Chapter 6

Intrinsic and Extrinsic Factors That Influence Epigenetics

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CONTENTS

6.1 INTRODUCTION

The original definition of *Epigenetics* by Conrad Waddington was "The branch of biology which studies the causal interactions between genes and their products which bring phenotypes into being" (1). As can be seen, this original definition is already aimed at describing how factors involved in the development of organisms (i.e., the "epi" part of epigenetics) affect the expression of their genetic composition.

Nowadays, the focus of epigenetics has shifted to study accessory chemical modifications on the DNA that are able to regulate gene expression and survive mitotic events (2). However, epigenetic mechanisms still represent processes that bridge the gap between environmental influences and long-term regulation of gene expression, which is in turn involved in phenotype formation.

Every epigenetic process described to date has two basic components: The intrinsic machinery of the cells and the contribution of external factors. External factors will act on the epigenetic machineries by: (i) providing chemicals as substrate for the epigenetic reactions, (ii) affecting the functioning of enzymes that are involved in epigenetic reactions, or (iii) altering the binding of ligands to receptors that respond to environmental stimuli, and that subsequently trigger cascade of reactions that will ultimately affect the epigenetic machinery.

Epigenetic machineries are involved in any aspect of the body's function. In the following sections we will describe the role of intrinsic (endogenous) factors in influencing epigenetic regulation related to reproductive health, followed by a description of extrinsic (environmentally available) factors that, in one way or another, alter the functioning of epigenetic machineries in the developing and adult organism and thereby may affect its reproductive capacity. First, however, we provide some basic information about epigenetic mechanisms and processes.

6.2 DEVELOPMENTAL PERIODS OF EPIGENETIC REPROGRAMMING

At the interface of the interaction between intrinsic and extrinsic factors are developmental periods of "epigenetic reprogramming". Special susceptibility for the action of environmental compounds occurs during these periods that involve transient albeit major epigenetic rearrangements. Two waves of extensive epigenetic reprogramming are described to date in mammals. One is after fertilization, where an initial reduction in DNA methylation is followed by re-methylation at the time of blastocyst implantation (3). This epigenetic reprogramming is crucial for the differentiation of somatic cells. Another period of epigenetic reprogramming occurs during the migration of primordial germ cells (PGCs) toward their final establishment in the gonads (4). During this migration a major demethylation of the genome also occurs followed by re-methylation (3–5). This epigenetic reprogramming is crucial for the differentiation of somatic cells.

Disruptions in epigenetic reprograming triggered by environmental factors have different consequences depending on whether blastocysts or germ cells are affected. When environmental exposures (such as endocrine disruptors) affect preimplantation embryos, important somatic phenotypic and epigenetic effects will be produced in the individuals emerging from these embryos (6,7). However, when the epigenetic reprogramming of

primordial germ cells is affected, the epigenetic disruption could affect individuals in the next generations (8–10). Although in the following generations epigenetic reprogramming will also occur, thereby erasing most of the epigenetic marks brought from previous generations, epigenetic marks will persist in genomic regions known as "escapees" (11). Such regions could be responsible for the transgenerational transmission of phenotypic effects induced by environmental insults.

6.3 INTRINSIC FACTORS INFLUENCING EPIGENETIC PROCESSES AND REGULATING REPRODUCTIVE HEALTH

Many endogenous factors shown to alter epigenetic mechanisms relate to the endocrine system, which have distinct roles at different ontogenetic stages. Due to this, we have separated the description of the endogenous factors on epigenetic systems according to different developmental and reproductive stages: Pre- and early postnatal development, puberty, and adulthood.

Pre- and Early Postnatal Development

The development of fetal germ cells is mediated by the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the fetal gonadotrope-precursor cells (GnPCs) located at the developing anterior pituitary. Studies in mouse models have shown that germ cell proliferation and differentiation is similar in both sexes until 10.5 days post coitum (dpc). Thereafter, germ cell development starts to become sex-specific (12).

