Chapter 4
Complex Phenotypes: Epigenetic Manifestation of Environmental Exposures

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Abstract Environmental influences in early development alter the epigenome and lead to complex phenotypes and disease susceptibility throughout the life course. Five primary factors, including nutrition, behavior, stress, toxins, and stochasticity, act to influence the epigenome during this critical period. To illustrate how changes in early environment can dramatically affect the epigenome, we provide examples from diverse members of the animal kingdom, spanning insects to human. Specific to mammalian early embryogenesis, DNA methylation, and other epigenetic marks are reset at two specific times in distinct cell lineages leading to epigenetic programming of gametic and somatic cells. These two waves of genomic demethylation and reestablishment of methylation frame the sensitive times for early environmental influences. Evaluating the complex effects of environmental exposures on the developing epigenome requires novel and comprehensive approaches. In this chapter we outline a strategy for the evaluation of environmentally induced epigenetic effects across animal models and human samples, highlighting the necessity for careful assessment of dose and resulting phenotypic changes across the life course. Herein we review the history, environmental factors, critical time points, and vulnerable genomic structures of epigenome–environment interactions. We also provide a framework to further explore epigenomic changes and translate this knowledge from mouse to man.

Keywords Epigenetics • Development • Environment • DNA methylation • Plasticity • Environmental epigenomics

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Abbreviations

BPA	Bisphenol A
CDK	Cyclin-dependent kinase
DMR	Differentially methylated region
DOHaD	Developmental origins of health and disease
GR	Glucocorticoid receptor
IAP	Intracisternal A-particle
LTR	Long terminal repeat
PGC	Primordial germ cells
PRC2	Polycomb repressive complex 2
RAMs	Regions of altered methylation
tRNA	Transfer RNA

4.1 Introduction

The developmental environment is emerging as an influential predictor of subsequent phenotypes and disease risk in later life. The "developmental origins of health and disease" (DOHaD) hypothesis posits that gene–environment interactions during early life result in long-lasting effects and points to epigenetic inheritance as a prime mechanism (Barker et al. 2002). Epigenetics is the study of changes in gene expression that are heritable from cell to cell, hence through cell lineage development, or in rare cases, transgenerationally from parent to offspring to grand-offspring (Youngson and Whitelaw 2008). Increasingly, we are recognizing that early environmental influences on the epigenome are diverse and include dietary (total caloric intake, specific nutrient level, phytochemicals), physical (behavior, temperature, species density, stress), chemical (toxins, endocrine disruptors, pharmaceuticals), or unknown (stochastic, random) effects. Thus, the convergence of environmental toxicology and epigenetic gene regulation is particularly important during the earliest stages of development when epigenetic modifications, such as DNA methylation, are the most sensitive to perturbation.

In this chapter, we introduce the reader to complex phenotypes emerging from environmental perturbations on the developmental epigenome. Firstly, we discuss five relevant environmental influences on epigenetic modifications in development. Here, we showcase examples in animals from insect to human where the environment influences the epigenome through early developmental exposures. Secondly, we review the timing of epigenomic reprogramming, focusing on the post-fertilization and germ cell differentiation stages in male and female offspring. Thirdly, we elucidate the major genome structures and mechanistic targets most vulnerable to environmental perturbations. Finally, we introduce strategies for evaluating

environmental effects on the developing epigenome, and for detecting and verifying epigenetic modifications using combined approaches in animal models and human clinical and epidemiological populations.

4.2 Developmental Influences on the Epigenome

The importance of the early environment in modifying developmental trajectory is not new. For example, in 1809 Jean-Baptiste Lamarck proposed that the increased use of a body part would cause a heritable increase in the size of that body part (Lamarck 1809). Mechanistically, Baptiste hypothesized that organisms have a "tendency to progression" in which offspring inherit traits acquired by the habits of their parents (Gould 2002). Likewise in the early twentieth century, early environmental manipulations were attempted by now-discredited Soviet biologist Trofim Lysenko in his claims that crop yields could be adapted to cold climates by exposing seeds to cold temperatures (Soyfer 2001). This culminated in Lysenko's experimental feeding of special diets to gestating cattle hybrids to produce offspring with greater milk productivity (News of Science 1957). Such misconceptions surrounding the environmental influence on developmental trajectory, therefore, persisted for decades despite early refutations such as August Weismann's experiment in cutting the tails off of rats over five generations while never observing the birth of a tailless rat (Weismann 1891). Epigenetics was revived as a modern science by Conrad Waddington in 1947 and by the end of the twentieth century had adopted its current definition (Jablonka and Lamb 2002).

