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Long-term alterations to DNA methylation as a biomarker of prenatal alcohol exposure: From mouse models to human children with fetal alcohol spectrum disorders

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ABSTRACT

Rodent models of Fetal Alcohol Spectrum Disorders (FASD) have revealed that prenatal alcohol exposure (PAE) results in differential DNA cytosine methylation in the developing brain. The resulting genome-wide methylation changes are enriched in genes with neurodevelopmental functions. The profile of differential methylation is dynamic and present in some form for life. The methylation changes are transmitted across subsequent mitotic divisions, where they are maintained and further modified over time. More recent follow up has identified a profile of the differential methylation in the buccal swabs of young children born with FASD. While distinct from the profile observed in brain tissue from rodent models, there are similarities. These include changes in genes belonging to a number of neurodevelopmental and behavioral pathways. Specifically, there is increased methylation at the clustered protocadherin genes and deregulation of genomically imprinted genes, even though no single gene is affected in all patients studied to date. These novel results suggest further development of a methylation based strategy could enable early and accurate diagnostics and therapeutics, which have remained a challenge in FASD research. There are two aspects of this challenge that must be addressed in the immediate future: First, the long-term differential methylomics observed in rodent models must be functionally confirmed. Second, the similarities in differential methylation must be further established in humans at a methylomic level and overcome a number of technical limitations. While a cure for FASD is challenging, there is an opportunity for the development of early diagnostics and attenuations towards a higher quality of life.

Alcohol consumption during any stage of pregnancy can result in the development of fetal alcohol spectrum disorders (FASD) (Singh, Laufer, & Kapalanga, 2014; Williams & Smith, 2015). FASD is an umbrella term for a heterogeneous spectrum of related developmental disorders that are caused by prenatal alcohol exposure (PAE), including fetal alcohol syndrome (FAS) at the extreme (Jones, Smith, Ulleland, & Streissguth, 1973; Lemoine, Harousseau, Borteyru, & Menuet, 1968; Williams & Smith, 2015). FAS shares commonalities with other psychiatric disorders including growth deficiency and central nervous system impairment. FAS is distinguished from other psychiatric disorders by a history of maternal alcohol consumption during pregnancy and three distinct facial features (Clarren & Smith, 1978). The first feature is a smooth philtrum: the vertical groove between the nose and upper lip is flattened. The second feature is a thin vermilion: a thin upper lip. The third feature consists of small palpebral fissures: a reduced width of the eye. However, these facial characteristics used to distinguish FAS and partial FAS (pFAS) do not typically apply to the lower part of the FASD spectrum, which is characterized by neurobehavioral and neuroanatomical alterations without the distinctive facial features.

The Institute of Medicine (IOM) of the National Academies has four diagnostic categories for the disorders that constitute FASD: FAS, pFAS, alcohol-related neurodevelopmental disorder (ARND), and alcohol-related birth defects (ARBD) (Chudley et al., 2005;

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Stratton, Howe, & Battaglia, 1996). The latest edition of the diagnostic and statistical manual of mental disorders (DSM–5) now includes the psychiatric diagnosis "Neurobehavioral Disorder Associated with Prenatal Alcohol Exposure (ND-PAE)" (American Psychiatric Association, 2013). ND-PAE considers the neurobehavioral alterations to be independent from the facial characteristics and is listed in the DSM–5 appendix under "conditions for further study" (Doyle & Mattson, 2015). There are 428 comorbidities related to FASD (Popova et al., 2016). FASD is associated with congenital malformations to the ocular, auditory, skeletal, cardiac, and renal systems. The clinical heterogeneity and uniqueness of each case appears to be driven by the combination of dosage and timing of PAE, other exposures, genetic background, sex, family history of PAE, as well as maternal genotype, nutrition and stress (Kleiber et al., 2014).

