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Epigenetic mechanisms in developmental programming of adult disease

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Abstract

Adverse insults during intrauterine life can result in permanent changes in the physiology and metabolism of the offspring, which in turn leads to an increased risk of disease in adulthood. This is an adaptational response by the fetus to changes in the environmental signals that it receives during early life to ensure its survival and prepare itself for postnatal life. Increasing evidence suggests that the epigenetic regulation of gene expression patterns has a crucial role in the developmental programming of adult disease. This review summarizes recent studies of epigenetic mechanisms and focuses particularly on studies that explore identifiable epigenetic biomarkers in the promoters of specific disease-associated genes. Such biomarkers would enable early recognition of children who might be at risk of developing adult disease with fetal origins.

Introduction

During the late 1980s, the ‘developmental origins of adult disease hypothesis’, often called the ‘Barker hypothesis’, was originally proposed by Barker and colleagues, based on a series of retrospective epidemiological studies, which described the potential relationship of fetal growth restriction and cardiovascular and/or metabolic disease in adult life [1,2]. Since then, substantial experimental evidence from different mammalian species has reliably supported the hypothesis that an adverse *in utero* environment has a programming role in postnatal physiology and pathophysiology. It has been demonstrated that prenatal undernutrition [3] and hypoxia [4,5], as well as other intrauterine insults, such as exposure to toxins [6,7], result in an increased risk of developing cardiovascular and metabolic disorders in later life, including hyperphagia, obesity, endocrine and metabolic abnormalities, type 2 diabetes mellitus, insulin resistance, hypertension and ischemic heart disease.

To explain how intrauterine malnutrition influences fetal development and the susceptibility of offspring to adult disease, the thrifty phenotype hypothesis was proposed [8,9]. When there is a change in the intrauterine environment, the fetus makes adaptations to ensure its survival. Once the adverse environment persists beyond the point of reversible adaptation, the fetus is forced to make an irreversible adaptation that will result in persistent alterations to physiological and metabolic homeostatic set points. After birth, if the adjusted set point does not match the postnatal environment, adult diseases will be induced.

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Although the Barker and thrifty phenotype hypotheses provide conceptual thoughts about the developmental programming of adult disease, it is still unclear how fetal developmental plasticity enables organisms to make adaptational responses to the fetal environment that can result in permanent adverse effects later in life. Recent studies have strongly suggested that epigenetic processes of gene expression patterns are influenced by the environment, and might have key roles in the developmental programming of adult disease [10]. In this review, we summarize current studies of the epigenetic mechanisms of developmental programming of adult disease and provide an explicit systematic blueprint of this field (Figure 1).

Epigenetic mechanisms of developmental programming of adult disease

Links between epigenetic process and developmental programming

Epigenetic modification relates to the stable and heritable patterns of gene expression that do not involve changes in DNA sequence [11]. Unlike genetic information, which is extremely stable, epigenetic modifications mark the effects of early environmental events and ensure sustained and reversible responses to transient stimuli, which result in modified gene expression patterns and phenotypes later in life. Several pieces of evidence support the hypothesis that epigenetic modifications are sensitive to environmental stimuli. First, monozygotic twins have an identical genotype but their phenotype is not usually identical. This manifests as discordances in the frequency or onset of disease. This could be explained by the accumulation of epigenetic changes that occur over their lifetimes; for example, in older twins, the overall content and distribution of methylated cytosines and histone acetylation are remarkably different, even though they were epigenetically indistinguishable at a younger age [12]. Studies of monozygotic twins have also revealed an association between epigenetic differences and discordance in diseases, such as bipolar disorder, Silver–Russell syndrome, Beckwith–Wiedemann syndrome and transient neonatal diabetes mellitus [13–16]. Second, studies in assisted reproduction technology (ART) directly demonstrate that the *in vitro* culture environment affects global patterns of DNA methylation, which can lead to the long-term alteration of the expression of genes involved in chronic metabolic disorders, such as obesity and type 2 diabetes mellitus [17]. Third, studies of metastable epialleles (i.e. alleles that are variably expressed in genetically identical individuals owing to epigenetic modifications established during early development; they are thought to be particularly vulnerable to environmental influences [18]) in mouse strains show the environmental effect on the epigenetic regulation of gene expression and function, especially the epigenetic effect of the *in vitro* environment on the later health of offspring. In the agouti viable yellow (avY) mouse, hypomethylation of the agouti gene promoter increased its gene expression and resulted in a yellow coat color as well as obesity (the agouti gene regulates body weight at the level of the hypothalamus). Conversely, hypermethylation decreased the expression of agouti and resulted in mice with a brown coat color and normal weight. In the same strain of mice, if the pregnant dam was fed food containing restricted methyl donors and cofactors (such as folate), the results included agouti promoter hypomethylation, increased prevalence of yellow coat color, obesity and even cancer in her offspring [19]. By contrast, feeding the dam soy isoflavone genistein led to promoter hypermethylation, decreased the prevalence of obesity and gave her offspring a brown coat [20].

These findings illustrate the fact that environmental signals early during the developmental period might produce an inherited epigenetic modification with potential long-term consequences. Epigenetic aberrations serve as a memory of exposure to inadequate or inappropriate chemical and/or nutritional or nonchemical environments during early life. Thus, identifying the specific features and functions of the epigenetic build-up at these stages and determining the mechanisms by which environmental factors might affect them

in the long term will be a major milestone in the investigation of the intrauterine origin of adult disease, and could also reveal potential intervention points and means.

DNA methylation and its reprogramming process during fetal development

Epigenetic modifications to chromatin include 5' methylation of the cytosine residue in CpG dinucleotides of DNA, covalent modifications (including methylation, acetylation, phosphorylation and ubiquitination) of histones and the gene-regulating and chromatin-organizing activities of noncoding RNAs. These epigenetic modifications change the binding of transcription activators and repressors to specific gene promoters, and/or alter the large-scale conformation and function of chromatin itself, which modulates gene expression [21].

