

Epigenetic studies in Developmental Origins of Health and Disease: pitfalls and key considerations for study design and interpretation

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The field of Developmental Origins of Health and Disease (DOHaD) seeks to understand the relationships between early-life environmental exposures and long-term health and disease. Until recently, the molecular mechanisms underlying these phenomena were poorly understood; however, epigenetics has been proposed to bridge the gap between the environment and phenotype. Epigenetics involves the study of heritable changes in gene expression, which occur without changes to the underlying DNA sequence. Different types of epigenetic modifications include DNA methylation, post-translational histone modifications and non-coding RNAs. Increasingly, changes to the epigenome have been associated with early-life exposures in both humans and animal models, offering both an explanation for how the environment may programme long-term health, as well as molecular changes that could be developed as biomarkers of exposure and/or future disease. As such, epigenetic studies in DOHaD hold much promise; however, there are a number of factors which should be considered when designing and interpreting such studies. These include the impact of the genome on the epigenome, the tissue-specificity of epigenetic marks, the stability (or lack thereof) of epigenetic changes over time and the importance of associating epigenetic changes with changes in transcription or translation to demonstrate functional consequences. In this review, we discuss each of these key concepts and provide practical strategies to mitigate some common pitfalls with the aim of providing a useful guide for future epigenetic studies in DOHaD.

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Introduction

Early-life environmental exposures are thought to influence organ development and physiology such that there is an increased risk of disease in later life.^{1,2} Growing evidence suggests that early-life exposures can also impact the epigenome. Epigenetics has been defined in a number of ways^{3–5} and the field has yet to reach a generally accepted consensus. For the purposes of this review, we define epigenetics as the study of heritable changes in gene expression, which occur without changes to the underlying DNA sequence.⁶ Epigenetic marks have the capacity to be stably inherited through successive mitotic cell divisions, providing a possible molecular ‘memory’ of the exposure, and can be associated with altered gene expression, thereby affecting phenotype. As such, epigenetics has the potential to both further our understanding of the mechanisms which underlie the link between early-life exposures and later health outcomes, and to produce novel molecular biomarkers of past exposure and/or future disease.⁷ Here, we cover essential concepts for epigenetic studies in Developmental Origins of Health and Disease (DOHaD),

identify potential hazards in study design and interpretation, and highlight strategies for the generation of informative and meaningful results.

Epigenetics

DNA methylation

Within mammals, cytosine methylation is the most well-characterized DNA modification. Cytosine methylation occurs most frequently within cytosine phosphate guanine (CpG) dinucleotides, with 70–80% of CpGs within the human genome methylated.⁸ Non-CpG cytosine methylation also occurs but is tissue-specific, with higher levels reported in oocytes,⁹ pluripotent cells¹⁰ and various regions of the brain.^{11–13}

Cytosine methylation is catalyzed by the highly conserved DNA methyltransferase (DNMT) family of proteins.^{14–16} DNMT1 has a higher affinity for hemi-methylated than unmethylated DNA and is responsible for propagating methylation after DNA replication, thus acting to maintain methylation states.¹⁴ DNMT3A and DNMT3B are essential for the establishment of new, or *de novo* methylation marks.¹⁵ Although DNMT3L lacks catalytic activity,¹⁷ it can bind to and stimulate the catalytic activity of DNMT3A and DNMT3B.^{18,19}

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Until recently, little was known about how DNA was demethylated. Several mechanisms had been proposed for the active demethylation of DNA, including those involving DNA deamination by methyl-CpG-binding domain protein 4 (MBD4) and glycosylation^{20,21} and cytosine deamination by DNMTs.^{22,23} There is a growing body of literature supporting another mechanism mediated by the Ten-Eleven Translocation family of proteins, which sequentially hydroxylate 5-methylcytosine to 5-hydroxymethylcytosine, 5-formylcytosine and finally to 5-carboxylcytosine.^{24–26} Through thymine DNA glycosylation followed by base excision repair, 5-carboxylcytosine is then converted back to the unmodified cytosine.²⁵

Passive, replication-dependent DNA demethylation can also occur.²⁷ In the early preimplantation mouse embryo, it was observed that chromosome methylation was iteratively lost with each cycle of DNA replication.²⁸ This followed an earlier observation that DNMT1 was excluded from the cell nucleus in the very early stages of embryogenesis following fertilization, and only observable in the nucleus from the eight-cell stage.^{29,30} The absence of a maintenance DNMT from the nucleus would result in a passive reduction in global methylation state with every cell division.

