same standard of care and confidentiality. It is, however, promising that Google has taken steps to enforce the corporate privacy rules that it has set forth for its clients, some of whom are not subject to Canadian privacy law, as they are based outside of Canada. Still, this does not prohibit a company based in a country with lax privacy protection to take advantage and overstep the personal boundaries of end-users in the pursuit of profit.

Privacy measures to regulate the access these companies have as well as acts and laws to regulate the ways in which they utilize epigenetic data are vital. “The prospect of internet-based marketing corporations using access to research data and to electronic health records as an opportunity to market more products seems both manipulative and cynical.” (Clarke et al. 2012). A further concern with advertising agencies utilizing personal epigenetic data is that they can present the public with infomercial-type advertising that most people will lack the critical tools to evaluate. If individuals are unable to distinguish between credible epigenetic medicines or services versus scams, unethical companies will enjoy a golden opportunity to profit by taking advantage of the ill-informed and unaware.

9.5 Conclusions

Epigenetics presents challenges that are distinct from any other branch of health: there are presently so many additional and commonly accessible means to assess one’s epigenetic status and health predisposition. Many of these avenues of assessment are unregulated and exposed. Privacy regarding a person’s epigenetic and long term health is open to inference and interpretation in ways that that other aspects of your health and life are not. Privacy advocates, individuals, and legislators need to take rapid action if they are to protect our access and opportunity to enjoy good jobs, healthcare, insurance, security, and privacy.

Provocative Questions

- In regions less concerned with individual rights, use of open sourced epigenetic information could propagate human rights violations. How can this be controlled or discouraged?

- Will lack of de-identification safeguards lead to discrimination and the imposition of unfair government standards?
- Can the Wild West of cross-jurisdictional data flow be regulated to protect privacy?
- How can individual responsibility for personal and extended familial epigenetic data protection be balanced with rights to freedom of expression?
- Online governance inherently undermines the principles of the internet and entrenched ideals of **net neutrality.** Regulatory activity tends to be reactive, not proactive. How can this be changed quickly in order to contain the possible damage?
- Some of the largest curators of data storage are not arms' length and disinterested parties – they are multinational corporations with vested interests and are uniquely positioned to exploit consumer data. How can this be controlled internationally?
- Can private scientific networks be sufficiently protected against intrusion?
- How can consumers be endowed with tools to detect and correct errors in their profiles?
- How can the comprehensive and extensive nature of the privacy threat be conveyed without eliciting a knee jerk reaction?
- With the growing privatization of many social services and a move towards business-focused models of running public institutions, is any organization truly focussed on public service and well-being?

Possible Solutions

- Protection of epigenetic privacy will be most effective through proactive measures. Direct linking of privacy

requests have been proposed in the scientific community as a way of giving the information owner greater control over their data (Lohr, 2013).

- Epigenetic privacy provisions must be flexible to accommodate the rapid pace of technical and scientific change.
- Education will be the greatest tool to protect privacy. It is not enough to educate the public - researchers and government officials must also be trained on the implications of use.
- Data storage security must be made a priority.
- Individuals must also understand their responsibilities must understand the potential risks, show some restraint, and consider the privacy implications of their actions for extended family and descendants.

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Chapter 10

Where Do We Go From Here?

Michael Crawford

Abstract

Recent discoveries in the emerging field of epigenetics will impinge in a profound way upon reproductive, medical, and environmental ethics. These discussions have implications for the role of the state in our homes and in our lives. What we do in our lives, how we live, the opportunities that we are afforded - these manifest as epigenetic traits and dispositions that can be modified and transmitted to offspring without changes being made to our DNA sequences. Environment, diet, toxins, and even human relations can alter an epigenetic imprint for generations. Inevitably, political, legal, and social discourse will focus upon where to balance individual rights and obligations versus societal costs and imperatives. I will argue that the complexity, longevity and time lag of epigenetic effects mitigate against a simple allocation of responsibility for injury or health. To clarify the scope of legislative and policy challenges, I will suggest the limitations of tort law to deal with injury, and deliver some of the perspectives, temptations, and directions for ethical solutions that will be brought to bear.

