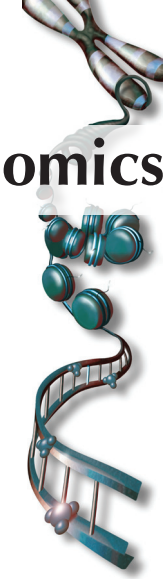
Review

Epigenomics



Effects of prenatal exposure to endocrine disruptors and toxic metals on the fetal epigenome

Exposure to environmental contaminants during pregnancy has been linked to adverse outcomes at birth and later in life. The link between prenatal exposures and latent health outcomes suggests that these exposures may result in long-term epigenetic reprogramming. Toxic metals and endocrine disruptors are two major classes of contaminants that are ubiquitously present in the environment and represent threats to human health. In this review, we present evidence that prenatal exposures to these contaminants result in fetal epigenomic changes, including altered global DNA methylation, gene-specific CpG methylation and microRNA expression. Importantly, these changes may have functional cellular consequences, impacting health outcomes later in life. Therefore, these epigenetic changes represent a critical mechanism that warrants further study.

First draft submitted: 2 September 2016; Accepted for publication: 8 December 2016; Published online: 17 February 2017

Keywords: DNA methylation • endocrine disruptors • environmental exposure • epigenetics • fetal epigenome • *in utero* • prenatal • toxic metals

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The fetal epigenome represents a critical developmental target, as epigenetic modifications are thought to underlie a range of disease outcomes, from cancer to neurodevelopmental disease [1,2]. There is increasing evidence that epigenetic reprogramming in early life may persist, influencing both development and susceptibility to disease later in life. In relation to the prenatal period, exposures are of particular concern as this time frame represents a susceptible developmental window during which critical patterns of DNA methylation and gene expression are being established [1]. Evidence that exposures during the prenatal period may result in epigenetic alterations supports the Developmental Origins of Health and Disease (DOHaD) hypothesis. This hypothesis, originally proposed by David Barker, posits that conditions experienced *in utero* play a role in fetal programming with important consequences for later life outcomes [3–5]. Critically, epigenetic modifications, particularly CpG methylation, are responsive to environmental factors, allowing these exposures to leave 'environmental footprints' on DNA [6–9].

There are three major forms of epigenetic modifications, namely DNA methylation, microRNA (miRNA) expression, and histone modifications [10]. This review focuses primarily on two of these modifications, namely, DNA methylation and miRNAs. Briefly, DNA methylation involves 'tagging' nucleotide bases with methyl groups, particularly at the 5′ position of cytosine (5′-mC). Depending on the genomic location, such methylation can either activate or silence transcription. A proposed mechanism by which this occurs is via transcription factor binding to DNA. In contrast, miRNAs are short, nontranslated RNAs that bind to and inhibit translation of mRNAs with reciprocal sequences [11,12]. Notably, these epigenetic modifications may 'cross-talk'

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with one another. For instance, evidence suggests that there is a relationship between DNA methylation and histone modifications, such that sites of histone modifications and DNA methylation are highly associated with one another [13]. Additionally, experimental evidence has demonstrated that manipulating histone acetylation induces alterations in DNA methylation, and *vice versa* [14,15]. Together, these epigenetic regulators control the timing and level of gene expression, fundamentally controlling cellular and physiologic function.

Toxic metals and endocrine disruptors are ubiquitously found in the environment and have been measured and quantified in the fetus, supporting that *in utero* exposure to these chemicals occurs [16,17]. Given the link between prenatal exposures to toxic metals, endocrine disruptors and diseases occurring later in life, epigenetic modifications are believed, at least in part, to underlie these effects [18]. This review summarizes the current literature related to four toxic metals where prenatal exposure is common and early life exposure yields both early developmental and later life effects, namely arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg). It also details studies related to five endocrine disruptors, specifically bisphenol-A (BPA), dichlorodiphenyltrichloroethane (DDT), polybrominated diphenyl ethers (PBDE), polychlorinated biphenyls (PCB) and phthalates. This review provides an overview of existing research that explores the relationship between prenatal exposure to both metals and endocrine disruptors in relation to the fetal epigenome, along with connections between observed epigenetic reprogramming and adverse health outcomes.

Toxic metals

Toxic metals, in contrast to essential metals, are those that are harmful to human health. For the toxic metals reviewed here, there is substantive literature linking early life exposures to persistent adverse health effects. In the following section, we summarize studies detailing the relationship between prenatal exposure to four toxic metals – As, Cd, Pb and Hg – and the fetal epigenome. Notably, all of the toxic metals discussed are known to cross the placental barrier, to some extent [19–21]. This suggests that the placenta serves as an incomplete barrier, resulting in direct fetal exposure to these compounds. The studies detailed below are summarized in Supplementary Table 1.

Arsenic

Inorganic arsenic (iAs) is a toxic metalloid ubiquitous in the environment with exposure related to both cancer and noncancer end points [22]. Notably, exposure via contaminated drinking water is a major route of concern. More than 100 million people are at risk of exposure that exceeds the World Health Organization's recommended limit of 10 parts per billion (ppb) [22]. iAs and its methylated metabolites are known to cross the placenta and are found within cord blood at similar levels as those in the mother [19]. Prenatal iAs exposure is linked to a wide range of adverse health outcomes that may be present at birth or those that emerge later in life, the latter suggesting long-term epigenomic reprogramming of the fetus [23]. It has been hypothesized that iAs impacts DNA methylation by creating competition for methyl donors, leading to dysregulation of DNA methylation processes [24,25]. However, this theory cannot account for the observation of gene-specific methylation patterns following iAs exposure. Instead, a separate hypothesis suggests that iAs, and other environmental contaminants, may disrupt transcription factor occupancy, including access of the methylation machinery to areas of the genome, giving rise to gene-specific patterning of CpG methylation [9].

The relationship between prenatal iAs exposure and global DNA methylation has been examined in several different populations, including those in Bangladesh, Mexico and the USA. Global methylation is often measured by examining methylation at CpG sites located within transposable elements, such as LINE1 or Alu, which comprise up to 30% of the human genome [26]. Changes in global hypomethylation are of concern as it is thought to create genomic instability, yielding an environment in which mutations may arise from the activities of transposable elements or where tumor-promoting genes may be overexpressed. In Bangladesh, where iAs exposures can range up to 2.5 parts per million (ppm), mixed results have been noted with respect to LINE1 methylation and no relationships have been noted between iAs exposure and luminometric methylation assay (LUMA) methylation or Alu methylation [22,27–29]. Similarly, results from an exposed population in Thailand indicate no relationship between cord blood LINE1 methylation and iAs exposure [30]. However, results from a Bangladesh-based nutritional intervention suggest a sex-dependent effect of maternal urinary As on cord blood methylation, noting nonsignificant hypermethylation in Alu, LINE1 and LUMA methylation in males, but hypomethylation in females. Results from this same study have also reported a positive association between maternal urinary total As and global methylation of cord blood, measured using a methyl incorporation assay [29]. These results suggest that impact of prenatal iAs exposure on global methylation may depend on fetal sex.