Methylation appears to be the most dominant and best-studied epigenetic modification of DNA. DNA methylation in mammalian and other vertebrates generally occurs on position 5 of the pyrimidine ring of cytosine nucleotides in the symmetrical dinucleotide CpG sequence [22]. The overall frequency of CpG dinucleotides in the vertebrate genome is approximately 20% and the clustered CpG-rich areas (so-called CpG islands) usually occur in the 5' end and the promoter regions of genes [23]. Approximately half of all transcribed genes have CpG islands, which includes all genes that are widely expressed; approximately 40% of genes are expressed in a tissue-specific manner. Of CpGs located outside of CpG islands, 80% are methylated, but CpG islands found in the promoter regions of active genes are normally not methylated [23]. DNA methylation of CpG islands is usually associated with transcriptional silencing of the associated gene [24]. In addition, DNA methylation in some non-CpG island CpG dinucleotides sometimes also has an important role in regulating the expression of certain genes, depending on the regulating role of the epigenetically modified loci in the promoter. Whereas DNA methylation that occurs in the promoter regions generally leads to gene repression by inhibiting transcription factor binding either directly or indirectly via the recruitment of methyl-CpG-binding domains (MBDs), the increased methylation in the binding sites of gene repressors results in an increase in gene activity. Additionally, DNA methylation-mediated downregulation of the repressors themselves might lead to an increase in the activities of the target genes of those repressors. Furthermore, DNA methylation can result in increased gene activities through its regulation of miRNA expression.

DNA methylation is a reversible and dynamic process [26], catalyzed by DNA methyltransferases (DNMTs). The major *de novo* methyltransferases, DNMT3a and DNMT3b, are principally responsible for establishing cytosine methylation at previously unmethylated CpG sites [26], particularly during embryogenesis. Genome-wide alterations in methylation by mutations in DNMTs or knockouts of the methylase genes cause a significant reduction in the level of methylated cytosines, abnormal development and embryonic lethality [27]. Other than DNMT3a and DNMT3b, DNMT1 recognizes methylation associated with the parental strand and imposes this pattern on the newly formed daughter strand of DNA; thus, methylation patterns imposed on the genome at precursor cells could be maintained by DNMT1, which is referred to as the 'symmetrical methylation rule' [28]. Reduction of DNMT1 expression can result in genome-wide hypomethylation in all tissues and leads to tumor development in later life [29].

During the process of DNA methylation, methylated-CpG dinucleotides are recognized by a family of MBD proteins, including MBD1–4 and methyl-CpG-binding protein 2 (MeCP2). MeCP2 binds to single methylated-CpG dinucleotides and causes chromatin remodeling and gene silencing by recruiting chromatin-remodeling corepressor complexes to the regions of DNA that bind MeCP2 [30]. Inactivating mutations in either DNMT3b or MeCP2 are

associated with immune deficiency, centromeric instability and facial anomalies (ICF) syndrome and Rett syndrome [31–34].

Given that the epigenetic information can be modified and faithfully transmitted during cell development and differentiation, questions arise as to how and when the epigenetic regulation of gene expression patterns serve as a link between prenatal environmental cues, gene expression and later health. The fact that DNA methylation patterns experience a reprogramming period during gametogenesis and early embryo genesis provides a crucial window for the epigenetic-mediated developmental programming of adult disease [35]. The first phase of methylation reprogramming occurs immediately after fertilization, when the entire paternal genome (except for paternally imprinted genes, heterochromatin around centromeres and some repetitive elements) is rapidly demethylated [36]. This naturally demethylation process then slows down generally and keeps until the morula stage [36]. Simultaneously, the maternal genome undergoes a relatively slow demethylation compared with the paternal genome [37]. After five cell cycles at the first differentiate event, *de novo* methylation begins. After this point, the methylation level is higher in the inner cell mass that gives rise to somatic tissues than in the trophoctoderm, which forms the future placenta [37]. At a later stage of development, the asymmetry pattern of DNA methylation in somatic tissues is maintained and there are tissue-specific patterns of both DNA methylation [38] and histone modification [39,40]. After *de novo* remethylation, the primordial germ cells migrate through the allantois to the developing germinal ridges, where they eventually differentiate into mature gametes, thus completing this cycle of epigenetic reprogramming. During gametogenesis, primordial germ cells undergo another DNA methylation reprogramming process. Before their migration to the genital ridge, new patterns of methylation are established (before birth in the male germ line and after birth in the female line). The early primordial germ cells then erase their somatic-like epigenetic patterns [41]. This demethylation process completes around day 12–13 after fertilization. Once this occurs, the remethylation starts again and shows a sex-specific timing. In the male, *de novo* methylation begins at day 15.5–18.5 of gestation, continues postnatally and is completed in pachytene spermatocytes [42]. Little is known about overall DNA remethylation during oogenesis, and most studies performed to date have concentrated on imprinted genes. In females, imprints are acquired at different times before ovulation in maturing oocytes [43]. A growing body of evidence suggests that epigenetic gene regulation is influenced by the environment and is one of the molecular mechanisms with an important role in the foundation of developmental programming [10]. The differential timing of remethylation during gametogenesis might have implications for studies of sex-dependant changes caused by prenatal insults, which we review below.

Early embryogenesis is therefore a crucial period for the establishment of epigenotypes. As key nutrients are needed for the methylation process, dietary excess or deficiencies might alter the epigenetic patterns, as might as other environmental factors, such as glucocorticoid (GC) exposure, maternal cigarette smoking, maternal hypoxia or maternal drug abuse. These environmentally induced epigenetic alterations can be maintained throughout a lifetime, influencing gene expression patterns and causing changes in phenotypic traits [44].