Endophenotype is a psychiatric genetic concept that deconstructs a complex behavioral phenotype into components with a clearer genetic connection (Gottesman & Gould, 2003). The endophenotypes of FASD overlap with other psychiatric disorders, particularly attention deficit hyperactivity disorder (ADHD). While an individual can be diagnosed with both disorders, they are distinct, and misdiagnosis of one can lead to inappropriate medication and therapy (Greenbaum, Stevens, Nash, Koren, & Rovet, 2009; O'Malley & Nanson, 2002; Peadon & Elliott, 2010). Endophenotypes commonly associated with FASD include impairments in cognition, learning, executive function, judgment, attention, and social adaptation (Green et al., 2014; Jirikowic, Olson, & Kartin, 2008; Mattson & Riley, 1998). The molecular mechanisms behind FASD endophenotypes have been difficult to ascertain in humans; however, animal models have greatly aided research (Gil-Mohapel, Boehme, Kainer, & Christie, 2010; Kiecker, 2016). The results suggest that FASD endophenotypes are a result of cellular and molecular aberrations to neurodevelopment that are caused by PAE (Resendiz, Mason, Lo, & Zhou, 2014).

1. Epigenetic mechanisms may underlie the altered gene expression of PAE

In addition to altering gene expression, PAE alters epigenetic marks (Basavarajappa & Subbanna, 2016; Haycock, 2009; Kobor & Weinberg, 2011). Epigenetic marks maintain gene expression profiles related to development and tissue specificity without altering the DNA sequence and are heritable in dividing cells. Epigenetic marks are distinct from transcription factors; however, they influence each other because gene expression is initiated by transcription factors recruiting RNA polymerase. Epigenetic marks include DNA cytosine methylation (Smith & Meissner, 2013), select histone post-translational modifications (Gurard-Levin & Almouzni, 2014), and a diverse array of non-coding RNA (ncRNA) species (Pauli, Rinn, & Schier, 2011; Rinn, 2014). These marks enable spatial and temporal control of gene expression by acting together to create distinct states of variation that have unique functions in the brain (Sweatt, Meaney, Nestler, & Akbarian, 2012). While each mammalian cell has a (nearly) identical genome, there are layers of diversity that give each cell type a unique epigenome that is reflective of ontogeny.

One of the most examined mechanisms of the epigenome is DNA methylation: a highly dynamic modification that is used to store additional information on nucleotides. The most widely studied DNA methylation mark is 5-methylcytosine (5 mC). During embryonic development, there are two waves of genome-wide DNA de-methylation (Seisenberger et al., 2013). The first follows the fertilization of the preimplantation embryo, and the second occurs during the establishment of primordial germ cells in the embryo that will go on to form sperm and oocytes. The first wave of DNA de-methylation retains genomic imprinting, and the second resets it. After the waves of de-methylation, methylation is established *de novo*, maintained across cell division, and also locally modified throughout development as cells become committed to their lineage (Hemberger, Dean, & Reik, 2009). DNA methyltransferases (DNMTs) are an enzyme family capable of establishing and maintaining DNA methylation. In vertebrates, DNMTs are involved in establishing methylation at CpG dinucleotides and other sequences in the genome. CpG islands are a regulatory feature composed of greater than 50% GC content and range from 300 to 3000 bp (Fatemi et al., 2005; Gardiner-Garden & Frommer, 1987). When methylated, CpG islands typically result in the repression of gene expression and are seen in promoters or the first exon of ~40% of mammalian genes (Brenet et al., 2011; Fatemi et al., 2005). Furthermore, ~70% of human gene promoters have a higher CpG content than is expected when compared to the rest of the genome (Saxonov, Berg, & Brutlag, 2006). Intragenic, or gene body, CpG islands may have a role as alternative tissue-specific promoters (Maunakea et al., 2010). Finally, CpG islands are also mutational hotspots due to the tendency of 5 mC to spontaneously undergo deamination and mutate to thymine. Genome-wide scans of CpG sites have revealed that CpG island shores, rather than CpG islands, may be the primary sites of normal tissue-specific differentially methylated regions (DMRs) (Doi et al., 2009; Irizarry et al., 2009; Yasui et al., 2007). CpG island shores are ~250 bp in size, up to 2 kb away from a CpG island, and typically outside of gene promoters. DMRs in CpG island shores often show a strong inverse relationship with gene expression. CpG island shores are associated with developmental genes and are conserved across mice and humans. Additionally, there are CpG island shelves that are located 2–4 kb from a CpG island and also the open sea, which refers to genomic regions that show no significant CpG enrichment (Sandoval et al., 2011). Overall, DNA methylation is a widely used epigenetic mark in mammals and occurs at a number of distinct genomic features.