Numerous studies have also examined the relationship between prenatal iAs exposure and methylation of gene-specific regions of DNA. Targeted gene analyses

have identified differential methylation of *p16* and *p53* promoter regions, providing evidence that iAs dysregulates critical tumor suppressor pathways in cord blood [27,30]. Several studies examining genome-wide methylation have reported few differentially methylated CpG sites following false discovery rate correction [28,31,32]. However, among the most significant probes identified in these analyses, enrichment for genes involved in pathways related to juvenile diabetes, cancers, infectious diseases and inflammatory disorders has been noted, providing evidence, at the molecular level, for the relationship between prenatal iAs exposure and such outcomes [28,32]. Yet, other studies have identified more robust changes in the newborn epigenome. For instance, research from the Biomarkers of Exposure to ARsenic (BEAR) pregnancy cohort in Mexico identified over 2000 differentially methylated CpG sites. Methylation for a set of these was functional, predicting altered gene expression in the cord blood as well as showing an association with birth outcomes. Notably, among the genes identified was the imprinted gene *KCNQ1* [8]. When considering methylation changes across a variety of fetal tissues, Cardenas *et al.* identified numerous differentially methylated genes associated with iAs in the placenta and umbilical artery. In particular, the genes identified here were enriched for pathways similar to those noted above, reinforcing these previous observations [33]. Similarly, while a recent study from the New Hampshire Birth Cohort did not find significant relationships between maternal urinary total As and placental methylation, and only one significant probe when considering maternal toenail iAs, they reported over 100 differentially methylated CpG sites in the placenta associated with placental As [34]. Taken together, these results suggest that there are tissue-specific effects of iAs exposure on the fetal epigenome. Moreover, the effects of iAs exposure vary across individuals and populations, likely as a result of genotype and/or nutritional factors [35,36]. Future research should investigate whether differential susceptibility to iAs is linked to the epigenome, contributing to the differences observed between the various Bangaldeshi, Mexican and USA populations.

As observed with studies focusing on global DNA methylation, sex-based differences have also been noted for gene-specific methylation. In particular, increasing iAs exposure in male infants was more strongly associated with cord blood CpG methylation than in females. Moreover, iAs exposure in males was associated with DNA hypomethylation, while female infants tended to display hypermethylation. Pathway analysis of the most significantly affected genes also revealed that male infants displayed gene-specific methylation at sites that were most significantly enriched for cancer pathways, while females were most significantly enriched for inflammatory diseases [28]. Future research should be carried out to further elucidate the mechanistic underpinnings of sex-based differences in iAs-induced disease.

Two studies have examined the relationship between prenatal iAs exposure and miRNA expression in the placenta. Research from the formerly established National Children's Study (NCS), a prospective cohort of children across the US, did not note a relationship between placental iAs and miRNA expression [37]. In contrast, results from the BEAR cohort included differential expression of miRNAs that mediate signaling pathways related to iAs-associated diseases, including cancer, inflammatory disease, respiratory disease and metabolic disease, among others [38]. Moreover, these miRNAs were predicted to target 20% of the observed differential mRNA expression in this population, indicating that iAs-associated miRNA dysregulation may have functional consequences for downstream gene expression that may be related to disease outcomes [38].

Cadmium

Cd is found in the environment both as a natural component of the earth's crust and as a result of anthropogenic activities. Specifically, Cd may be found in batteries, electronic waste, pesticides and tobacco smoke, among other sources [39]. Cd emissions have been declining globally, although areas of concern remain near smelters, e-waste sites, and other areas polluted by industry [39]. While Cd crosses the placenta, it does not cross as readily as other toxic metals because metallothionein sequesters Cd within the placenta. Therefore, the placenta provides a partial barrier against this metal [20]. As a result, Cd levels in cord blood are typically lower than maternal Cd levels [40]. Nevertheless, *in utero* Cd exposure has been linked to a range of adverse health effects, including impaired growth and neurodevelopment [41–43]. To date, one study has examined the relationship between global DNA methylation and prenatal Cd exposure. Boeke *et al.* demonstrated that there is an inverse relationship between maternal Cd exposure and LINE1 methylation in cord blood [44]. These observations have also been made in adult populations, suggesting that similar changes could be occurring within fetuses following prenatal Cd exposure [45].

In a nested cohort from the Center for Environmental Health Initiative (CEHI) Healthy Pregnancy, Healthy Baby study in North Carolina, DNA methylation occurring within gene-specific regions of cord blood DNA was examined in relationship to maternal blood Cd collected at delivery [7]. Over 60 genes were identified as differentially methylated, with a major-

ity of them exhibiting hypermethylation. Overlap between differentially methylated genes identified within the cord blood and within maternal blood were also observed [7]. Taken together with the relationship between Cd and global methylation, these results suggest that prenatal Cd exposure is associated with both global hypomethylation and gene-specific hypermethylation. However, in the Maternal and Infant Nutrition Interventions, Matlab (MINIMat) cohort in Bangladesh, no CpG sites were identified as significant following multiple test corrections [46]. Instead, sex-specific effects were noted with the most significant probes displaying hypermethylation in males, but hypomethylation in females. Interestingly, in girls, the most significant probes were enriched for genes relating to bone mineralization and morphology, possibly shedding light on the sex-specific effects of Cd exposure, especially as it relates to the susceptibility of female populations to Cd-induced bone outcomes [46]. Other studies on Cd-associated fetal epigenomic alterations have also observed sex-specific effects [47,48]. Of note, the relationship between maternal blood Cd and the methylation of imprinted genes was altered in a sex-dependent manner in infants from the Newborn Epigenetic STudy (NEST) in North Carolina [47]. These relationships were also dependent on levels of circulating zinc and iron, indicating that Cd-associated epigenomic disruptions may be modified by maternal levels of essential metals.

Lastly, several studies have reported on Cd-induced miRNA dysregulation. Placental Cd levels were significantly associated with increased placental expression of miR-1537 [37]. Little is known about this miRNA, however, it has been suggested to play a role in cancer [49,50]. Interestingly, a recent study examining the relationship between preeclampsia and Cd exposure identified a set of miRNAs common to both preeclampsia and Cd-exposure. These miRNAs also regulate genes involved in the TGF-β signaling pathway [51]. Given the epidemiological links between placental Cd levels and preeclampsia, these results provide evidence, at the molecular level, for a relationship between exposure and disease [52].