Other epigenetic modification mechanisms and their crosstalk with DNA methylation

In eukaryotic cells, the basic unit of chromatin is the nucleosome, which comprises 146 bp of DNA wrapped around eight histone proteins; in turn, each histone protein comprises two copies each of histone (H)2A, H2B, H3 and H4 [45]. Modulation of the structure of chromatin is crucial for the regulation of gene expression because it determines the accessibility and the sequential recruitment of regulatory factors to the underlying DNA. Histones undergo a variety of reversible post-translational modifications, including acetylation and methylation of conserved lysine and arginine residues on the amino terminal

tail domain, phosphorylation of serines and threonines, ubiquitinylation and ADP ribosylation [46]. The most common histone modifications are acetylation and methylation of lysine residues in the amino termini of H3 and H4. Histone acetylation, for instance, is generally associated with euchromatin, a relaxed, higher order chromatin structure, and this is thought to permit or facilitate the access of gene regulatory proteins, which include transcription and elongation factors. By contrast, hypoacetylated histone is associated with heterochromatin, a highly condensed inactive chromatin structure [46]. Histone methylation can be a marker for both active and inactive regions of chromatin. Methylation of lysine 9 on the N terminus of histone H3 (H3K9) is a hallmark of silent DNA and is globally distributed throughout heterochromatic regions, such as centromeres and telomeres. By contrast, methylation of lysine 4 of histone H3 (H3K4) denotes activity and is found predominantly at promoters of active genes [47]. Because lysine methylation can be monomeric, dimeric or trimeric, and histones might also be subject to other post-translational modifications, such as phosphorylation [48], this enormous variation leads to many possible combinations of different modifications. This might constitute a 'histone code' [49], which can be read and interpreted by different cellular factors.

miRNAs, a class of small noncoding RNAs, are also important players in the epigenetic control of gene expression [50]. miRNA genes are transcribed into primary miRNAs and then processed to precursor miRNAs. An endoribonuclease dicer cleaves precursor miRNAs into the final short fragments called matured miRNAs. miRNAs bind to their target mRNAs and lead to degradation of the target or translational suppression of the target genes [51].

The interactions of DNA methylation, histone modifications and miRNA might serve as a self-reinforcing network to regulate gene expression patterns in a sophisticated feedback manner (Figure 2). First, DNA methylation-induced chromatin silencing might be facilitated by the coupling action of covalent modifications of histone and *vice versa* [52]. For example, H3K9 methylation is a prerequisite for DNA methylation [53], and DNA methylation might also trigger H3K9 methylation [54]. Interactions among histone deacetylases (HDACs), histone methyltransferases and methylcytosine-binding proteins lead to the recruitment of DNMTs [55], although it is not yet clear what initiates the recruitment of the different epigenetic modifiers to their specific target sequences. The relation and consequences of DNA methylation and histone modifications might be more complicated than is currently thought. Individual histone modification, DNA methylation or their interactions might mediate bidirectional effects on gene transcriptional outcomes and the epigenetic marks might not be uniformly associated with 'on' or 'off' transcriptional states but instead with the potential for phenotypic plasticity [56]. Furthermore, the true causal effects of epigenetic modifications in the developmental programming of phenotypic and functional changes remain to be determined [57]. Second, miRNAs have been shown to provide post-transcriptional regulation of chromatin via targeting the enzymes involved, including histone methyltransferases, MeCPs, chromo-domain-containing proteins, HDACs and DNMT enzymes [51,58–60]. Furthermore, the miRNA-initiated transcriptional gene silencing complex can guide the histone methyltransferase to specific chromatic regions and form silenced heterochromatin [61]. Third, miRNA genes are also targeted by epigenetic modification. For example, an aberrant pattern of methylation of CpG islands near or within miRNA genes could result in misregulated expression of key miRNAs and ultimately in pathogenic alterations, including tumorigenesis [62]. Lastly, primary miRNAs are either transcribed by RNA polymerase (Pol) II as independent transcriptional units or coexpressed from the intronic regions of host gene transcripts that might serve as a feedback regulation loop of miRNA regulating the expression of host genes as well as its own.

Techniques for detecting epigenetic mechanisms

The most commonly used techniques for detecting DNA methylation are bisulfite genomic sequencing [63] and methylation-specific PCR [64]. Bisulfite genomic sequencing is a useful approach for a comprehensive investigation of the methylation status of CpG islands in multiple clones, whereas methylation-specific PCR is used for studying CpG sites in non-CpG island, sequence-specific transcription factor binding sites. HPLC is another reliable and reproducible method for measuring DNA methylation levels in tissues. This method has been recently improved by reducing the starting DNA requirement from 50 mg to 3 mg, which enables the method to be used with low DNA-yield samples [65]. Additionally, DNA methylation microarrays have been used to examine extensive DNA methylation in large segments of DNA [66]. Other methods used to measure DNA methylation include DNA fingerprinting [67], methylation-specific quantum dot fluorescence resonance energy transfer (MS-qFRET) [68], high performance capillary electrophoresis (HPCE) [69] and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry [70]. For detecting histone modifications, chromatin immunoprecipitation (ChIP) and ChIP on single nucleotide polymorphisms (SNP)-chip are widely used [71]. Similar to DNA methylation, techniques for measuring histone modifications are developing rapidly. New techniques, such as pathology tissue-ChIP (PAT-ChIP) [72] and the purification of histone ubiquitin ligases have been used recently [73]. Furthermore, a method using nanofluidics and multi-color fluorescence microscopy has been used to detect DNA and histone modifications simultaneously in individual chromatin fragments at approximately 10 Mbp/min [74]. This technique provides an unprecedented opportunity for genome-wide, simultaneous analyses of multiple epigenetic marks on single molecules using femtomole quantities of samples. Methods for detecting miRNA include computational approaches for miRNA target prediction and molecular biology-based analysis methods, such as cloning, northern blotting, RT-PCR and microarrays [75]. Currently, new techniques based on electronic and optical signal transduction appear to be promising candidates for use in emerging miRNA analysis applications [75].

Evidence of epigenetic mechanisms in developmental programming of adult disease

Epigenetic mechanisms in malnutrition models—It has been demonstrated that intrauterine growth restriction (IUGR) is accompanied by changes in the quantity and activity of enzymes responsible for modifying chromatin. In the IUGR rat model, global decreases in DNA methylation and increases in H3 acetylation on lysine 9 (K9) and K14 are observed in the brain at birth [76]. These changes are accompanied by a concomitant decrease in DNMT1, MeCP2 and HDAC1 [76]. Liver chromatin modifications are also induced by IUGR and present as persistent increases in acetylation of H3K9 and K14 [77], as well as reduced hepatic expression of DNMT1 [78].