2. Differential methylation identified in rodent models

DNA methylation is the most studied marker for epigenomic dysregulation, including PAE-induced dysregulation (Haycock, 2009). In 1991, Garro et al. observed that binge PAE exposure in pregnant mice resulted in global hypomethylation of fetal DNA (Garro, McBeth, Lima, & Lieber, 1991). The group also observed that low levels of acetaldehyde, a metabolite of ethanol, were able to interfere with DNA methyltransferase activity *in vitro*. In 1999, Maier et al. found that PAE can result in increased methylation and decreased expression of brain-derived neurotrophic factor (*Bdnf*) in the developing olfactory bulb of rats exposed during the equivalent of the first two trimesters of human pregnancy (Maier, Cramer, West, & Sohrabji, 1999). Together, these studies suggested that PAE alters DNA methylation.

In 2009 Haycock & Ramsay examined the effect of PAE on genomic imprinting in pre-implantation mouse embryos by examining the *H19* differentially methylated region (DMR) (Haycock & Ramsay, 2009). They observed that while PAE embryos were severely growth restricted, hypomethylation of the paternal allele at this specific region was only detectable in the placenta. Genomic imprinting is an epigenetic process that utilizes DNA methylation and other marks to enable parent-of-origin specific monoallelic expression of genes related to growth and development. Thus, the study suggests that genomic imprinting is deregulated by PAE.

Later in 2009, Liu et al. examined the genome-wide effects of ethanol in a B6 whole embryo culture (Liu, Balaraman, Wang, Nephew, & Zhou, 2009). Gene expression arrays and methylated

DNA immunoprecipitation (MeDIP) arrays (MeDIP-Chip) revealed that genes of promoters with a high CpG content had decreased methylation and increased expression from binge exposure with an enrichment for genes on chromosomes 7, 10, and X. A 10-fold increase of methylation was observed on chromosomes 10 and X in exposed embryos with neural tube defects when compared to those without. The alterations to methylation were seen in a large number of genes associated with olfaction as well as genes involved in development, chromatin, and genomic imprinting. The altered methylation was associated with changes in the expression of 84 genes. This study suggests that PAE induced alterations to methylation are not random. The findings also demonstrate that most genes showing significantly altered methylation do not show significantly altered expression when examined.

A number of studies have examined the mechanism behind PAE induced alterations to DNA methylation. In 2010, Kaminen-Ahola et al. used the Agouti viable yellow (A^{vy}) B6 mouse model, which provides a visual phenotype when methylation is altered at a single gene (Kaminen-Ahola et al., 2010). This revealed that voluntary maternal consumption of 10% alcohol causes hypermethylation. This study showed that PAE at the embryonic stage can result in a long-lasting alteration to DNA methylation. In 2011, Downing et al. examined methylation of the imprinted *Igf2* locus in mouse embryos and observed a subtle ~8% decrease to methylation at a single CpG site with associated transcripts showing a −1.5 fold change (Downing et al., 2011). Notably, placing mothers on a methyl-supplemented diet attenuated some of the PAE endophenotypes. Later in 2011, Zhou et al. examined the effects of alcohol exposure on rat neural stem cell culture and found altered migration, neuron formation, and growth that was similar in an effect to the DNA methyltransferase inhibitor 5-aza-cytidine (Zhou et al., 2011). During differentiation, it was found that alcohol exposure prevented the reprogramming of genes related to neurodevelopment, neuronal receptors, and olfaction. The specific sites showing altered methylation were correlated with transcription factors related to neurodevelopment. Overall, these studies link PAE induced differential DNA methylation to birth defects.