CpG-rich regions, called CpG islands, are often found at the 5' promoter region of genes,³¹ and methylation of these regions is associated with transcriptional silencing.^{32,33} Promoter methylation is believed to prevent transcriptional initiation.³⁴ In contrast, intragenic CpG methylation in mammalian cells has little effect on transcriptional initiation, instead discouraging transcriptional elongation.³⁵ Intragenic methylation can also aid in exon recognition, playing a role in the regulation of alternative splicing.^{36,37}

DNA methylation appears to be responsive to the environment, with alterations in DNA methylation patterns reported in both humans and animals following a range of adverse early-life exposures, including those of malnutrition, alcohol, choline and arsenic.^{38–44} The DOHaD field has historically focused on promoter DNA methylation; however, the use of unbiased genome-wide screens for DNA methylation has identified associations between early-life exposures and methylation of non-promoter regions such as enhancers⁴⁵ as well as intergenic regions.^{46,47} Mechanistically, there is some evidence that early-life exposures to alcohol and choline deficiency in rodents can alter the expression of the maintenance methyltransferase *Dnmt1*.^{41,48,49} One-carbon metabolism is a network of pathways involved in a number of functions, including the synthesis of methionine which can subsequently be adenosylated to *S*-adenosyl methionine – a major source of methyl groups necessary for DNA methylation.⁵⁰ One-carbon metabolism may also mediate environmentally induced changes to DNA methylation as it can be perturbed by early-life exposures to alcohol⁵¹ and maternal smoking⁵² as well as by alterations in gestational maternal intake of methyl donors such as choline and folate.^{48,53–55} Disrupting either *Dnmt1* levels or one-carbon metabolism would be expected to impact DNA

methylation genome-wide. Although global alterations to DNA methylation have been reported following certain exposures,⁵⁵ many exposures fail to induce such changes, instead resulting in locus-specific effects.^{45,48,56} How perturbations to *Dnmt1* expression or one-carbon metabolism could induce locus-specific methylation changes remains unclear, and further study is required to understand the mechanisms by which the environment influences the methylome.

Post-translational histone modifications

In the nucleus, DNA is packaged into chromatin, the individual building blocks of which are nucleosomes. Within the nucleosome, DNA is wrapped around a protein octamer, comprising of two each of histone H2A, histone H2B, histone H3 and histone H4.⁵⁷ Although the C-terminal domains of histones are critical for the maintenance of nucleosome structure,⁵⁷ the N-terminal tails function to alter the accessibility of the associated DNA.⁵⁸ The N-terminal tail of any histone can, at specific amino acid positions, undergo chemical modifications including acetylation, methylation,⁵⁹ phosphorylation,⁶⁰ ubiquitylation,⁶¹ carbonylation,⁶² poly (ADP-ribosyl)ation⁶³ or sumoylation.⁶⁴ These modifications are thought to alter chromatin structure by affecting electrostatic interactions between the DNA and histones, making them either more, or less tightly packaged and permissive of transcription. In addition, modified histones can be recognized by and directly interact with various proteins which can further modify the histones and/or affect chromatin structure.⁶⁵ For example, the recognition of histone H3 lysine 4 trimethylation (H3K4me3) by inhibitor of growth 2 results in the recruitment and stabilization of the mSin3a-histone deacetylase 1 (HDAC1) complex at the gene promoter.⁶⁶ In contrast, SWItch/sucrose non-fermentable is a chromatin remodeling complex which recognizes acetylated histones.⁶⁷

The histone code hypothesis posits that the combinatorial identity and position of each N-terminal tail modification acts as a code, controlling transcription in a highly specific manner.⁶⁸ Individually, H3K4me3 at gene promoters is associated with transcriptional activation,⁶⁹ whereas trimethylation of lysine 27 at histone H3 (H3K27me3) is associated with transcriptional repression.⁷⁰ However, bivalent domains that have both activating (H3K4me3) and repressive (H3K27me3) marks simultaneously also exist.⁷¹ Occurring near gene promoters, bivalent domains are thought to poise genes for expression.⁷¹

