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REVIEW

Influence of environmental exposure on human epigenetic regulation

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ABSTRACT

Environmental toxicants can alter epigenetic regulatory features such as DNA methylation and microRNA expression. As the sensitivity of epigenomic regulatory features may be greatest during the *in utero* period, when critical windows are narrow, and when epigenomic profiles are being set, this review will highlight research focused on that period. I will focus on work in human populations, where the impact of environmental toxicants *in utero*, including cigarette smoke and toxic trace metals such as arsenic, mercury and manganese, on genome-wide, gene-specific DNA methylation has been assessed. In particular, arsenic is highlighted, as this metalloid has been the focus of a number of studies and its detoxification mechanisms are well understood. Importantly, the tissues and cells being examined must be considered in context in order to interpret the findings of these studies. For example, by studying the placenta, it is possible to identify potential epigenetic adaptations of key genes and pathways that may alter the developmental course in line with the developmental origins of health and disease paradigm. Alternatively, studies of newborn cord blood can be used to examine how environmental exposure *in utero* can impact the composition of cells within the peripheral blood, leading to immunological effects of exposure. The results suggest that in humans, like other vertebrates, there is a susceptibility for epigenomic alteration by the environment during intrauterine development, and this may represent a mechanism of plasticity of the organism in response to its environment as well as a mechanism through which long-term health consequences can be shaped.

KEY WORDS: Environmental epidemiology, Epigenomics, DNA methylation, miRNA, Histone modification

Introduction: critical periods for epigenetic effects

Environmental contaminants and toxicants are widespread throughout the world, and can include various materials and chemicals, from byproducts of combustion to contaminating trace metals and residual organic compounds used in daily life. Exposure to varying amounts and types of these contaminants is linked to impacts on human health and the fields of environmental health and toxicology have focused on bettering the understanding of how these contaminants impact biological processes so that their health impacts can be attenuated or prevented. In the early 2000s, researchers began to examine how environmental exposures and factors may operate at the cellular level not only through genotoxic mechanisms but also through influencing genomic regulation through epigenetic mechanisms. Following animal research pioneered by Jirtle, Skinner and colleagues, epidemiologists and environmental health researchers began to examine the impact of environmental exposure on epigenetic mechanisms in human populations. This review will highlight some of the work done in human environmental epigenetics, considering the implications of the work and the areas for future exploration.

Until recently, most studies of environmentally associated epigenetic effects on humans have been performed in adults, and only in the past several years has the focus moved into the developmental and childhood periods. Of course, the *in utero* developmental period represents only one potential critical period during which the natural environment can impact epigenetic regulation and alter the expression of phenotypes. The 'fetal origins' or Developmental Origins of Health and Disease (DOHaD) hypothesis emerged from a series of studies that demonstrated that measures of birth size are associated with long-term chronic disease risk. The majority of studies have focused on cardiovascular disease and metabolic syndromes (Barker, 1998; Barker and Osmond, 1988; Barker et al., 1989), linking antenatal environmental factors, including diet, xenobiotic exposures, stress and lifestyle factors, to altered fetal growth and, through programming, to permanent biological and physiological changes in the offspring. It is generally accepted that birth size is not at the heart of these disorders but rather is a proxy for a complex interplay of the underlying etiological mechanisms and that there are common factors that influence intrauterine growth as well as adult physiological systems (Welberg and Seckl, 2001). What these studies do suggest, though, is that a significant portion of the risk for common disease and also likely healthy phenotypes may be determined at birth, and, in fact, this fetal programming effect is likely an evolutionary mechanism that allows the organism an opportunity to adapt on a more proximal time scale in order to prepare for the environment it is about to experience.

When considering molecular mechanisms through which long-term programming can occur, epigenetic mechanisms become readily apparent as the key players mediating these effects. Distinct cellular phenotypes are the result of specific gene expression events that are controlled through epigenetic mechanisms. Thus, the entire process of lineage-dependent differentiation characterizing *in utero* development is the result of a complex series of cell-specific epigenetic events, including re-programming of epigenetic patterns such as DNA methylation in a cell-specific context. This differentiation occurs as a series of crucial windows in early fetal development, and the epigenetic mechanisms responsible for differentiation can be influenced by environmental factors.