There are currently a variety of recognized developmental influences resulting in lifelong phenotypic change, mediated by epigenetic gene regulation. The five early developmental influences described here, nutrition, behavior, stress, toxins, and stochasticity interact to influence methylation and other epigenetic marks that in turn affect life-stage phenotype and disease (Fig. 4.1). As indicated by the wide number of animal species discussed (from insects to humans), it is likely that the capacity for epigenetic plasticity is evolutionarily selected. Therefore, it is likely that many more instances of environmental epigenetic influences remain to be elucidated (Jablonka and Raz 2009). Of important note, however, is that not all animals use DNA methylation to repress gene function; for example, the model organisms, fruit fly (*Drosophila melanogaster*) and roundworm (*Caenorhabditis elegans*). To ultimately succeed in identifying environmental factors that affect the epigenome and lead to complex phenotype and disease, researchers must integrate the layers of epigenetic changes in response to mixtures of environmental exposures, paying attention to the times of sensitivity and the model system of evaluation.

Fig. 4.1 Environmental factors working individually and in concert. Five environmental influences that affect the developing embryo and its primordial germ cells (represented by the *pink* and *blue dots*). Each of these factors can act through a variety of mechanisms, and result in an array of changes in epigenetic marks

4.2.1 Nutrition

Nutrition in early life is never guaranteed or consistent. In bees (*Apis mellifera*), early life nutritionally induced changes are the underlying mediators of queen and worker honeybee differentiation. Bee larva fed royal jelly, a diet specially enhanced with royalactin proteins, shifts development to the queen phenotype, and shows similar effects in the fruit fly (*Drosophila melanogaster*) (Kamakura 2011). DNA methylation is a primary mechanism by which royal jelly acts on the genome through the diet (Kucharski et al. 2008). For example, methylation of the promoter region of *dynactin p62* is decreased in worker bee heads as compared to bodies and averages 10 % lower methylation in the queen bee's complementary tissues, an effect that has been experimentally induced by siRNA-mediated silencing of *Dnmt3*. Additionally, in utero supplementation of animals with methyl-donor rich diets permanently shifts the coat color pattern of mice carrying the *Agouti* viable yellow allele (Waterland and Jirtle 2003) and increases methylation and suppresses transcription of the *Runx3* gene in lung tissue (Hollingsworth et al. 2008). Similarly, dietary supplementation with genistein, the major phytoestrogen in soy, interacts with the methyl-donor pathway to similarly shift the coat color distribution of *Agouti* viable yellow mice, which have emerged as a model biosensor for in utero effects on the fetal epigenome (Dolinoy et al. 2006).

Whole genome hypomethylation is also seen in animals receiving folate deficient diets during gestation and lactation (McKay et al. 2010). For example, in mice, an early postnatal methyl-donor deficient diet reduced methylation at the imprinted gene, *Igf2* (Waterland et al. 2006b). In fact, in utero malnutrition in rodents not only directly affects the expression and methylation of several genes, such as glucocorticoid receptor, *Nr3c1*, and peroxisome proliferator-activated receptor alpha, *Pparα*, but also the neonatal response to leptin. Moreover, these epigenetic effects persist later in life and affect the ultimate adult phenotype, adiposity (Gluckman et al. 2007). Humans are also affected by early life nutritional status as shown in the DNA methylation changes at the *IGF2* locus in whole blood from individuals subject to the Dutch hunger winter (Heijmans et al. 2008). Human

longevity also appears correlated to food abundance available to our grandparents during their prepubertal growth, an effect hypothesized to be epigenetic in origin, although the direct epigenetic mechanism remains unknown (Kaati et al. 2007). Candidate gene studies followed by whole epigenome analysis will become important to environmental epigenomics especially in the translation from mouse to human research.

4.2.2 Behavior and Stress

Behavior- and stress-induced changes are likewise widespread from insects to mammals. The desert locust (*Schistocerca gregaria*) produces more offspring of the gregarious swarming phenotype when bred in crowded conditions (Maeno and Tanaka 2010). Similarly, rats show persistent DNA methylation changes of the glucocorticoid receptor and many other loci in the hippocampus due to high versus low levels of maternal care in the first week of life (McGowan et al. 2011). Falling under both behavior and stress, humans abused in early life also show increased DNA methylation at the *NR3C1* glucocorticoid receptor promoter in the hippocampus (McGowan et al. 2009).

Stress induces epigenetically controlled phenotypic changes in many animals. Insects show widely varying genomic methylation levels between species and changes within a species during development, suggesting a role for methylation in gene–environment interactions (Kronforst et al. 2008). The pea aphid (*Acyrthosiphon pisum*) has a functional DNA methylation system, and under stress from crowded conditions or predators will produce more winged offspring (Weisser et al. 1999). The crowded mothers express more DNMT enzyme, and the winged offspring show increased methylation at the *ApJHBP* locus (Walsh et al. 2010). This illustrates the importance of verifying concordant changes in message level and translation with DNA methylation. Emerging evidence also suggests that regulation of epigenetic marks is associated with stress and environmental response genes in the pacific oyster (*Crassostrea gigas*) and in the basal chordate *Ciona intestinalis* (Gavery and Roberts 2010; Sasaki and Satoh 2007). Furthermore, after being in close proximity to cats, rats exhibit symptoms of post-traumatic stress syndrome concomitant with increases of methylation in the *Bdnf* gene in the hippocampus (Roth et al. 2011). Interestingly, increased methylation of this same gene is also seen in human suicide victims (Keller et al. 2010).