10.1 A Bioethical Agenda For Consideration

None of us chose our parents, no matter how much we might like to take the credit or avoid the blame. We enjoyed

control neither over our birth, early childhood economic, familial, educational, nor nutritional environment. Who then bears responsibility for repairing the health, economic, and environmental disparities that can exert long-term reproductive consequences? Whose interest is served by improvements that will better subsequent generations? Parents? Children? The state?

Clearly, we all should all do our best to respect and nurture our bodies. They are the vessels that we ride into old age, and they are the machines that help to build future generations. For our own sakes, as well as for our progeny, we need to respect and care for ourselves as best we can. Exerting the necessary discipline is often hard, but if epigenetics tells us anything, it is that we might not be as autonomous and unencumbered in our proclivities and predispositions as we might formerly have supposed.

At present, the legal system does not explicitly recognize epigenetic effects let alone the interaction that exists between epigenetic imprinting and the environment. However, the environment has always influenced the genome and the health of individuals, and it is becoming clear that improvements to the environment can translate to improvements in human health and prosperity (Dupras et al., 2012; Guthman and Mansfield, 2012). With epigenetics we have a window to see the mechanism by which environmental wellbeing is connected to individual health: the complex interactions between the two could offer a tool to improve societal health (Dupras et al., 2012). Such a mindset would link improved individual health to environmental stewardship as well as the propagation of better medical and environmental health through future generations (Rothstein, 2013).

With advances in diagnostics and epigenetic testing, the detrimental effects of the environment could be identified and possibly even corrected before they propagate to future

generations (Loi et al., 2013). With increased attention to the prevention of disease, rather than the treatment of conditions after development, the remedy of disadvantages early in life should take precedence. The emphasis should shift to the early treatment of social, educational, dietary, and genomic challenges: epigenetic interactions are likely to amplify health deficits that are the consequence of these any of these area over time and across generations (Loi et al., 2013). However, this will require a shift in strategy. Preventive treatments or "wellness" and education programs have the potential to drastically improve the quality of health care offered to patients yet they may introduce new forms of inequality. If epigenetic assessment and diagnosis is not affordable and accessible, the likelihood is that greater inequities regarding quality, efficacy, and availability of health will arise (Rothstein, 2013). As a consequence, enjoyment of health, life, and opportunities will become stratified, and productivity of the community at large will be hampered.

There is still much to be determined with regard to the place of epigenetics in the legal system, tax system, and health care systems. A change in perspective, extensive consultation, and increased public knowledge of epigenetics is necessary for changes to take place.

We can illustrate the convoluted complexion of the challenge, and the inadequacies of current frameworks to contend with epigenetic issues, using the example of tort law discussed in Chapter 8. In tort law, the difficulties that attend identification of the origin of an imprint will prove daunting in assigning liability. For example, people will inherit genetic and epigenetic predispositions from their parents, and then they will themselves add to this historical baggage via behaviour, experiences, and choices. Quite aside from the fact that a child has yet to successfully sue a parent for wrongful life (indeed since some jurisdictions have ruled it repugnant to

assign a compensatory value for birth-associated defects that would place a differential value on lives) (Karpin, 2010), demonstrating a causal relationship between an individual's actions and their consequences is too fraught with variables to survive close scrutiny. For example, obesity has often been regarded as the product of over-indulgence on the part of parents or individuals. Similarly, addicts, while arguably at least partially the product of their environment, have been regarded as the authors of their own dissolution. Recently it has become clear that there are genetic and epigenetic factors that need to be considered (Delport and Pollard, 2010; Byrnes et al., 2013): people who indulge in self-harming behavior may be the victim of proclivities and impulses that have a more complex etiology than formerly supposed.

While individuals cannot be excluded from exerting significant control over their epigenetic status, neither can they be assigned sole responsibility. Given that assignment of blame for epigenetic damage to individuals and businesses is likely to prove difficult under tort law, similarly, one must ask how ethical considerations can be gauged and how practical assignments of responsibility can be allocated? Where does the balance lie between state and citizen? Indeed epigenetic causes and effects are so complex, so temporally extended in their manifestations, and so subject to multiple influences, that for reasons of both political as well as practical, it might be better to ignore where, in the spectrum of interactions between individuals, the state, and businesses, ethical responsibility actually lies. Perhaps we need to focus instead upon how ethics can guide our practical responses.