Though epigenetic studies in DOHaD have primarily focused on DNA methylation, post-translational histone modifications are also subject to the influence of the early-life environment.^{41,72–74} The mechanism by which this occurs; however, is yet to be fully understood. Gestational choline deficiency has been identified to alter the expression of genes involved in the conferral of histone modifications, including the histone lysine methyltransferase *Set domain bifurcated 1* (*Setdb1*) and *histone methyltransferase G9a* (*Kmt1c*).^{41,75} One-carbon metabolism may also be involved as *S*-adenosyl

methionine, which in addition to being required for DNA methylation, also contributes to the post-translational methylation of histone tails.⁵⁰ Given that early-life exposures can influence one-carbon metabolism,^{48,51–55} there is potential for these exposures to have consequences on histone as well as DNA methylation but, again, widespread changes might be expected. In support of this, global changes to histone methylation have been reported in rodents following various early-life exposures, including those of gestational choline deficiency⁷⁶ and nicotine exposure.⁷⁷ In contrast, locus-specific effects were observed when H3K4me3 was assayed by chromatin immunoprecipitation and next generation sequencing in the dentate gyrus of inbred C57BL/6 mice following an early-life exposure to arsenic.⁷⁸ Further, a maternal high-fat diet produced coding region-specific changes in histone H3 lysine 9 trimethylation at the rat *Wingless-type MMTV integration site family member 1* (*Wnt1*) gene in offspring liver,⁷⁹ suggesting the presence of mechanisms which allow for conferral of locus- and region-specificity. Therefore, while it is evident that changes in post-translational histone modifications are associated with early-life exposures, further study is required to elucidate both how this occurs and how it impacts offspring health.

Non-coding RNAs

Non-coding RNAs can also affect gene expression, either by transcriptional or post-transcriptional mechanisms. Long non-coding RNAs influence gene expression using a wide array of mechanisms.⁸⁰ For example, the *Antisense Igf2r RNA* (*Air*) long non-coding RNA accumulates at the *Slc22a3* promoter and recruits the histone H3K9 methyltransferase G9a protein, thereby inducing locus-specific transcriptional repression in the placenta.⁸¹ For a more detailed discussion on the various mechanisms by which long non-coding RNAs influence transcription, we direct the reader to the review by Wang *et al.*⁸⁰

Small non-coding RNAs can interact with nascent transcripts as well as with single- and double-stranded DNA in a sequence-specific manner.⁸² The major categories of small non-coding RNAs include endogenous short interfering RNAs (which are presently poorly characterized in mammals⁸³), P-element induced wimpy testis-interacting RNAs (PIWI-interacting RNAs or piRNAs; expressed primarily in germ cells⁸⁴), and microRNAs (miRNAs) (relatively well characterized and expressed in many tissues⁸⁵).⁸⁶ Of these small non-coding RNAs, miRNAs have been the subject of particular interest within the DOHaD field and as such, the remainder of this discussion will focus on miRNAs.

miRNAs are ~22 nucleotides in length, and bind to the 3' untranslated region (UTR) of target messenger RNAs (mRNA) in order to post-transcriptionally regulate their stability and/or translation into protein.⁸⁷ miRNAs regulate target mRNA levels by cleavage⁸⁸ or degradation.⁸⁹ Although some studies have reported exclusive effects of miRNAs on protein translation,^{90,91} others have reported instances whereby miRNAs first inhibit protein translation, and are then

subsequently involved in mRNA deadenylation and decay.⁹² The extent of base-pair complementarity between the miRNA and target mRNA can influence whether a given miRNAs inhibits or aids translation.⁹³ Further, *Let-7* and a synthetic miRNA (miRcxcr4) were identified to upregulate translation at certain points during the cell cycle, but at other times, the same miRNAs repressed translation of the same target reporter construct.⁹⁴

miRNAs were previously estimated to regulate between 20 and 30% of human genes.^{95,96} However, since the initial estimates were generated, a large number of new miRNAs have been discovered, rendering the figures relatively conservative. Each miRNA has been estimated to target 100–200 mRNAs, with miRNAs likely to act coordinately to aid in the regulation of any given target gene.^{97,98}

An increasing number of studies within the DOHaD field are reporting changes in the expression of both long non-coding RNAs⁴² and miRNAs^{99–101} following an early-life exposure. A number of studies have also begun to identify circulating miRNAs, in plasma and serum, as potential biomarkers of various early-life exposures.^{101–104} Interest in non-coding RNAs in the context of DOHaD is relatively recent, and consequently little is known about either the mechanisms by which they are regulated or the downstream functional consequences.

Interactions between epigenetic modalities

In DOHaD studies, the epigenetic modalities of DNA methylation, histone modifications and non-coding RNAs are often considered in isolation, but there is substantial evidence that they regulate gene expression in concert with each other.