In mice, stress in early life results in increased adult brain expression of arginine vasopressin (AVP) protein correlating with increased methylation of the *Avp* gene in neurons (Murgatroyd et al. 2009). Stress from maternal separation in mice results in depressive behavior coupled with both increased and decreased DNA methylation in a number of genes (Franklin et al. 2010). Interestingly, these mice can transmit the DNA methylation pattern and phenotype transgenerationally through

the male line (Franklin et al. 2010). Early life stress in humans is also linked with gene expression changes for a polymorphic form of serotonin receptor (Caspi et al. 2003).

4.2.3 Chemical Toxicants

Chemical toxins are widely dispersed and have been shown to impact epigenetic processes and marks following early life exposures. Water fleas (*Daphnia magna*) show decreased global DNA methylation when reared in the presence of vinclozolin, a fungicide and endocrine disruptor (Vandegehuchte et al. 2010). Moving from insects to mammals, female rats exposed to vinclozolin during gestation produce male offspring with methylation changes in numerous genes in their sperm. In the female offspring there is a greater incidence of tumor formation and pregnancy abnormalities, which in some cases persists transgenerationally through the germ line (Anway et al. 2005; Guerrero-Bosagna et al. 2010; Nilsson et al. 2008). A whole epigenome approach will offer a more complete understanding of these changes. Because F1 and F2 germ cells are present in the exposed gestating female, they are exposed to vinclozolin during early development. Thus, the effects on the epigenome can be inherited transgenerationally (Fig. 4.2).

While a complete review of the full suite of chemicals affecting the developing epigenome is beyond the scope of this chapter, a number of classes of chemicals beyond pesticides have also been elucidated to act on the epigenome. For example, bisphenol A (BPA), a widely studied endocrine disruptor, is ubiquitous in our environment and is repeatedly shown to affect DNA methylation in multiple rodent tissues, such as liver and brain (Dolinoy et al. 2010; Yaoi et al. 2008). In primates, early exposure to the metal lead (Pb) results in decreased DNA methyltransferase activity in the brain even 23 years later, again underscoring the importance of correlating message level to methylation (Wu et al. 2008). Given the emerging weight of evidence linking developmental toxicant exposures to later disease states in animal models via methylation, a clear path from dosage experiments in model organisms to candidate genes in human studies is particularly crucial.

4.2.4 Stochasticity

Lastly, stochastically or randomly placed methylation marks laid down in early development have been observed at several loci. The model biosensor, the viable yellow (A^{vy}) mouse, varies from brown, pseudoagouti, to yellow fur coloration due to randomly established levels of DNA methylation at a recently inserted intracisternal A-particle (IAP) element within the 5′ end of the *Agouti* gene. DNA methylation can vary by over 80 % at several CpG sites within this IAP between animals (Dolinoy et al. 2010). Similarly the $Cabp^{IAP}$ locus in C57BL/6 mice also contains a contraoriented IAP element in intron 6, capable of stochastic DNA

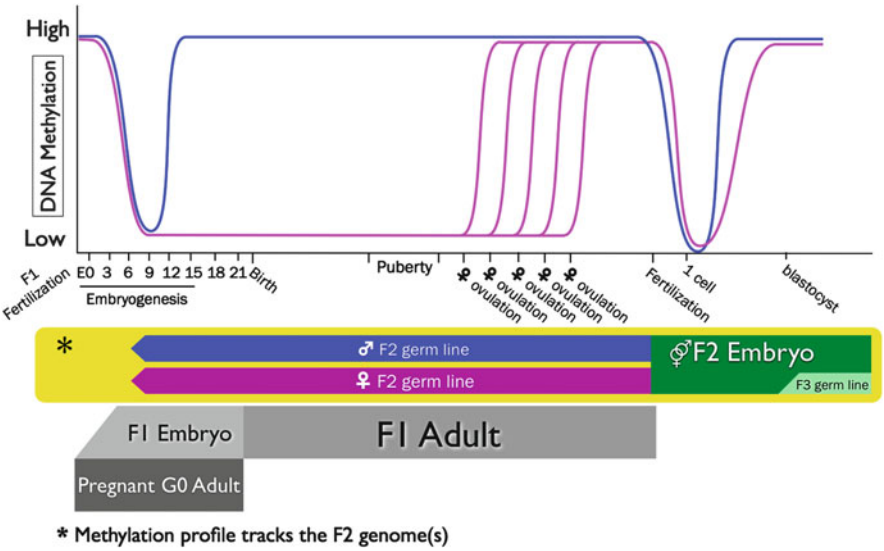

Fig. 4.2 Methylation reprogramming in a single genome occurs in two waves. Global demethylation events from the perspective of the mouse F2 genome from germ cells to newly conceived F2 embryo. Within the pregnant G0 mouse, the F1 embryo generates a group of cells destined to become its gametes, which will form the F2 generation. These primordial germ cells (*PGC*) begin to migrate to the genital ridge around embryonic day 7.25 in the mouse. During this time they become demethylated in preparation to adopt the somatic methylation pattern, and for imprinted genes, the gender-specific methylation pattern to match the genotype of the individual in which they reside. In males, methylation is reestablished by E14. In females the PGC remain largely unmethylated until maturation in the F1 adult during each estrous cycle. During fertilization, the F2 gametes combine and undergo the second, more complete, wave of demethylation in preparation to establish somatic methylation patterns (with the exception of the F3 PGCs). Any environmental influences on the pregnant G0 adult can affect the development and adult disease susceptibility of both the F1 and F2 generations as their somatic and germline methylation patterns are being established, respectively