Maria Hedlund makes an interesting observation: she points out when epigenetic responsibility is assigned retrospectively, individuals will tend to be identified and blamed (Hedlund, 2012). By contrast, she argues that when responsibility is viewed prospectively, the structural and

contextual nature of a problem demands action by political and social organs.

Let us reduce the equation solely to factors of economics and practicality: an argument can be made for shifting some of the onus of responsiveness and responsibility for epigenetic health from individuals to businesses and the state. The long-term consequences of epigenetic perturbation are costly, so by facilitating good practices, businesses and states can position themselves to control heath care costs and to minimize future encumbrances upon labour availability and efficiency. Given the difficulty of demonstrating a simple causal link between individual lifestyle choices and imprinting effects, interventions that are facilitative rather than proscriptive seem most likely to succeed and to provide efficiencies for both health and societal success.

Facilitative experiments have met with success in the past, albeit with no knowledge of the epigenetic consequences of the programs at the time. The Finnish "Baby Box" program was installed in 1939 with the objective of reducing infant mortality among the poor. The program is still running and delivers a box of useful goods to parents on condition that they seek free prenatal medical advice and examinations. The program was universalized in 1948 (Lee, 2013; Söderblom and Kela, 2013). Parents have the option to ignore the program, and even to receive monetary compensation in place of the boxed goods. Nevertheless, nearly everybody uses the program, and parents receive both a high standard of prenatal and delivery healthcare as well as a wealth of baby clothes, diapers, blankets etc.. The boxes are regarded as a "good deal" and the rate of infant and mortality has dropped. Similarly, in the State of Hawaii, intervention was forced when a cohort of impoverished teens was identified as poorly served by the existing QUEST/Medicaid programs. The cycle of addiction, obesity, diabetes, and teen pregnancy presented both

immediate and long term risks that were addressed by extending post partum health coverage from 8 weeks to 6 months or more (Partika et al., 2008).

10.2 An Agenda for Consideration

So where do ethicists, social engineers, scientists, business people, and government meet to allocate resources and responsibility? Where might ethics help to guide our response? The comprehensive and overlapping nature of epigenetics effects mitigates against a piecemeal approach. Gesche argues for a carefully integrated approach to analysis and policy development: she argues that it will require transformation of public health policy (Gesche, 2010). We'd argue that the scope needs expansion further still and that it would help to commence with discussions that pertain to purely practical measures:

1. Government agencies need to coordinate centrally to gather and then disseminate information and advice. A Standing Committee should serve as a nexus for information and policy development, and all stakeholders should have a means to articulate concerns. This necessitates an inter-disciplinary approach, and presumably one guided by professional ethicists. Given the breadth, complexity, and ever-changing nature of the information landscape, such a committee needs to be constituted not merely of well-educated representatives of science, social policy, medicine, and economics, but to have additional positions that are occupied by topically relevant short-term "floaters." Epigenetics will continue to surprise, so the participants need to be flexible and engaged in learning on the fly. Operational principles of such a Committee would have to reflect the need for this evolution of education and compositional flexibility.

2. Governments, through their agencies, need to undertake a rapid and fulsome practical education of their

agents, as well as of the media, and public at large. To this end, initially, open participation conferences with plenary speakers from multiple disciplines (ethics, science, government, economics, healthcare delivery, business, law) might be a good place to start. More especially, practical information needs to drill down to family physicians, expectant mothers, and children in elementary school on upwards.

3. Individuals need help to make good lifestyle decisions. Options to facilitate this beyond merely educating might include: better labeling of food contents; "sin taxes" on foods that are commercially prepared and/or comprising high fat content and/or high glycemic index; tax credits for participation in clubs and organizations that promote fitness (especially for pre-pubescent children); better public transit combined with more expensive parking for cars etc.; in-fill and public space development in cities to render them more walk-able and accessible for shopping, living, playing.