DNA methylation and histone modifications

The co-dependent nature of DNA methylation and histone modifications was nicely demonstrated when Zhang *et al.*¹⁰⁵ observed that in order to achieve complete demethylation and activity of the *luteinizing hormone receptor* promoter *in vitro*, the addition of both a histone deacetylase inhibitor (trichostatin A) and a DNA demethylating reagent (5-azacytidine) were required. These results built on earlier findings in which the binding of methyl-CpG-binding protein 2 (MeCP2) to methylated DNA was shown to recruit histone deacetylases to impact locally on histone acetylation and chromatin structure.^{106–108}

Likewise, both histone modifications themselves as well as the proteins responsible for conferring histone modifications can impact DNA methylation. For example, protein arginine methyltransferase 5 (PRMT5) confers symmetric methylation of arginine 3 at histone H4 (H4R3me2s) which then acts as a binding target for DNMT3A.¹⁰⁹ Oocytes deficient in a H3K4 demethylase (KDM1B) exhibited genome-wide DNA hypomethylation, suggesting a critical role of H3K4 demethylation in DNA methylation regulation; however, the exact mechanism for this has not yet been elucidated.¹¹⁰ The histone methyltransferase Enhancer of Zeste homolog 2 (EZH2)

directly recruits DNMTs,¹¹¹ and DNMT3L can bind to histone H3 when its lysine 4 is unmethylated, inducing *de novo* DNA methylation by DNMT3A2.¹¹²

For further information on the complex interplay between DNA methylation and histone modifications, we direct the reader to several reviews.^{113–115}

DNA methylation and non-coding RNAs

DNA methylation can regulate the expression of non-coding RNAs including miRNAs.¹¹⁶ In turn, DNA methylation itself can also be influenced by various types of non-coding RNAs. DNA methylation can be directed in a sequence-specific manner through the direct interaction of DNMTs with long non-coding RNAs, including *Tsix* (the antisense transcript of *Xist*) and numerous promoter-associated non-coding RNAs.^{117–121} The imprinted *H19* non-coding RNA also indirectly regulates the activity of DNMT3B by binding to *S*-adenosylhomocysteine hydrolase, thereby interfering with the hydrolysis of *S*-adenosylhomocysteine – an inhibitor of DNMT3B.¹²²

Short non-coding RNAs can also influence DNA methylation. When MitoPLD, a protein involved in primary piRNA synthesis was mutated in mice, the *de novo* DNA methylation of the *RAS protein-specific guanine nucleotide-releasing factor 1* (*Rasgrf1*) differentially methylated region (DMR) in spermatogonia was impaired, suggesting a role for piRNAs in *de novo* DNA methylation.¹²³ Similarly, the PIWI proteins MILI and MIWI2, which interact with piRNAs, were essential for the establishment of *de novo* methylation of retrotransposons in male fetal germ cells.¹²⁴ A number of miRNAs, which include miR-148a and miR-152, are able to directly target the expression of *Dnmt1*.^{125,126}

Histone modifications and non-coding RNAs

Although histone modifications can regulate the expression of non-coding RNAs^{127,128}, non-coding RNAs themselves are also capable of directing histone modifications.^{129,130} The long non-coding RNA, HOTAIR, facilitates the conferral of histone modifications to the *Homeobox D cluster* by acting as a scaffold for both the polycomb repressive complex 2 (PRC2) and the Lysine-specific demethylase 1 (LSD1)/coRepressor element-1 silencing transcription factor (coREST)/RE1-silencing transcription factor (REST) complex, which in turn recruit enzymes to trimethylate histone H3 lysine 27 and demethylate histone H3 lysine 4, respectively.^{131,132} A number of histone modifying enzymes, including HDAC1¹³³ and EZH2,¹³⁴ have also been identified to be direct targets of miRNAs.

For further information regarding the interaction between non-coding RNAs and the other epigenetic modalities, we direct the reader to other reviews.^{129,130}

Epigenetic reprogramming in mammals

There are two major developmental periods, preimplantation development and gametogenesis, when the epigenome is erased

and reset genome-wide in a process called epigenetic reprogramming.^{135–138} It has been proposed that the epigenome is most susceptible to environmental exposures during these periods of epigenetic reprogramming.³⁹

In preimplantation development, DNA methylation changes include an initial global demethylation event post-fertilization,¹³⁹ in which paternally derived DNA undergoes active demethylation,¹⁴⁰ whereas maternally derived DNA undergoes replication-dependent passive demethylation.²⁸ Methylation is then re-established *de novo* from implantation onwards (approximately gestational day 4.5 in the mouse), with somatic tissues becoming increasingly methylated.¹⁴¹ This reprogramming of the epigenome in the preimplantation embryo is necessary to allow cells of the early embryo to achieve a state of pluripotency,¹⁴² and to then set up distinct patterns of gene expression that are associated with differentiation and cell fate determination.