Following fertilization, there is a rapid wave of epigenomic changes. These include changes in the character and modification of histones, particularly those in the paternal germline as the sperm protamines are replaced by histones present in the oocyte, leading to a relative enrichment of histone variant H3.3, and depletion in modifications such as H3K9me2, H3K9me3 and H3K27me3 (Puschendorf et al., 2008). This replacement is followed

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immediately by loss of genomic DNA methylation in the paternal genome, occurring through an active demethylation process (Mayer et al., 2000; Oswald et al., 2000). The maternal genome also undergoes demethylation, although through a passive process related to an absence of the maintenance DNA methyltransferase Dnmt1 (Ratnam et al., 2002), and potentially to the presence of the oocyte-derived protein PGC7/Stella (Nakamura et al., 2007). Importantly, this demethylation occurs incompletely, as particular genomic regions, including regions involved in genomic imprinting, have been identified to maintain some degree of DNA methylation (Borgel et al., 2010; Smallwood et al., 2011). At least partly, this demethylation is responsible for the totipotency of cells in the morula, while re-establishment of appropriate epigenetic patterning has already begun by the blastocyst stage, allowing for the differentiation of the trophectoderm, which will develop into the extraembryonic tissues from the inner cell mass that will form the fetus (Santos et al., 2005). From this point, DNA methylation is re-established and responsible for cell lineage-dependent differentiation leading to the formation of each of the fully differentiated cells of the offspring (Borgel et al., 2010).

This highly orchestrated process of de-programming and reprogramming of the genome in a lineage-specific fashion during the developmental period provides an opportunity for elaboration of the genome beyond its encoded sequence in order to provide plasticity for adaptation to the environment. This temporality also introduces a critical period during which environmental insults or short-term but influential environmental factors can become biologically embedded in the offspring, potentially resulting in maladaptive phenotypes. Extensive research efforts in understanding which environmental factors can produce epigenetic variation and the types of variation elicited by these exposures is a central topic in children's environmental health and epidemiology research.

Any period of rapid growth and/or additional cellular differentiation has the potential to be a critical period during which environmental factors can impact epigenetic mechanisms. For example, the postnatal period has, at least in animal models, been shown to be a susceptible period for environmental effects on neuronal epigenetics (Weaver et al., 2004). Similarly, puberty may constitute a period of susceptibility for alteration of the epigenome (Biro and Deardorff, 2013). Additionally, there is a body of literature examining the impact of aging on human epigenetics (Bell et al., 2012; Christensen et al., 2009; Florath et al., 2014; Garagnani et al., 2012; Hannum et al., 2013; Horvath et al., 2012; Johansson et al., 2013; Langevin et al., 2011; Madrigano et al., 2012; McClay et al., 2014; Rakyan et al., 2010; Salpea et al., 2012), which could indicate the potential importance of a lifetime of environmental exposures on epigenetic mechanisms and potentially an increased susceptibility for these alterations in an aging population.

Examples of epigenetic variation and specific environmental exposures

The following sections focus on examples of environmental exposures coming from the natural environment and the link between these exposures and epigenetic variation. In particular, exposure to environmental metals in various contexts is most prominent in this literature, as exposure to these metals is known to have important health impacts, and, in many cases, the mechanisms through which these health effects occur at the cellular and molecular level are poorly understood. The majority of studies in human populations have focused on DNA methylation, and only in recent years have additional epigenetic mechanisms including histone modification and the expression patterns of microRNA (miRNA) begun to be explored. This section will highlight each potential epigenetic mechanism and the work relating specific environmental exposures to the mode of epigenetic regulation.

DNA methylation

A major focus of the literature linking environmental exposures to DNA methylation in humans has been on exposure to inorganic arsenic. As described below, this is partly due to the potential toxicity, relatively common occurrence and interesting mechanism of action of this toxicant, and this metalloid serves as an important example of the potential for epigenetic effects of environmental exposures.