methylation (Druker 2004). The Axin-fused ($Axin^{Fu}$) mouse has a dramatic kinky tail phenotype caused by stochastic DNA methylation of another intronic IAP element (Vasicek et al. 1997). Thus, IAP elements are important proxies for genome-wide methylation determination as well as for understanding methylation resetting during development, as will be discussed in more detail in Sect. 4.4.1.

These alleles are termed "metastable epialleles," as they are variably expressed in genetically identical organisms due to epigenetic modifications that are established during early development (Rakyan et al. 2002). The epigenetic marks are variable between individuals but consistent and stable in their patterns within a mouse throughout its life, implying that the level of methylation is set early in development and stable for life (Weinhouse et al. 2011). The distribution of variable expressivity is shifted at these metastable epialleles following maternal exposure to nutritional and environmental factors (Cooney et al. 2002; Dolinoy et al. 2006, 2007; Kaminen-Ahola et al. 2010; Waterland and Jirtle 2003; Waterland

et al. 2006b). In humans, we see variation in methylation in monozygotic twins where spinal deformation was associated with increased *AXIN1* methylation in one twin with lack of methylation and associated physical anomaly in the other one (Oates et al. 2006). It is likely that the underlying stochastic distribution of methylation at metastable epialleles may be affected by as yet uncharacterized environmental factors.

4.3 Time Points of DNA Methylation Lability

DNA methylation is primarily a stable repressive mark; however, its regulation is more dynamic than previously believed and can be actively removed at specific loci and genome-wide at several stages during development (Wu and Zhang 2010). In the context of early environmentally modifiable epigenetic marks, it is important to determine the windows of greatest susceptibility. The epigenome is most vulnerable to environmental factors during embryogenesis because the DNA synthetic rate is high, and the elaborate DNA methylation patterning required for normal tissue development is established. During adulthood, somatic tissues vary widely in cellular turnover rate and environmental exposures (see Sect. 4.3.3). The mammalian genome undergoes two waves of global DNA demethylation followed by de novo methylation, as illustrated in Fig. 4.2 using the mouse as a representative mammalian animal model (Bernal and Jirtle 2010). In mammals, the mother, G0, hosts the development of the F1 offspring from zygote stage to birth. During the development of the F1 offspring, a separate lineage of cells within the F1, called the primordial germ cells (PGCs), migrate and differentiate into gamete precursor cells that will eventually become the F2 generation. By convention, the first wave of methylation resetting refers to the reprogramming of the epigenome within these PGCs, and the second wave refers to the reprogramming that happens shortly after zygote formation. Exposure of a pregnant mother can affect methylation status of both the first wave (in the F2 PGCs) and the second wave (in the post-fertilization F1 pluripotent somatic cells).

4.3.1 Germ Cell Methylation Reprogramming

In mice, the first wave of reprogramming occurs in primordial germ cells (PGCs) during and after their migration to the genital ridge (Yamazaki et al. 2003). This demethylation is complete in the mouse before birth and allows resetting of imprinted genes in the PGCs to match the sex of the host in which they now find themselves (Lees-Murdock and Walsh 2008; Sasaki and Matsui 2008). A number of repetitive elements, including IAPs, are also protected from demethylation to varying extents during this reprogramming wave in developing oocytes (Hajkova

et al. 2002). These genetic loci are more susceptible to loss of heterozygosity and increased methylation instability, respectively (Dolinoy and Jirtle 2008).

In male mouse fetuses, PGCs differentiate into prospermatogonia, enter mitotic arrest, and reestablish methylation before birth (Kota and Feil 2010; Reik et al. 2001). Consequently, this window is especially important for disruptions to loci that escape demethylation as well as resetting of global methylation in male offspring with any effects likely to be seen in the F2 generation (Guerrero-Bosagna et al. 2010; Hanel and Wevrick 2001). Nonmammalian animals, in general, do not have imprinted genes (Kaneda et al. 2004). Developmental exposures may affect the growth of the offspring by inheritance without necessarily having a lasting impact on the parent.

After sexual maturation in all male mammals, the prospermatogonia complete meiosis and differentiate into mature sperm, and during this process, the chromosomes are almost entirely stripped of histones and repackaged with highly basic protamines. Because the protamines do not contain any modifiable tails, any epigenetic information carried on histones is unable to be passed through the male germ line (Balhorn 2007). A small number of histones are retained in mammalian sperm; however, it is unknown whether they play a role in passing on any epigenetic information to the resulting zygote (Gaucher et al. 2010; Kota and Feil 2010).

