

The Role of Environmental Exposures and the Epigenome in Health and Disease

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Running Title: Environmental Exposures and the Epigenome in Health

Abstract

The genetic material of every organism exists within the context of regulatory networks that govern gene expression, collectively called the epigenome. Epigenetics has taken center stage in the study of diseases such as cancer and diabetes, but its integration into the field of environmental health is still emerging. As the Environmental Mutagenesis and Genomics Society (EMGS) celebrates its 50th Anniversary this year, we have come together to review and summarize the seminal advances in the field of environmental epigenomics. Specifically, we focus on the role epigenetics may play in multi and trans-generational transmission of environmentally induced health effects. We also summarize state of the art techniques for evaluating the epigenome, environmental epigenetic analysis, and the emerging field of epigenome editing. Finally, we evaluate transposon epigenetics as they relate to environmental exposures and explore the role of non-coding RNA as biomarkers of environmental exposures. While the field has advanced over the past several decades, including being recognized by EMGS with its own Special Interest Group, recently renamed Epigenomics, we are excited about the opportunities for environmental epigenetic science in the next 50 years.

Keywords: environmental epigenetics; transposons, DNA methylation; piRNA; TaRGET II

Introduction: The Role of the Environmental Epigenomics in Health and Disease

Epigenetics is the collective term for the study of mitotically heritable and potentially reversible changes in gene expression unrelated to changes to the DNA sequence itself. Epigenetic marks include chromatin modifications (e.g. histone protein acetylation, methylation, and ubiquitination), non-coding long and small RNA (e.g. lncRNA, miRNA, piRNA), and alterations to DNA itself (e.g. DNA methylation and hydroxymethylation). Epigenetics has taken center stage in the study of chronic diseases such as cancer, obesity, diabetes, and neurodegeneration; however, its integration into the field of environmental health sciences and toxicology is only a few decades old. For example, the developmental origins of health and disease (DOHaD) work of David Barker and

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colleagues showed that a child's environment during gestation and soon after birth influences his or her risk of developing disease later in life (Bateson et al. 2004). The molecular basis, however, for this implausible mode of inheritance of disease risk was unknown until 2003, when Drs. Randy Jirtle and Robert Waterland published a seminal paper in *Molecular and Cellular Biology* (Waterland and Jirtle 2003). The stunning physical differences in the genetically identical mice seen in **Figure 1** are the result of the epigenetic status of a metastable epiallele (shown is the murine A^{vy} viable yellow *Agouti* locus). In this model, Jirtle and colleagues demonstrated that maternal dietary supplementation with methyl donors including betaine, methionine, and folic acid, alters the offspring coat color distribution of the offspring. More importantly, they identified DNA methylation of a transposable element upstream of the *Agouti* gene as the molecular basis for this shift (Waterland and Jirtle 2003). This observation marked the advent of a new field of scientific investigation: environmental epigenomics.

Despite a growing interest in the integration of epigenetics into toxicology and translational sciences, recent advances of the field have not yet fully incorporated epigenetics into research, risk assessment, or epigenome editing applications. To honor the 50th Anniversary of the Environmental Mutagenesis and Genomics Society (EMGS), we provide a primer on the principles and practices critical to understanding the role of the epigenome in regulating gene expression and the resulting response of cells, tissues, and individuals to environmental exposures. We first discuss how the work of Jirtle and Waterland has been extended to evaluate multi- and trans-generational effects of perinatal exposures. We next focus on the complexity of tissue and even cell type epigenomic specificity by highlighting the National Institute of Environmental Health Sciences' (NIEHS) multi-phased Toxicant Exposures and Responses by Genomic and Epigenomic Regulators of Transcription (TaRGET) Program (Wang et al. 2018). As epigenetics encompasses genes, non-coding elements, regulatory regions, and transposons, we next describe the use of transposons as epigenetic biomarkers. We finish with a discussion of the emerging need to expand beyond DNA methylation in environmental epigenetics research to also include non-coding RNAs as biomarkers of exposures and mechanisms of disease risk. Finally, new advances in epigenome editing, including our recent work on the potential of piRNA DNA methylation editing, will be explored for environmental health research (Perera et al. 2019).

Multi- and Trans-Generational Transmission of Environmentally-Induced Health Effects

Gestation as a Sensitive Period for Environmentally Induced Epigenetic Changes
Epigenetic regulation can be modified by the environment and these changes contribute to growth, development, and disease risk (Faulk and Dolinoy 2011). The epigenome, and DNA methylation in particular, is especially sensitive to environmental perturbation in the early stages of gestation when epigenetic patterns that can be inherited across subsequent cell divisions are being set up. Hundreds of studies in human cohorts and animal models have shown associations between the gestational environment and

epigenetic change in the offspring. These studies provide evidence for epigenetic change as one mechanism underlying the DOHaD hypothesis (Barouki et al. 2018). For example, differential DNA methylation is associated with gestational exposures to toxicants (e.g., lead, arsenic, bisphenol A, cigarette smoke (Cardenas et al. 2015; Goodrich et al. 2015; Anderson et al. 2016; Joubert et al. 2016; Wu et al. 2017; Alavian-Ghavanini et al. 2018; Junge et al. 2018), maternal stress (Mulligan et al. 2012; Non et al. 2014), and maternal diet (Waterland and Jirtle 2003; Dolinoy et al. 2006; Burdge et al. 2007; Amarasekera et al. 2014; Gonzalez-Nahm et al. 2017). Understanding the influence of gestational environmental exposures on the offspring epigenome is thus an important part of risk assessment for toxicants. In addition to epigenetic effects by gestational environment on the F1 offspring, there is evidence for F2 grandoffspring epigenetic changes (Rehan et al. 2012; Mitchell et al. 2016) since the fetal germ cells are directly exposed during this time (**Figure 2**), and even F3-F4 transgenerational effects on subsequent generations that were never exposed (Anway et al. 2005; Manikkam et al. 2014). This section will highlight several examples of changes to offspring, grandoffspring, or great-grandoffspring DNA methylation following gestational exposures and will briefly describe common approaches for DNA methylation analysis that can be applied to studies in this area.

Examples of Multigenerational Epigenetic Effects

As mentioned above, perhaps the most striking rodent examples of the impact of the early gestational environment on offspring phenotype through epigenetic change stem from the A^{vy} mouse model (Figure 1). A^{vy} is a metastable epiallele, an allele that is variably expressed among individuals due to epigenetic modifications established early in development (Rakyan et al. 2002). As such, metastable epialleles are particularly vulnerable to early environmental influences, including diet, toxicants, and radiation. In the A^{vy} mouse model, coat color throughout life and obesity status and tumor susceptibility in adulthood correlate with whether the A^{vy} allele is turned on or off through DNA methylation and histone modifications in early development. The epigenetic status of this locus has been shown to be associated with early gestational exposures to genistein, a methyl-donor enriched diet, bisphenol A, lead, and other dietary agents or environmental toxicants, all of which continue to impact the offspring's health later in the life course (Waterland and Jirtle 2003; Dolinoy et al. 2006; Dolinoy et al. 2007; Kaminen-Ahola et al. 2010; Bernal et al. 2013; Faulk et al. 2013b).