Arsenic is listed amongst the top 10 priority hazards on the US Centers for Disease Control and Prevention, Agency for Toxic Substances and Disease Registry (ATSDR) priority list. Particularly amongst those utilizing private or unregulated wells as their water source, this metalloid can be a naturally occurring drinking water contaminant throughout the world. Arsenic's overt toxicity is relatively well characterized, and its effects at high doses in endemic populations broadly reported. Chronic exposure is linked to skin, bladder, lung, liver and kidney carcinogenesis as well as skin hyperkeratoses, and cardiovascular and immunological effects [National Research Council (US) Subcommittee on Arsenic in Drinking Water, 2001]. Prenatal arsenic exposures, in some cases at low levels of exposure, have been linked to reduced birth weight (Cnattingius, 2004; Guan et al., 2012; Hopenhayn et al., 2003; Huyck et al., 2007; Llanos and Ronco, 2009; Rahman et al., 2009; Xu et al., 2011; Yang et al., 2003) although other studies have not observed this association (Kwok et al., 2006; Saha et al., 2012), or have linked exposure to spontaneous abortion or neonatal death, thereby eliminating more subtle effects at birth (Myers et al., 2010; Sohel et al., 2010). Although the overt phenotypes resulting from arsenic exposure are well documented, the mechanistic basis of its toxicity remains unclear, as this metalloid is not overtly genotoxic.

Although arsenic is not a known nutritional requirement for any species, the metabolism of this metalloid has evolved throughout the plant and animal kingdoms including in humans. In higher organisms, including humans, inorganic arsenic is first methylated to monomethylarsonic acid (MMA) and then to dimethylarsinic acid (DMA) catalyzed by arsenic methyltransferases and requiring the co-factor *S*-adenosylmethionine and the presence of glutathione (Roy and Saha, 2002). *S*-Adenosylmethionine is the universal methyl donor of the cell and is required for methylation reactions of cellular nucleic acids, lipids and proteins, including those modifications driving epigenetic regulation. DMA is subsequently excreted in the urine. Arsenic's relatively poor genotoxicity combined with the reliance on *S*-adenosylmethionine for arsenic metabolism has brought particular interest to this metalloid as an epigenetic toxicant.

Arsenic exposure has been associated with DNA methylation alterations in non-pathological as well as tumor tissues and there are some data suggesting that changes in miRNA expression and histone tail modifications are also associated with exposure to arsenic. A study of human adults with high water arsenic exposure found a positive relationship between global methylation of lymphocytes and urinary arsenic levels, which was modified by folate intake (Pilsner et al., 2007). Studies of infants exposed to arsenic *in utero* have demonstrated small increases in cord blood methylation of the *LINE1* repetitive region and *CDKN2A* gene promoter but no change to the *TP53* promoter region (Kile et al., 2012), while others have observed increased methylation of *TP53* gene promoter methylation with no change to repetitive element methylation (Intarasunanont et

al., 2012). The discrepancies may arise as a result of different measures of arsenic exposure and their relevance: the former study utilized drinking water and maternal urine measures (Kile et al., 2012), which represent short-term and potentially variable exposure, while the latter (Intarasunanont et al., 2012) relied on toenail measures, which represent longer term measures during *in utero* development. Maternal urinary arsenic levels have been associated with an overall increase in global 5-methylcytosine levels in offspring cord blood, as well as sex-specific differential relationships between measures of maternal arsenic and various measures of the extent of DNA methylation in infant cord (Pilsner et al., 2012). Our recent epigenome-wide analysis of cord blood DNA methylation and its relationship to *in utero* arsenic exposure demonstrated generalized hypermethylation of CpG loci within CpG islands related to increasing arsenic exposure, and the possibility that arsenic is eliciting alterations in the proportions of specific immune cell subsets, specifically an increased proportion of CD8+ T-lymphocytes (Koestler et al., 2013). A study of a population of Andean women showed the haplotypes of the arsenic-3-methyltransferase (AS3MT) gene modulate the effects of arsenic on DNA methylation of this gene and other genes near this locus, suggesting potential coordinated targeting or selection of methylation in response to exposure (Engström et al., 2013). Similarly, in a Mexican adult population, differences in arsenic metabolism reflected by the concentration of various arsenic metabolites in urine was related to variation in the DNA methylation profiles identified in genome-wide scans of peripheral blood (Bailey et al., 2013).