Lead

As observed with the ongoing struggles in Flint, Michigan, Pb continues to be a toxic metal of critical importance in children's health [53,54]. In urban centers, children are often exposed via Pb-based paint that remains in homes, particularly within low socioeconomic areas [55]. However, maternal exposure is also of importance as Pb is known to cross the placenta, although the mechanisms underlying placental transfer remain unknown [20]. While exposures have generally been decreasing over the course of the last century, even levels below the established regulatory limits (5 μg/dL) have been associated with impaired neurodevelopment in children [56,57]. Given the latent and persistent effects of early life exposure to Pb, epigenetic reprogramming has been posited to contribute to these effects. Maternal bone Pb levels have been significantly associated with LINE1 and Alu methylation, although cord blood Pb has not [58,59]. Maternal bone Pb represents cumulative exposure, suggesting that maternal cumulative Pb exposure is associated with altered global methylation. Supporting these observations, it has been previously noted that maternal bone Pb is better at predicting adverse health outcomes in infants and children than cord blood Pb [60].

In genic regions, results from the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) study suggest that methylation of the imprinted genes *IGF2* and *HSD11B2* was positively associated with *in utero* Pb exposure, with a sex-dependent effect and stronger relationship in girls. However, results were not significant following multiple test correction [59]. Additional studies have focused on the sex-specific effects of Pb-induced epigenetic disruptions of the fetus. Using cord blood and blood spots, Sen *et al.* examined differentially methylated regions of the genome that may serve as sex-dependent or independent biomarkers of Pb exposure [61,62]. Interestingly, different patterns were noted when observing CpG methylation in cord blood versus infant blood spots. In cord blood, males showed a greater number of sites with altered CpG methylation [61]. However, in blood spots, females showed greater disruption in CpG methylation. Given that males tend to be more susceptible to the effects of Pb exposure, researchers concluded that disruptions in CpG methylation may be adaptive in blood [62]. Notably, Sen *et al.* has also reported that maternal Pb exposure induces epigenetic changes in the germ line of their offspring, impacting DNA methylation in the F2 in humans. These results provide evidence that Pb exposure may induce multigenerational epigenomic changes [63].

In the NCS, placental Pb levels were negatively associated with expression of several miRNAs, including miR-190b [37]. Interestingly, miR-190b targets several neurotrophins, including *NRG3*, which is related to impulsivity. In mice, miR-190b expression is positively associated with impulsivity behaviors, a behavioral trait that has also been associated with Pb exposure [64]. Taken together, this suggests that Pb-associated miRNA changes in the placenta may relate to the neurological effects seen in developing children by targeting neurodevelopmentally- and behavior-related signaling pathways.

Mercury

Long known to be linked to adverse neurological outcomes, Hg exposure during pregnancy is associated with impaired attention, visuospatial and motor functioning, among other outcomes [65]. Within the US, approximately 15% of childbearing-age women have elevated blood Hg levels of concern, suggesting that over 600,000 children may be born with increased risks of such neurological outcomes [66]. Importantly, Hg bioaccumulates within the fetus, suggesting an active transport mechanism across the placenta, although the mechanisms for such transport are unknown [21]. No studies were identified that examined the relationship between prenatal exposures to Hg and global DNA methylation. However, two studies from birth cohorts in the USA have explored the relationship between *in utero* Hg exposure and gene-specific methylation in cord blood. Using independent test and validation populations, the relationship between cord blood Hg and CpG methylation was examined using several different methods [67]. It was shown that *TCEANC2* was significantly differentially methylated in both the test and validation populations. However, given the association between *TCEANC2* and blood cell composition, these results could be attributed to either Hg-induced changes in methylation at that loci or Hg-induced changes in blood cell composition [67]. Therefore, these observations should be interpreted with caution. In a second study, prenatal Hg exposure was significantly associated with genome-wide CpG methylation, with a majority of significant probes displaying a nonmonotonic relationship between exposure and methylation. Notably, a subset of the Hg-associated probes were also significantly associated with high-risk status for adverse neurobehavioral outcomes, indicating that dysregulated CpG methylation may have functional consequence for Hg-associated outcomes [68].

Lastly, results from the NCS indicate that placental Hg levels are significantly associated with changes in miRNA expression in the placenta. In particular, Hg levels were associated with a large number of miRNAs within the let-7 family, which is critical for proper developmental timing in animal models [69]. Further examination of the relationship between let-7 miRNAs and Hg exposure may shed further light on the teratogenic and other developmental effects of prenatal and early life Hg exposure.

Endocrine disruptors

Early life exposure to endocrine disruptors, which interfere with endocrine signaling and development, remains a topic of concern. Endocrine disruptors are ubiquitous in the environment, representing a wide range of chemicals, such as pesticides, plasticizers or other synthetic compounds used in industrial settings. While we have separated metals and endocrine disruptors in this review, it is important to note that some toxic metals also act as endocrine disruptors [70]. In the following section, the literature focusing on the relationship between prenatal exposure to five endocrine disruptors and the fetal epigenome is reviewed. Specifically, exposures to BPA, DDT, PBDE, PCB and phthalates are highlighted. As noted above with respect to toxic metals, all of the endocrine disruptors discussed here are known to cross the placental barrier [71–74]. The studies detailed below are summarized in Supplementary Table 2.

Bisphenol-A

BPA, an industrial compound with significant estrogenic effects, is used in a variety of products as a component of polycarbonate plastics or epoxy resins [75]. Results from the Maternal-Infant Research on Environmental Chemicals (MIREC) study has demonstrated that BPA levels among pregnant women do not appear to differ markedly from those in the general population [76]. It has been posited that 'free' BPA is able to cross the placenta, while glucuronidated BPA cannot. Thus, the maternal capacity for clearance depends on the maternal clearance of BPA as well as the fetal capacity for conjugation, which may 'trap' glucuronidated BPA in the fetal compartment and prolong fetal exposure [71]. In a study that focused on the assessment of BPA exposure and effects in fetal tissue, fetal liver tissue was analyzed from the Washington Birth Defects Research Laboratory Fetal Biobank. Using next-generation sequencing, liver BPA levels were determined to be significantly associated with indicators of global methylation. Specifically, higher BPA levels were associated with greater numbers of hypomethylated repeat sequences, suggesting global hypomethylation. They also noted that, based on differential methylation of CpG islands, shores and shelves, there was an overall trend of hypomethylation across the genome [77]. Further research using fetal liver tissue has also identified specific relationships between BPA and repetitive DNA elements, where nonmonotonic relationships between BPA exposure and DNA methylation were observed in several repeat families, including LINE1 [78]. Nonmonotonic relationships have previously been observed in prenatal mouse exposure models both with respect to both epigenomic and metabolic end points [79–81]. However, previous research, using both LUMA and pyrosequencing, did not identify a relationship between global methylation and BPA exposure in fetal liver or kidney, but did note a positive relationship between BPA exposure and LINE1 methylation in the placenta [82]. These results

suggest that indicators of global methylation, namely repetitive DNA elements, are associated with prenatal BPA exposure in a dose- and tissue-specific manner. It is also important to note that repeat DNA elements respond differentially to environmental exposures, therefore, changes in Alu and LINE1 may represent methylation changes occurring in different parts of the methylome [26,83].