Imprinted genes, which are resistant to preimplantation epigenetic reprogramming, have been the subject of a number of studies in the DOHaD field.¹³⁹ Although most genes are expressed from both the maternally and paternally derived alleles (biallelic expression), imprinted genes are expressed monoallelically – that is, exclusively from either the maternally or paternally derived allele.¹³⁹ Imprinting is a known epigenetic process; many of these genes have well-characterized DMRs that are associated with and thought to control monoallelic expression. The finding that imprinted gene DMR methylation in somatic tissues can be altered by gestational environmental exposures^{143,144} appears at odds with their resistance to pre-implantation epigenetic reprogramming; however, it is possible that the exposure compromises this resistance. Interestingly, other studies have identified no changes in imprinted gene DMR methylation in response to gestational environmental perturbations, despite identifying changes in expression,^{145,146} leading to speculation that these expression changes are due to transcription-factor-mediated mechanisms rather than epigenetic mechanisms.¹⁴⁶ Indeed, the idea that imprinted regions are of no greater importance than any other genomic region in the epigenetic response to early-life exposures has been extensively discussed in a recent review.¹⁴⁷

Later in development, a second major epigenetic reprogramming event occurs during gametogenesis.¹³⁹ This reprogramming event involves the removal of parent-of-origin DNA methylation from imprinted loci, allowing for the establishment of new sex-specific methylation patterns, such that the alleles are imprinted with either a maternal pattern (in oocytes) or a paternal pattern (in sperm). This erasure of parental imprints begins during the migration of primordial germ cells to the genital ridge (from approximately gestational day 9.5 to 11.5 in the mouse).¹⁴⁸ In murine male germ cells, DNA remethylation occurs when these primordial germ cells become prospermatogonia (from approximately gestational day 13 in the mouse) and is completed by birth.¹⁴⁹ In contrast, DNA remethylation in female germ cells does not commence until after birth, occurring during preovulatory oocyte growth

and maturation.^{135,150} Given that the timing of DNA remethylation is sexually dimorphic in nature, sensitivity to environmental exposures may also differ between sperm and oocytes. Furthermore, gametogenesis may be a period during which imprinted genes may be most sensitive to exposures. Specifically, differences in imprinting may be most likely to occur in offspring derived from males exposed during late gestation or offspring derived from females exposed in the preconceptional period (during oocyte maturation).

Given the difficulty of obtaining germ cell and/or preimplantation embryo samples from humans, much of the knowledge pertaining to epigenetic reprogramming has been obtained using murine models. Nonetheless, a small number of studies utilizing human samples have found that many of these processes appear to be conserved between species.^{151,152}

Mammalian epigenetic reprogramming is a complex phenomenon, and while a very brief overview is provided here, we direct the reader to more comprehensive reviews for further information.^{135–138}

Considerations for epigenetic studies in DOHaD

As much promise as epigenetics has in unraveling the molecular mechanisms underlying adverse health outcomes following early-life environmental exposures, there are a number of challenges when conducting these studies. In this section, we discuss the central challenges in epigenetic studies in both humans and animal models, how they may impact the interpretation of results and highlight strategies to mitigate some of these issues (summarized in Tables 1 and 2).

The epigenome is influenced by the genome

In addition to the extrinsic influence of the environment, the epigenetic landscape is also shaped intrinsically by the underlying DNA sequence. For example, the comparison of adolescent and middle-aged monozygotic and dizygotic twins

suggested a greater contribution of genetics than environmental factors to DNA methylation at the imprinted *Insulin-like growth factor 2 (IGF2)* DMR.¹⁵³ Genetic influences on DNA methylation have also been reported genome-wide. When two well-characterized inbred mouse strains, C57BL/6 and BALB/c, with several hundred differentially methylated loci were mated, the F1 hybrid (C57BL/6xBALB/c) offspring exhibited strain-specific methylation patterns on each allele, likely driven by the local genomic context in *cis*.¹⁵⁴

In humans, widespread associations between single nucleotide polymorphisms and DNA methylation have been reported in methylation quantitative trait loci studies.^{155–157} The underlying mechanisms as to how these genetic variants influence methylation remain poorly understood; however it has been proposed that the creation or disruption of CpG sites,¹⁵⁵ or perturbations in transcription factor binding¹⁵⁶ may be involved. Another way in which the genome can influence the epigenome is via functional mutations within genes which contribute to the establishment or maintenance of epigenetic marks. For example, the 5, 10-methylenetetrahydrofolate reductase (*MTHFR*) gene encodes an enzyme critical for the supply of methyl donors for reactions such as DNA methylation. The C677T polymorphism within the human *MTHFR* gene results in reduced MTHFR activity¹⁵⁸ and is associated with genomic DNA hypomethylation in peripheral leucocytes.^{159,160} Similarly, the R271Q polymorphism within the *DNMT3L* gene in humans is another example of a polymorphism resulting in DNA hypomethylation.¹⁶¹ There is limited literature to suggest that histone modifications too may be dependent on the local genomic context.¹⁶² Consequently, in DOHaD studies utilizing genetically heterogeneous populations such as humans or outbred animals, it can be difficult to distinguish between epigenetic changes driven by the environment and those driven by genetic differences, unrelated to the environment.