Continuous maternal preference drinking also produces offspring with altered DNA methylation. In 2013, Chen et al. used immunostaining to visualize altered DNA methylation programming in the developing C57BL/6J mouse hippocampus (Chen, Ozturk, & Zhou, 2013). In 2015, Marjonen et al. examined a voluntary maternal preference drinking model of PAE and found that early gestational alcohol exposure results in alterations to gene expression in the adolescent post-natal day (PND) 28 hippocampus, bone marrow, and main olfactory epithelium (Marjonen et al., 2015). The group observed gene expression changes related to olfaction, immune response, and two keratin associated proteins in the hippocampus. They also observed differential expression of a histone and miRNAs. Genes showing differential expression belonged to similar families reported by our group (Kleiber, Laufer, Wright, Diehl, & Singh, 2012). Marjonen et al. also used bisulfite sequencing and found alterations to DNA methylation in CpG islands upstream of some of the genes. Furthermore, magnetic resonance imaging (MRI) of PND 60 PAE adult mice revealed an enlargement of the left hippocampus and decreased volume of the left olfactory bulb. While the differential expression in the hippocampus was low and not possible to confirm by quantitative reverse transcription PCR (qPCR), some of the gene expression alterations identified by the gene expression array of the hippocampus were also confirmed in the bone marrow or main olfactory epithelium by qPCR. These observations suggest that differences present in adults with PAE are a long-term result of earlier alterations in embryonic stem cell populations, which differentiate into a number of tissues and maintain some signature of developmental alterations.

DNA methylation alterations induced by PAE are also transmitted transgenerationally. In 2012, Govorko et al. examined a rat model of continuous maternal preference drinking from GD7 to GD21. The group examined *Pomc*, a gene that produces multiple peptide hormones, and found that adult (PND60-80) F1 offspring had alterations in the hypothalamus, which is involved in stress reaction (Govorko, Bekdash, Zhang, & Sarkar, 2012, 2016). The neurons showed increased CpG methylation at the *Pomc* promoter, suppressed gene expression, and a resulting phenotype of altered stress response. Alterations to DNA and histone methyltransferase gene expression levels were also seen. The alterations to the methylation, expression, and function of *Pomc* were also found to be transmitted to both the F2 and F3 generations by the male germline with sperm showing alterations to *Pomc* methylation. These findings show that the DNA methylation alterations induced by PAE can transmit not only through mitosis but also through meiosis.

Overall, rodent model studies suggest that binge or continuous PAE at any developmental time point can result in both short-term and long-term alterations to DNA methylation. These alterations appear to be dependent on the dosage and timing of exposure as well as the cell type(s) and developmental time point examined.

3. Mechanisms of PAE induced alterations to the epigenetic landscape

PAE appears to alter a number of epigenetic marks in overlapping and distinct regions of the genome. The altered loci are enriched for functions related to cellular fate commitment events that occur during neurodevelopment. Thus, it appears that PAE results not only in immediate toxicity, but also creates distinct epigenotypes that may influence later developmental events. Conrad Waddington proposed the concept of the epigenotype (Waddington, 2012), which led to his theory of canalization and the epigenetic landscape (Bird, 2007; Reik, 2007; Waddington, 1942). The epigenetic landscape is an abstract concept that describes the dynamic epigenomic profiles of stem cells during development. As cells differentiate, they become increasingly committed to certain lineages and are poised (or primed) at the epigenetic level for later events and certain responses (Lesch, Dokshin, Young, McCarrey, & Page, 2013; Waddington, 1942). The brain is highly dependent on the epigenome for neurodevelopmental processes (LaSalle, Powell, & Yasui, 2013). Developmental trajectories are also sensitive to the environment, where exposures may alter quality of life long after the initial exposure and immediate toxicology (LaSalle, 2013). This effect appears to be due to an altered epigenetic programming related to future development that is continuing to exert an effect as well as a shift in vulnerable cell populations. Therefore, early life exposures may continue to exert an effect on adult hippocampal neurogenesis. One third of neurons in the hippocampus are subject to exchange over a lifetime, with an annual turnover rate of 1.75% in the renewing fraction of adult humans that is also similar in mice (Spalding et al., 2013). Ultimately, the above suggests that some PAE endophenotypes may be maintained by long-term alterations from the initial exposure.