Maternal diet during pregnancy can also elicit changes within the epigenome. Studies of offspring of low protein (LP)-fed dams have identified changes in DNA methylation of the genes encoding the GC receptor (GR) and the peroxisome proliferator-activated receptor- α (PPAR α), leading to the increased expression of their corresponding transcripts in the liver [79]. Bogdarina *et al.* used a LP diet rat model to study the intrauterine programming of hypertension. They demonstrated decreased methylation of the gene promoter for angiotensin receptor, type 1b (Agtr1b) [80]. Correlating with an increase in the expression of the Agtr1b mRNA transcript and receptor protein expression, these changes are thought to augment the regulation of blood pressure and contribute to the development of hypertension later in life. A more recent study in mice reported that a maternal LP diet can differentially methylate over 200 promoter regions within the fetal liver, including the liver-X-receptor α (*Lxra*) gene [81]. Follow-up studies in the offspring showed a reduction in the levels of hepatic DNA promoter methylation at the gene encoding the GR, a reduction in H3

dimethylation and increases in H3 acetylation at this locus, which were associated with increased transcription [78].

Maternal calorie restriction has been associated with hypermethylation of the oncogene H-Ras in adult offspring [82]. Changes in histone modifications of offspring have also been demonstrated in maternal calorie restriction models, including losses in acetylation and increases in dimethylation of H3 at the glucose transporter type 4 (GLUT4) locus. These changes persist into adulthood, and are thought to lead to the development of type 2 diabetes mellitus in the adult offspring [83]. Pancreas/duodenum homeobox protein 1 (Pdx-1) has been shown to be epigenetically regulated in fetuses from intrauterine arterial ligation dams with deacetylation of core histones and a reduction in upstream transcription factor-1 (USF-1) binding [84]. With aging, these offspring exhibited further changes in the histone marks and increased DNA methylation of the Pdx-1 locus, which were both associated with a progressive reduction in Pdx-1 expression [84]. Global changes in DNA methylation were observed in sheep offspring of dams fed with alterations in vitamin B and methionine during the periconceptional period [85]. In addition, supplementation of the diet of pregnant mice with methyl donors altered the methylation of genes implicated in allergic airway disease [86]. It has been shown that the effects of neonatal leptin on hepatic gene expression and epigenetic status in adult offspring are directionally dependent on the nutritional status of the animal *in utero* [87]. Additionally, studies in sheep demonstrated that periconceptional undernutrition resulted in epigenetic changes in the hypothalamic proopiomelanocortin and GR genes in the fetus [88].

Aaggard-Tillery *et al.* [89] used a nonhuman primate model to investigate the effects of maternal diet on alternations to the epigenome that might be related to obesity. The authors identified obesity and hyperacetylation of H3K14 in the liver of offspring that were fed with a high-fat diet (35% fat compared with a control diet of 13% fat) [89]. In a separate study, they found that decreasing the high fat diet from 35% to 32% during pregnancy maintained obesity in offspring but the epigenetic changes were lost [90]. Recently, it has been shown in rats that a chronic high-fat diet in fathers caused hypomethylation of the interleukin 13 receptor, alpha 2 (*Il13ra2*) gene and programming of β -cell dysfunction in F1 female offspring, demonstrating a concept of non-genetic, intergenerational transmission of metabolic sequelae of a high-fat diet from father to offspring [91].

Recent studies in humans have also shown long-term effects of maternal diet on the epigenome of the offspring. It has been demonstrated that individuals exposed to the Dutch famine of 1944 (the so-called 'hunger winter') have lower levels of methylation of the insulin-like growth factor 2 (*IGF2*) gene in adulthood [92]. Furthermore, Tobi *et al.* [93] identified an additional six loci that were differentially methylated after prenatal exposure to the famine; all six loci were implicated in either growth or metabolic and cardiovascular phenotypes.

Epigenetic mechanisms in other prenatal insult models—Hypoxia is a common intrauterine stress. A fetus might experience prolonged hypoxic stress under a variety of conditions, including pregnancy at high altitude, pregnancy with anemia, placental insufficiency, cord compression, preeclampsia, heart, lung and kidney disease, or hemoglobinopathy. Animal studies have suggested a possible link between fetal hypoxia and increased risk of cardiovascular disease in the resulting offspring [94–102]. Studies in rats have found that maternal hypoxia resulted in an increase in cardiac vulnerability to ischemia and reperfusion injury in male offspring [99,102,103]. In addition, it has been demonstrated that downregulation of protein kinase C epsilon [PKC ϵ (the gene is *PRKCE*)] protein expression in the hearts of adult offspring is a mechanism for increased heart susceptibility to ischemia and reperfusion injury in animals exposed to hypoxia before birth [104]. A

recent study showed that prenatal hypoxia caused repression of *PRKCE* in the heart via increased CpG methylation of specificity protein 1 (SP1) and early growth response protein 1 (Egr-1) binding sites at the *PRKCE* promoter [105]. This provides clear evidence of a novel mechanism of methylation in non-CpG island, sequence-specific transcription factor binding sites in the subtle epigenetic modifications of gene expression patterns in developmental programming of cardiac function in response to an adverse intrauterine environment. Although how hypoxia directly causes the increased methylation of the PKC ϵ promoter remains unclear, recent studies suggest a link between prolonged oxidative stress and aberrant DNA methylation patterns [106]. Given the fact that cardiomyocytes are major producers of reactive oxygen species owing to their high metabolic demand, it is plausible that increased oxidative stress is a mechanism in the hypoxia-induced methylation and repression of *PRKCE* in the heart.