In contrast to global methylation detailed above, Faulk *et al.* also examined genome-wide CpG methylation in relation to BPA exposure, identifying and validating differential methylation within the snRNA cluster around *SNORD116*, a maternally imprinted locus [77]. One study has tested the relationship between BPA exposure and miRNA expression, but no significant relationships were observed [37].

Dichlorodiphenyltrichloroethane

DDT is a persistent organic pollutant. While DDT was used extensively from the 1940s until 1973, it is no longer in commercial use in the US, although it remains in the environment as a legacy contaminant [84]. Dichlorodiphenyldichloroethylene (DDE), a common breakdown product of DDT that may form in the environment or within the body, is often examined alongside DDT as it has been associated with adverse health outcomes in human populations [85,86]. While DDT and DDE both have long half-lives in the environment and the human body, DDE was observed to be the predominant form found in pregnant women participating in the National Health and Nutrition Examination Survey [87]. While levels of DDT are lower within fetal than maternal tissues, there is evidence that DDT is actively transported across the placenta as fetal levels are higher than expected via passive diffusion [72]. Prenatal exposure to DDT and DDE has been associated with a range of early and later life health outcomes, including fetal growth measures, adolescent neurodevelopment, indicators of female reproductive functioning, and lung functioning [88–93]. When examining the association between DDT, DDE, and global methylation of the fetal epigenome, results from the Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS) study indicate that maternal serum DDT and DDE levels are inversely associated with Alu methylation in cord blood [83]. However, additional research from this cohort, using LUMA to measure global CpG methylation, did not report a relationship between placental DDE levels and global methylation [94]. The inconsistency between these results originating from the same cohort suggest that DDT has tissue-specific effects on the fetal epigenome, as was noted previously with BPA. Alternatively, the discrepancy may also be due to the use of different methods of assessing DNA methylation. As previously mentioned, LINE1 and Alu elements may be differentially impacted by environmental exposures and may capture changes occurring at only a portion of sites within the genome [26]. Thus results from LINE1 and Alu elements may differ from LUMA methylation, which instead measures a ratio of unmethylated to methylated CpG sites across the genome.

In relation to DDE exposure, the CHAMACOS investigators have also examined placental methylation at CpG islands affiliated with two imprinted genes, *IGF2* and *H19*. No significant associations were noted between placental DDE levels and methylation at these loci [94]. Additionally, no relationship has been found between exposure to DDE and placental miRNA expression [37].

Polybrominated diphenyl ethers

Two studies have examined fetal global methylation in association with exposure to PBDE, a class of compounds used as flame retardants in products ranging from clothing to household building materials [95]. Evidence suggests that there is relatively free transfer of PBDE across the placenta, particularly for low-brominated congeners [73]. Notably, infants and young children have been observed to have higher body burden of PBDE than adults as a result of this placental transfer, as well as breast feeding and exposure to household dust [96]. Research originating from the CHAMACOS study assayed LINE1 and Alu methylation in DNA derived from cord blood. No association was noted between these indicators of global methylation and third trimester maternal blood PBDE. However, interactions between maternal blood PBDE and blood DDT and DDE were noted [83]. These results will be discussed in more detail, below, in the context of co-exposures. A second study, examined placental LUMA methylation in correlation with placental PBDE levels. Here, a significantly positive relationship between placental PBDE and global methylation was observed [94]. Additionally, loss of imprinting at *IGF2* and *H19* was also examined in relationship to PBDE, although no significant results were reported [94].

In light of the immune-related effects of prenatal PBDE exposure, the relationship between methylation of *TNF*-α and PBDE exposure was investigated in the Boston Birth Cohort [97]. Maternal serum levels were significantly associated with a decrease in CpG methylation of the promoter region of *TNF*-α in DNA isolated from cord blood. Interestingly, this relationship exhibited a threshold effect, where levels below approximately 5 ng/g-lipid displayed no change in *TNF*-α methylation. Above this threshold, there was a negative association between PBDE and CpG meth-

ylation of *TNF*-α. Moreover, this effect appeared to be modified by the sex of the fetus, with the same pattern noted in females, but not males [97].

A single study has examined the relationship between placental PBDE levels and miRNA expression. No relationship was found with total placental PBDE, however, PBDE 209 was positively associated with miR-188–5p expression, while PBDE 99 was negatively associated with let-7c expression [37].

Polychlorinated biphenyls

As with DDT, PCB are chemicals that are no longer in commercial production in the USA. Previously used widely as coolants, these chemicals persist in the environment [98]. Research has demonstrated that PCB levels are higher than expected in fetal tissues based on passive diffusion alone, suggesting an active transport mechanism across the placenta [72]. Moreover, biomonitoring of pregnant women suggests that serum levels of PCB may increase over the course of pregnancy as lipid stores are mobilized [99]. Results from the NCS did not identify a relationship between LUMA methylation in the placenta and placental PCB levels [94]. At a gene-specific level, no relationship was noted between PCB levels and methylation at the *IGF2* and *H19* loci, although an inverse relationship between *H19* expression and PCB levels was reported [94]. These results suggest that *in utero* exposure to PCB may influence the expression of *H19* via mechanisms that are independent from CpG methylation.

Additional studies from the NCS examined miRNA expression in the placenta in relation to placental PCB levels. Notably, total PCB, PCB 52 and PCB 101 were significantly positively associated with miR-1537 expression [37].

Phthalates

Phthalates, like BPA, represent a class of ubiquitously used industrial compounds that are found widely in personal care items and household products [100–103]. There are numerous routes of exposure to phthalates, with the compounds being inhaled, ingested and dermally absorbed. Urinary metabolites are often used as indicators of exposure representing all of these possible routes, although their stability as a biomarker is short because phthalates are quickly metabolized and excreted from the body. Despite this rapid metabolism and excretion, phthalates are still detected in the amniotic fluid, indicating that it crosses the placenta and enters the fetal compartment [74]. However, perfusion studies indicate that placental transfer of phthalates may be slow [104]. As with BPA, the MIREC study has also demonstrated that phthalate levels in pregnant women are comparable to those measured in the general population [76]. There are many different phthalates used in industry, but several are more commonly detected and used in epidemiological studies. These include di(2-ethylhexyl) phthalate, diethyl phthalate and dibutyl phthalate, among others [105].