In contrast to males, F1 female mammalian PGCs complete meiosis I while still in the developing embryo, followed by cell arrest until puberty (Kota and Feil 2010). Thus, in human females, for example, the oocytes remain in a haploid demethylated state for years. Therefore, the window of possible disruption to the establishment of methylation patterns in oocytes is much longer and repeatedly occurs during the maturation of each egg throughout fertility (Fig. 4.2) (Sasaki and Matsui 2008).

4.3.2 Zygotic Methylation Reprogramming

The second wave of global demethylation occurs shortly after fertilization and before implantation. The male pronucleus is stripped of the protamines, while the DNA is actively demethylated and repackaged with newly synthesized histones in the zygote (Nonchev and Tsanev 1990; Santos et al. 2002). The female complement of chromosomes becomes demethylated via a passive mechanism during replication (Hemberger et al. 2009). Not every gene is demethylated since the oocyte contains egg-specific isoforms of DNMT1, and the early embryo synthesizes its own somatic DNMT1 isoform. The presence of these maintenance methyltransferases is required to ensure the preservation of gametically derived differential methylation for imprinted genes, particularly during global demethylation of most other regions of the genome (Cirio et al. 2008). Application of whole epigenome methods in model organisms will complete the list of candidate targets for environmental sensitivity.

The zygotic round of demethylation is less comprehensive than the reprogramming in PGCs, with imprinting control regions retaining differential DNA methylation depending on their parent-of-origin and some classes of repetitive elements also retaining their methylation (Lees-Murdock and Walsh 2008). This wave of methylation cycling sets the pattern for all somatic cells in the resulting embryo and adult except for the PGCs, which will form the gametes for the next generation. The embryo (F1) would be most vulnerable during this window of time to environmental influences that disrupt the reestablishment of DNA methylation. Thus, any epigenetic disruptions early in development would affect tissues in all three germ layers (Weinhouse et al. 2011).

4.3.3 Somatic Methylation Lability

The major global demethylation event that occurs in the somatic cells of adults is associated with aging and disease states (Calvanese et al. 2009). As mammals age, they undergo gradual genome-wide DNA hypomethylation concomitant with hypermethylation at normally unmethylated CpG islands (Murgatroyd et al. 2010). Additionally, adult tissues undergo widely varying life spans and cellular turnover rates. Neurons can last a lifetime, while gut epithelial cells live only a few days (Creamer et al. 1961). Cell types have differing exposure profiles as well, with some exposed more directly to toxins and nutrients like the gut and intestinal tract. Other tissues are exposed primarily to chemical metabolites, and some, like liver, are exposed to both. Location, length of exposure, and cellular life span all play a role in the dynamics of DNA methylation change for a given organ over time. Since cancer is a heterogeneous disease, it displays inconsistent methylation profiles, but in general, the genome is widely hypomethylated as compared to normal tissue with the notable exception of hypermethylation of tumor suppressor gene promoter regions (Feinberg 2007).

4.4 Vulnerable Genomic Structures

The genome is not homogenous in content, expression, or epigenetic marks; some loci are more likely to resist reprogramming or to be environmentally sensitive. As discussed in Sects. 4.2 and 4.3, repetitive elements and imprinted genes can be incompletely reprogrammed or unusually labile, respectively. Below we examine these types of loci in greater detail.

4.4.1 Repetitive Elements

Human and mouse genomes are comprised of 46 % and 39 % repetitive elements, respectively. These elements fall under several different types based on their methods of duplication including cut-and-paste, copy-and-paste, tandem duplication, and gene conversion (Cordaux and Batzer 2009). From an evolutionary perspective, selection acts to limit the spread of these potentially genome destabilizing elements. Epigenetics, particularly DNA methylation, plays a major role in their suppression. As an example, the long terminal repeat (LTR) class of elements is widely active in mice with several thousand copies accumulating and still being quite active in reshaping the genome today (Qin et al. 2010). These elements are generally silenced to a high degree in normal somatic cells; however, they can act as gene promoters in both mouse and human (Bernal and Jirtle 2010; Cohen et al. 2009). Additionally, the same elements can exhibit differing species-specific methylation patterns (Carbone et al. 2009). Elements of different classes regain methylation at different times as well. For examples, some LTR-class IAPs can remain unmethylated in the female oocyte until maturation, whereas LINE-1 elements are remethylated much sooner, around the time of birth (Lees-Murdock and Walsh 2008). Sex-specific differences are also evident in these same elements, with both classes becoming methylated in male germ cells immediately following the global demethylation event in migrating primordial germ cells. Clearly, the genomic identity of a region plays a large role in the timing in the laying down of epigenetic marks.