Epidemiological studies have employed genome-wide screening approaches across multiple tissues to identify potential metastable epialleles in humans and to determine their response to the environment (Waterland et al. 2010a; Dominguez-Salas et al. 2014; Silver et al. 2015). For example, a natural experiment in rural Gambia compared individuals with periconceptual exposure to the dry versus rainy season, which largely impacts diet, and found differential methylation of the *VTRNA2-1* epiallele, which persisted into later childhood (Silver et al. 2015). Genomic imprinting is an epigenetic process that allows a subset of autosomal genes to be expressed exclusively from one allele, in a parent-of-origin-specific manner. Thus, imprinted genes are another set of

candidates to consider in environmental epigenetics due to their unique characteristics, including parent-of-origin dependent regulation and key functions in early life growth (Lawson et al. 2013). Importantly, DNA methylation of many imprinted genes is responsive to the gestational environment. For example, DNA methylation at well-characterized differentially methylated regions, including the imprinting control regions of *IGF2* and *H19* are associated with prenatal exposures to smoking, phthalates, folate, malnutrition, and more in epidemiological studies (Heijmans et al. 2008; Murphy et al. 2012; Tobi et al. 2012; Hoyo et al. 2014; LaRocca et al. 2014). There is also evidence that this epigenetic alteration is persistent – even into late adulthood (Heijmans et al. 2008) – and contributes to adverse health outcomes including low birth weight, adolescent adiposity, and adiposity in post-menopausal women (Huang et al. 2012b; Murphy et al. 2012; Song et al. 2018).

Rodent and human studies have provided evidence for the theory that primordial germ cells are uniquely susceptible to epigenetic change by grandmaternal exposures, and these exposures can thus impact health of not only the F1 but also the F2 generation (Sales et al. 2017). In epidemiological studies, grandmother smoking on the maternal side was associated with autistic traits (Golding et al. 2017), asthma with allergies (Accordini et al. 2018), and obesity (Ding et al. 2017) among F2 grandchildren. In a rat model, following F0 exposure to nicotine during gestation, the F1 and F2 generations both displayed alterations in lung function that were consistent with effects on myofibroblast differentiation. Importantly, changes to both global DNA methylation and histone acetylation were observed in lung and gonad tissues of the F1, suggesting an epigenetic underpinning for F2 effects (Rehan et al. 2012).

Examples of Transgenerational Epigenetic Effects

Evidence for environmentally-induced transgenerational effects via germline epimutations that transmit beyond the exposed generations is recently coming to light (Skinner 2015). Transgenerational reproductive toxicity including decreased epididymal sperm count and reduced sperm motility were first reported in four generations of male rats following F0 pregnancy exposure to the fungicide vinclozolin and the pesticide methoxychlor (Anway et al. 2005). These transgenerational effects were associated with inherited epigenetic marks, including DNA methylation in the male germline (Anway et al. 2005; Manikkam et al. 2014). Exposure-specific sperm DNA methylation profiles transmitted through the F3 generation have been reported in rats for a pesticide mixture, a plastics mixture, jet fuel, and dioxin (Manikkam et al. 2012). Overall, these studies draw attention to the need to assess whether other common exposures lead to transgenerational epimutations and health effects. The well-characterized model organisms, zebrafish and *C. elegans*, may serve as valuable screening species for transgenerational effects and their epigenetic underpinnings of a wider array of exposures given the fast generational turnover in these species (Greer et al. 2011; Carvan et al. 2017; Cavalieri and Spinelli 2017; Camacho et al. 2018).

Methods for DNA Methylation Analysis

Candidate Gene or Locus-specific Analysis

Identifying DNA methylation changes associated with early life exposures has the potential to serve as biomarkers of exposure and/or mechanistic links to increased disease risk across generations and will improve toxicological risk assessment. Common approaches for DNA methylation analysis in human or animal models include quantifying DNA methylation at specific genes, transposable elements (TE), or across all or most genes (epigenome-wide analyses). Many popular methods for gene-specific analysis rely upon sodium bisulfite treatment of the DNA first, which converts unmethylated cytosine residues to uracil but leaves methylated cytosines unchanged (Grunau et al. 2001). These methods include cloning followed by sequencing (Zhang et al. 2009), methylation-specific quantitative PCR (Eads et al. 2000), pyrosequencing (Busato et al. 2018), and more recently, targeted bisulfite sequencing which takes advantage of next generation sequencing technology (Bernstein et al. 2015; Wendt et al. 2018). In addition to their utility for specific genes, pyrosequencing is widely used to measure both human and mouse TE methylation (e.g., LINE1, Alu). The EpiTYPER assay is another popular method for gene-specific analysis which involves enzyme digestion after amplification of bisulfite-converted DNA, creating fragments of different mass based on methylation status which are then analyzed via mass spectrometry (Suchiman et al. 2015).

Epigenome-wide Analysis

The most common tools used in epigenome-wide association studies in human populations have been the Illumina Infinium series of probe-based arrays which quantify DNA methylation at single CpG site resolution, needing as little as 250 ng of bisulfite converted DNA as input. The first two versions of this platform, the Infinium HumanMethylation27 and HumanMethylation450 BeadChips provided coverage at >27,000 and >480,000 CpG sites. In December 2015, the latest version of the Infinium array, the MethylationEPIC BeadChip, was released which quantifies DNA methylation at >850,000 CpG sites across all known genes and in intergenic and key regulatory regions (e.g., gene promoters, enhancers (Moran et al. 2016)). Apart from the Infinium arrays, next generation sequencing-based methods are gaining popularity as these methods can be used for any species with a mapped genome and costs are decreasing (Bock et al. 2010; Zhao et al. 2014; Ziller et al. 2015). In addition to bisulfite-conversion based sequencing methods, antibody based methods can be used to semi-quantitatively measure methylation at transposons or genome-wide. Referred to as methylated DNA-immunoprecipitation (Me-DIP), these methods involve using anti-5mC antibodies to immunoprecipitate methylated DNA (Zhao et al. 2014; Staunstrup et al. 2016). Antibody-based approaches can also be utilized to evaluate DNA hydroxymethylation, which is also emerging as an environmentally-responsive modification (Kochmanski et al. 2018a; Kochmanski et al. 2018c).