Maternal cigarette smoking is another major health concern worldwide and has been associated with adverse pregnancy outcomes for the mother, the fetus and the newborn child. The adverse consequences have been well identified in epidemiological studies, including intrauterine growth restriction, sudden infant death syndrome and cardiovascular disease in offspring [107–109]. As one of the main toxic components in cigarette smoke, nicotine is a major contributor to the development of cardiovascular disorders. It has been demonstrated that antenatal nicotine exposure caused *PRKCE* repression in the developing heart through an increase in methylation of the Egr-1 binding site at the *PRKCE* promoter [110,111]. It appears that nicotine has no direct effect but instead stimulates the release of sympathetic neurotransmitter norepinephrine in the fetal heart, resulting in *PRKCE* repression. Interestingly, maternal hypoxia caused an increase in methylation at both the SP1 and Egr-1 binding sites in the fetal heart, yet the direct effect of hypoxia on cardiomyocytes resulted in increased methylation only at the SP1 binding sites [105]. These findings are intriguing and suggest a lack of a direct effect of hypoxia on methylation at the Egr-1 binding site. Given the fact that fetal hypoxia also increases sympathetic activity [112], it is possible that the increased Egr-1 methylation at the *PRKCE* promoter in fetal hearts seen in the rat model of maternal hypoxia was mediated in part by an increase in sympathetic neurotransmitter norepinephrine content in the heart in response to hypoxic stress. Although these studies demonstrate the different patterns of promoter methylation between maternal hypoxia and nicotine treatments, they also reveal a possible common mechanism of norepinephrine-mediated increase in methylation of the Egr-1 binding site at the *PRKCE* promoter in the developing heart.

Another well-known insult to the developing fetus is caused by maternal cocaine administration. Antenatal cocaine exposure causes the downregulation of PKC ϵ in the heart, resulting in heightened ischemic injury and reduced preconditioning-mediated protection in the heart [7,113]. Similar to the findings of maternal hypoxia and fetal nicotine exposure, it has been shown that cocaine increased CpG methylation of certain loci at the *PRKCE* promoter, leading to decreased transcription factor binding to the promoter and *PRKCE* repression in the heart [114,115].

Epigenetic mechanisms in the placenta—The placenta is the principal metabolic, respiratory, excretory and endocrine organ of the fetus; there is substantial molecular variation across its fetal and maternal compartments and it affects fetal growth [116]. There are approximately 80 known imprinted genes (approximately 200 predicted), a large percentage of which are expressed in trophoblasts and regulate placental growth [117]. In a study of IUGR and non-IUGR placentas, an altered expression pattern of imprinted genes, including increased pleckstrin homology-like domain, family A, member 2 (*PHLDA2*) and decreased mesoderm-specific transcript homolog protein (*MEST*), in placental response to maternal vascular underperfusion, was investigated [118]. The unbalanced expression of

these two oppositely imprinted genes is one component of the adaptive response of placental tissue to chronic maternal vascular underperfusion associated with IUGR. This provides support for the hypothesis that maternally expressed genes, such as *PHLDA2* and cyclin-dependent kinase inhibitor 1C (*Cdkn1c*) restrict fetal and placental growth to the energetic advantage of the mother, whereas paternally expressed genes, such as *MEST* and *IGF2*, augment fetal and placental development at the expense of maternal resources. In the study of IUGR, there was no evidence of altered DNA methylation in imprinting centers of *PHLDA2* and *MEST*, which led McNinn *et al.* [118] to conclude that ‘the high *PHLDA2*/*MEST* mRNA ratios in this subset of IUGR may reflect altered DNA methylation in as yet uncharacterized cis acting regulatory sequences, but more likely reflects conventional transcriptional dysregulation by transacting factors in placental cytotrophoblasts’. Although the mechanisms involved in acute regulation of placental 11 β -hydroxysteroid dehydrogenase-2 (11 β HSD2) are not well known, evidence suggests that epigenetic mechanisms modify expression of the gene encoding 11 β -HSD2 [119].

Sex differences in the developmental programming of adult disease

The sex differences in the developmental programming of adult disease have been well observed. Epidemiological studies have shown that the association between low birth weight (LBW) and chronic kidney disease is observed in men, but not in women [120,121]. Another similar inverse association is also observed between birth weight and blood pressure in both men and women [122]. Sex-specific differences in response to fetal insults are also seen in experimental studies, with a protective status observed in the females [123]. The male offspring of LP-fed dams underwent an age-dependent loss in glucose tolerance, showing impaired glucose tolerance by 15 months of age and type 2 diabetes mellitus and insulin resistance by 17 months of age [124]; by contrast, the female offspring developed hyperinsulinemia and impaired glucose tolerance at a much later age (21 months) [125]. In response to moderate protein restriction administered during gestation in the rat, a reduction in nephron number associated with hypertension was observed only in male offspring [126]; severe protein restriction was required to induce adverse programming effects in female offspring [127]. Similarly, vascular dysfunction was enhanced only in the male offspring of nutrient-restricted dams [128]. Hypertension programmed in response to placental insufficiency in the rat resulted in hypertension in adult males, but not in females [129]. In sheep, blood pressure was higher only in male offspring after fetal exposure to GCs [130]. Only male offspring exhibited serious cardiovascular dysfunction in response to fetal hypoxia, nicotine and cocaine exposure [7,103,131].

Therefore, sex differences in developmental programming are observed, with female offspring exhibiting a protected status regardless of the species or specific fetal insults. Although only a few studies have begun to elucidate the mechanisms leading to sex differences in the developmental programming of adult disease, the protective phenomena observed in females in response to prenatal insults are likely to involve multiple mechanisms at different developmental stages. The first is the crucial role of sex hormones developed postnatally, which are probably associated with sex-dependent changes. Testosterone has been demonstrated to be a key factor in hypertension programmed in adult male growth-restricted offspring [132]. By contrast, estradiol is implicated to have a protective role against hypertension in adult female growth-restricted offspring [129]. For example, in the offspring of rats, increases in blood pressure induced by a maternal LP diet were influenced by estrogen levels [133]. The mechanism by which sex hormones contribute to sex differences in developmental programming might also involve modulation of some crucial regulatory systems. For example, in the renin angiotensin system (RAS), the expression of angiotensin I converting enzyme 2 (*ACE2*) mRNA was enhanced in normotensive adult female growth-restricted offspring to protect them from hypertension. Ovariectomy

normalizes ACE2 mRNA expression to control levels and induces hypertension in female growth-restricted offspring [129]. Thus, modulation of RAS by estradiol might be one mechanism that contributes to sex differences in the developmental programming of hypertension.