Embryonic LH production appears to be under intricate epigenetic control. It has recently been shown that expression of the *Lh* gene in mice is orchestrated by the active DNA demethylation involving Tet1 and Tet2 enzymes (13). Interestingly, mice lacking Tet1/2 are viable but show abnormal ovarian development and reduced fertility (14) pointing toward an important role of Tet enzymes in ovarian development. In addition, the regulation of Tet1 expression and activity in differentiating GnPCs is regulated by liganded estrogen and androgen receptors, and the gonadotropin-releasing hormone (GnRH) through activation of protein kinase A (PKA) (13), suggesting that proper hormonal signaling is decisive in the epigenetic control of ovarian development.

A key factor responsible for the initiation of male sexual differentiation is the Y-chromosome encoded transcription factor SRY (sex-determining on the Y chromosome), which controls the expression of different genes involved in male gonadal development and thus the increase in perinatal testosterone levels (15,16). In the developing mouse testes, DNA demethylation of a regulatory region of the *Sry* gene occurs at 11.5 dpc and correlates with increased expression of *Sry* (17). This demethylation is testis specific and is believed to be mediated by GADD45 (growth arrest DNA damage-inducible 45) proteins that recruit DNA

repair proteins to replace methylated cytosines by unmethylated ones (15). Interestingly, GADD45 proteins are also known to be involved in stress response (18), which could imply that environmental stress can affect early gonadal development and testosterone production. Increased gonadal sex hormones levels are needed for developmental reprogramming of the hypothalamus in a sexual dimorphic manner, for the development of reproductive organs, and for imprinting of sexual dimorphic behavior. In the prenatal brain of male rodents, it has been shown that aromatase (*Cyp19a1*) readily converts (aromatizes) testosterone into estrogen (E2) that is responsible for imprinting male-typical behavior (19,20). During this period, increase in the transcription-activating mark histone 3 (H3) acetylation is found at the *Cyp19a1* gene in males, coinciding with their testosterone surge (21).

Another critical window for hormonal influence is the neonatal period when sex-differentiated development of the hypothalamus occurs. It has been shown that variations in transient hormonal surges associated with maternal care given with a preference to male over female neonatal pups has lasting effects on DNA methylation of the estrogen receptor alpha (ERα) promoter in the hypothalamus and thus on adult sexual behavior (22,23).

Puberty

The initiation of mammalian puberty is orchestrated by a myriad of complex interactions involving different cell types and organs that activate large and interconnected gene networks. Although the molecular mechanisms are still largely obscure, from a neuronal perspective it is known that puberty is triggered by trans-synaptic (24,25) and glial (26) interactions with hypothalamic neurons that release GnRH. Kisseptins are neuropeptides that have a major role in GnRH release. Kisspeptins are transcribed from the *KISS1* gene and bind to the kisseptin receptor (*GPR54/KISS1R*) on GnRH neurons of the hypothalamus (24,27,28). GnRH, in turn, triggers the release of LH and FSH from the anterior pituitary, leading to downstream effects in hormonal levels related to pubertal progression, development of secondary sexual characteristics, and ovulation in females. De-methylation of a regulatory region of the *GNRH* gene seems to be one of the mechanisms of regulation of GnRH release during puberty (29).

Both *KISS1* and *GNRH* are suggested to be under hormonally dependent epigenetic control. For example, peripubertal increases in gonadal estrogen (E2) levels (in female rodents) promote the binding of the estrogen receptor alpha (ERα) to the *Kiss1* promoter (30,31). This binding, which appears to be mediated by reduced H3 acetylation, promotes a peripubertal GnRH surge (30,31). Interestingly, as mentioned above, *ERα* is itself under epigenetic control, with both DNA methylation (32) and histone deacetylase (HDAC) modifications in the promoter (21) being fundamental in regulating *ERα* expression and sexual development.