The NIEHS TaRGET II Consortium and Environmental Epigenomics

Current Challenges in Environmental Epigenomics Studies

Although we have made substantial strides in our understanding of how environmental exposures impact the epigenome and human health, several factors continue to pose significant challenges and limitations. These include tissue specificity of environmental exposures, tissue and cellular heterogeneity, sexually dimorphic effects, and the effects of age and stress on exposure-related epigenetic changes and health outcomes. First, human environmental epigenomics studies are limited by tissue accessibility, as access to most disease-relevant tissues targeted by environmental exposures is not possible. Thus, human studies typically rely on surrogate tissues, including blood, hair, and skin, to gain insight into the effects of environmental exposures on inaccessible target tissues. However, the extent to which epigenetic changes in surrogate tissues reflect those in target tissues is currently unclear. Second, it is increasingly clear that there is significant heterogeneity within tissues, and even within individual cell types, with respect to gene expression and patterns of epigenetic marks (Cheow et al. 2016; Cusanovich et al. 2018; Ben-Moshe and Itzkovitz 2019). Thus, assessment of tissue-specific effects of environmental exposures may be masked by such heterogeneity. Moreover, environmental exposures may induce changes in the cellular composition of tissues (Trevino and Katz 2018; Hung et al. 2019), complicating the interpretation of epigenetic studies. These challenges may be addressed through emerging methods in single cell analyses (Cheow et al. 2016). Third, significant evidence demonstrates that the effects of toxicant exposures are highly sex-specific (Faulk et al. 2013b; Bansal et al. 2017; Kundakovic 2017; Neier et al. 2019; Winterbottom et al. 2019). Given that there are also significant sex disparities in the prevalence and outcomes of many diseases associated with environmental exposures (Graham 2015; DeSantis et al. 2017), an understanding of how environment differentially affects disease risk in a sex-specific fashion is critical. Finally, although cross sectional studies have clearly demonstrated environment-induced epigenetic changes (Montrose et al. 2018; Curtis et al. 2019; Winterbottom et al. 2019), whether these changes persist across time in both surrogate and target tissues is not well understood. Notably, environmental exposures can alter the trajectory of age-related epigenetic changes (Fraga et al. 2005; Faulk et al. 2014; Krauskopf et al. 2018). However, it is currently unclear whether these interactions between environment and aging are uniform across tissues. It will therefore be important to consider age-environment interactions in the design of human toxicoepigenetics studies.

The NIEHS TaRGET Consortium

In order to address these important issues, and to aid in the design and analysis of human environmental epigenetics studies, the NIEHS developed the TaRGET program. The first phase of this program, TaRGET I, was formed in 2012 to investigate how environmental exposures affect the cellular machinery critical for establishment of normal epigenetic patterning (Prins et al. 2014; Wang et al. 2016). During the second phase of the project, a multi-institutional consortium, TaRGET II, was established to investigate the conservation of environment-induced epigenetic signatures across multiple target and surrogate tissues and cells (Wang et al. 2018). Using a common mouse model and a well-defined treatment paradigm, consortium members are investigating the effects of several perinatal environmental exposures, including lead

(Pb), diethylhexyl phthalate (DEHP), bisphenol A (BPA), arsenic (As), dioxin (TCDD), and particulate matter (PM_{2.5}) on the epigenome and transcriptome in multiple target and surrogate tissues (Wang et al. 2018) (**Figure 3**). These analyses are conducted at three separate time points during the lifespan of the offspring, including the early postnatal period, early adulthood, and late adulthood, in order to investigate whether perinatal toxicant-induced epigenetic programming persists as the animals age. Collectively, work from this project is anticipated to provide valuable insight into the tissue-, cell-, and sex-specific effects of environmental exposures on epigenetic programming across the lifespan. These findings will aid in the design and interpretation of human population-based epigenomics studies, which will be the focus of the TaRGET III and TaRGET IV phases of the project.

Pb Exposure and Tissue-specific DNA Methylation

As part of the TaRGET consortium, we are investigating the effects of perinatal Pb exposure on epigenetic and transcriptional programming of offspring mice, using the consortium mouse model of environmental exposures. We recently investigated the effects of perinatal Pb exposure on DNA methylation in mouse liver and blood, to determine whether common signatures exist between the two tissues, whether these signatures are sex-specific, and whether they persist into adulthood (Abstract presented at Society of Toxicology Annual Meeting, Baltimore, MD, 2019). For these studies, dams were exposed to Pb acetate (32 ppm) as outlined previously (Faulk et al. 2013b) via drinking water for two weeks prior to mating, and Pb exposure continued throughout pregnancy and lactation. Offspring were weaned at three weeks of age, and Pb exposure ceased at this time point. Between 3 weeks and 5 months of age, offspring were given standard chow and Pb-free drinking water. Six male and six female mice were euthanized at 5 months of age, and blood and liver were collected according to TaRGET II consortium-approved protocols. Thus, this two-stage study design allows for the assessment of transient as well as persistent epigenetic modifications, as transient changes may readily occur immediately following exposures but may not persist over time when the exposure is removed.

To determine the effects of Pb exposure on DNA methylation, within TaRGET II, we utilized enhanced reduced-representation bisulfite sequencing (ERRBS), which allows the detection of base pair resolution DNA methylation at CpG-rich loci (Garrett-Bakelman et al. 2015). Notably, although Pb exposure ceased at 3 weeks of age, our studies revealed thousands of differentially methylated regions (DMRs) that persisted into adulthood. DMRs were highly sex-specific, with few regions overlapping between blood and liver. In females, 3 DMRs overlapped between blood and liver, although the changes in methylation with Pb exposure were in the opposite direction in the two tissues. In males, there were 4 DMRs that overlapped between blood and liver, with two loci, mapping to *Grifin* and *Plekhhg3*, which exhibited concordant changes in methylation with Pb exposure. These findings suggest that perinatal Pb exposure results in stable changes in DNA methylation in the blood and liver of offspring mice, with few sites directly overlapping between the two tissues or across sexes. Ongoing studies are

focused on determining whether other epigenetic signatures in the blood may reflect Pb-induced changes in liver and other target tissues, including brain.

It is notable that Pb-induced changes in DNA methylation persisted into adulthood, although the functional consequences of these changes are currently unclear. Notably, recent work suggests that early-life environment can induce epigenetic changes that are “silent”, but render the target genes hyper-responsive to hormonal cues later in life (Greathouse et al. 2012; Wang et al. 2018). Indeed, Wang et al. demonstrated that early life exposure to BPA induced reprogramming of the activating H3K4me3 histone mark at prostate cancer-associated genes that persisted into adulthood (Wang et al. 2016). Intriguingly, several of the BPA-reprogrammed genes exhibited no change in basal expression; however, they were hyper-responsive to subsequent hormonal stimulation (Wang et al. 2016). These findings suggest that changes in epigenetic marks may provide a more reliable signature of early-life environmental exposures than changes in gene expression. The data also suggest that the functional consequences of toxicant-induced epigenetic programming may only manifest upon subsequent environmental insult. Thus, in future studies it will be important to investigate how subsequent environmental exposures, nutritional perturbations, and stressors later in life interact with early epigenetic programming to initiate or exacerbate disease. Future research is required to understand the persistence of environmentally induced alterations on epigenome in order to determine transient versus persistent epigenetic changes associated with environmental exposures.

Transposons as Epigenetic Targets

Transposons

Transposable elements (TEs) are small regions of DNA that have evolved the ability to copy themselves throughout the genome. Referred to as repetitive elements, mobile DNA, or transposons they are found in all animals. Varying in size from 250 nt to over 7000 nt they can encode protein machinery that enables their jumping ability (Huang et al. 2012a). These mobile elements have several origins, including endogenous retroviruses that have incorporated themselves into the genome, or gene fusions that have gained the ability to copy themselves. Subsequently, they have spread throughout the genome. They are delineated in classes, such as short or long interspersed elements (SINEs and LINEs) which dominate the genome, and subcategorized into families and subfamilies based on sequence similarity and presumed copying origin (Bao et al. 2015).