Although it is plausible to suggest a primary role of sex hormones developed postnatally for the sex dichotomy seen in the developmental programming of adult disease, recent studies demonstrate that fetal insults cause sex-dependent changes in gene expression patterns in fetuses and neonates before sexual maturity [105,115]. This suggests that the sex difference in developmental programming occurs earlier during fetal development. Given the fact that male and female fetuses are probably exposed to similar concentrations of steroid hormones *in utero*, another mechanism is that the sex difference observed might be caused, in part, by the greater expression of estrogen receptor α (ER α) and β (ER β) in female fetuses. This is indeed the case in the heart [105]. The greater expression levels of both ER α and ER β in the heart of female fetuses protect the SP1 binding sites at the *PRKCE* promoter from being methylated by hypoxia and maintain the cardiovascular protective role of PKC ϵ [105]. Furthermore, it has been reported that ER α can cause DNA demethylation [26], which might contribute to the protection of promoter methylation in female fetuses. The sex-specific reprogramming of DNA methylation patterns during gametogenesis and early embryo genesis [35] might also contribute to the sex differences in the fetal epigenetic response to prenatal insults. Additionally, the gene expressions in the placenta are also sex specific. It is known that some genes expressed in the placenta are normally maternally silenced and/or paternally expressed genes that promote growth, whereas others are normally maternally expressed and/or paternally silenced that limit growth, which might also be a potential mechanism that contributes to the sex-dependent development of adult disease caused by prenatal insults. In human pregnancy, the placenta of a female fetus might impart a certain degree of protection from GC excess owing to increased GC inactivation compared with males [134]. Animal studies also showed that sex-specific placental function has a role in the development of anxiety-related behaviors in male offspring resulting from stress during early gestation [135].

Inheritance of developmental programming of adult disease

An increasing number of epidemiological studies indicate that the effects of intrauterine insults can be passed on to subsequent generations, without further exposure of the F1 generation. For example, birth size is reduced in the offspring of women who themselves had a LBW [136]. Similar findings were obtained in the study of the Dutch ‘hunger winter’, in which women who were severely undernourished during the first trimester of pregnancy gave birth to babies who were on average of normal birth weight, but who themselves went on to give birth to smaller babies in the next generation [137]. Owing to the limitations of clinical studies, the evidence for true transgenerational epigenetic inheritance in humans remains to be determined. Animal studies showed that it takes three generations for fetal growth and development to return to normal after 10–12 generations of a LP diet [138]. Offspring of females administered dexamethasone during the last week of pregnancy exhibited LBW and dysregulated glucose homeostasis in adulthood, in association with alterations in hepatic gluconeogenic enzymes [139]. When these F1 animals were mated, their offspring (F2) also had LBW, disrupted glucose homeostasis and elevated liver phosphoenolpyruvate carboxykinase (PEPCK) in the absence of any gestational manipulation [140]. However, the F3 generation is unaffected.

There are several possible explanations for the intergenerational effects induced by prenatal insults. First, the environment of the uterus of undernourished mothers might affect the developing reproductive tract of the fetus. Indeed, mothers who were small at birth have

reduced uterine and ovarian size [141]. It is proposed that smaller uterine size might impose a greater 'maternal constraint' on the fetus, thereby reducing its growth; although there is not yet any direct evidence for such an effect. Second, the transgenerational effect could be a result of alterations in primordial germ cell formation in the F1 generation. The prenatal insults are likely to affect both somatic and germ cell differentiation, leading to the exposed offspring developing the observed phenotype but also programming gametes and affecting the next generation of offspring. Third, changes in the epigenome during development might be passed on to subsequent generations [142]. For these effects to be inherited, such exposure must induce either stable chromosomal alterations or involve epigenetic modification that is maintained through germ cell maturation [143]. Evidence from rodent studies showed that nutritional and endocrinological interventions in pregnant animals (F0) resulted in phenotypic and/or epigenetic changes that persisted for at least two generations (F1 and F2) [140,144]. Altered methylation patterns of PPAR α induced by prenatal nutrition were found in juvenile rats (day 34) and persisted throughout their lifespan (day 80) [145]. Moreover, these methylation patterns of the hepatic PPAR α and GR were observed in the F1 and F2 generations [144]. In view of epigenetics as a possible mechanism of genetic imprinting of adult disease, future prenatal and gestational nutritional recommendations might need to be formulated on the basis of region, culture and risk assessment criteria.

The mechanisms by which epigenetic information (whether in terms of environmentally imposed methylation patterns or genomic imprinting) survives the reprogramming that occurs during embryo genesis and gametogenesis remain unresolved. A role for miRNA in spermatozoa has been proposed for the non-mendelian inheritance of one trait in the mouse, but the wider applicability of this mechanism remains uncertain [146]. Nonetheless, the transgenerational effects of prenatal insult are not passed on forever, as they are eventually diminished through several generations [138,140]. The involved mechanisms are not clear but might partially be to the result of the reversibility of epigenetic modification by the environment.

Reversibility of epigenetic changes in the developmental programming of adult disease and therapeutic opportunities