Histone post-translational modifications

The genotoxic effects of arsenic may also be mediated by altered chromatin. Ramirez et al. have shown that treatment with sodium arsenite in human hepatocarcinoma cells resulted in global increases in histone acetylation (Ramirez et al., 2008). In a substudy of a folate trial in highly arsenic-exposed Bangladeshi adults, total urinary arsenic levels were correlated with peripheral blood mononuclear cell global levels of histone H3 lysine 9 (K9) di-methylation and inversely correlated with histone H3-K9 acetylation, and sex led to opposite relationships between both urinary and water arsenic levels and global post-translational histone modifications (Chervona et al., 2012). This is consistent with a study of male adult steel plant workers, which linked plant air levels of nickel, arsenic and iron to increases in histone H3-K9 acetylation and histone H3-K9 di-methylation (Cantone et al., 2011). By demonstrating that related and overlapping epigenetic mechanisms are also susceptible to alteration by arsenic exposure, these findings lend support to the studies linking DNA methylation and arsenic exposure in human populations. They also suggest that sex, possibly through the action of sex hormones, can influence these relationships and may play an important role in the epigenetic effects of environmental exposure, possibly through the action of sex hormones.

miRNA expression

The expression patterns of miRNA and their susceptibility to environmental exposures is a growing area of research, with only a small number of publications interrogating this epigenetic mechanism. In one of the first studies on the susceptibility of miRNA expression to exogenous agents, we demonstrated that treatment of human lymphoblastoid cells with sodium arsenite led to global increases in miRNA expression, similar to those that might be observed with cellular nutritional stress such as folate deficiency; further, we identified these differences in human peripheral blood samples (Marsit et al., 2006). In the occupational setting of a steel factory, week-long workplace exposure to particulate matter containing arsenic, iron, nickel, lead, cadmium, chromium and manganese led to increases in miR-222 and miR-21 expression in peripheral blood (Bollati et al., 2010). However, a study of children aged 12–19 years found decreased expression of both miR-21 and miR-221 associated with increasing urinary arsenic and lead levels (Kong et al., 2012), but no associations between these miRNA levels and mercury, cadmium or lead levels. In a Mexican pregnancy cohort, maternal total urinary arsenic was associated with the increased expression of 12 miRNAs in infant cord blood identified through genome-wide miRNA analysis, and this study suggested that these miRNA alterations can lead to gene expression changes in these samples (Rager et al., 2013).

Genomic imprinting

Imprinted genes, in particular, have been highlighted as ideal epigenetic environmental sensors (Hoyo et al., 2009). Methylation of imprinting control regions (ICRs) and ICR-like elements (ILEs) controls the allele-specific expression of imprinted gene clusters ranging from one to nine genes (Lewis and Reik, 2006). ICRs may be located several kilobase pairs (kbp) away from a specific imprinted gene cluster, in non-coding genomic areas or in the promoter or gene body of other imprinted/not imprinted genes (Lewis and Reik, 2006). ILEs, in contrast, refer to the promoter region of imprinted genes that are not clustered (Bliek et al., 2009; Lewis and Reik, 2006; Monk et al., 2008; Paulsen et al., 2001). The fact that imprinting marks are established early in development also suggests that any alteration is likely to be detectable in most tissues and to have wide-ranging effects (Jirtle and Skinner, 2007). This has been demonstrated empirically in a study of Dutch famine survivors who experienced severe caloric restriction *in utero* and demonstrated hypomethylation of the imprinted IGF2 differentially methylated region (DMR) in peripheral blood, six decades later (Heijmans et al., 2008).

A new study of twins also demonstrated discordant ICR methylation among monozygotic twins implicating environmental influence on imprinting control (Ollikainen and Craig, 2011). In both human and animal studies, specific environmental exposures, including bisphenol-A, cigarette smoke and ethanol, have been linked to genomic imprinting alterations in various tissues (Aros et al., 2011; Haycock and Ramsay, 2009; Murphy et al., 2012; Prins et al., 2008; Stouder et al., 2011; Zhang et al., 2012). Dietary factors, specifically folate supplementation in pregnant women, have been linked to reduced methylation of the imprinted IGF2 gene's ICRs in cord blood, an effect most pronounced in male infants (Hoyo et al., 2011). There is also some controversial literature on the impact of assisted reproductive technologies and *in vitro* fertilization on imprinting status, which has been suggested to be related to exposures experienced by the embryo during *in vitro* culture (DeBaun et al., 2003; Oliver et al., 2012; Puumala et al., 2012; Rancourt et al., 2012; Shi et al., 2011; Wong et al., 2011).