With respect to the fetal methylome, several studies have assessed the impact of prenatal exposure to phthalates on global methylation, gene-specific methylation and miRNA expression. Urinary levels of low-molecular-weight (LMW) phthalate metabolites and a diethyl phthalate metabolite, mono-ethyl phthalate, have been negatively associated with Alu methylation in cord blood using maternal-infant dyads from the CHAMACOS study [106]. These results suggest global hypomethylation of the cord blood following phthalate exposure and are supported by previous research originating from a Chinese cohort of pregnant women, which demonstrates that maternal urinary levels of di(2-ethylhexyl) phthalate were associated with hypomethylation of LINE1 in the placenta [107]. Taken together, these results suggest that prenatal phthalate exposures result in global hypomethylation of fetal DNA as assessed within placental tissue and cord blood.

Only one study was identified that examined gene-specific methylation in relationship to prenatal phthalate exposure [108]. Namely, CpG methylation of *H19* and *IGF2* was examined in relationship to first-trimester maternal urinary phthalate measures. Both cumulative and LMW phthalates were inversely associated with *H19* and *IGF2* methylation. These results indicate a possible dysregulation of these imprinted genes that play a critical role in development [108].

A single study has examined the impact of *in utero* phthalate exposure and placental miRNA expression. Namely, maternal urinary LMW phthalates were significantly associated with decreased expression of miR-185. However, *in silico* predicted downstream mRNA targets were not differentially expressed, indicating that other mechanisms contribute to gene expression [109].

Mixtures

In the environment, human populations are more likely to be exposed to mixtures of toxic substances, rather than single contaminants. Yet, few studies have been published that examine the effects of compound mixtures on the fetal epigenome, despite the fact that the chemicals reviewed here are often found within cord blood together [16,110]. Those that were identified examine interactions between compounds, such as DDT and PBDE, or how cumulative exposure to a group of varied chemicals, such as phenols, may correspond to epigenomic changes in the fetus [83,109,111–113].

Toxic metal mixtures

Among infants exposed prenatally to toxic metals, a single study has examined the effects of exposure to iAs and Hg on the fetal epigenome. While no significant probes were identified after Bonferroni correction, a greater number of probes were observed with a $p < 0.0001$ when considering the interaction than when considering Hg alone [111]. These results may indicate that there are relationships between exposure to toxic metals and the fetal epigenome that may not be identified until co-exposures are considered. More research into metals mixtures is warranted. For instance, while no studies have examined the impact of co-exposures to iAs and Cd on the fetal epigenome, recent research has identified that both contaminants significantly disrupt genes involved in the innate and adaptive immune system, particularly those involved in glucocorticoid signaling [114]. These results suggest that iAs and Cd may similarly affect immune-related pathways, supporting the possibility of synergism.

Endocrine disruptor mixtures

As mentioned previously, the CHAMACOS study reported interactions between prenatal DDE and PBDE exposure [83]. When stratified based on maternal serum DDE, PBDE was associated with hypomethylation of LINE1 in the fetus among those with low maternal DDE exposure. In those subjects with high maternal DDE exposure, PBDE levels were associated with LINE1 hypermethylation. The same relationships were noted for DDE exposure when the study population was stratified by PBDE levels. Notably, no associations were found between DDE, PBDE and LINE1 methylation until they were considered as co-exposures [83]. These results suggest that relationships between prenatal exposures and the fetal epigenome may not be fully elucidated until co-exposures are considered and underscore the need for continued research into how endocrine disruptors may interact with one another.

A number of endocrine disruptors exhibit estrogenic activity, including BPA, DDT/DDE and PCB [115]. Total effective xenoestrogen burden (TEXB) represents a biomarker for cumulative xenoestrogen burden and has been used to assess co-exposures to such chemicals [116]. TEXB has been associated with LINE methylation in the placenta, with males displaying hypomethylation of LINE and Alu elements [112]. However, when assessing the relationship between TEXB and CpG methylation, no significantly differentially methylated probes were identified [113].

Lastly, in placental samples from the Harvard Epigenetic Birth Cohort (HEBC) and the Predictors of Preeclampsia Study (POPS), the sum of maternal urinary phenols was significantly negatively associated with expression of miR-142 [109]. Cumulative exposure to nonparaben phenols, such as BPA and triclosan, was also significantly inversely associated with miR-15a-5p expression. Interestingly, this relationship exhibited a sex-dependent effect, with female infants exhibiting a significant negative relationship between total phenol exposure and miR-15–5p expression in the placenta. However, downstream gene expression analysis revealed that none of the *in silico* predicted mRNA targets were differentially expressed, nor were these miRNAs noted to be associated with any birth outcomes [109]. These results indicate that miRNA expression in the placenta is affected by cumulative phenol exposure, however, it is likely that multiple mechanisms are responsible for controlling downstream gene expression.

Contributions of animal models & *in vitro* experiments

The bulk of this review focuses on evidence that *in utero* exposure to toxic metals and endocrine disruptors are associated with epigenomic reprogramming of human fetal tissues that, ultimately, provide mechanistic evidence for the DOHaD hypothesis. Animal models have the potential to contribute substantially to our understanding of the mechanisms underlying these effects. First, animal models facilitate the examination of epigenomic changes that occur within tissues where disease originates, rather than accessible fetal tissues like the placenta or cord blood. For instance, if exposure occurs following zygote formation, tissue-specific epigenomic changes result in susceptible tissues. However, if exposure directly impacts gametes, then whole-organism epigenomic changes may result. Only tissues susceptible to these changes are thought to go on to develop disease during development. Thus, animal models may be used to examine epigenomic changes occurring within disease-relevant tissues in order to determine the role of environmentally induced reprogramming in disease. Moreover, they may aid in determining whether epigenomic reprogramming occurring in readily accessible tissues serves only as a biomarker of disease, or whether they may be directly related to disease development.