4.4.2 Imprinting

Epigenetic systems in mammals may have developed as a consequence of totipotency and the need to activate genes in only certain cell types despite the fact that all cells share the same genetic components (Jablonka and Lamb 2002). One of the most extensively studied epigenetic phenomena in mammals is genomic imprinting, in which one parental allele is epigenetically altered resulting in parent-of-origin modification of gene transcription (Murphy and Jirtle 2003; Reik and Walter 2001). Imprinted genes were first hypothesized following nuclear transplantation studies conducted by Surani and colleagues in the 1980s in which diploid androgenotes derived from two male pronuclei and diploid gynogenotes derived from two female pronuclei developed improperly (Barton et al. 1984; Surani et al. 1984). It was not until 1991, however, that the first imprinted genes were identified. Since the demonstration that *insulin-like growth factor 2* (*Igf2*), a potent growth factor (DeChiara et al. 1991), and *insulin-like growth factor 2 receptor* (*Igf2r*) (Barlow et al. 1991) are imprinted, approximately 80 imprinted genes have been identified in mice and humans, with 29, or about one third being imprinted in both species (Morison et al. 2005).

Since imprinted genes are functionally haploid, the health consequences of genomic imprinting are potentially disastrous. Monoallelic expression eliminates the protection that diploidy normally affords against deleterious effects of recessive mutations. The most widely accepted theory of imprinting evolution, "the conflict hypothesis," posits that imprinting arose because of a genetic tug-of-war between the parents to control the amount of nutrients extracted from the mother by her offspring (Haig and Graham 1991; Wilkins and Haig 2003). Work in the early 2000s showed that imprinting evolved approximately 180 million years ago following the divergence of Prototherian (i.e., monotremes) from Therian (i.e., marsupials and eutherians) mammals (Killian et al. 2000; Murphy and Jirtle 2003). Thus, genomic imprinting arose in mammals with the evolution of the placenta and the advent of viviparity.

Imprinted genes and their associated regulatory components may be particularly sensitive to developmental environmental perturbations on the epigenome. In fact, as discussed above, individuals conceived during the Dutch hunger winter at the end of World War II, were shown 60 years later to have altered DNA methylation at the *IGF2* locus (Heijmans et al. 2008). Imprinted genes are associated with differentially methylated regions, termed DMRs (Ferguson-Smith 2011). Since DMRs, unlike most regions of the genome, must retain methylation on a single copy on one chromosome with the sister chromosomal locus hypomethylated, they are naturally sensitive to methylation disruption (Bernal and Jirtle 2010). The classic example of complementary genes being imprinted is the maternally expressed *Igf2r* and paternally expressed *Igf2* genes in mice. Although these regions are believed to have evolved rapidly, the *Igf2r* DMR is maintained in humans despite the now biallelic expression of the gene (Kalscheuer et al. 1993). Moreover, the imprinting status is often, but not always, preserved between species (Ferguson-Smith 2011; Weidman et al. 2004). The monoallelic expression of imprinted genes can also be specific to a tissue or developmental time point. Consequently, developmental dysregulation by toxins or other environmental exposures can vary in tissue and time-dependent manner further complicating risk assessment.

4.5 Mechanistic Targets of Environmental Exposure

Mounting evidence suggests that environmental pressures can exert effects on multiple levels of gene regulation. The weight of evidence is currently focusing on gene transcription and chromatin structure controlling access to transcriptional machinery.

4.5.1 Gene Transcription

The original function of DNA methylation is likely to be host defense against rogue transposable elements but has since been co-opted to also serve a gene regulatory function (Zemach and Zilberman 2010). Gene transcription can be suppressed by the DNA methylation of CpG islands in promoters as well as histone modification and nucleosome placement. For example, the methyl group of the 5-methyl cytosine extends into the major groove of DNA, inhibiting transcription by interfering with transcription factor binding proteins. In addition, DNMTs and methylated DNA interact with higher-order chromatin proteins to affect histone modifications and further compact chromatin. Thus, DNA methylation and histone modifications act synergistically to maintain silencing and inhibit access to transcription factors.

As introduced previously, the insertion of a metastable IAP in the *Agouti* gene in the A^{vy} mouse strain provides a locus for variable DNA methylation to affect transcription. The $Cabp^{IAP}$ and $Axin^{Fu}$ loci also show a correlation of increased methylation and decreased expression affected by environmental exposures. These loci provide a basis for the investigation of genome-wide changes due to methylation, and a number of studies find novel metastable epialleles with variable methylation (Luedi et al. 2005; Waterland et al. 2010). Repetitive element insertions can also be the cause of several human diseases (Callinan and Batzer 2006). Although they can disrupt gene transcription, they can also be silenced by surveillance mechanisms, as we see in the case of the A^{vy} mouse IAP element. Environmentally induced derepression of these repetitive elements is an active area of research.