In both humans and outbred animals, genetic differences can be accounted for, if not controlled for, by

Table 1. Strategies to address common challenges in epigenetic studies

Challenges	Possible solutions
Genetic heterogeneity	<ul style="list-style-type: none"> • Use inbred strains of animals • Identify genetic polymorphisms: <ul style="list-style-type: none"> ◦ Identify known single nucleotide polymorphisms using online databases ◦ Genotyping samples ◦ Whole-genome sequencing
Functional consequences	<ul style="list-style-type: none"> • Assay messenger RNA and protein levels as well as phenotype
Cell- and tissue-specificity	<ul style="list-style-type: none"> • Assay epigenetic modifications in multiple tissues to determine tissue-specificity or lack thereof • Purify and assay single types of cells • Define the cellular composition of the tissue to be assayed • Use bioinformatics algorithms to adjust for cellular heterogeneity
Temporal-specificity	<ul style="list-style-type: none"> • Evaluate temporal stability or lack thereof

Table 2. A selection of recent DOHaD publications that have addressed two or more of the challenges highlighted in this review. Black boxes denote the challenge addressed by the study

First author (year)	Species (strain)	Early-life exposure	Epigenetic modification	Genetic heterogeneity	Cell- or tissue-specificity	Temporal-specificity	Functional consequences
Novakovic (2014)	Human	Smoking	DNA methylation				
Breton (2014)	Human	Tobacco smoke	DNA methylation				
Kundakovic (2013)	Mouse (BALB/c)	Bisphenol A	DNA methylation				
Silver (2015)	Human	Peri-conceptual environment	DNA methylation				
Mehedint (2009)	Mouse (C57BL/6)	Choline deficiency	DNA methylation, histone modifications				
Zhang (2015)	Mouse (C57BL/6J)	Ethanol	DNA methylation, histone modifications, ncRNAs				
El Hajj (2013)	Human	Gestational diabetes mellitus	DNA methylation				
Amarasekera (2014)	Human	Folate	DNA methylation, histone modifications				
Kile (2014)	Human	Arsenic	DNA methylation				
Downing (2011)	Mouse (C57BL/6)	Ethanol/methyl-supplementation	DNA methylation				
Laufer (2013)	Mouse (C57BL/6J)	Ethanol	DNA methylation, ncRNAs				
Basil (2014)	Mouse (C57BL/6N)	Immune activation	DNA methylation				
Thompson (2010)	Rat (Sprague-Dawley)	Intrauterine growth restriction	DNA methylation				
Begum (2013)	Sheep (Romney)	Undernutrition	DNA methylation, histone modifications				

assaying genotype.^{162–165} In rodents, genetic differences can be minimized through the use of inbred strains. A recent study identified few genetic or epigenetic differences of gross magnitude between C57BL/6 littermates.¹⁶⁶ However, even this approach is not without problems. Using inbred mice, Shea *et al.*¹⁶⁷ recently identified variation in DNA methylation at ribosomal DNA repeats in C57BL/6 sperm, which seemingly correlated with paternal diet. Upon further analysis, the difference in methylation was found to be an artifact of copy number variation at this repetitive element, and unrelated to paternal diet.¹⁶⁷ Taken together, the evidence supports the importance of considering the contribution of genetic variation to epigenetic variation, even in inbred animals.