Studies examining one-carbon metabolism have provided insight into how PAE initiates disruptions to DNA methylation, while also demonstrating the preventable nature of some long-term alterations. Alcohol exposure during embryonic development affects the transfer of folate from the mother to the developing embryo (Hutson, Stade, Lehotay, Collier, & Kapur, 2012). This is of significance to the developing embryo because folate is a methyl donor and essential in establishing and maintaining DNA methylation. The lack of folate has the potential to cause aberrant epigenetic profiles. Treatments that contain methyl donors, such as

choline, have been able to attenuate some of the effects of PAE (Zeisel, 2011). Of particular interest is the observation that co-incubation of alcohol-exposed mouse embryos with folic acid, a synthetic form of folate, was able to prevent altered expression of a miRNA and target gene (Wang et al., 2009). Such results argue that the alcohol-induced molecular cascade might involve DNA methylation. In 2013, Bekdash et al. examined the hypothalamus of adult offspring of a voluntary maternal consumption rat model and found a decrease in histone modifications related to open chromatin and an increase in DNA methylation and a histone modification related to closed chromatin (Bekdash, Zhang, & Sarkar, 2013). Furthermore, they observed increased methylation of the *Pomc* promoter and a decrease in *Pomc* mRNA expression. Notably, these alterations could be attenuated with choline supplementation during PAE. Using the same model in 2014, Gangisetty et al. demonstrated a mechanistic role for MeCP2 in altering *Pomc* expression upon PAE (Gangisetty, Bekdash, Maglakelidze, & Sarkar, 2014). Overall, these studies suggest that altered DNA methylation is mechanistically responsible for some PAE induced differential gene expression and that the prevention, or even potentially the reversal, of these alterations can attenuate some of the endophenotypes.

Given the above observations, it appears that PAE can alter DNA methylation in specific regulatory sequences. For example, CTCF is a highly conserved ubiquitous 11-zinc-finger protein with multiple functions in 3D chromatin organization and gene regulation, including chromatin insulator activity as well as transcriptional activation and repression (Williams & Flavell, 2008). CTCF binds to a set of signal sequences that are sensitive to methylation (Filippova, 2007) and mediates chromosomal interactions by forming chromatin loops (Ling et al., 2006). There are over 100, 000 CTCF-binding sites in the mouse genome (Shen et al., 2012). CTCF binding sites are not limited to promoters; they include intergenic regions, such as enhancers. CTCF-binding sites involved in the DMR of *H19* have shown significant differential methylation in PAE placental tissue (Haycock & Ramsay, 2009) and in the sperm of alcohol-consuming mice (Knezovich & Ramsay, 2012). These results argue that the observed effect of PAE on gene expression may be caused by the effect of PAE on DNA methylation at specific regulatory sequences, which include imprinted regions.

A number of studies of PAE have observed methylation changes occurring in genes that are known to be genomically imprinted (Dietz, Masterson, Sittig, Redei, & Herzing, 2012; Liu et al., 2009; Shukla, Sittig, Ullmann, & Redei, 2011; Sittig, Shukla, Herzing, & Redei, 2011). Genomic imprinting enables parent-of-origin specific monoallelic expression of a select set of genes that are important in early development, particularly neurodevelopment (Kernohan & Bérubé, 2010). These genes are crucial during not only neurodevelopment but are also crucial for the normal functioning of the brain (Davies, Isles, Humby, & Wilkinson, 2008). Interestingly, ~30% of imprinted genes are hypothesized to be ncRNAs, including miRNAs (Morison, Ramsay, & Spencer, 2005). The above observations suggest that PAE is best explained as a sequence of events in response to ethanol that may begin with differential methylation of imprinted genes encoding for ncRNAs, which regulate gene expression. For example, moderate and voluntary maternal PAE alters adult levels of a vesicular glutamate transporter in the mouse hippocampus (Zhang, Ho, Vega, Burne, & Chong, 2015). This glutamate transporter showed increased mRNA levels that were correlated with decreased DNA methylation and increased H3K4me3 (a histone post-translational modification related to open chromatin) at the transporter promoter. However, while mRNA levels of the transporter were increased, the protein levels were decreased, which suggested post-transcriptional regulation. The discrepancy between mRNA and protein was found to be caused by the increase of an imprinted miRNA from the *Sfmbt2* cluster, and further functional experimentation showed that the miRNA binds to the 3′UTR of the transporter mRNA. This observation highlights the complexity of the interactions that shape the epigenetic landscape as well as their potential for disruption by PAE. Ultimately, it appears that the epigenetic landscape is one of the prime mechanism(s) for the long-term deregulation of brain gene expression following PAE.