Adulthood

Increasing evidence points toward nuclear receptor (NR) transcription factors, including the sex hormone receptors, as having an important role in epigenetic regulation of gene expression. NRs can recruit both chromatin-remodeling co-activators with histone acetylase (HAT) and deacetylase (HDAC) activities (33,34) and direct *de novo* DNA methylation and de-methylation to regulatory regions (34,35). The mechanisms underlying NR-induced DNA de-methylation is still unclear, however, recent reports show evidence that at least ERα (36), ERβ (37), retinoic acid receptor alpha (RARα) (38), and androgen receptor (AR) (39) can direct DNA de-methylation to specific genomic loci by interacting with thymine DNA glycosylase (TDG). TDG belongs to the base excision repair machinery and is part of the final step in DNA de-methylation by replacing deaminated methylcytosines to unmethylated cytosines. In the case of ERα, the TDG-ERα interaction is dependent on E2 activation (36), however, for ERβ this does not seem to be the case (37). Instead, it can be speculated that antagonistic ligands may be more important in modulating the interaction between ERβ and TDG. In view of the essential roles that sex hormones play in sexual differentiation and reproduction, deregulations in sex hormone signaling may directly impose lasting effects on the epigenome, not only in the affected individual, but also in the offspring.

In females, the dynamic changes in sex-hormone levels during the menstrual cycle, for example fluctuating E2 levels, appear to occur through the involvement of hormonal action on epigenetic mechanisms. These dynamic hormonal changes are regulated by the equally dynamic expression of *Cyp19a1* and the steroidogenic acute regulatory protein (*Star*), which is also involved in progesterone synthesis (40). Interestingly, the action of both enzymes, which are constantly unmethylated, are under the control of LH surges that trigger highly dynamic histone marks within their promoter regions (41–43). While *Cyp19a1* is rapidly suppressed after the LH surge, *Star* is rapidly upregulated in relation to luteinization following ovulation.

In adult males, spermatogenesis is dependent on high levels of free testosterone and is sensitive to drops in these levels (44). Additionally, spermatogenesis relies heavily on proper DNA methylation and chromatin remodeling of regulatory elements of testis genes (45,46,47). Interestingly, testosterone and FSH differentially affect sperm chromatin remodeling through epigenetic mechanisms and transcription factors that ultimately hamper the replacement of histones with protamines (48). Testosterone deficits interfere with the expression of proteins involved in the biogenesis of small non-coding RNAs, the levels of histone deacetylases (HDAC1 and 6), and generate modifications in histones such as h2b and the testis specific th3 (48). FSH deficits, in turn, affect the turnover of ubiquitylated histones and inhibit DNA repair mechanisms, leading to sperm DNA damage (48).

6.4 ENVIRONMENTAL EPIGENETICS: THE STUDY OF HOW EXTRINSIC FACTORS INFLUENCE EPIGENETIC MARKS

The terms "Environmental Epigenetics" and "Environmental Epigenomics" were first mentioned in the 2007 review article "Environmental Epigenomics and Disease Susceptibility" by Jirtle and Skinner (49). At the time, this review summarized many of the important studies that led to the conceptual definition of the terms. The trend of correlating environmental exposures and epigenetic changes, however, started much earlier, and it could be consider that the seminal paper was the 1998 review "Epigenetics and Epimutagens: Some New Perspectives on Cancer, Germline Effects and Endocrine Disrupters" by MacPhee (50). In this paper, MacPhee argues that previously published estrogen-dependent effects on the expression and DNA methylation of the vitellogenin promoter in laying hens (51) could be mimicked by the action of endocrine disruptors (EDCs), that is, compounds that alter the function of the endocrine system in organisms. In a visionary fashion, MacPhee stated: "*Other epigenetic changes (inappropriate methylation, generalised hypomethylation) associated with exposure to environmental agents may also be recognised more readily in the future.*" Three years later, John McLachlan also suggested that estrogens or endocrine disrupting chemicals could play a role in the programming or imprinting of genes through persistent changes in DNA methylation (52).

In the following years, endocrine disruptors started to become the main environmental influence known to affect epigenetic changes, and have since been one of the strongest drivers of the field of "Environmental Epigenetics." Meanwhile, other environmental factors started to be studied and gained importance in relation to epigenetic effects. These include pharmacological compounds known as demethylating agents, nutritional compounds that provide the substrate needed (methyl groups) for DNA methylation reactions, or inorganic chemicals.