Although epigenetic DNA modifications are typically thought of as being stable once established, pharmacological manipulations of acetylation and methylation patterns have been shown in low- and high-lactation glooming (LG) offspring. Low GR expression in low-LG offspring can be reversed by the central infusion of a HDAC inhibitor, trichostatin A (TSA), which increases acetylation of H3, reduces methylation of the exon 17 region and increases NGFI-A (EGR-1) binding in the adult rat brain [147]. The higher levels of GR in high-LG offspring can be reduced by the administration of the methyl donor *S*-adenosyl-methionine (SAM), through increased DNA methylation of exon 17 [148]. The combined results of these studies imply that there is a degree of plasticity that remains in the adult for alterations in gene expression by epigenetic modification. Recent laboratory studies have explored the reversibility of induced phenotypic effects and whether aberrant phenotypes induced *in utero* or during early development can be rescued. Research exploring methods of restoring aberrant phenotypes has led to promising speculation that, ultimately, susceptible patients might be identified by means of screening for epigenetic markers during early life, and that customized interventions might then be instituted. Given that the epigenetic control of key genes is central to the developmental priming of the metabolic and cardiovascular diseases, epigenetics provides a probable target for pharmacological therapies. Several compounds have been identified that are able to regulate DNMT or HDAC activity. For example, the plant-derived isoflavone genistein can reactivate methylation-silenced genes. Similarly, administration of leptin to neonates of undernourished dams has been shown to reverse epigenetic changes and several otherwise

primed metabolic sequelae, including weight gain and hyperinsulinemia, and also increased total body adiposity [149]. As a methyl donor, folate supplementation of LP-fed dams prevented the elevation of blood pressure, impaired endothelium-dependent vasodilatation and reduced nitric oxide synthase mRNA levels [150]. Fish oil administered to LP-fed dams minimized the elevation of blood pressure and the reduced microcirculation in the myocardium in the offspring [151]. Omega-3 supplementation of pregnant dams that received dexamethasone prevented intrarenal ACE activation in the offspring [152]. Vitamin D supplementation in infancy is associated with a reduced incidence of preeclampsia [153]. Insufficient vitamin D intake is also incriminated in the short height and reduced pelvic diameter in mothers of LBW babies with increased risk of hypertension [154]. Corrective effects on phenotypic changes, gene expression and associated methylation changes in PPAR α have been reported after exogenous leptin administration to the neonatal offspring of undernourished rats [155]. Other studies suggest that hyperleptinemia and hypertension are reversed by dietary intervention with *n*-3 fatty acids and that altered behavioral responses can be reversed by pharmacological manipulation of epigenetic status [156].

Given that nutritional factors, including deficiency or supplementation of dietary methyl donors, can have a profound impact on pregnancy outcome and offspring health through epigenetic mechanisms, it is important to consider carefully dietary intake of supplements and methyl donors during pregnancy. Adequate intake of folates throughout the life span is associated with a lower risk of cardiovascular disease and certain cancers. However, the long-term effect of folic acid supplementation on susceptibility to chronic diseases has not yet been clearly defined. Therefore, despite the promising prospect of epigenetic therapy, there are still many concerns regarding the clinical approach in humans. First, animal studies inducing global demethylation have indicated that, although the risk of colorectal cancers is reduced, the risk of lymphoma and sarcoma is simultaneously increased [157]. This indicates the potential carcinogenicity and mutagenicity of current epigenetic therapies. In addition, HDAC inhibitors and DNA methylation inhibitors are cytotoxic agents and induce cell-cycle arrest and apoptosis by upregulating the cyclin-dependent kinase inhibitors p21 and/or p53 [158]. Given that current epigenetic therapy compounds produce nonspecific modification of genes and transposable elements, their adverse effects on other gene expression are a major concern. As we have reviewed here, epigenetic modulation of gene expression is a complicated network involving DNA methylation, histone modification and miRNA regulation, and each disorder-related abnormal epigenetic pattern has its own characterization; therefore, finding the most sensitive adjustable epigenetic target is a main challenge for researchers. Although more work is needed to characterize all of these effects and the mechanisms involved, current findings emphasize that epigenetic intervention at crucial periods during development is still an area of potential pharmacological therapeutic benefit.

Concluding remarks

In summary, prenatal insults, including malnutrition, hypoxia, GC exposure, smoking and drug abuse, can not only restrict the growth of fetus, but also cause serious impacts on their health in later postnatal life, such as increased risk of metabolic and cardiovascular disease. Furthermore, owing to the possibility of transgenerational inheritance of the prenatal insult-induced detrimental effects, more than one generation could be affected. Fortunately, the phenomenon of the developmental programming of adult disease has been demonstrated by epidemiological and experimental studies and has attracted extensive attention from sociologists, obstetrician-gynecologists, pediatricians and researchers. In addition to avoiding possible prenatal insults, a timely and valid therapy approach is also important for correcting the existing consequences induced by prenatal insults to improve the later health of offspring. The developing technology of epigenetic manipulation could help to screen for

epigenetic biomarkers during early life, and the reversibility of epigenetic action provides a promising window for designing therapy invention. Although current understanding of the epigenetic mechanisms in developmental programming of adult disease is in its infancy, it is one of the most promising research areas for improving reproductive health.

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Biographies

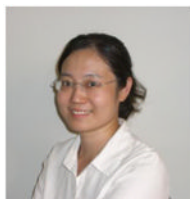
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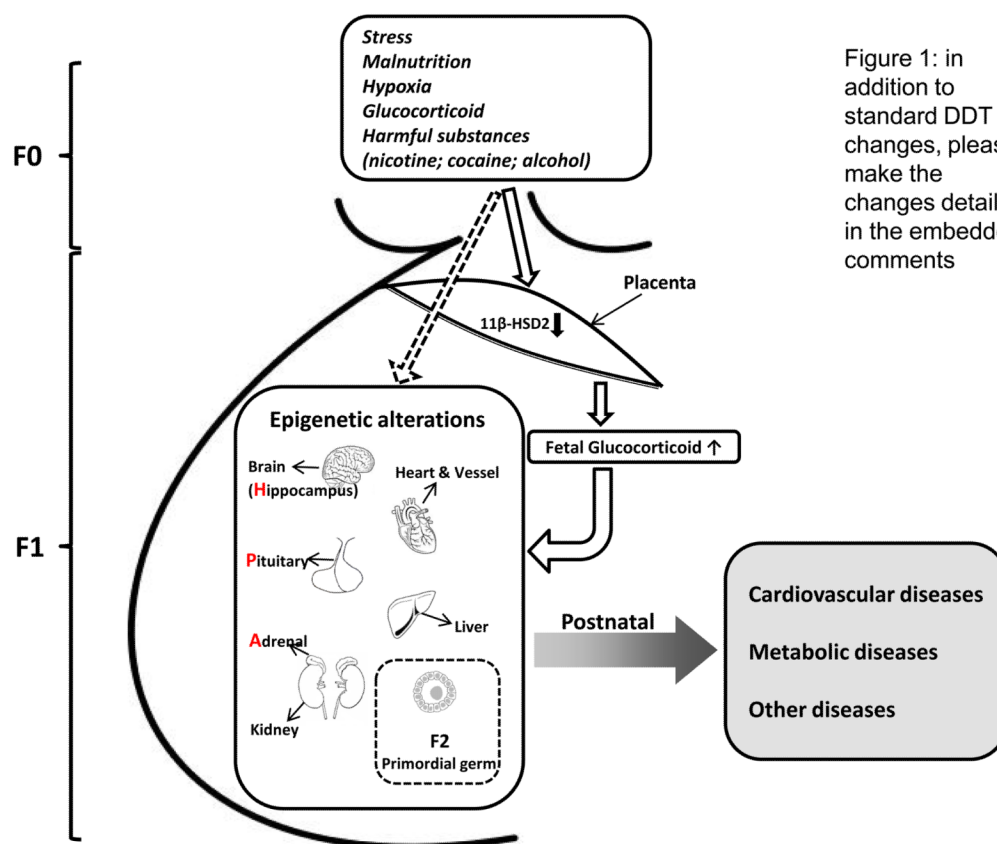