Our group has examined long-term alterations to the epigenome of the adult B6 brain (PND60-70) across a number of paradigms of prenatal alcohol exposure (Laufer et al., 2013). A voluntary and continuous maternal preference-drinking paradigm was examined for alterations to DNA methylation as well as non-coding RNA and gene expression, while three separate binge time points that correspond to the different trimesters of human pregnancy were examined for alterations to non-coding RNA and gene expression. Alterations in ncRNA expression were observed from three imprinted clusters of ncRNA: *Snrpn-Ube3a*, *Dlk1-Dio3*, and *Sfmbt2* (Laufer et al., 2013). Different copies of *Snord115*, which is in the *Snrpn-Ube3a* locus and involved in alternative splicing of the *5htr2c* serotonin receptor (Laufer & Singh, 2012), showed increased expression in all paradigms. Altered methylation was seen at the above three imprinted clusters of ncRNA and many more imprinted regions in the continuous preference drinking paradigm (Laufer, 2016; Laufer et al., 2013). Furthermore, a number of cellular signaling pathways showed differential methylation, including CDK5 signaling, PTEN/AKT/PI3K/mTOR signaling, calcium signaling, and hippo signaling. Ultimately, the results suggest that PAE leaves a long-term epigenomic profile in adult mice that is related to neurodevelopment.

Finally, because we examined gene expression, miRNA expression, and DNA methylation, the different datasets were integrated to observe any biological relationships. Notably, a larger scale of alteration was seen to DNA methylation as compared to gene and ncRNA expression. Furthermore, few genes (~16%) and (~18%) ncRNAs showing altered expression also showed altered DNA methylation. The lack of correlation between altered epigenetic marks and transcription has been reported in FASD literature (Veazey, Parnell, Miranda, & Golding, 2015). The lack of correlation presumably reflects on the combinatorial nature of the epigenome, where multiple modifications are needed for effect. Furthermore, the lack of correlation also reflects on the fact that the gene expression tested is from mice in a resting steady state and that differential expression could be seen under other activating conditions (Lussier et al., 2015). Finally, it likely also represents a 'footprint' of alterations to past transcriptional events because the time point examined is long after exposure (Kleiber et al., 2014).

4. Differential methylation identified in humans

Unlike mouse model research, reports on the epigenetic profiles of humans with FASD are rather limited. Four research groups had published *in vivo* examinations of the association between FASD and DNA methylation at select loci in humans. Lee et al. examined a Korean cohort of families, where it was found that there are changes in the unsorted peripheral blood of parents and also the cord blood of FASD children (Lee et al., 2015). There was a significant reduction of methylation to the promoter of a dopamine transporter (*SLC6A3*) in heavy alcohol consuming fathers and their offspring. The *SLC6A3* promoter also showed a significant decrease of promoter methylation in mothers who consumed both moderate and heavy amounts of alcohol. The methylation of a serotonin transporter (*SLC6A4*) promoter was significantly decreased in children from both heavy and moderate alcohol consuming mothers. Methyl CpG binding protein 2 (*MECP2*) showed increased

promoter methylation in the offspring of mothers with moderate alcohol consumption. Masemola et al., who examined saliva in a South-African cohort (Masemola, van der Merwe, Lombard, Viljoen, & Ramsay, 2015). There is advantage to the examination of saliva (buccal epithelial cells) because the cells share an ontogenetic relationship to neuronal cell types via the ectoderm. The aforementioned study focused on confirmed cases of FAS, and did not examine FASD, and the differential methylation examined was from a few selected imprinting control regions (ICRs). Some of the pyrosequenced regions were not informative, but KvDMR1 showed a 7.09% decrease in methylation. However, the functional relevance of such a small alteration in methylation toward transcriptional regulation remains an open question. Overall, the above pioneering investigations demonstrate that altered DNA methylation at specific loci can distinguish children with FASD from matched controls.