Mechanisms

There remain significant questions regarding the mechanisms through which environmental exposures drive or select epigenetic alteration. In fact, dependent upon context and exposure, different mechanisms can contribute to epigenetic alterations. Such contextual situations can include genomic characteristics such as relative location (repeat, promoter, other), existing methylation state, nucleosome position, local and regional CpG density and

transcription factor binding sites. It is important to recognize, though, that contextual elements may differentially affect the likelihood of exposure-related alterations. For example, the chromatin architecture, even simply considering heterochromatin versus euchromatin, may impact the ability of exposures that directly contact DNA or transcription factors and various binding elements to lead to epigenetic changes.

We must also keep in mind that we do not yet have a complete understanding of *de novo* and maintenance methylation mechanisms in development and even throughout the lifespan. Nonetheless, several mechanisms for environmental exposure-related epigenetic alterations should be considered.

A number of reports have linked reactive oxygen species and oxidative stress either due to exposure or as a result of inflammatory response as key factors in the mechanism leading to epigenetic alteration. Specifically, 5-hydroxymethylcytosine can be generated by oxidation of 5-methylcytosine (Masuda et al., 1975), and both 5-methylcytosine, adjacent to 8-oxoguanine, and 5-hydroxymethylcytosine have been shown to inhibit binding of methyl-CpG binding protein 2, a critical epigenetic regulator that recruits cytosine methyltransferases and histone deacetylases (Valinluck et al., 2004). Additionally, 5-hydroxymethylcytosine is not recognized as 5-methylcytosine by the maintenance methyltransferase DNMT1 and, hence, may lead to aberrant loss of methylation during cell replication (Valinluck and Sowers, 2007). Halogenated cytosines can form via neutrophil and eosinophil peroxidase-derived bursts and can be mistaken by DNMT1 as 5-methylcytosine during replication, thus providing a potential mechanism for inflammation-induced aberrant hypermethylation (Henderson et al., 2003; Valinluck and Sowers, 2007).

DNA methylation spreading, a process whereby CpG methylation from within the gene migrates into the promoter as a result of the loss of protective boundary elements that normally protect promoters from CpG island methylation (Sun and Elgin, 1999; Turker, 2002; Turker and Bestor, 1997), is also a potential mechanism for altered methylation occurring outside the developmental window. Alternatively, this spreading can occur subsequent to a stochastic methylation alteration potentially resulting from aging, which can then allow the initiation of a spreading process that eventually confers an altered expression phenotype. This drift of normal epigenomes, which may initially occur without functional effect, may confer significantly increased risk of conversion to a pathological phenotype by enhancing both the likelihood and frequency of methylation events that ultimately result in altered expression or genomic stability. In the context of acquired 'non-functional' CpG methylation in the promoter region of an aged individual, continued stochastic methylation events (e.g. 'methylation spreading') increase the chance of methylation-induced silencing at that promoter (or silencing of another locus through action at a distance via silencing of other important regions such as enhancers) and, hence, progression to a pathological phenotype.

Genomic hypomethylation, in addition to specific gene hypermethylation, is also an important form of epigenetic alteration and is a common feature of environmentally induced altered methylation (Ehrlich, 2002). This DNA hypomethylation, dependent on context, may lead to the expression of normally silenced transposons, resulting in an increase in transposition events and increases in genomic instability (Florl et al., 1999). Hypermethylation, then, may be occurring as a defense against retrotransposition and the resulting instability, as methylation is thought to suppress homologous recombination (Colot and Rossignol, 1999; Domínguez-Bendala and McWhir, 2004; Maloisel and Rossignol, 1998). There remain significant questions regarding the mechanisms responsible for altering DNA methylation and chromatin modification in response to the natural environment.