Epigenetic modifications are cell- and tissue-specific

Although each individual tends to have minimal genetic variation across tissues, the epigenome is reflective of the different and often dynamic transcriptional identities of tissues, and even individual cells. As such, any given individual, despite having only one genome, can have numerous epigenomes.^{12,168–173} When the methylomes of six regions from the brain as well as that of whole blood were compared in nine human donors by immunoprecipitation of methylated DNA followed by next generation sequencing, greater differences were observed between tissues within an individual than between the same tissue across individuals.¹⁷¹ Even within tissues, cell-to-cell variation in DNA methylation has been reported.^{174,175}

Similarly, when epigenetic changes are identified in one tissue following an early-life exposure, comparable changes are not assured in other tissues. Maternal smoking during

pregnancy was associated with hypomethylation of the *Aryl hydrocarbon receptor repressor (AHRR)* gene in newborn cord blood mononuclear cells, but not in buccal epithelium or placental tissue.¹⁷⁶ This example of a tissue-specific response is not an isolated case, with methylation of long interspersed element 1 (LINE1) repeats, measured as a proxy for global DNA methylation, hypomethylated in the hypothalamus but not the striatum in offspring of a mouse model of prenatal maternal immune activation.¹⁷⁷ Furthermore, Kundakovic *et al.*¹⁷⁸ observed an increase in DNA methylation within the *estrogen receptor 1 (Esr1)* gene in the prefrontal cortex, but not hypothalamus of male offspring from an inbred BALB/c mouse model of prenatal bisphenol A exposure. In the offspring from an ovine model of maternal undernutrition, despite the differential methylation of the glucocorticoid receptor gene in various regions of the brain, no evidence of differential methylation of this gene was identified in leucocytes.¹⁷⁹ Therefore, caution is recommended particularly when inferring the epigenetic state of a disease-relevant, but inaccessible tissue based on the epigenetic state of another more accessible tissue.

The relatively accessible nature of whole blood makes it a commonly assayed tissue in many human studies of DNA methylation following an early-life environmental exposure.^{56,180,181} There is however, a growing body of literature reporting considerable epigenetic heterogeneity within whole blood, reflective of its diverse cellular composition.^{182,183} When cellular composition was corrected for *in silico* in five published studies examining age-related DNA methylation changes, cellular composition explained a greater proportion of the reported epigenetic variation in these studies than did age.¹⁸⁴ Subsequently, Bauer *et al.*¹⁸⁵ found that a

previously reported association between tobacco smoking and DNA methylation at the *G protein-coupled receptor 15 (GPR15)* locus was in fact an artifact of increased numbers of CD3+ T-cells in the smoking population. Therefore, any study of epigenetic changes in whole blood or white blood cells should consider the cellular composition of these samples.

There are a number of approaches available to reduce the confounding influence of cellular heterogeneity. One solution is to sort samples into individual cell types; however, this will significantly reduce the amount of tissue available for analysis. As an alternative to this, a number of computational methods have been developed to estimate and account for differences in cellular distributions in heterogeneous tissues such as blood and brain, utilizing previously defined DNA methylation signatures for each cell type.^{186–190} These methods have been applied in some studies examining DNA methylation following early-life exposures.^{43,44,181} Future DOHaD studies will undoubtedly continue to assay blood and other heterogeneous tissues; however, the consideration of cellular heterogeneity should aid the meaningful interpretation of any epigenetic changes identified.

Epigenetic modifications are not necessarily stable over time

In addition to being influenced by genetics and cellular identity, the epigenome may also change with time. Global DNA methylation in the livers of male C57BL/6 mice was found to gradually decline from the ages of 6–24 months,¹⁹¹ suggesting that aging can alter methylation profiles. A similar study of DNA methylation differences in lymphocytes between monozygotic twins ranging in age from 3 to 74 found that while younger twins had relatively few epigenetic differences, the magnitude of difference within twin pairs increased with age across multiple tissues.¹⁹² In this study, the contribution of genetic heterogeneity to epigenetic variation was reduced through the utilization of monozygotic twins.¹⁹² Similar trends in methylation differences were also observed in four separate tissue types, suggesting that the impact of cellular composition on the epigenetic profiles was minimal.¹⁹² It is possible, even likely, that in addition to aging, the observed methylation changes reflect differences in postnatal exposures between twins over life. Regardless of the underlying causes, these studies draw attention to the idea that DNA methylation patterns can change over time.

The stability of epigenetic modifications over time also appears to be gene-dependent. When methylation of DNA isolated from saliva samples from adolescent monozygotic twin pairs were assayed before and after a period of several months, Levesque *et al.*¹⁹³ found that even in this relatively short period of time, the methylation of 46 genes was unstable, whereas 226 genes were identified to be temporally stable. Another study reported similar outcomes, with DNA methylation at five of eight candidate loci studied longitudinally over 11–20 years deemed stable in both whole blood and buccal cells.¹⁹⁴ In this study, the authors were able to account for genetic

heterogeneity by assaying for sequence variation, and for cellular heterogeneity using computational methods.¹⁹⁴

Most DOHaD studies assay for epigenetic changes in samples collected at a single time-point,^{38,40–43,45–47} often far removed from the environmental exposure itself. The evidence presented above highlights the importance of assaying for epigenetic changes on multiple occasions, and demonstrating stability over time, especially if the epigenetic marks are proposed to confer a memory of the exposure or to serve as biomarkers.