Mammalian genomes consist of approximately 50% of sequence derived from transposons, as opposed to just ~1% of sequence coding for genes (Platt et al. 2018). Despite the consistency of this fraction across mammals, the composition of the transposon sequence varies widely between species. Generally, there is a balance between TE proliferation and host genome suppression, often through epigenetic mechanisms (Canapa et al. 2015). Most mammals have at least one actively

transposing family of TEs. They are inherited from parent to offspring, rarely transferring horizontally between species. While “selfish,” due to their ability to promote their own transmission at the expense of other genes, these TEs can often be adapted to serve the host as a source of raw variation and mutation (Dupressoir et al. 2012). Some elements are enriched in CpG sites or other useful features for evolution, so their proliferation throughout the genome can be adaptive.

Humans are illustrative of transposon dynamics in the genome. The most abundant TE by number in the human genome is the *Alu* element, a short ~300 nt element that does not encode its own transposition machinery and is present in about 1.2 million copies in primate genomes (Deininger 2011). It relies upon the reverse transcriptase protein for mobilization, encoded by the most abundant transposon by percentage nucleotide in the human genome, the LINE1 element. The LINE1 is >6000 nt and is present in >100,000 copies in the human genome making up 18% of the genome, but most are 5' truncated, with around 100 LINE1s suspected to be capable of activity and just 6 “hot” LINEs provide over 80% of the observed transposition in cell culture (McLaughlin 2018). Both elements can contribute to disease by several mechanisms including transpositional mutation breaking a gene, by causing homology mediated deletions, or most relevant here, by disruption of chromatin or nearby gene expression (Beck et al. 2011; Rebollo et al. 2011). *Alus* are primate specific, while LINE1s are found across all vertebrates. Both are used as epigenetic biomarkers of exposure and disease status.

Epigenetic Relevance of TE Insertions

In mammals and other organisms, the fifth carbon of cytosines in a CpG context can be methylated. Deamination of methylated cytosine results in a thymine base. Transitions from C to T account for up to 42% of all mutations in the human genome, far more than any other transition or transversion, most occurring at a methylated CpG sites (Gojobori et al. 1982). The rapid turnover of CpG sites to either TpG or CpA has important implications in evolution. Whereas humans have a 1% sequence divergence from chimpanzees on average, at CpG sites the mutation rate rises to 15% (Chimpanzee and Analysis 2005). Over evolutionary time, mammalian genomes have lost CpG sites from an expected frequency of 6.25% to an observed frequency of less than 1% of dinucleotides. Since DNA methylation is crucial for gene regulation, CpG sites at high density CpG islands (CGIs) near gene promoters must be replenished somehow (McLain and Faulk 2018). Transposons are typically silenced by DNA methylation at CpG sites to prevent their movement from disrupting the genome. However, non-random TE insertions appear to provide new CpG sites via their ability to copy paste sequence throughout the genome. In humans, the *Alu* element is both enriched in CpG sites and found at higher density near CGIs and therefore replenishes CpG sites, providing flexibility in methylation status near genes (Gu et al. 2016).

Transposons as Epialleles

In mice, the intracisternal A particle (IAP) element is an endogenous retrovirally derived transposon and is responsible for ~10% of de novo mutations found in inbred strains.

The high mobilization activity of IAP has resulted in high polymorphism as well, with 60% being private to specific strains (Zhang et al. 2008). The IAP is capped on both ends by long terminal repeats (LTRs) that are relatively CpG dense and have bidirectional promoter activity. Typically, these CpG sites are silenced through DNA methylation, however several IAPs have been shown to be variably methylated between mice, with consistent methylation across tissues, hallmarks of metastable epialleles. The A^y mouse differential coat colors (**Figure 1**) are driven by promoter activity at insufficiently silenced CpG sites within the LTR of an IAP insertion. Generally, most IAPs are completely reset between generations and only rarely escape reprogramming (Kazachenka et al. 2018). Several studies in humans have also identified potential epialleles hosting differential methylation in various classes of TEs (Waterland et al. 2010b; Faulk et al. 2016).

Benefits of Transposons as Biomarkers

TEs have several characteristics that make them useful as biomarkers of exposure and disease status. Many classes are found widely throughout the genome and there are multiple copies per genome. They are not under selective constraint. They are usually heavily methylated, so any change in methylation status is suggestive of strong environmental impact. In practice, their sequence similarity allows thousands of loci to be amplified in a single PCR reaction with a single set of primers. Measuring the DNA methylation of these amplicons gives an average methylation value for all TEs of a specific class, usually *Alu* in humans or LINE1 in humans and mice. Given their relatively uniform spread across the genome, their methylation serves as a proxy for global methylation across the genome.

Epidemiological Biomarkers in Human Exposure

When studying populations for environmental exposures that are associated with disease, two main transposon assays have been used. The most widely used measure are taken with LINE1 and *Alu* elements, originally developed by Yang et al. to measure methylation in a PCR pool using primers matching consensus sequences of these two families (Yang et al. 2006). The LINE1 pyrosequencing assay was initially used to measure genomic instability in cancer but has been adapted for epidemiological studies (Estecio et al. 2007). The *Alu* and LINE1 assays, while both considered global, have different methylation levels by tissue, and have distinct responses to environmental exposures (Price et al. 2012). For example, both *Alu* and LINE 1 correlate to preterm birth (Burris et al. 2012), whereas LINE1 but not *Alu* was affected by exposure to prenatal air pollution (Breton et al. 2016). While too numerous to list here, these epigenetic biomarkers have shown response to mercury (Narvaez et al. 2017), traffic particles (Baccarelli et al. 2009), industrial environments (Alvarado-Cruz et al. 2017), aging and ischemic heart disease (Baccarelli et al. 2010), carbon nanotubes (Ghosh et al. 2017), and polycyclic aromatic hydrocarbons (Lee et al. 2017a). The *Alu* assay has been used to study response to persistent organic pollutants in Koreans (Kim et al. 2010; Lee et al. 2017b), and Inuit (Rusiecki et al. 2008). Nearly all studies of toxicant exposures report hypomethylation of LINE1. Fewer studies report *Alu* methylation responses.