Nowadays most scientists in related disciplines would agree that environmental factors are able to influence the establishment of epigenetic mechanisms, and that this process can occur through many different biological pathways. Here we describe four groups of environmental compounds for which there is abundant evidence of related epigenetic effects: endocrine disruptors; nutritional factors; pharmacological compounds; inorganic compounds.

Endocrine Disruptors and Epigenetic Changes

We have recently extensively reviewed the literature related to the connection between EDCs and epigenetic changes (53). This connection, as previously mentioned, has been a driving force for the field of environmental epigenetics, especially regarding transgenerational effects. Nowadays, the literature reporting actions of EDCs on epigenetic mechanisms is extensive. For example, EDCs are shown to regulate numerous endocrine

related genes through DNA methylation, which includes well-known receptors such as to estrogen, progesterone, glucocorticoids, mineralocorticoids, retinoic acid, oxytocin, follicle-stimulating hormone, thyroid-stimulating hormone, and the insulin-like growth factor (53). EDCs reported to induce epigenetic effects include DES, BPA, Benzo[a]pyrene, Vinclozolin, n-butylparaben, DEHP, PCBs, and TCDD, among others (53).

Although the exact mechanism by which EDC promote epigenetic changes is still not fully elucidated, recent research has given hints on potential mechanisms. Because EDCs mimic the action of endogenous hormones, they can, in theory, interfere with endocrine response both at the physiological and molecular levels. Such interference is reported to have reproductive effects. Well-known examples of detrimental reproductive effects are those produced by exposures to DES (54), BPA (55), and vinclozolin (56). Once EDCs bind to cytosolic receptors that belong to the nuclear hormone receptors (NHRs) superfamily, either they can trigger responses through the classical genomic pathway or through the non-genomic pathway (57). The genomic pathway involves nuclear translocation and the further binding of the ligand-activated hormone receptors to hormone-responsive elements in the genome, while the non-genomic pathway involves the rapid and transient induction of membrane-initiated signaling pathways that activate kinase cascades (57). EDCs appear to act on hormone receptors through both pathways (57). EDCs can also mimic hormonal action that takes place directly on genomic regions known as "response elements" such as the estrogen response element (ERE) (58,59). For example, Bhan et al. (60) have shown that the binding of EDCs to an ERE within the promoter of a non-coding RNA (HOTAIR) enables the binding of histones methylases that will modify the chromatin and activate gene expression (60).

Nutritional Factors and Epigenetic Changes

Nutrition is a critical environmental component influencing the epigenome. This is particularly important for DNA methylation, which requires the presence of methyl-group substrates, commonly derived from the diet. Dietary sources of methyl groups include folic acid, betaine, zinc, and vitamin B_{12}, which ultimately participate in the metabolism of methionine and S-adenosil methionine (SAM) (61). SAM is formed from methyl groups derived from choline, methionine or methyl-tetrahydrofolate, and is the primary methyl donor for the various methyltransferase enzymes in organisms (62). The amount of folates in the diet can directly influence their levels in the blood (63).

Possibly the best known animal model for studying the effects of dietary methyl donors on DNA methylation is the agouti mouse. Using this model, changes in methylation in the Avy allele can be easily detected through changes in the coat color. Specifically, the level of DNA methylation in an intracisternal A particle (IAP) retro-transposon located upstream of the Avy allele correlates with coat color shifts from yellow-agouti to yellow (64). Changes

in maternal consumption of methyl groups lead to coat color variations in the offspring, which correlates with the methylation status of the Avy allele (64,65). Other experiments have taken advantage of other properties of the Avy allele, such as its association with obesity (66,67).

Another model exploiting phenotypic traits to reflect DNA methylation in IAP elements uses IAPs located upstream of the promoter of Axin fused. In this case, high Axin fused DNA methylation in the tail is associated with a straight tail phenotype, while low levels correlate with a kinky tail phenotype (68). Both tail axin fused DNA methylation and kinky/straight phenotype correlate with the pre- and post-natal availability of methyl groups.