4. Should incremental health service cost increases or service restrictions apply to individuals who abuse themselves, or who refuse to control unhealthy behaviour? Seeking help should be rewarded, and failure to succeed should not necessarily result in a sanction. Educative support would have to account for options that might be excluded by economic or cultural context. There is a geographical, economic, and cultural dimension to epigenetics - no absolute metrics are likely to be practical or effective. The ethical debate and social policies that derive will need to be managed in a sensitive manner.

5. Should states reconsider the thresholds at which they intervene to protect children from abuse, and to encourage full parental engagement and support? The definition of abuse should be re-examined beyond the scope of purely physical.

Diet and parenting are clearly of trans-generational, not merely familial importance.

6. Consideration should be given to maternal health and dietary support both pre-term and post delivery, and to investment in feeding and food education programs at schools where the habits of a lifetime may still prove open to modification. Given the onerous financial and human cost of the growing plague of obesity and diabetes, the cost of such early life social supports seems paltry by comparison.

7. Consider developing a directed taxation structure whereby companies that produce new categories of materials, drugs, and waste, help to build an autonomous and protected investment fund for the purpose of offsetting potential generational consequences of unforeseen health issues.

8. Ensure that companies are regulated and insured to cover the true costs of environmental degradation. It is hard to predict in advance what wastes will have an effect, so all effluents and emissions should be factored into an accounting. Who could have predicted that PCBs or endocrine mimics would exert trans-generational effects?

9. Research in humans needs to redress a recent gender bias in the literature. Paternal and parental care studies in rodents demonstrate that females are not necessarily privileged in conveying an imprint to offspring. The recent preponderance of focus upon human maternal effects risks burdening women with an unfair share of responsibility for generational health. The effects of pre-conception activities upon later fetal health might also impinge upon our perception of how to balance individual rights and long-term reproductive obligations.

10. Medical, social, environmental, and economic policy experts need to conference to both learn about epigenetics, and to begin developing policies to mitigate extant as well as developing challenges. Little recognition has been

paid to this field or to the long-term health and economic costs that will arise. By way of example, if, as rodent studies suggest, the presence of endocrine disruptors in our food and drinking water exerts a subliminal and persistent effect, we could be in for interesting times. How and where to effectively marshal resources to maintain and improve equitable access to good health will be a complex problem, and initiating discussions pertaining to health and social ethics is a good place to start.

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Glossary

acetylation involves the addition of an acetyl group (C_2H_3O) to a molecule. In epigenetics, acetylation of the tail of histone H1 is associated with a lax nucleosomal conformation that is conducive to gene activity.

acute myelogenous leukemia – also known as Acute myeloid leukemia or acute nonlymphocytic leukemia (ANLL). It the most common type of blood system cancer. White blood cells proliferate in the bone marrow and inhibit the production of other blood cell types.

Alcoholism – type I The tendency is to drink excessively in response to a stress. Alcoholics of this type can avoid abuse for extended periods of time, but can fall into an abuse spiral. Type I alcoholics usually feel guilt and fear of their addiction.

Alcoholism – type II This category of alcoholics tend to start drinking from an early age and often have an older male family member who also abuse alcohol. Drinking tends to be continuous, and considerable control over ingestion can be exerted.

algorithm: computer science; programming logic to efficiently provide output based on input.

Alu elements or sequences are, like LINE elements, remnants of retroviral invasion, and they constitute roughly 11% of the genomebut are somewhat shorter in length than LINES.

apoptosis is a technical term that describes genetically programed cell death in response to injury, senescence, or as part of normal embryological development.

autism spectrum disorder (ASD) encompasses neurodevelopmental disorders that result in varying degrees of social or communication deficits, sensory and cognitive disorders, and a tendency to repetitive or stereotyped behavior.

big data: a large, complex collection of data that requires special processing considerations due its size. Computational analysis of these data sets attempt to reveal trends or behaviors, especially with regard to human commerce or behavior.

bioinformatics: intersection between computer science and biology; using computational methods to analyze biological information**.**

bioinformatic tools: computer software developed for the processing and analysis of biological systems and data