An additional issue to be considered mechanistically is the role of sexual dimorphism, which is becoming apparent in some studies of environmentally related epigenetic alterations. Sexually dimorphic associations may arise from different susceptibilities to exposure by sex, or by the influence of sex chromosomes or sex hormones on exposure or epigenetic programming, and, particularly during development, sexual differences in cell, tissue and organ development (Gabory et al., 2009). In fact, even transgenerational inheritance of epigenetic alterations may occur in a sexually dimorphic manner (Taylor et al., 2005) and there are known sex-related differences in epigenetic reprogramming (Morgan et al., 2005). This highlights the importance of considering differences in epigenetic variation by sex and in aiming to understand the mechanisms that drive these differences.

Challenges in epigenetic environmental epidemiology

Cellular composition and heterogeneity

The key role that epigenetic mechanisms, such as DNA methylation, play in cellular differentiation and determination also leads to a fundamental challenge in the examination of epigenetic alterations related to the environment in human population studies. Unlike genetic studies, where genotype can be consistently determined from any available diploid cell, the epigenome is tissue, cell and context specific, and so these considerations must be taken into account to understand environmental impacts on epigenetic mechanisms. Unlike animal models, where target tissues can be accessed and purification to the cellular level accomplished, human studies are more limited in tissue accessibility, and, dependent on sample size, may not be able to reliably focus at the cellular level, which is required for observation of cell type-specific phenomena.

These tissues themselves represent a complex mixture of various, differentiated cellular components, each with their own inherent epigenomes. The challenges associated with cellular mixtures in epigenetic epidemiology have become a significant source of hesitation in moving the field forward (Verma et al., 2014). Ultimately, purification to specific cells of interest to allow for specific examinations of epigenetic variation within a cell is ideal, but is feasible, generally, in only freshly collected samples and in situations where such advanced cell biological techniques can be implemented rapidly. Many studies of exposed populations, though, occur in developing regions or in areas without such immediate access, or rely on samples collected years or decades prior to the study. Biostatistical methodologies making use of referent cell populations and genome-scale DNA methylation assessments are now allowing for estimation of the contribution of cell mixture in assessments from peripheral blood (Houseman et al., 2012), and emerging techniques are enabling the control of confounding effects of cell mixture even if the underlying populations of cells are not known (Houseman et al., 2014; Zou et al., 2014). These advancements will open the door for additional genome-wide studies of DNA methylation in relation to various environmental factors and exposures.

Assays of epigenetic features in human populations

The vast majority of human epidemiological studies of epigenetic variation in response to the environment have focused on DNA methylation. This is because of the stable nature of DNA methylation, and the availability of various high-throughput technologies, including bisulfite sequencing and PCR, as well as

array-based methodologies to determine the presence of methylated cytosines.

Studies of DNA methylation in human samples have focused on assays of 'global' methylation extent, considering the overall content of 5-methylcytosine present in a DNA samples, or on the assessment of gene/site-specific DNA methylation using targeted sequencing or high-throughput genome-scale approaches. Alterations to 'global' methylation are thought by many to represent a passive dosimeter, which is susceptible to alteration by various environmental insults (Schulz et al., 2006), and a large body of literature is emerging linking these alterations with various exposures, as well as with disease outcomes both in retrospective and now prospective studies (Baccarelli et al., 2010a; Baccarelli et al., 2010b; Baccarelli et al., 2009; Bollati et al., 2007; Bollati et al., 2009; Pavanello et al., 2009; Tarantini et al., 2009; Wilhelm et al., 2010). A number of methods have been developed to allow the quantification of the global methylcytosine content of DNA samples. Total methylcytosine can be directly measured using chromatographic methods following digestion of the DNA into single nucleotides. Although these methods are highly quantitative, they generally require large amounts of DNA and highly specialized equipment, and are generally difficult to standardize for use on large sample collections, like those obtained in an epidemiological context. Thus, many research groups have opted for PCR-based methods that capitalize on the bisulfite modification chemistry. In 2005, Weisenberger et al. published data describing methylation at satellite repeats, *LINE-1* and *ALU* elements as being reasonably well correlated with total methyl-cytosine content using a quantitative PCR-based approach (Weisenberger et al., 2005). This effort followed a report by Yang et al. (Yang et al., 2004) describing the initial use of bisulfite sequencing of *LINE-1* and *ALU* elements for determination of global methylation. These studies prompted a new line of investigation, using quantitative methylation at repetitive elements in lymphocytes to evaluate the association of variation in environmental exposures with levels of what became accepted as a measure of 'global DNA methylation'. The true relationship of methylation of these repetitive sequences and total methylcytosine content has more recently been called into question, with groups unable to replicate the correlations between total methylcytosine content and *LINE-1* methylation measured with bisulfite pyrosequencing (Choi et al., 2009). Others have questioned whether the underlying biology of these ancient retroviral elements may be influencing their methylation extent, and whether interpreting these findings merely as markers of global levels is truly representative of the underlying biology of these alterations (reviewed in Nelson et al., 2011). Certainly, the issues of cellular composition and heterogeneity are also potentially important confounders to studies of global methylation in blood or tissues, as it is possible and likely that individual cells may have subtle variations in global or repetitive element methylation that may then influence the observed measures dependent on the underlying distribution of cells.