Use as Biomarkers in Mice

There is considerable interest in the use of TEs as biomarkers environmental exposures that alter DNA methylation. Studies have examined methylation of a subset (Faulk et al. 2013a) or the entire genomic complement of TE methylation in mice (Kazachenka et al. 2018). Locus-specific measurements of TE DNA methylation allow detection of differential methylation for individual TE insertions and can be considered causal for disruption of nearby gene expression (Rebollo et al. 2011). Potentially, incompletely silenced TEs could transcribe in unintended ways; indeed studies have shown hundreds of protein isoforms that express a fusion of a TE and a native gene (Ekram et al. 2012). Locally, several IAP elements show methylation response to perinatal lead exposure (Montrose et al. 2017) and phthalates (Neier et al. 2019). Globally, IAP elements have been used as genome-wide epigenetic biomarkers, showing hypomethylation in transcription factor binding sites (Shimosuga et al. 2017). Prenatal inflammation can impact murine LINE1 methylation in the brain (Basil et al. 2014). Radiation induces hypomethylation at LINE1 and the SINE B1 in mouse hematopoietic progenitors (Miousse et al. 2014). In contrast, at least one study showed low dose radiation does not affect methylation of several TEs including IAP, B1, and LINE1 longitudinally (Newman et al. 2014). Similarly, a study by Tommasi et al. found no methylation changes in these same element families upon exposure to secondhand smoke (Tommasi et al. 2012). However, trichloroethylene exposure does alter IAP methylation in immune cells, which is of great interest given its widespread use as an obstetric analgesic (Gilbert et al. 2012). Regarding longitudinal studies, LINE1 and IAP elements have been used to measure epigenetic drift over time (Faulk et al. 2014; Kochmanski et al. 2017), during aging (Barbot et al. 2002) and by dietary exposure (Kochmanski et al. 2018b).

Caveats of TEs as Epigenetic Biomarkers

The nature of repetitive elements produces several caveats that should be considered when analyzing TE methylation data. Most importantly, the lack of conserved CpG sites between various copies of a family of TEs can mask the true methylation level. Assays are built to compare CpG site methylation by using a consensus sequence that is the average of thousands of TEs of a particular family. Often individual TE copies will have mutations at the location of CpG sites when compared to the consensus. In sequencing methods, for example, a read may come from a family member that has a TpG mutation SNP at CpG site annotated in the consensus. This position would be erroneously read as an unmethylated CpG site. Since methylation is often calculated as percentage C to non-C, over thousands of elements or reads, methylation level can appear anomalously low at specific CpG sites. The true methylation level at CpG sites that exist in TE copies is usually very high in reality. For *Alu* elements, Yang et al. found only 36% of potential CpG sites maintained in aggregate PCR products (Yang et al. 2004). Of these, 85% were methylated. Another major difficulty arises from the similar, but non-identical, nature of TE family members. Performing multiple alignments can be difficult, leading to undercounting of true TE copies.

Despite these caveats, methylation assessment between studies is comparable, and deviance from control group methylation level usually indicates an environmental cause. Therefore, the continued use of TEs as epigenetic biomarkers has a bright future, as long as the underlying biology of TEs is understood by those who measure them.

Non-coding RNA and Epigenome Editing Tools as Potential Interventions

An Introduction to non-coding RNA

Although non-coding RNA (ncRNA) was previously considered as products of “junk DNA”, recent scientific advancements have concluded that ncRNA are functional RNA molecules that arise from DNA, which do not translate to specific proteins (Boland 2017). These ncRNAs are critical for biological functions as they are involved in complex biochemical and epigenetic regulatory mechanisms (Angrish et al. 2018). The genome encodes for long ncRNAs (lncRNAs), which are non-protein coding transcripts >200 nucleotides and small ncRNAs (sncRNAs), which are of much shorter lengths in comparison. lncRNAs are functionally distinct from sncRNA and are involved in biological processes such as genomic imprinting, X-chromosome inactivation, and development (Kung et al. 2013; Dhanoa et al. 2018).

On the other hand, sncRNAs include several regulatory RNA species such as microRNA (miRNA) that are involved in mRNA transcription, short interfering RNAs (siRNAs) that are involved in transcriptional repression, PIWI-interacting RNAs (piRNAs) that are involved in transposon silencing, and small nucleolar RNAs (snoRNAs) that interact with other RNA and proteins for gene regulation. Based on current knowledge, miRNAs and siRNAs are roughly ~20 nucleotides in length, and are associated with Ago proteins to silence genes via transcriptional and translational silencing mechanisms within the RNA interference (RNAi) pathway, while germline piRNAs are typically 24-32 nucleotides in length, in comparison (Lin 2007). In contrast, PIWI proteins are important for piRNA biogenesis and forming the piRNA-induced silencing complex (piRISC) to induce DNA methylation for silencing of transposable elements in the germline (Tan et al. 2015). Therefore, the PIWI-piRNA interactions are functionally distinct from that of other sncRNAs (Han et al. 2017a), including several structural attributions that are distinct from other sncRNAs: preference for a 5' uridine signature, presence of an adenosine signature at the 10th position, a 2'-O-methylation modification at the 3' ends, and clustering within a 20-90 kb length region (Lin 2007; Zuo et al. 2016).

Circular RNAs (circRNAs) are another class of ncRNA species, which are a product of back-splicing of linear pre-mRNA that join together a donor site with an upstream acceptor site (Salzman 2016). In some cases, circRNAs serve as molecular sponges that regulate transcription by removing miRNAs (Carrara et al. 2018). They are associated with cancer, Alzheimer's disease, diabetes, cellular stress, aging, and genomic imprinting (Han et al. 2017b; Qu et al. 2017; Perera et al. 2018). Although miRNAs are by far the most studied class of sncRNA species in many fields including

cancer, environmental toxicology, and risk assessment, future research in other sncRNA species may lead to similar breakthroughs based on their distinct cellular functions.

ncRNA Biomarkers Relevant for Human Health and Disease

The fields of genomics, toxicogenetics, and environmental epigenetics have greatly benefitted from the technological advancements and increasing power of sequencing, transcriptomics, and bioinformatics over the past few years. It provides much needed information to better understand the genetics and environmental factors that influence human health and disease. Thus, the discovery of ncRNA biomarkers related to a wide array of human disease stages and environmental exposures contribute towards identifying novel ncRNAs for precision medicine (Karlsson and Baccarelli 2016; Roy et al. 2018). A few, recent studies in the field of environmental toxicology have provided evidence for lncRNA biomarkers associated with response to environmental stressors such as EDCs, metals, cigarette smoke extracts, and genotoxic agents (Bhan et al. 2014; Bi et al. 2015; Zhou et al. 2015; Karlsson and Baccarelli 2016), where genes are regulated by complex cellular and molecular mechanisms which are yet unknown.

SncRNA and circRNA biomarkers are often times preferred over lncRNAs since they are small, versatile, resistant to degradation, and widely available in biospecimens (Bahn et al. 2015). The most extensively studied ncRNA biomarkers are miRNAs, which are associated with EDC exposures (De Felice et al. 2015), metals (Sanders et al. 2015), pollution (Krauskopf et al. 2018), and several human diseases (Siddeek et al. 2014; De Felice et al. 2015; Sanders et al. 2015; Ehrlich et al. 2016; LaRocca et al. 2016; Romano et al. 2017; Krauskopf et al. 2018). The piRNA biomarkers may also be considered for future studies due to their expression in human blood, stability (due to the 2'-O-methylation modification at its 3' end), and tissue-specificity (Yang et al. 2015; Zuo et al. 2016). Although piRNAs are extensively studied and highly expressed from the germline, recent studies have provided evidence for piRNA expression in the soma (Lin 2007; Zuo et al. 2016; Perera et al. 2019). So far, piRNAs have shown associations with chemical exposures such as dichlorodiphenyltrichloroethane (DDT) and vinclozolin (Nilsson et al. 2018; Sai et al. 2018; Skinner et al. 2018) and human health and disease (i.e. cancer) (Han et al. 2017a; Chalbatani et al. 2019).