4.5.2 Chromatin Remodeling Complexes

Chromatin modifying complexes act throughout the genome changing the higher-order chromatin structure to inhibit or increase access to transcriptional machinery. The methyltransferase *EZH2* catalyzes H3K27 trimethlyation as part of the PRC2, and it can also be phosphorylated by cyclin-dependent kinases (CDK) (Hansen et al. 2008). After phosphorylation it loses the ability to bind to noncoding RNAs and is thus unable to place the H3K27me3 mark in a sequence-specific manner (Zeng et al. 2011). Importantly, this mark is propagated from cell to cell, preserving the epigenetic silencing of marked regions. Any environmental influences that alter the activity of *EZH2*, or its upstream CDK enzymes, can thereby have widespread developmental effects lasting into adulthood. Placental mammals also have *REX1*, a DNA-binding transcription factor thought to target chromatin remodeling complexes (Kim et al. 2007). In mouse *Rex1* null mutants, DNA methylation profiles at the DMRs of several imprinted loci are disrupted in adult tissues despite the fact that *Rex1* is only expressed very early in development (Kim et al. 2011).

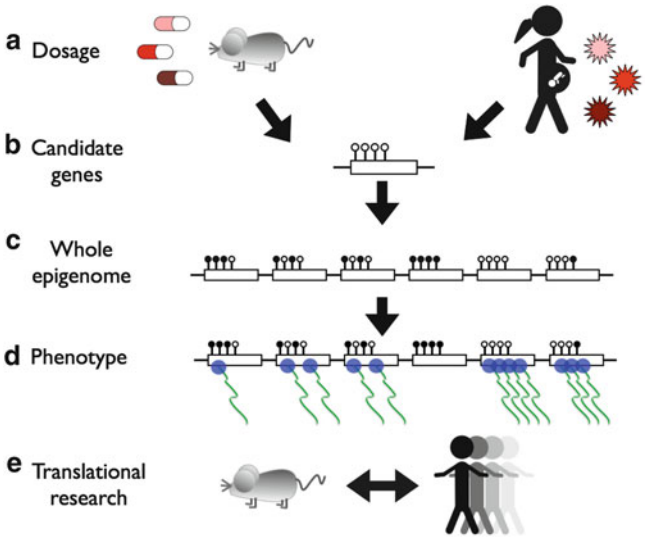

Fig. 4.3 Comprehensive strategy for the analysis of environmental disruption on the developing epigenome from mice to men. (**a**) Multiple dosages in animal model studies better reflect relevant human exposure ranges allowing for the detection of non-monotonic epigenomic effects, often characteristic of endocrine active compounds such as bisphenol A (BPA). (**b**) Candidate gene assays are quick, reproducible, and relatively inexpensive and are able to be used for epidemiological research. Conversely, this approach alone is limited in the ability to identify new targets of environmentally influenced epigenetic changes. (**c**) Epigenome-wide studies, enabled by advances in technology, provide a comprehensive overview of DNA methylation changes over the entire genome, enabling the discovery of new regions of metastability. (**d**) Phenotype can be measured quantitatively by message level and correlated to genomic DNA methylation. An environmentally induced change in an epigenetic mark does not necessarily reflect a corresponding change in message, such as mRNA levels. (**e**) Translational research from animal models to human clinical samples to human populations is necessary to locate and quantify environmentally induced changes (e.g. BPA) on the epigenome

4.6 Mapping Environmental Influences on the Developing Epigenome: A Representative Approach from Mice to Men

Until recently, most attempts to elucidate the effects on the epigenome following nutritional and environmental exposure were either candidate gene driven or based on epigenetic techniques with limited genome coverage/sensitivity, restricted in dose–response assessment, or confined to animal models. Using bisphenol A (BPA) as a representative early environmental exposure, we have developed a comprehensive strategy for evaluating effects on the developing epigenome (Fig. 4.3). This approach combines multi-dose studies in animal models with careful environmental characterization of human clinical samples and extended analysis of epidemiological-characterized human population samples. Integral to this strategy and important for identifying biomarkers for disease risk and progression is the requirement that

environmentally induced changes on the developmental epigenome be correlated with subsequent gene expression and phenotypic effects across the life course.

BPA is a high-production volume monomer used in the manufacture of polycarbonate plastic and epoxy resins. It is present in many commonly used products including food and beverage containers, baby bottles, dental composites, and thermal receipt paper. Furthermore, BPA is associated with epigenetic alterations following developmental exposure (Dolinoy et al. 2007; Ho et al. 2006; Yaoi et al. 2008). In the rat model, Ho et al. observed multiple changes in gene-specific DNA methylation patterns in the adult male prostate, including hypomethylation of the phosphodiesterase type 4 variant 4 (*Pde4d4*) gene following neonatal exposure to both estradiol and low-level BPA (10 μg/kg of body weight BPA). Decreased *Pde4d4* methylation is associated with a marked increase in prostate cancer risk. Using the viable yellow *Agouti* (A^{vy}) mouse model, we showed that maternal dietary exposure to moderate levels of BPA (50 mg BPA/kg diet) results in decreased DNA methylation at the A^{vy}, and $Cabp^{IAP}$ metastable epialleles (Dolinoy et al. 2007). Finally, using restriction-enzyme based methylation technology, Yaoi and colleagues reported both hyper- and hypomethylation at *NotI* loci in murine offspring forebrain following gestational exposure to 20 μg/kg of body weight BPA (Yaoi et al. 2008). These recent attempts, including our own, were limited to (1) a candidate gene driven approach, (2) restricted dose–response assessment, (3) less than comprehensive timing and expression effects, and finally (4) animal models.