Gene-specific DNA methylation can be assessed using targeted approaches. Herman and colleagues' development of methylation-specific PCR in 1996 (Herman et al., 1996) opened up the assessment of DNA methylation at specific CpG sites to studies of human populations – they based their method on the sequencing subsequent to sodium bisulfite modification pioneered by Frommer et al. (Frommer et al., 1992). These methods have evolved to be highly quantitative, either using real-time PCR for quantitative methylation-specific PCR of specific CpG sites or through the use of pyrosequencing or mass spectrometry to quantify the extent of methylation in short sequence reads (Dupont et al., 2004; Ehrich et al., 2005). In addition, the scale of the assessment of DNA methylation has grown through the use of various platforms and techniques, now achieving genome-scale coverage with high-throughput sequencing and high-density array-based technologies (reviewed in Laird, 2010).

These technologies still suffer the same difficulties as all epigenetic studies, and studies utilizing these genome-scale technologies in particular are susceptible to the influence of cellular composition, although the statistical methods to control for these issues are particularly designed for use in these high-throughput settings (Houseman et al., 2012; Houseman et al., 2014). There are also platform-specific issues that must be taken into account. For example, the array-based approaches require significant efforts for quality assurance and quality control, as well as normalization for technical variability introduced in the assessments (Harper et al., 2013; Wilhelm-Benartzi et al., 2013). Genome-wide bisulfite sequencing remains technically challenging, as the bisulfite modification reduces specificity so genome alignment presents a computation burden, and accurate calls require deep read depths leading to a cost that remains prohibitive for large, population-based studies.

As a result of the development of these new technologies and a better understanding of their limitations and strengths, there is a growing surge in epigenome-wide association studies (EWAS) to link site-specific DNA methylation to phenotypic outcomes as well as to environmental exposures. One feature, thus far, of these EWAS studies in non-pathological tissues has been that the findings of altered DNA methylation have been relatively small in scale, usually less than a 10% change in methylation by phenotype or exposure of interest at any identified site. Much of this may be related to the cell composition issue, as the actual change in DNA methylation may be limited to only specific cells within a sample, and so the overall effect within a sample is diluted. This can be compared with studies in human tumor samples, where effects can be magnified as a result of the clonality of the sample, although even here heterogeneity likely biases the effects to be smaller than what might be observed in a pure population. There has also been limited examination of the impact of these small changes on gene expression. Often the cost of performing both genome-scale DNA methylation and expression experiments in large populations leads to a deficit in these data. In addition, sampling and storage conditions may affect the assessment of gene expression, as RNA is much more unstable than DNA and DNA methylation, and so there is often greater variability observed in gene expression assessments, which may weaken the ability to detect relationships between methylation and expression. Ongoing efforts should continue to seek ways in which the functional effects of identified altered patterns of DNA methylation operate in order to better understand the biological effects of altered epigenetic regulation.