The relationship between epigenetics and gene expression can be ambiguous

Although DNA methylation patterns are often linked with transcriptional activity, this is not always the case. Indeed, in eight human tissues, 5'UTR DNA methylation status was inversely correlated with transcription for only 37% of the 43 genes analyzed.¹⁹⁵ Similarly, a paternal low-protein diet was associated with considerable changes in both mRNA and miRNA expression as well as DNA methylation in offspring livers; however, the genes at which promoter methylation was altered were not necessarily those which displayed differential expression.¹⁹⁶ In a separate study, the differential methylation of 181 gene promoters in pancreatic islets from patients with type 2 diabetes and healthy controls correlated with altered transcription for only 18% of the genes.¹⁹⁷ It must be acknowledged that an epigenetic effect on adjacent locus expression at a different time-point cannot be excluded, nor an effect on the expression of other, more distantly located, genomic loci. In these instances however, such outcomes would need to be demonstrated experimentally.

A disconnect between transcriptional activity and DNA methylation has been observed at imprinted loci as well. The differential expression, but not DNA methylation, of a number of imprinted genes was identified in the livers of offspring exposed to gestational protein restriction.¹⁴⁵ Further, when altered expression, but not methylation of the imprinted *paternally expressed 3 (Peg3)* gene was observed in a mouse model of maternal undernutrition, the authors speculated that this was likely due to transcription factor-mediated mechanisms, rather than epigenetic mechanisms *per se*.¹⁴⁶ It is however possible that other epigenetic marks, such as post-translational histone modifications, may still be contributing to these outcomes, even in the absence of changes to DNA methylation.

The finding that mRNA and protein levels are not always positively correlated,^{101,198,199} suggests that protein analyses may also be informative in epigenetic studies in DOHaD. The lack of correlation between mRNA and protein levels can potentially be explained by an epigenetic mechanism: miRNAs. In a model of gestational nutrient restriction, 23 miRNAs associated with the insulin-signaling pathway were identified to be differentially expressed in the liver of fetal lambs.²⁰⁰ The expression of these miRNAs were then found to correlate with target protein but not

mRNA levels,²⁰⁰ suggesting that the miRNAs act on target protein translation rather than mRNA stability.

Our recent study which assayed DNA methylation, histone modifications and miRNA expression, as well as both mRNA and protein levels illustrates the informative potential of broader experimental designs. In this study, hippocampal tissue was assayed from adult male C57BL/6J mice following an early gestational ethanol exposure.¹⁰¹ Both a reduction in DNA methylation and increase in H3K4me3 (a marker of active chromatin) were observed at the promoter region of a vesicular glutamate transporter gene, *Slc17a6*, in ethanol-exposed mice. As would be predicted, these epigenetic changes correlated with an increase in *Slc17a6* mRNA levels. However, when we assayed for the protein encoded by *Slc17a6*, there was a reduction in protein.¹⁰¹ We identified miR-467b-5p, a miRNA predicted *in silico* to target *Slc17a6*, to be differentially expressed in the same tissue, and experimentally validated this interaction using *in vitro* reporter assays. In this study, transcriptional output (mRNA) correlated with both promoter DNA methylation and histone modifications, following which at least one miRNA was proposed to regulate expression at a translational level, demonstrating that an early-life environmental exposure can exert complex, independent effects on gene expression.¹⁰¹

Finally, it is often difficult to discern whether epigenetic marks, such as DNA methylation and post-translational histone modifications which are associated with gene expression changes following an early-life environmental exposure, are a cause or a consequence of the change in transcription. As a result, the relationship between these epigenetic modifications and gene expression is often best described as correlative in nature.

Concluding remarks

The field of DOHaD has historically focused on characterizing the long-term health consequences of early-life environmental exposures. There is emerging evidence that these early-life exposures can affect the epigenome, which has the potential not only to impact gene expression and phenotype but may also be stably remembered for a lifetime. The assays available to investigate the epigenome are now well within the reach of all investigators. This review covers essential concepts in epigenetics which are relevant to the DOHaD field, and highlights potential pitfalls as well as key considerations for study design and interpretation. We look forward to an exciting new era of DOHaD studies which will bring us closer to understanding not only the impact of early-life environmental exposures on health and disease in later life, but also greater knowledge of the role of epigenetics in mediating such phenomena.

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Conflicts of Interest

None.

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