Bisphenol-A (BPA) is used in the production of plastics and can leach from containers into food products. BPA can act as an estrogen mimic.

brain-derived neurotrophic factor (BDNF) is a growth factor that is required for neural growth, stability, and synapse formation. Mis-regulation of BDNF is linked to depression, schizophrenia, addiction, and Alzheimer's disease.

chemopreventive reversers is a substance that reverses the effects of an environmental toxin.

chromatin: The "stuff" of chromosomes, chromatin is an assembly of DNA, RNA, and proteins such as histones. Chromatin is the higher order, flexible, and dynamic architecture of our genome.

chronic lymphocytic leukemia is a disease of the bone marrow stem cell population that contributes lymphocytes (white blood cells). The disease develops slowly with most tuning into an acute form of B-cell leukemia.

chronic physical aggression (CPA) describes a pattern of behaviour where young children display physical aggression and who fail to moderate it between the ages of three and four. The pattern of aggressive behavious persists throughout school years and into adulthood. It is associated with histories of child abuse, and/or family disfunction.

circRNA molecules do not encode proteins but help to regulate the activity of miRNAs which in turn regulate the transcription and translation of mRNAs thereby ultimately affecting proteins synthesis.

cirrhosis is a sign of advanced liver disease and can be the product of alcohol abuse, hepatitis or other diseases. It represents a scaring or regenerative response to stressors.

clear cell adenocarcinomas are a rare form of tumour, usually associated with the female uro-genital tract. The tumours usually have a glandular origin

cloud computing: large, connected networks of servers to allow fast data processing and storage through a single shell access point.

coeliac disease is an autoimmune disorder of the small intestine that is triggered by gluten-containing foods. It results in diarrhea, weight loss, anemia, fatigue, and disorders in other organs.

collective responsibility is a philosophical concept that defines a social and moral duty to accommodate or remedy larger scale and collective harms. Groups such as governments, businesses etc., might be argued to hold collective responsibility for harms to individuals or groups.

CpG sites of dinucleotides: shorthand for cytosine-phosphate-guanine, a DNA sequence that is a preferred site for DNA methylation.

CpG islands are CG-rich regions, usually located in the 5' upstream regulatory regions of genes. They are a frequent site of DNA methylation.

Crohn's disease or syndrome is an incurable inflammatory bowel condition that can result in pain, weight loss, diarrhea, fever, bowel obstruction,

rash, and a predisposition to bowel cancer.

cytokines are small secreted proteins used by cells to communicate. They are often associated with responses to stress or with cells of the immune system.

data mining: analyzing large sets of information for specific points of significance.

dataveillance: information technology; constant analysis and collection of data transferred over a network for purposes other than diagnostics.

decentralization: dispersing functions from a central point among many entities for shared responsibility.

developmental programming, also known as Barker's hypothesis posits that a mother's diet - and fetal exposure - during pregnancy will have an effect upon the onset of diseases later in the life of her offspring.

diethylstillbestrol (DES) is a synthetic non-steroidal estrogen. It has a history of use in cattle, and in humans as: a cancer treatment, chemical castration agent (Alan Turing), and it was erroneously prescribed as a fertility enhancer from the late 1930 until the 1970s.

de-identification: bioinformatics; removal of all markers that could be used to identify the owner of a biological data set.

direct-to-consumer genetic testing: purchasable services to analyze and receive information on one's genome. ie. 23andme.com

DNA methylation occurs when methyl groups are attached, usually to the C-nucleotide in GCG-rich regions of gene regulatory regions. DNA methylation is usually, but not always, associated with gene inactivation.

DNA methyltransferases (DNMTs) are the enzymes that catalyze addition of methyl groups to DNA. They tend to inactivate genes.