Figure 1: in addition to standard DDT changes, please make the changes detailed in the embedded comments

Figure 1. Epigenetic mechanisms in the developmental programming of adult diseases. An adverse *in utero* environment caused by maternal stress, malnutrition, hypoxia or exposure to harmful substances results in epigenetic modifications of gene expression patterns that can lead to diseases later in life. Additionally, alterations in placental 11- β -hydroxysteroid dehydrogenase-2 (11 β -HSD2) might also have an important role in maternal stress-mediated epigenetic modifications in the fetal hippocampus–pituitary–adrenal (HPA) axis via its regulation of glucocorticoid levels. The epigenetic alterations that originated *in utero* eventually result in a postnatal phenotype with heightened susceptibility to diseases. Furthermore, the epigenetic alterations that occur in primordial germ cells can be inherited and might influence subsequent generations.

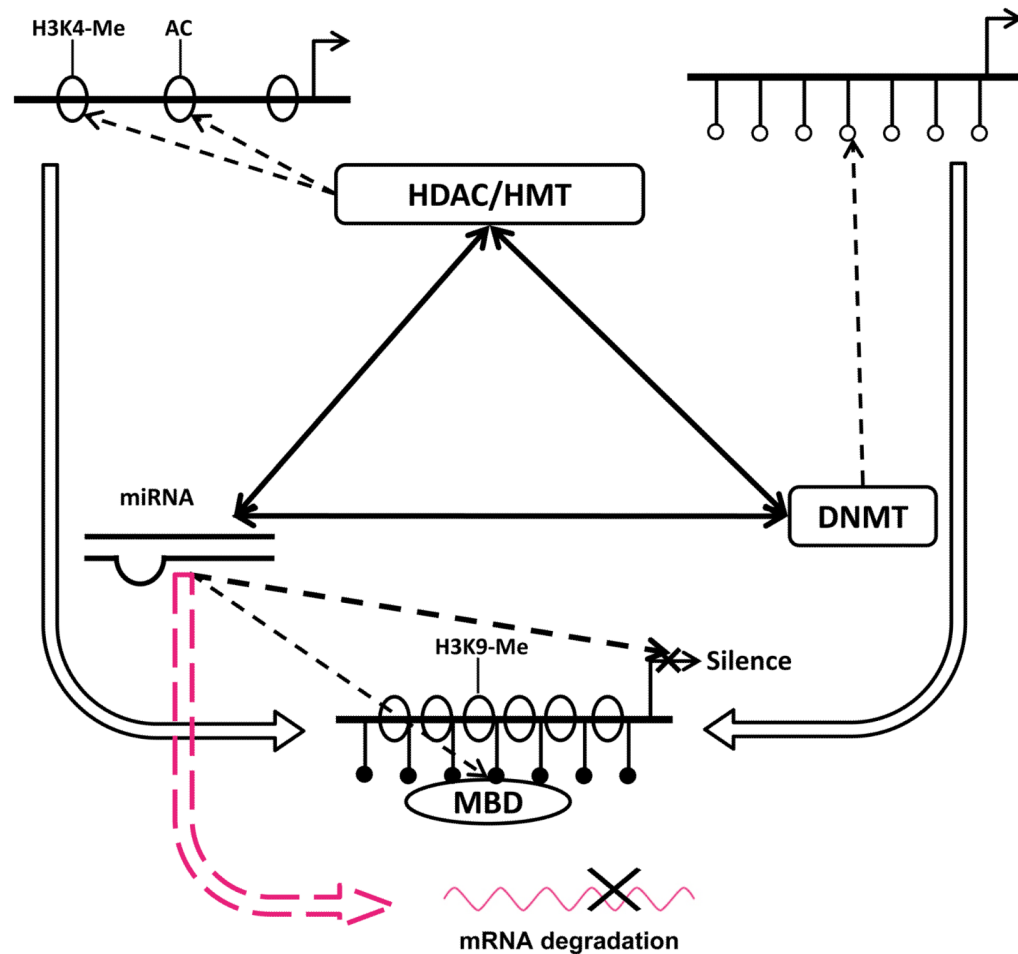


Figure 2.

The relationship between DNA methylation, histone modifications and miRNA. DNA methylation-caused chromatin silencing can be facilitated by the coupling action of covalent modifications of histone and vice versa. Interactions between histone deacetylases (HDAC), histone methyltransferases (HMT) and methyl CpG-binding proteins (MBD) lead to the recruitment of DNA methyltransferases (DNMT). miRNAs can suppress protein expression by increasing the degradation of mRNAs. Additionally, miRNAs can modulate gene transcriptional activities via their regulation of HMT, MBD, HDAC and DNMT. Furthermore, miRNA genes themselves are also targeted by epigenetic modifications. Abbreviations: AC, acetylation; H3K4, histone H3 lysine 4; H3K9, histone H3 lysine 9; Me, methylation.