Nutritional factors also influence the expression of Dnmts. In humans, increased *DNMT1* (the maintenance Dnmt) expression in observed in cervical intraepithelial neoplasia samples after mandatory fortification of grain products with folic acid in the United States (69). Concordantly, Dnmt1 has been shown to be reduced in the liver of rat offspring born to protein-restricted mothers (70). In addition to DNA methylation, dietary compounds have also been implicated in the modulation of other epigenetic systems, such as histone modifications (71) and non-coding RNAs (72).

In addition to folate groups, dietary flavonoids have also been associated with epigenetic changes. Flavonoids (or isoflavones) is a class of plant compounds that elicit estrogenic actions in animals (73), and hence are also called phytoestrogens. Dietary intake of phytoestrogens is known to produce reproductive effects in mammals (74–77) including humans (78), where isoflavones are reported to be transferred from mother to child through breastfeeding (79).

Initial experiments showed that administration of the phytoestrogens coumestrol and equol to newborn mice inactivated the proto-oncogene H-ras through increased DNA methylation (80). Later, it has been shown that consumption of high doses of the phytoestrogen genistein by 8-week-old mice induces altered DNA methylation patterns (81), while neonatal exposure of females to high genistein levels results in tissue-specific hypermethylation in the gene Nsbp1 (nucleosomal binding protein) in the uterus (82). Also in mice, gender-specific changes in DNA methylation of the Acta1 promoter in the liver are observed in response to a diet rich in the phytoestrogens genistein and daidzein (83). The Agouti mouse model has also been used to evidence the epigenetic effects of phytoestrogens. With this model, hypomethylation of the Avy allele induced by maternal exposure to BPA was shown to be inhibited by maternal dietary supplementation with either methyl-donors or genistein (84). Table 6.1 summarizes studies that investigate epigenetic effects induced via nutrition.

The epigenomic effects of dietary phytoestrogens are not limited to DNA methylation. In prostate cancer, genistein has a protective effect that takes place through the activation of tumor suppressor genes by histone modifications and chromatin remodeling (85). In breast cancer cells, genistein, in addition to reducing the expression of Dnmts, inhibits the

TABLE 6.1 Nutritional Factors and Epigenetic Changes

Nutritional Factor	Experimental Model	Effect	References
Methyl supplemented maternal diet	Mouse (agouti)	Increased longevity and DNA methylation in LTR repeats in liver and kidney	[64]
Methyl supplemented maternal diet	Mouse (agouti)	Altered coat color in mice exposed as embryos and their offspring. Coat color is dependent on methylation levels at the Avy allele	[65]
Maternal dietary genistein	Mouse (agouti)	Altered coat color in mice exposed as embryos and DNA methylation in LTR repeats in many tissues	[66]
Methyl supplemented maternal diet	Mouse (axin fu)	Increased DNA methylation in the Axin-fused allele, and reduction in the incidence of kinky tail phenotype	[68]
Folic acid supplementation	Human samples of cervical intraepithelial neoplasia	Increased DNMT1 expression	[69]
Dietary genistein	Mouse (C57BL/6J)	DNA methylation differences in a novel gene in prostate	[81]
Dietary genistein and daidzein	Mouse (C3H)	Suppression of gender-specific differences in body weight and in promoter DNA methylation in Acta1 (liver); advanced sexual maturation in females	[83]
Maternal diet with methylsupplementation and genistein	Mouse (agouti)	Neutralization of BPA-induced hypermethylation in Avy alleles	[84]

hTERT (human telomerase reverse transcriptase) gene by promoting hypomethylation in E2F-1 sites (thereby increased E2F-1 binding) and altering methylation in H3K9 and H3K4 histones in its promoter (86).

Pharmacological Compounds and Epigenetic Changes

The first pharmacological agent used to deliberately alter the epigenome was the demethylating agent 5-AzaC. 5-AzaC was initially tested as a treatment against leukemia in mice (87) and is currently approved by the FDA (since 2004) for the chemotherapeutical treatment of the myelodysplastic syndrome (88). In addition to 5-AzaC, there are currently a number of other epigenetic drugs approved for clinical use by the FDA (89): Decitabine (5-aza-2′-deoxycytidine) is also a hypomethylating agent with similar therapeutic applications as 5-AzaC for the treatment of myelodysplastic syndrome; Tranylcypromine and phenelzine are lysine demethylase inhibitors initially approved as anti-depressants, but currently also tested for cancer treatment; Trichostatin-A, Vorinostat, Panobinostat, and Belinostat are HDAC inhibitors (of the hydroxamic acids group) employed in the treatment