DNA sequencing determines the order of nucleotides that comprise a DNA fragment or a genome. The readout is then assembled and compared against a database to identify genes etc.

eclampsia defines a health condition in women either during or shortly after pregnancy where high blood pressure, organ failure, or high blood protein levels lead to convulsions.

endocrine typically describes hormones or the glands that produce them. Typically endocrine products are secreted into and circulated by the circulatory system, but they tend to have specific target tissues upon which they exert their effect.

end-user: computer science; the person who is meant to access a network or system after it has been fully developed and released to market.

environmental toxicants are toxic substances that we ingest from the environment via direct contact, food,

water, or air. Examples include mercury, PCBs, DDT, lead, asbestos, etc.

epididymal cysts are benign fluid-filled cysts that develop in the male reproductive tract at the top of the testes.

epiphenotypes encompass the morphologies, behaviours, or characteristics embodied in an organism by the superimposition of factors other than genes. Epiphenotypes are often considered to be more or less heritable.

erythroleukemic cells represent a less common form of blood system cancer where the progenitors of red blood cells are affected.

estrogens are steroidal hormones that are of primary importance in female reproductive development and reproductive function and behaviour. Their function is mediated by receptors that signal the activation of specific downstream genes.

euchromatin is the more loosely packed structure of chromatin, and it often encompasses genomic regions where genes are active.

feed-forward control loops result in the transmission of an external cue to elicit a defined response, but without monitoring or moderating how it reacts.

fetal programing, also known as Barker's hypothesis posits that a mother's healthy diet during pregnancy will help to control the onset of diseases later in the life of her offspring.

gametogenesis is the process by which the gametes, sperm or egg (oocyte), are derived from stem cell populations in the gonads.

glia, also known as neuroglial cells, support neurons in the central nervous system. Recent evidence suggests that they can participate in the transmission of nerve impulses, and that they secrete substances that modify or stimulate the connections made by neighboring neurons.

global methylation is the averaged estimate of DNA methylation that is determined by analyzing methylation, usually of DNA, detectable in blood samples. It does not predict the epigenetic status of specific genes, nor of specific cell types and tissues other than blood – is serves as a general marker of epigenetic health or age.

hematopoietic stem cells have the capacity to proliferate and differentiate to populate blood.

heterochromatin is the densely packed conformation of chromatin, and is usually associated with areas where genes are tightly packed and inactive.

hippocampus is a part of the limbic brain and plays a role in short and long term memory consolidation, and in spatial navigation. The hippocampus is especially sensitive to stress and its shrinkage is thought to play a role or be caused of post traumatic stress disorder.

histone acetylase or histone acetyle transferase (HAT) enzymes add acetyle groups to histones, often with

the result that associated DNA sequences (genes) become more active.

histone deacetylase (HDAC) enzymes remove acetyl groups from histones which often results in lower activity from the associated DNA sequence (gene).

histone acetylation usually occurs on the COOH-terminal tail of histones H1. It affects the intimacy of histone-DNA interacyion in nucleosomes, and this in turn regulates gene activity.

histone methylation occurs when methyl groups are added to histones. Within the context of chromatin structure, this can have the effect of either activating or repressing associated DNA sequences (genes).

histone methyltransferase adds methyl groups to histone proteins. This can have the consequence of either activating or repressing the activity of associated DNA sequences.

histone proteins: the five main classes of histone proteins (H1, H2A, H2B, H3A, H3B) assemble into groups of eight (octamers) and serve as the substrate around which DNA is wound into the higher order structures constituting chromatin.

hydatidiform moles, also known as trophoblastic disease, are a gestational anomaly that occurs when an egg loses its genome and is inseminated by one or more sperm. The consequence of a solely paternal genome is that there is over-development of chorionic pre-placental tissues, usually in combination with an absence of fetal tissue.

hypoplastic testes: under-developed or smaller than normal testes

immunoprecipitation employs antibodies that recognize a specific antigen to bind, immobilize, precipitate, and permit the identification, purification, or modification of a specific target molecule.

inferred data: information derived indirectly on an individual from relatable sources.

informed consent: permission received from an individual after they have been well educated regarding an investigation or procedure and its likely effects.

joint venture liability encompasses partners who combine resources in a business venture to make a profit. They share liability for failures of product integrity, efficacy, or safety.