Capitalizing on advances in unbiased epigenomic and high-throughput quantitative DNA methylation technologies, we have developed a comprehensive approach to identify the constellation of genomic loci with altered epigenetic status following dose-dependent in utero BPA exposure. Using a "tiered focusing approach," our strategy proceeds from unbiased broad DNA methylation analysis using methylation-based next generation sequencing technology to in-depth quantitative site-specific CpG methylation determination using the Sequenom MassARRAY and QIAgen pyrosequencing platforms. Innovative to this design, we employ this approach across both mice and humans in order to identify species specificity in lability from early exposure to environmental agents. Additional toxicologically relevant animal models, including rats and sheep are also being considered for this approach, resulting in a comparative epigenomic analysis across mammalian species. Using bioinformatics and biostatistical methods, we compare the regions of altered methylation (RAMs) following BPA exposure, and the cellular pathways in which the genes with nearby RAMs function.

Human BPA exposure, including time-dependent intra-individual variation, is an extremely active area of research as well as being controversial. Examination of 2,517 individuals ($\geq$6 years) from 2003 to 2004 NHANES survey showed urinary BPA concentrations ranging from 0.4 to 149 ug/L (mean 2.6 ug/L) (Calafat et al. 2008). Levels were lower in Mexican Americans compared to non-Hispanic blacks and whites and higher in women and children as well as individuals of low socioeconomic status (Calafat et al. 2008). A study of circulating blood BPA levels in pregnant women in Michigan indicated exposure levels between 0.5 and 22.3 ug/L (mean 5.9 ug/L) (Padmanabhan et al. 2008). These trends indicate that in utero

development and infancy may be particularly vulnerable time periods for exposure to BPA. Collaborating with analytical chemists, we are evaluating BPA concentrations in human placental and fetal tissue and employing epigenome-wide techniques to correlate human in utero BPA exposure levels with distinct methylation profiles. These samples represent a unique opportunity to not only measure BPA levels in tissue matrices but to also conduct epigenome-wide and transcriptomic analysis, an endeavor that requires relatively large amounts of DNA and RNA. Moving from human clinical samples to human population studies with well-characterized early environmental exposure data, as well as life-course demographic and nutrition data, is a crucial next step in this pipeline.

The strategy described herein utilizes BPA as a representative environmental toxicant that is easily applied to other environmental factors of interest. The elucidation of epigenomic loci dysregulated in a dose-dependent manner in both animal and human genomes will ultimately strengthen human health risk assessment and shape diagnostic and therapeutic strategies for disease. As mentioned above, the mouse is a tractable and popular model for human diseases; however, animal models for toxicology studies may not be the best choice for modeling the potential impact on the human genome if the repertoire of epigenetically labile genes is markedly species dependent. For example, recently Jirtle and colleagues employed machine-learning algorithms to identify epigenetically regulated imprinted genes throughout the genome. This approach uncovered 600 novel imprinted candidate genes in the mouse and 156 in the human (Luedi et al. 2005, 2007). Interestingly, humans are predicted to have not only fewer imprinted genes than mice, but also a markedly different repertoire. The divergence of imprinted genes, and potentially other epigenetically regulated loci such as metastable epialleles, between mouse and human could have serious consequences for the reliance on rodents as models of human disease. Further, the use of whole genome and unbiased deep sequencing approaches allows for the identification of epigenetic biomarkers for exposure that will be useful in enabling clinicians to identify at-risk individuals prior to disease onset.

4.7 Moving Forward

It is increasingly recognized that environmental exposure to chemical, nutritional, and behavioral factors alters gene expression and affects health and disease, by not only mutating promoter and coding regions of genes but also by altering gene expression through the modification of the epigenome. The investigation of early environmental effects can inform the fields of toxicology and environmental epidemiology by elucidating the mechanisms underlying developmental exposure and adult disease. Of the five environmental factors we highlighted as affecting development, nutrition is the best studied. Given the ubiquity of environmental toxins in our environment, a comprehensive plan is needed to assess their effects. Dosage levels, corresponding mRNA message levels, protein translation, and resulting

phenotypic consequences, ideally must all be determined in model organisms. Candidate gene approaches will be enhanced by concomitant whole epigenome technologies. Epidemiological studies must then translate these results from mouse to man.

In order to integrate epigenetic research into risk assessment or clinical practice, we must first understand the most sensitive time points in the resetting of epigenetic marks. These vulnerable periods are likely distinct for males and females as well as for offspring and grand-offspring and may require specialized preventive and/or corrective actions. Additionally, the molecular mechanisms linking these sensitive time points to the adult presentation of disease need to be fully characterized. Ultimately scientists must integrate the layers of epigenetic changes with the times of sensitivity to generate the best prescriptions for human health. Since epigenetic profiles, unlike genetic mutations, are potentially reversible, approaches for prevention and treatment, such as nutritional supplementation and/or pharmaceutical therapies, may have significant impact on human health and disease trajectories.

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