Second, animal models, along with cell lines, have also been crucially important in elucidating the mechanistic underpinnings of environmentally-induced epigenomic reprogramming. There is evidence for the ability of toxic metals and endocrine disruptors to impact the epigenetic machinery in several different ways. First, endocrine disruptors and toxic metals have direct impacts on steroid hormones and/or steroid hormone receptors, which can interact with and alter the activity of histone-modifying enzymes. For

example, in human cell lines, PCB exposure has been shown to modulate the activity of histone demethylases via androgen receptor binding [117]. Aside from direct activity on steroid hormone signaling, toxic metals and endocrine disruptors have also been shown to interact with DNA methyltransferases and ten-eleven translocation enzymes, which are responsible for methylating and demethylating CpG sites. For instance, Cd and As have been demonstrated to interact with DNA methyltransferases and ten-eleven translocations, altering their activity in *in vitro* experiments [118,119]. Much of this mechanistic evidence is reviewed in greater depth, elsewhere [120]. Additionally, some toxic metals and endocrine disruptors are known to interact with one-carbon metabolism, which produces methyl donors used for DNA methylation. For instance, iAs metabolism uses S-adenosylmethionine as a methyl donor [36]. Given that S-adenosylmethionine is also a methyl donor utilized for DNA methylation, it has been posited that iAs metabolism results in dysregulation of DNA methylation [36]. Importantly, while these mechanisms describe how toxic metals and endocrine disruptors may impact epigenetic machinery, they do not explain how such exposures result in gene-specific patterning of epigenomic markers [9]. Instead, the transcription factor occupancy theory provides an explanation by positing that environmental exposures alter transcription factor activity, changing the availability of DNA to epigenetic machinery and giving rise to gene-specific patterning of epigenomic markers [9].

When considering the persistence of the impacts of *in utero* exposure to toxic metals and endocrine disruptors, it is critical to consider the relative contribution of and interaction between pre- and postnatal exposures. As mentioned, the toxic metals and endocrine disruptors reviewed here represent ubiquitous exposures. Therefore, it is likely that populations experiencing *in utero* exposures to toxic metals and endocrine disruptors also experience chronic exposure throughout the life course. Animal models represent important model organisms in which exposure during defined developmental windows can be investigated [121]. With respect to iAs exposure, prenatal exposure paradigms have been used to establish the sensitivity of the prenatal developmental window compared with other periods of exposure [122,123]. In human populations, on the other hand, it is often impossible to differentiate between prenatal and postnatal exposures and their contributions to health outcomes [121]. Related, animal models also foster research examining the transgenerational impacts of exposure. As Sen *et al.* demonstrated, maternal Pb exposure may be related to DNA methylation in the blood of their grandchildren (F2), suggesting that Pb impacts the germ line of a developing fetus [63]. However, assessing truly transgenerational effects is unlikely to be feasible in human populations. In animal models, transgenerational impacts can be assessed by examining epigenomic changes occurring in the F3 generation, following exposure during the F1 prenatal period. While little work has been done with the toxic metals and endocrine disruptors reviewed here, exposure to a mixture of BPA and phthalates during pregnancy in rats has been observed to induce a range of adverse health outcomes, including ovarian/testis disease and obesity, in the F3 generation. Interestingly, the F3 sperm also had altered DNA methylation in promoter regions previously associated with the onset of obesity [124].

Discussion & future perspective

There is a growing body of evidence linking *in utero* and early life exposures to both toxic metals and endocrine disruptors to disorders present during early life and emerging later in life. A mechanistic basis underlying the associations between these exposures and adverse health outcomes have often been difficult to elucidate. However, current research suggests that the epigenome may provide this critical link. With modifications that are both responsive to the environment and may persist throughout the lifetime, epigenetics provides a mechanism for how environmental exposures create long-lasting biological changes in cellular functioning. While, the epigenome plays a critical role throughout the human lifetime, the prenatal period represents an especially sensitive developmental window during which epigenetic marks are first being established [1]. Notably, the specific window of environmental exposure during reproductive development is important in determining the effects observed [125]. For instance, if exposure occurs during a critical developmental period for reproductive system development, these tissues may be especially susceptible to epigenetic alterations and downstream adverse health outcomes [126]. Additionally, if germ cells are exposed to toxic metals or endocrine disruptors, exposure may yield epigenetic reprogramming within every cell of the offspring [125]. Then, during development, tissues sensitive to the resulting epigenomic reprogramming may have an elevated risk for disease development. Moreover, if germ cells are exposed, then multi- and transgenerational impacts may be observed [127]. In other words, timing of exposure during the *in utero* period may direct whole-organism and/or tissue-specific effects of toxic metals and endocrine disruptors on the fetal epigenome. The importance of timing of exposure to toxic metals and endocrine disruptors and fetal epigenomic reprogramming should be further explored.

Global methylation is often measured when assessing the impact of environmental exposures on the epigenome. Much of the literature reviewed here uses transposable elements (i.e., LINE1 or Alu) as indicators of global methylation. However, there is evidence that these elements respond differentially to environmental exposures and in a sequence-specific manner, suggesting that they may represent only a specific part of the methylome. For instance, evidence demonstrates that LINE1 methylation may only represent weak CpG island methylation, rather than methylation occurring in other areas of the genome [26]. Moreover, Price *et al.* maintains that the only true measure of global methylation is total 5′-mC content and that results between studies should only be compared when methylation has been assayed using the same technique [26]. This creates difficulties when comparing the existing data on prenatal exposure to endocrine disruptors, as few studies have utilized the same method and little replication has been conducted. Faulk *et al.* has examined the relationship between BPA and global methylation using next-generation sequencing in two separate studies, with both reporting hypomethylation based on transposable elements or overall genomic methylation [77,78]. Additionally, with respect to maternal phthalate exposure, Alu hypomethylation was noted in cord blood, while LINE1 hypomethylation was found in the placenta [106,107]. Taken together, prenatal BPA and phthalate exposure may lead to global hypomethylation and genomic instability in the fetal epigenome. Additionally, these results suggest that prenatal phthalate exposure may have tissue-specific effects on indicators of global methylation. We reviewed no other studies on endocrine disruptors that used the same method of assessing global methylation, and thus, we caution against cross-comparisons.

Unlike the literature on endocrine disruptors, research on toxic metals has used more consistent methods to measure both global and site-specific DNA methylation. Notably, high-throughput methods, such as the Illumina 450K BeadChip®, have been used routinely in the study of toxic metals-induced disease. Results from these assays facilitate gene-specific analysis and better enable analysis of downstream affected biological pathways, shedding light on cell functions that may be dysregulated. For instance, studies from multiple Bangladeshi cohorts have identified differential methylation in genes related to juvenile diabetes, cancers, immunodeficiency and neurological dysfunction, among others related to iAs-associated diseases [28,32,33]. Moreover, in a cross-study analysis of genes targeted for altered CpG methylation in infants with *in utero* contaminant exposure, many of the gene targets were identified as having common enriched transcription factor binding sites, providing evidence for common transcriptional controls in epigenetic patterning following contaminant exposures [9]. The identification of enriched transcription factor binding sites also provides further evidence for the transcription factor occupancy theory, indicating that altered binding of transcription factors to DNA may result in patterns of gene-specific hyper- and hypomethylation noted following environmental exposures [9].