LINE elements are Long Interspersed Nuclear Elements that represent roughly 20% of the human genome and likely constitute remnants of retroviral invasion.

lncRNA (long non-coding RNA) molecules represent a new and poorly understood class of RNA that do not encode proteins, but appear to act as regulators of gene transcription and translation.

luck-egalitarianism is a model of distributive justice that maintains that people ought to be held responsible only for those things that they exercise control, not over inherited economic, genetic, or other factors. Some

normalizing element must enter into the equation to make justice equitable.

luck-prioritarian models argue that the less fortunate (those with bad luck) ought to receive priority in any distribution or resources. Justice argues for resources where they will make the biggest difference.

mass spectrometry is a tool used in analytical chemistry that uses the abundance of gas phase ions and the mass-charge ratio of molecules to identify chemical modifications to, the amount, or the identity of samples.

methylation refers to the addition of a methyl group (CH_3) to other molecules such as DNA or histones. Highly methylated DNA tends to be inactive, while methylated histones can cause associated gene sequences to be either active or inactive.

microbiota describes the community of bacteria, yeast, viruses and other microscopic organisms that populate our bodies

miRNA molecules are a class of short RNA molecules that do not encode proteins help regulate the activity of other RNA transcripts.

motif: any DNA, RNA, or protein sequence that is shared by related species of molecule, and that is associated with a functional or operational significance. eg; a receptor binding site.

myelodysplatic syndrome is a disease of bone marrow resulting in a failure to produce enough blood cells – patients can become anemic.

ncRNA is any category of RNA that does not encode a protein. Examples include lncRNA, rRNA, tRNA, telRNA, miRNA, circRNA etc..

mRNA is the RNA transcript that transmits information from genes to ribosome where they encode the amino acid sequence of proteins. They are often edited (spliced) prior to being translated to protein. mRNA molecules are usually characterized by a poly-adenylated tail and a 5'-Cap.

multivariate – having multiple component parts or contributing factors that may or may not interact directly.

net neutrality: information technology; to view all data and access via the internet in an egalitarian manner so that special privileges will not be taken or granted with respect to its access or transfer.

neural tube is the embryonic precursor to the spinal cord and brain. Defects in its development and progression rank among the most common of birth defects.

nucleosome: one of the fundamental units that contributes to chromatin, the higher order organization of DNA. Each nucleosome is embodied by DNA wrapped around eight histones protein subunits.

nucleotide: one of the fundamental constitutents or building blocks of RNA and DNA. The molecule comprises a nitrogenous base, at least one

phosphate, and either a ribose or deoxyribose sugar.

oestrogens are steroidal hormones that are of primary importance in female reproductive development and reproductive function and behaviour. Their function is mediated by receptors that signal the activation of specific downstream genes.

packet-sniffing: information technology; analyze data transferred over a network for diagnostic and quality control purposes.

phosphorylation occurs when phosphate groups are added to a protein, usually by a kinase enzyme. Depending upon context, this can either activate or repress activity of the protein.

polymerase chain reaction (PCR) is a process whereby a thermo-stable DNA polymerase is used to reiteratively make copies of a DNA template sequence.

placental previa is an obstetrical condition where the placenta is placed near to the cervix and is prone to shearing or bleeding under the weight of the fetus.

preconception torts are a category of law suit in which the litigant makes claims for injuries sustained before they were even conceived and born. Instances might include suing for negligent advice given by a physician to presumptive parent regarding genetic risks s.

predictive analytics: the act of modeling consumer behaviour and personal events in order to understand future needs (Duhigg, 2012).

progesterone is a steroid hormone involved in the menstrual cycle, pregnancy, and human development and sexual maturation.

prospective responsibility acknowledges that in the real world, every time we go somewhere or o something, it comes with an attendant risk for which we must bear some responsibility. In epigenetics, the term has a pregnant meaning: both individuals and society share an obligation to provide circumstances that are conducive to healthy epigenomes, even pre-conception.