CircRNAs are worthy biomarkers since they are stable, resistant to exonucleases, present in liquid biopsies, and highly abundant with specificity towards tissues and stage of development (Carrara et al. 2018; Zhang et al. 2018). Thus, circRNAs are becoming increasingly popular candidates for diagnosis and clinical interventions in recent cancer research, and may serve as a potential ncRNA biomarkers of interest for future research (Zhang et al. 2018). Generating reliable ncRNA databases with precise detection methods for tissue-, sex-, and developmental stage-specific ncRNA species (i.e. miRNA, piRNA, and circRNA) is absolutely necessary to improve ncRNA biomarker identification. For instance, the publicly available databases for piRNA mostly consist of germline tissues with high incidences of non-specific sequences for somatic tissues (Tosar et al. 2018; Perera et al. 2019), thus serving as a limitation for biomarker discovery. The less

studied ncRNA species such as lncRNA, piRNA and circRNA species are potential epigenetic biosensors that may serve as predictors for disease risk and later-life health outcomes that could be developed to improve personalized medicine in the future.

Epigenome Editing Techniques

Epigenome editing/engineering is a technology that manipulates the epigenome without disrupting the actual DNA sequence to regulate desired gene expression, which makes it a safer therapy in comparison to genome editing. Current technologies that act on the epigenome can be categorized as global epigenetic modifiers and target-specific epigenetic modifiers, both of which act on epigenetic readers such as Methyl-CpG-binding proteins (MBPs), histone methylation binding proteins, histone acetylation binding proteins; writers such as DNA methyltransferases (DNMTs), histone methyltransferases (HMTs), histone acetyltransferases (HATs); and erasers such as DNA demethylases, histone demethylases, histone deacetylases (HDACs) (Gillette and Hill 2015).

Global epigenetic modifiers include pharmaceutical agents, such as azacytidine, that are widely used to inhibit DNMTs, resulting in global hypomethylation in dividing cells (Yang et al. 2010; Micevic et al. 2017). HDAC inhibitors such as vorinostat (suberoylanilide hydroxamic acid), impact chromatin by maintaining an “open chromatin” configuration, facilitating transcriptional activity (Gryder et al. 2012). Advantages of these agents lie in their well-characterized use as human therapeutics and for basic research in cell lines and animals. Disadvantages of the pharmaceutical approach include their pleiotropic effects caused by indiscriminate epigenomic activity and propensity to affect biochemical pathways separate from the epigenome. Despite the desperate need for technologies to precisely modulate the epigenome, it is unlikely that either small-molecule pharmaceuticals or the disruption of chromatin enzymes can be rapidly adapted for locus-specific targeting.

Emergence of Targeted Epigenome Editors

Artificial DNA binding domains (DBDs) fused with catalytic domains of epigenetic modifiers have been developed as useful tools to recognize specific DNA sequences that introduce or remove epigenetic marks (i.e. DNA methylation, histone modifications) at a given locus via Zinc-finger proteins (ZFP), transcriptional activator-like effector nucleases (TALENs), and clustered regulatory interspersed palindromic repeats (CRISPR) systems (**Figure 4**) (Waryah et al. 2018). A few critical parameters for epigenome editing are specificity and efficiency of the editor, along with stability of the edited state. With its initial discovery in 1985 (Miller et al. 1985), the first practical method used the DBDs of ZFPs to recruit DNA methylation using DNMTs. In this method, iterative phage display selects for a ZFP that recognizes a 9-12 bp motif and the resulting encoding sequence is ligated to the sequence for the methyltransferase domain (**Figure 4A**) (Xu and Bestor 1997). So far, ZFPs have been engineered in combination with other DNMTs (Li et al. 2007; Siddique et al. 2013), DNA demethylases such as Ten-Eleven translocation (TET) enzymes (Zhao and Chen 2013; Chen et al.

2014), and histone modifiers (Groner et al. 2010; Falahi et al. 2013). Major drawbacks for this system include high cost for protein domain construction, low specificity due to off-target effects, complexity (every zinc-finger domain must be custom evolved to target a specific sequence), and motif size limitation (Nemudryi et al. 2014; Waryah et al. 2018). Since their discovery in 2009 (Boch et al. 2009; Moscou and Bogdanove 2009), TALENs are the second method used as potential epigenome editors (**Figure 4B**) (Konermann et al. 2013), where Ten-eleven translocation methylcytosine dioxygenase 1 (TET1)/DNMT3a was used to modify DNA methylation (Maeder et al. 2013) and lysine-specific demethylase 1 (LSD1) was used to modify the histones (Mendenhall et al. 2013). The DBDs of the TALEN system contains 7-34 tandem repeats, with each repeat binding to the major groove of DNA. Even though TALENs have higher specificity compared to ZFN, this technology poses significant limitations in assembly and delivery due to its large number of tandem repeats.

The CRISPR and CRISPR-associated (Cas) system is the most recent and impactful genome editing technique (Gasiunas et al. 2012; Jinek et al. 2012), which has recently become desirable for epigenome editing. This system utilizes guide RNAs (gRNAs) that recognize ~20 nucleotide sequences to direct deactivated Cas9 (dCas9) attached to a desired epigenome modifying enzyme to its complementary DNA sequence for desired effects (**Figure 4C**) (Waryah et al. 2018). The CRISPR/Cas system has been used for targeted demethylation and methylation of genes using dCas9-TET1 and dCas9-DNMT3a, respectively (Liu et al. 2016; Kantor et al. 2018; Xie et al. 2018). The use of dCas9 attached to HATs (p300), HMTs (PRDM3), HDACs, and chromatin-modifying complexes (LSD1, KRAB) indicate the wide-applicability of the CRISPR/Cas9 system (Gilbert et al. 2013; Hilton et al. 2015; Cano-Rodriguez et al. 2016; Waryah et al. 2018). However, high incidence of off-target effects and time taken for screening is one of the main drawbacks of this system (Falahi et al. 2015). Nonetheless, use of ZFP, TALENs, and CRISPR/Cas9 represent a significant advance in the locus-specific manipulation toolkit and are considered standards by which to compare any new methods (Rots and Jeltsch 2018). Precisely targeting epigenetic marks at specific regions on the genome at a developmental-stage and tissue-specific manner still remains a challenge since the molecular mechanisms underlying epigenetic regulation in normal and disease states are still not well-understood.

Epigenetic changes that occur due to environmental exposures are also not well-understood for most exposures. To inform risk assessment of hazardous exposures and intervention efforts to reverse toxic effects and disease risk, systematic evaluation and rigorous analysis of the epigenome by common exposures is needed. This investigation should involve all aspects of epigenetic regulation including DNA methylation at gene promoters, histone modifications, RNA-mediated gene silencing mechanisms, genomic imprinting, and X-inactivation that are induced by sncRNAs (miRNA, piRNA, circRNA, and siRNA). Since recent advancements in miRNA- and siRNA-based ncRNA therapeutic approaches have provided much promise (Aagaard and Rossi 2007; Burnett et al. 2011; Aliabadi et al. 2016), using a similar ncRNA-based technology to modify the

epigenome as a potential intervention is indeed an avenue for future studies. For instance, the piRNA class of ncRNA represents a fascinating adaptive mechanism and “ready-made” tool for innovation in locus-specific repression through DNA methylation (**Figure 4D**) (Mani and Juliano 2013; Fu et al. 2014). Therefore, it is important to conduct research using in vitro cell cultures, tissue samples and vivo animal models to evaluate the persistence of epigenetic changes with ncRNA delivery, dosage, and specificity.