Far less research has been conducted regarding the impacts of prenatal environmental exposures on fetal miRNA expression. Only four studies were identified that examined the relationship between prenatal exposure to metals and endocrine disruptors on fetal miRNA expression. However, the results reviewed here demonstrate their utility. For instance, prenatal iAs exposure was associated with 12 dysregulated miRNAs that were enriched for disease pathways related to iAs-associated disease (e.g., immune function) and predicted functional changes in downstream gene expression [38]. With respect to prenatal Pb exposure, dysregulated miRNAs in infants was significantly associated with neurobehavior-related signaling, providing further evidence for the role of epigenetics in Pb-induced neurodevelopmental outcomes [37,64]. Taken together, miRNA dysregulation has explanatory value when considered in the context of *in utero* exposures and research should be expanded upon. It is of particular importance given the observation, in some studies, that DNA methylation only corresponds to functional changes in gene expression at a subset of probes [8].

Exposure to toxic metals and endocrine disruptors is associated with sex-specific effects. For instance, *in utero* BPA exposure in females is more strongly associated with preterm birth, BMI, obesity and body fat compared with males [128,129]. Likewise, much of the evidence reviewed here notes a sex-dependent nature of the relationship between such exposures and the fetal epigenome. Given the difficulties in elucidating the mechanisms underlying this phenomenon, this research provides evidence that there is sex-dependent cellular reprogramming that may later influence and/or determine the phenotypes noted in exposed populations. As mentioned above, in a Bangladesh-based birth cohort, Cd exposure in females was associated with methylation of genes enriched for pathways related to bone mineralization and morphology. Notably, in populations with Cd exposure, women are especially susceptible to osteoporosis and bone fractures [130]. Additionally, *in utero* Cd exposure has been inversely associated with femur length in females, but not males [131]. The mechanisms underlying these sex-dependent changes are unknown. However, it is possible that funda-

mental differences between placentas derived from male or female fetuses may result in sexually dimorphic responses to environmental exposures [132]. It has recently been shown that gene-specific methylation differs between male and female placentas and these genes are enriched for immune function, transport of substances across the placenta, and transcription factors [133]. Further substantiating these findings, female- and male-derived placentas have differential expression levels of immune-related genes. Specifically, male placentas have enriched expression of genes related to inflammation and immune functioning, while female placentas have enriched expression of immune-regulating genes [134,135]. These immunologic differences between male and female fetuses may result in greater vulnerability in male fetuses when faced with adverse environmental exposures *in utero* [132]. Additionally, differences in the expression of glucocorticoid receptors have been observed based on fetal sex, which may result in differential fetal susceptibility to the maternal stress and/or immune response following exposure to environmental contaminants [136,137]. Together, these may ultimately contribute to sex-specific epigenomic reprogramming and health outcomes. While the mechanism underlying sex-dependent epigenetic reprogramming remains unknown, these results demonstrate the promise of using epigenetics to further understand the sex-dependent effects of environmentally induced diseases.

Interactions between these environmental exposures and genomic imprinting has also been explored in several studies. As mentioned above, LaRocca *et al.* found that maternal phthalate exposure was associated with methylation of the promoter regions of both *IGF2* and *H19*, imprinted genes with roles in both pre- and postnatal development and cancer [138–140]. Likewise, results from iAs- and/or Cd-exposed infants reveals differential methylation of several imprinted genes, including *KCNQ1*, *PEG3,* and *PLAG1* [8,47]. Interestingly, it has been shown that methylation at imprinted loci are stable across a wide range of human tissues in infants, suggesting that the observed changes in imprinted genes may be representative of changes occurring throughout the fetus [141]. The ability for such exposures to alter genomic imprinting is concerning given the tight regulation of imprinting during development and the impact that the expression of imprinted genes has on health throughout the lifespan [142]. Thus, the relationships between heavy metals, endocrine disruptors and methylation of imprinted genes should be further examined.