Rawlsian-egalitarian model of justice is a variation of luck egalitarianism that requires the playing field be leveled so that everybody can compete on equal footing for opportunities. Social inequalities need to be configured to benefit the least advantaged, but not so much that they remove incentive to strive.

re-identification: analysis of anonymous data and cross-referencing with other data sources to find similarities and markers that can identify the original producer of the information.

remarketing campaigns: recording end-user behaviour in order to effectively advertise to the user based on their suspected interests

ResearchGate: “Facebook of Science” (Gujarathi & Costa, 2014); a social medial network that facilitates global

interaction between those in the STEM fields.

retrotransposons are mobile viral remnants that retain the capacity to rtranscribe from our DNA (genome) into RNA, then reverse transcribe back to DNA, and finally to reinsert into the genome, often at a different location.

retroviral elements derive from retroviruses., a category of RNA virus that encodes, among other products, the enzymes necessary to reverse transcribe to DNA and then to integrate parasitically into a host's genome.

rRNA are RNA molecules that constitute part of the ribosomal complex – a larger assembly of RNA and proteins that serve to build polypeptide chains (proteins)

single nucleotide polymorphisms (SNPs) embody some of the DNA sequence variation between individuals. They are used as markers that associate with specific gene alleles (ie; the normal or the mutant allele for a trait)

sires are the male parent in animals

statutory of limits or statutes of limitations and repose define a period of time beyond which a person or agency cannot be prosecuted. The idea is to ensure that a trial can rely upon fresh evidence that has not deteriorated with the passage of time. Limits also ensure a reasonable expectation that individuals, agencies and businesses can enjoy an environment that is free of unforeseeable long term liabilities.

stress tests are used in rodent studies and involve restraining the animal in a tube for a period of time, usually ranging from 15 to 30 minutes.

striatum is a subcortical region of the brain responsible for regulating fine motor coordination.

swim stress is a test commonly used to elicit or study neurological function or endocrine stress responses in rodents. Essentially it is a monitored tank filled with water in which the animals must swim to keep their head above the surface.

telomeres are structures that protect the ends of chromosomes and that are comprised of repetitive DNA and RNA sequences as well as proteins. Telomeres generally shorten with age leading to chromosomal instability.

telRNA molecules are a class of non-coding RNA that binds to the ends of chromosomes (telomeres) to preserve chromosomal integrity.

tort law is the civil branch of law where rights, obligations and remedies are argued. Litigants sue for remedy or relief from harms that are alleged to be the responsibility of the defendant.

transparency: business and social sciences; openness of actions that facilitates honesty and accountability.

tRNA are the species of RNA molecules that shuttle amino acids to ribosomes to help in the construction of new polypeptides (proteins).

trophoblast comprises the outer layer of cells in a pre-implantation embryo. These cells are the first to differentiate and will contribute to development of the placenta.

trophoblastic disease, also known as **hydatidiform moles**, are a gestational anomaly that occurs when an egg loses its genome and is inseminated by one or more sperm. The consequence of a solely paternal genome is that there is over-development of chorionic pre-placental tissues, usually in combination with an absence of fetal tissue.

victim attenuation – a concept of diminished effect or liability due to distance between cause and effect. This term can cover indirect injuries, or injuries that do not become readily apparent due to transmission across generation.

white hat (hacking): computer science; purposeful but not malicious violations of network security usually to identify and correct network vulnerabilities.

X-Chromosome inactivation. The human X chromosome contains many more genes than the Y chromosome. Human males are normally XY, and females XX. In order to maintain a similar dosage of active genes in females, during early development, one of the two X chromosomes is inactivated by making it extremely compact (heterochromatic) thereby rendering the majority of its genes inactive. The resultant structure is called a Barr body.

xenobiotics are foreign chemicals or substances that are not normally produced in nature or ingested under normal circumstances. The term is sometimes used to describe compounds that are detectable at high levels when they are normally only found at trace concentrations.

Index

ABOUT THE STUDY GROUP

This book arose as a collaboration among students of disciplines as diverse as biological sciences, literature, political science, psychology, and computer science. The group met at the University of Windsor to workshop topics to highlight, in accessible language, the social, political, ethical, and legal ramifications of epigenetics. The aim was to translate the science to a comprehensive, exhaustive, and up-to-date resource for policy and law makers, teachers, other students, and for the public at large.