Conclusions and Future Directions

As we have discussed, a comprehensive understanding of the tissue- and even cell-specific epigenetic effects of toxicant exposures, their interactions with other stressors later in life, and their persistence across the lifespan, is an important and lofty goal for the next 50 years of the EMGS. We are a Society founded with deep interest and expertise in environmental impacts on mutagenesis, and thus focusing on transposons that make up so much of the genome is crucial to understanding the mechanistic link between the environment and heritable changes in gene expression. Transposons serve as both biomarkers and drivers of epigenetic changes and underlie the most well-known epigenetic phenotypes in animals. Future human studies of environmental exposures should place special emphasis on including transposon epigenetic analysis as both an endpoint and explanatory mechanism. The discovery of disease-associated biomarkers have rapidly moved recent scientific research from basic to translational sciences, which now includes miRNA-based therapies that target-specific interventions (Yu et al. 2018). Meanwhile, the current knowledge of less popular ncRNA biomarkers (lncRNA, piRNA, circRNA) association in cell-, tissue-, sex-, and developmental stage-specific environmental exposures or disease states still needs to be carefully examined.

As relatively few chemicals have been evaluated for effects on the epigenome, the toxicopigenetic effects of a broader array of environmental exposures, especially exposures during critical periods of development, should be prioritized for comprehensive assessment. The identification of epigenetically-labile genes, such as additional metastable epialleles or genes that under environmental deflection with age could be prioritized for initial screening. Furthermore, environmentally-associated epigenetic changes need to be related to long-term health in order to delineate whether changes are on the causal pathway to toxicity and disease or simply biomarkers of past exposure without phenotypic implications. Similarly, there is the need to acquire knowledge of persistence, duration of persistence, whether altered epigenome status can be revertible to prior pre-exposed status, and temporary versus long term health or other manifestations of persistent versus revertible effects. Special attention should be paid to the evaluation of multi- and transgenerational effects of toxicant exposures. Collectively, this information needs to be incorporated into risk assessment and policy. With respect to epigenetic effects robustly associated with environmental exposures and health outcomes, epigenome-editing interventions could be developed to mitigate health risk.

Author Contributions

Drs. Bambarendage Perera, Christopher Faulk, Laurie Svoboda, Jaclyn Goodrich and Dana Dolinoy critically reviewed the relevant literature, contributed to the interpretation of that information, and drafted the manuscript. Dr. Dolinoy focused on reviewing the role of environmental epigenomics in health and disease, while Dr. Goodrich focused on the literature involved in multi- and trans-generational transmission of environmentally-induced health effects, Dr. Svoboda focused on NIEHS consortia and environmental epigenomics literature, Dr. Faulk focused on reviewing transposons as epigenetic targets, and Dr. Perera focused on ncRNAs and potential epigenome editing tools as interventions. All authors read and approved the final version of the manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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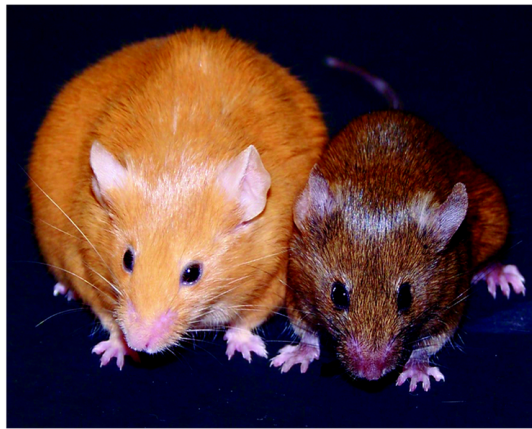
Figure Legends

Figure 1. One-year-old female genetically identical viable yellow agouti mice (A^{vy}). Maternal dietary supplementation with methyl donors such as folic acid, choline, and betaine (Waterland and Jirtle 2003) or the phytoestrogen, genistein (Dolinoy et al. 2006), shifts the coat color of the offspring from yellow to brown, and reduces the incidence of obesity, diabetes, and cancer. Furthermore, these maternal dietary supplements can guard the epigenome from the hypomethylating effects of bisphenol A (BPA), a common endocrine disrupting chemical used in the production of polycarbonate plastic and epoxy resins (Dolinoy et al. 2007).

Figure 2. Direct and Indirect Effects of Exposures Across Multiple Generations. Among mammals, maternal exposures during pregnancy may directly influence the mother (F0) and child (F1). Future grandchildren (F2) may be impacted as the primordial germ cells are also directly exposed. Effects are considered transgenerational when they are observed in generations that were not directly exposed (i.e. F3, F4, and beyond in this human example).

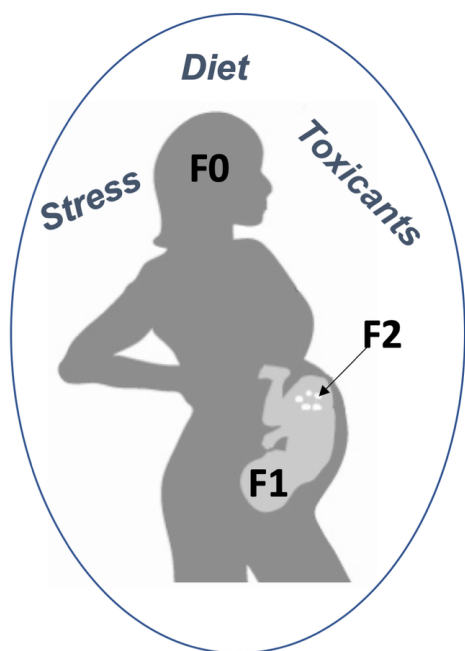
Figure 3. Conceptual diagram for environmental epigenomics studies. Environmental epigenomics studies utilize surrogate tissues as proxies for epigenetic changes in target tissues and determine whether these changes correlate with disease. Several types of epigenetic changes may represent biomarkers of environmental exposures and adverse health outcomes.

Figure 4. Targeted epigenome editors. Epigenetic marks represent DNA methylation changes and histone modifications. The open popsicles indicate unmethylated DNA, while closed popsicles represent methylated DNA, while the unmodified histone (green open dot), is changed to modified histone (red dot) via epigenetic editing. A) ZFP-based epigenome editing. B) TALEN-based epigenome editing. C) CRISPR/dCas9 based epigenome editing. (sgRNA: single guide RNA; dCas9: deactivated Cas9; NGG/NCC: PAM sequence). The effector protein signifies a DNA/histone modifying protein that is responsible for the desired epigenetic change. D) potential ncRNA-based epigenetic modification. Based on current research that indicates piRNA presence and activity in somatic tissues (Perera et al. 2019), other ncRNA species may be used as potential candidates for epigenome editing. For instance, mature piRNA that contain a 2'-O-methylation modification at its 3' end and its associated PIWIL proteins may be used to specifically methylate DNA.