TABLE 6.2 Current Approval Status of Pharmacological Agents

Drug (s)	Epigenetic Mechanism	Uses	Approval Status
5-AzaC	DNA methylation	Treatment of myelodysplastic syndrome	FDA approved
Tranylcypromine and phenelzine	Lysine demethylase inhibitors	Anti-depressants	FDA approved
		Cancer treatment	Being tested
Trichostatin-A, Vorinostat, Panobinostat, and Belinostat	Histone deacetylase inhibitors	Treatment of lymphoma and leukemia	FDA approved
Mocetinostat	Histone deacetylase inhibitor	Treatment of myelodysplastic syndrome	FDA approved
Romidepsin	Histone deacetylase inhibitor	Treatment of cutaneous T-cell lymphoma, after patients have had systemic therapy	FDA approved
Miravirsen and RG-101	miRNAs	Treatment of hepatitis C	Clinical trials
MRX34	miRNAs	Treatment of cancer	Clinical trials

of lymphoma and leukemia; Mocetinostat is an HDAC inhibitor from the benzamides group also employed for the treatment of myelodysplastic syndrome; Romidepsin is an HDAC I and II inhibitor with cyclic tetrapeptide antibiotic and antineoplastic activity approved for the treatment of patients with cutaneous T-cell lymphoma, used after they have been administered with systemic therapy (89). In addition, three epigenetic drugs based on the action of miRNAs have entered clinical trials: Miravirsen and RG-101 for the treatment of hepatitis C, and MRX34 for the treatment of cancer (89). Table 6.2 summarizes the current status of pharmacological agents that alter the epigenome.

Inorganic Compounds and Epigenetic Changes

Special attention is currently given to the epigenetic effects of inorganic compounds due to increasing knowledge about the consequences of exposure of human populations to heavy metals. One of the first inorganic elements that has been related with epigenetic effects is arsenic, due to its reported role in the metabolism of methyl groups (90). In mice in which hepatocellular carcinoma has been induced by exposure to arsenic in utero, altered estrogen signaling plays a role, in which arsenic induces overexpression of ERα and hypomethylation in regions of the ERα promoter in the liver (91). In mouse Leydig (MLTC-1) cells arsenic exposure induces upregulation of 3β-HSD (3β-hydroxysteroid dehydrogenase) through the suppression of histone H3K9 di- and tri-methylation (92). Another inorganic element of recent concern is cadmium due to its carcinogenic properties and adverse health effects in relation to smoking. In humans, high cadmium levels detected in urine samples (associated with smoking status) correlated with hypomethylation in MGMT gene independent of gender, hypomethylation in MT2A and DNMT3B in women, and LINE-1 hypermethylation

in men (93). In human bronchial epithelial cells that undergo cadmium-induced malignant transformation, Dnmts get progressively overexpressed, which increases global DNA methylation, while the expression of DNA repair genes is progressively reduced (94).

6.5 CONCLUDING REMARKS

Timely epigenetic events mediate proper development in organisms and contribute to the formation of healthy individuals. These events are highly plastic, allowing the organism to cope with variations in its surrounding environment. However, such plasticity also implies that developmental windows of increased epigenetic remodeling may be sensitive to the action of environmental exposures that will generate detrimental outcomes. Nutritional factors, EDCs (man-made or natural) or various pharmacological agents are currently known to act on epigenetic processes, thereby interfering with the epigenetic machineries. In parallel, it is important to consider the known detrimental effects EDCs exert on reproduction, such as those generated by exposure to BPA and phytoestrogens. Increasing evidence points toward endocrine signaling, particularly signaling involving sex hormones, as being very sensitive to epigenetic remodeling by extrinsic factors. This is of special concern since sex-hormone signaling is not only needed for the normal functioning of the adult organism but also for its reproductive ability. Future research on the interaction between intrinsic and extrinsic epigenetic regulation is therefore warranted.

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