Finally, studies of histone alterations in large, population studies has been generally limited to examinations of 'global' levels of modified histones, as such assessments can be scaled for reproducible assessments in large sample sizes using ELISA techniques. Examinations of non-coding RNA, such as miRNA have also been performed in human samples, on a more limited scale, but this area of research is one of growth. Incorporation of these additional layers of epigenetic regulation in integrated analyses of human populations will be essential to provide a complete understanding of the epigenetic impact of environmental contaminants.

Additional factors influencing epigenetics

All human studies can be challenged by issues of underlying confounding factors, which can often be difficult if not impossible

to measure or to control. For example, underlying genetic variability inherent in the human population can play an important role in epigenetic programming and variation. These genetic differences can include variations reflected in race or ethnicity, but also even more subtle genetic heterogeneity. Studies linking genetic variability and race or ethnicity and epigenetic mechanisms have been limited. Where studies have examined genetic variation and epigenetics, they have generally focused on candidate genes involved in metabolism of folate or the pathways involved in the epigenetic mechanism, or in some cases on specific relationships between genetic variants within a gene and their epigenetic consequences (Bromer et al., 2013; Klengel et al., 2013; Lesseur et al., 2013; Paquette et al., 2013). There have been suggestions of differences in DNA methylation by race and ethnicity (Terry et al., 2008), although strong examinations that can control for the confounding factors of environment and race have not yet been undertaken, and are an important area to consider.

In addition, most studies linking environmental exposures and epigenetic mechanisms have done so considering single exposures. Of course, even under the most controlled conditions, humans experience highly variable mixtures of exposures from the natural and man-made environment. Considering this complexity has garnered enthusiasm in light of the growing technologies and field of 'exposome research' although this has yet to be rigorously applied to epigenetic studies (Buck Louis et al., 2013; Rappaport, 2012; Wild et al., 2013).

Future efforts in human environmental epigenetic research

One area of increasing interest in epigenetic research is the concept of transgenerational inheritance. Initially, it was believed that because of both the reprogramming of epigenetic patterns in gametogenesis and the erasure of DNA methylation and other marks during the early phases of embryogenesis, epigenetic regulation would not be passed on to successive generations. Yet, it is well known that genomic imprinting, the patterning of parent-of-origin allele-specific expression of a subset of mammalian genes is established in the germline and is maintained in all somatic lineages (Jaenisch, 1997). Thus, mechanisms must exist to maintain some level of epigenetic patterning across successive generations.

It is important to differentiate transgenerational from intergenerational inheritance. The latter would represent effects on offspring of the parents' environment, including the effects of nutrition or environment exposures during pregnancy on the developing fetus and its germline, and thus would encompass effects in the F_1 and F_2 generations. True transgenerational effects, in contrast, would be observable in the F_3 generation and beyond (Daxinger and Whitelaw, 2012; Ferguson-Smith and Patti, 2011; Lim and Brunet, 2013). This strict consideration will make it difficult if not impossible to conclusively demonstrate the existence of transgenerational inheritance in humans, where control or at least adequate assessment of the environments experienced over three generations is not feasible. Studies in plants and in model organisms where experimental design can allow for exquisite control across multiple generations are truly the only way in which the issue of transgenerational inheritance can be appropriately assessed (Heard and Martienssen, 2014). Studies in model systems will eventually shed light on the key mechanisms involved in this type of inheritance and then the presence and prevalence of those mechanisms can be assessed in human populations in more tractable time frames to provide evidence, but not definitive proof, of the existence of transgenerational inheritance.

There is a growing interest in the study of environmental exposures and epigenetic alterations, including exposures from the natural and man-made environment, as well as the environment more broadly, including the impact of psychosocial stressors and community context. As this research moves forward, it would be of use to consider these environments even more broadly. Given that epigenetic variation may denote a mode of short-term adaptation to the environment, considering significant environmental issues such as climate change on the epigenome would be of great interest. Although challenging for human studies, more focused work on transgenerational effects will also help to elucidate the potential role of epigenetic mechanisms in short-term adaptation.

In summary, human population and epidemiological studies can play an important role in understanding the impact of the environment on the epigenome. Through cross-disciplinary research, careful study design and novel application of new molecular technologies, it will be possible to begin to better understand these impacts and to put the role of epigenetic variation in humans in a wider context.

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