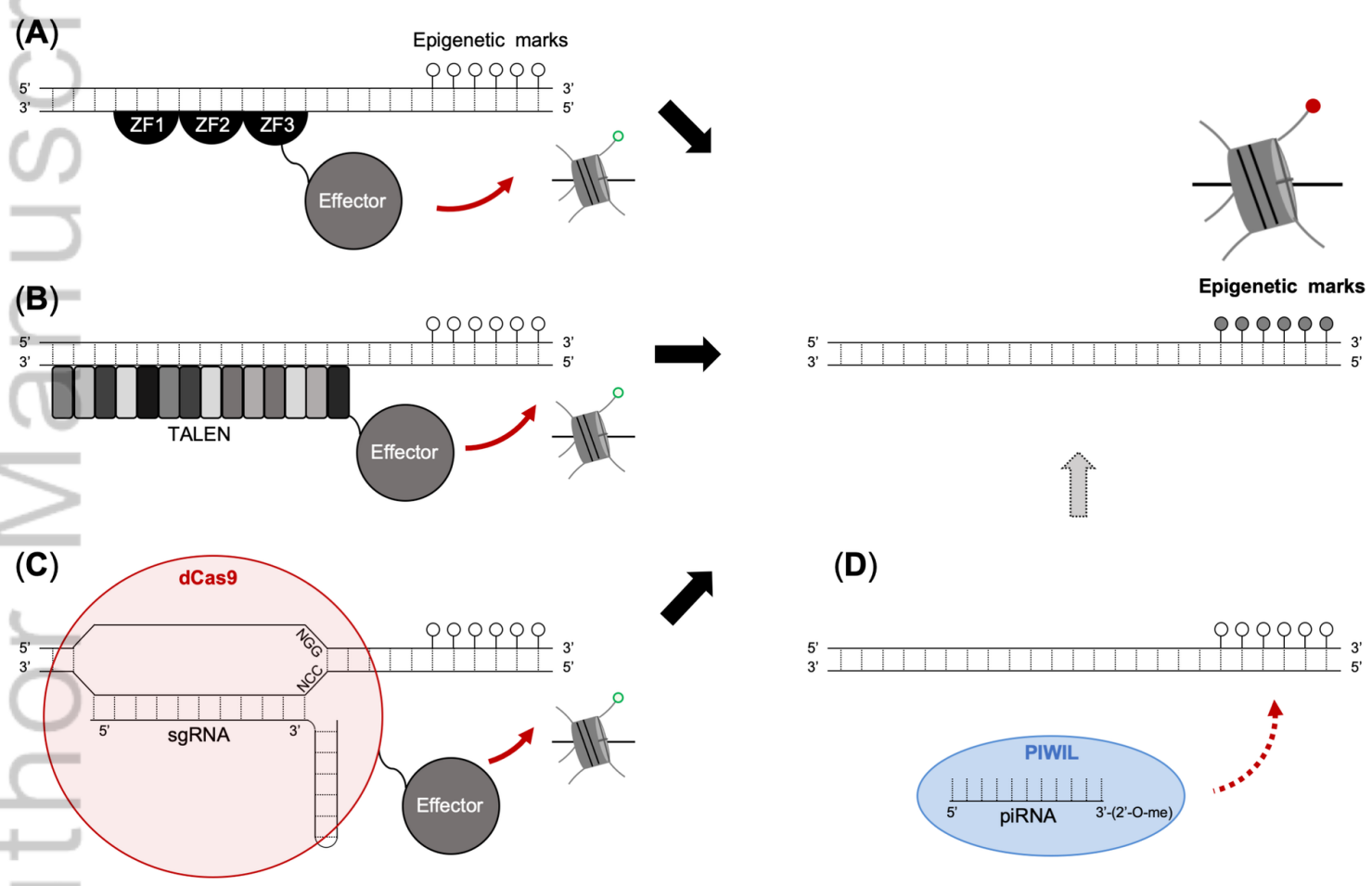
Genetically identical agouti sisters

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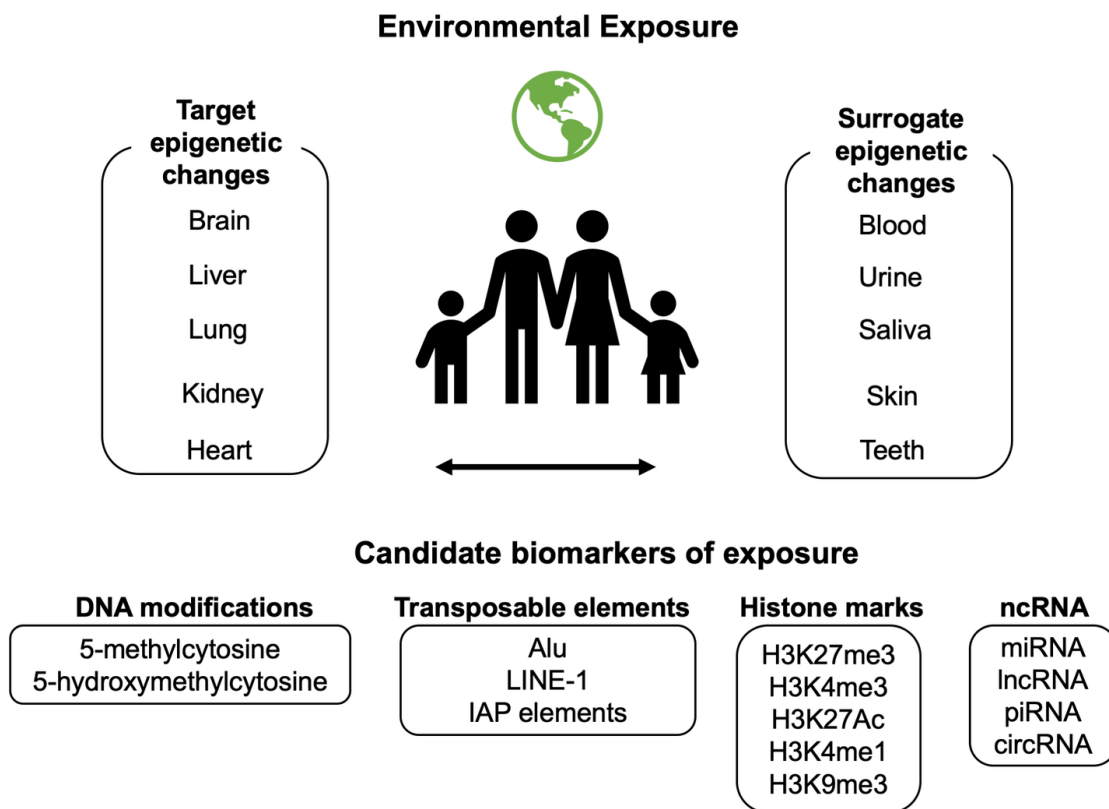


Maternal exposure during pregnancy

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