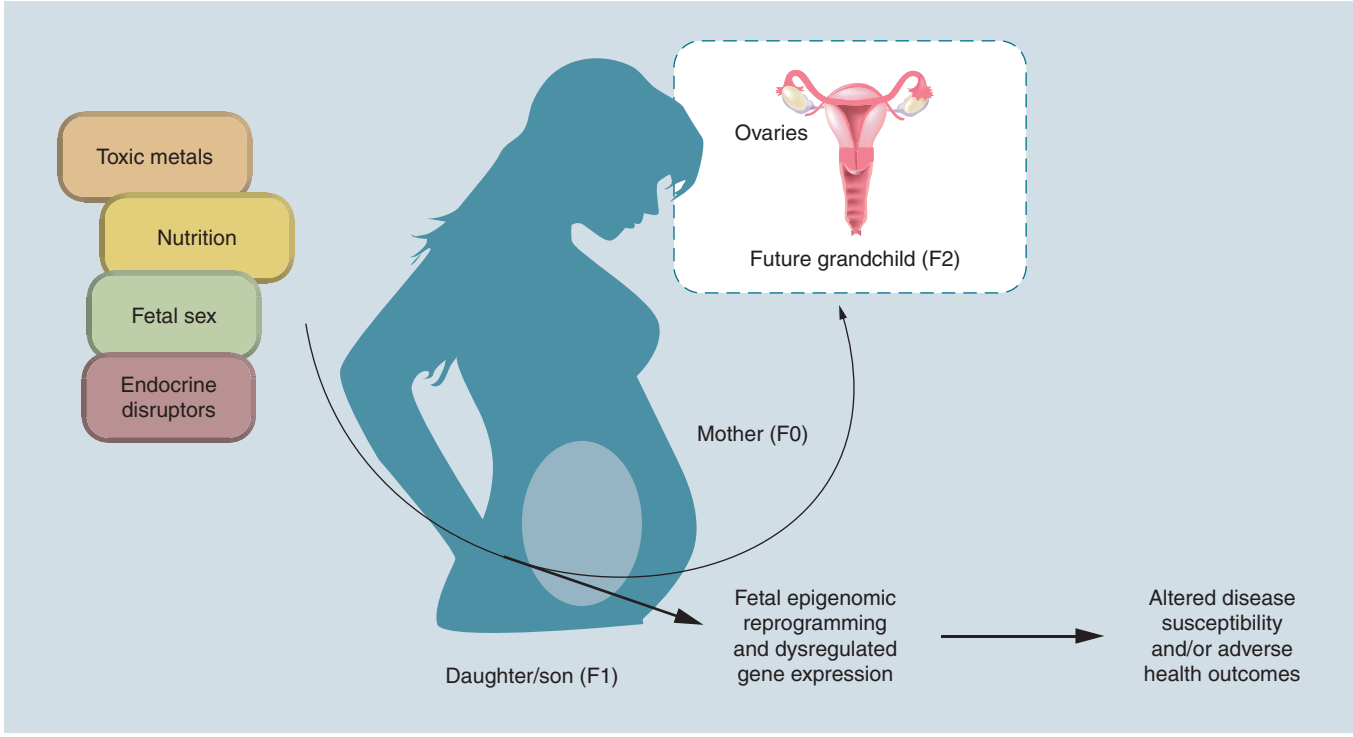

Figure 1. Schematic of environmentally-induced fetal epigenomic reprogramming. Exposure to toxic metals and endocrine disruptors occurring during the prenatal period may result in fetal epigenomic reprogramming. The impacts of exposure on the fetal epigenome depend, in part, on factors such as nutrition and fetal sex. There is evidence that fetal epigenomic reprogramming is associated with altered disease susceptibility and may increase the incidence of adverse health outcomes in exposed populations. Finally, if germ cells are exposed to toxic metals and/or endocrine disruptors and undergo epigenomic reprogramming, multi- and transgenerational effects may be noted following prenatal exposure in the F1.

As discussed above, humans are exposed to multi-contaminant mixtures, including metals and endocrine disruptors. In support of this, these compounds have been identified in cord blood together [16,110]. Thus, research focusing on exposure to a single contaminant does not adequately represent the true human experience. Notably, Huen *et al.* did not identify a relationship between DNA methylation and exposures to single classes of persistent organic pollutants. However, when considered together, significant relationships between exposure to DDT/DDE, PBDE and LINE1 methylation emerged [106]. These results indicate that the impact of environmental exposures on the epigenome may depend on simultaneous exposures, underscoring the need for continued research into mixtures. Additionally, it is important to point out that while described as two separate classes of contaminants in this review, toxic metals and endocrine disruptors are not mutually exclusive. Several toxic metals, including iAs, Cd, Pb and Hg have complex modes of action that include endocrine disrupting effects [70]. Despite the overlaps between these toxic metals and endocrine disruptors, no studies were identified that examined co-exposure to metals and endocrine disruptors.

In addition to considering co-exposures, another important factor underlying epigenomic reprogramming is nutritional status. Imbalances in micronutrients involved in one-carbon metabolism have been tied to dysregulated CpG methylation, resulting from their role as methyl donors [143]. In line with these observations, maternal nutrition also has a significant impact on the fetal epigenome, as well as developmental outcomes in offspring [144]. The relationship between micronutrients, environmental exposures and epigenomic reprogramming has been well-documented with respect to iAs exposure in human populations. For instance, in

Executive summary

Background

- There are three major epigenetic modifications including: DNA (CpG) methylation, microRNA expression and histone modifications.
- The fetal epigenome is impacted by environmental exposures, including toxic metals and endocrine disruptors.

Toxic metals

- Prenatal exposure to arsenic, cadmium, lead and mercury is associated with epigenomic alterations in the offspring.
- The epigenetic changes resulting from *in utero* toxic metal exposure are associated with functional changes in gene expression, birth outcomes and later life health outcomes.

Endocrine disruptors

- Exposure to bisphenol-a, dichlorodiphenyltrichloroethane, polybrominated diphenyl ethers, polychlorinated biphenyls and phthalates is associated with epigenetic changes in the offspring.
- Exposure to endocrine disruptors has been investigated with respect to global methylation, with tissue-specific effects identified. Less research is available with respect to gene-specific CpG methylation and miRNA expression.

Co-exposures/mixtures

- In human populations, exposures are likely to occur in mixtures.
- Relationships between exposure and the fetal epigenome may not be fully elucidated until mixtures are considered.
- Few studies have examined the effects of co-exposures to metals and/or endocrine disruptors on the fetal epigenome and further research is needed.

Contributions of animal models & *in vitro* experiments

- Animal models provide an important tool for discerning between the contributions of pre- and postnatal exposure to toxic metals and endocrine disruptors to epigenomic reprogramming.
- Animal models allow for assessment of target tissues and serve as model organisms in which transgenerational impacts of environmental exposures can be more readily examined.
- Animal models and *in vitro* experiments enable examination of mechanistic underpinnings of epigenomic reprogramming induced by exposure to toxic metals and endocrine disruption.

Conclusion & future perspective

- Prenatal exposures to toxic metals and endocrine disruptors are associated with birth outcomes and later life health effects.
- Exposure-induced epigenetic changes may underlie these effects as these compounds have been associated with changes to global methylation, CpG methylation and miRNA expression.
- Uncertainties remain about the mechanistic linkage between epigenetic changes observed in fetal tissues and adverse outcomes that may develop in distinct tissues.

populations with chronic iAs exposure, nutrition is a significant modifier of the relationship between exposure, epigenomic reprogramming, and health outcomes [145–147]. Importantly, folate supplementation has also been observed to reduce levels of more harmful arsenical species, indicating its potential as an intervention to mitigate the harms of iAs exposure [148]. Relationships between exposure to nutritional factors, such as folate and Vitamin D, have also been observed with respect to endocrine disruptors [149,150]. These relationships suggest that such nutritional factors may also modify the relationship between exposure to other toxic metals, endocrine disruptors and epigenomic reprogramming. This complicates the relationship between exposure to toxic metals, endocrine disruptors and the fetal epigenome and the ability to disentangle these effects. However, it also demonstrates that nutrition is an important factor to consider when studying the relationship between toxic metals, endocrine disruptors and the fetal epigenome. Moreover, it is especially important to consider when generalizing these results across populations. Changes to the epigenome provide an explanation for the persistent health effects that are observed following *in utero* exposures to environmental contaminants, such as toxic metals and endocrine disruptors. The research reviewed here demonstrates that the fetal epigenome displays changes associated with such exposures and is summarized in Figure 1. Some of these changes have also been associated with dysregulated downstream signaling and adverse birth outcomes, demonstrating the link between environmentally induced epigenetic reprogramming and adverse outcomes. However, questions remain about the functional consequences of these observations. Only a few studies reviewed here examined the relationship between differentially regulated epigenetic marks and downstream gene expression. Moreover, further research is needed to determine how CpG methylation and/or miRNA expression within the placenta or cord blood relate to disorders emerging within separate tissues. More work is needed to strengthen the links between exposure-associated epigenomic changes and adverse health outcomes.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/epi-2016-0112

Acknowledgements

The authors would like to thank C Reed for her assistance with the figures.

Financial & competing interests disclosure

This research was supported by grants from the National Institute of Environmental Health Sciences (T32ES007018 and P42ES005948). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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