Our group recently examined buccal epithelial cells from a small but carefully matched cohort of Canadian children using the Illumina HumanMethylation450 BeadChip, which is referred to as the 450K array (Laufer, 2016; Laufer et al., 2015). A CpG from *COLEC11* was the second most significant probe on the 450K array and was also confirmed by pyrosequencing. *COLEC11* encodes a guidance cue for neural crest migration and mutations produce a spectrum composed of craniofacial abnormalities and learning disabilities known as 3 MC syndrome (Rooryck et al., 2011). The CL-K1 protein encoded by *COLEC11* is part of the lectin complement pathway, which functions in the immune process of complement activation (Wallis, Mitchell, Schmid, Schwaeble, & Keeble, 2010). In a separate study, methylation in the peripheral blood from a group of patients with intellectual disability and normal molecular karyotype was examined by the 450K array and confirmed by pyrosequencing (Kolarova et al., 2015). Notably, a marker could not be found for general intellectual disability, and thus an individual-based approach was taken. Hypermethylation of *COLEC11* was observed and confirmed in a single patient with a thin upper lip vermillion, long flat philtrum, moderate developmental delay, generalized epilepsy, cerebral movement disorder with ataxia, and blepharophimosis. The aforementioned endophenotypes overlap with FASD. However, a deeper investigation by our group, which utilized pyrosequencing and a targeted next-generation sequencing sodium bisulfite sequencing panel, revealed that the methylation signature of *COLEC11* could be confounded by additional environmental and genetic variables (Laufer, 2016). Therefore, it is not yet suitable a biomarker for PAE but may be of particular relevance for future functional studies.

Our human study also revealed that, in both children with FASD and PAE mice, there are some similarities in the epigenetic alterations. These similarities are apparent at the level of genomic imprinting, where *SNORD115* and *SNORD116* are altered, as well as at the level of pathways and ontologies, where the functions are related to neurodevelopment (Laufer, 2016). Notably, the clustered protocadherins, which are involved in establishing individual neuronal identity, showed a broad increase in methylation at select regions that appears to reduce their expression (Laufer, 2016; Laufer et al., 2015). Furthermore, the observations across species were based on different technologies. The mouse observations were from methylated DNA immunoprecipitation arrays (MeDIP-CHIP) that tiled larger regions in the hundreds of basepairs, while the human observations were from the 450K array, which analyzes individual CpG sites. Thus, the findings suggest the possibility of a large-scale increase to methylation in the brains of children with FASD as both the brain and buccal epithelium are derived from the ectoderm. We have also found that the clustered protocadherins show increased histone methylation in the hippocampus of adult B6 PAE mice from a human third trimester equivalent binge exposure paradigm (Chater-Diehl, Laufer, Castellani, Alberry, & Singh, 2016). Both open chromatin related H3K4me3 and closed chromatin related H3K27me3 were increased. While the enriched histone post-translational modifications typically act in opposing fashions, this enrichment may also represent bivalent chromatin, which allows for genes silenced in embryonic stem cells to be poised for later activation during differentiation (Bernstein et al., 2006; Vastenhouw & Schier, 2012). This hypothesis is further supported by the observation that DNA methylation and DNA demethylation are involved in the establishment and maintenance of bivalent chromatin (Kong et al., 2016).

An independent group has also replicated some of our observations. Portales-Casamar et al. published an examination of genome-wide DNA methylation patterns in the buccal epithelial cells of a larger cohort (Portales-Casamar et al., 2016). They compared 110 children with FASD to 96 control children between the ages of 5–18. In addition to utilizing the 450K array and pyrosequencing, a genotyping array was used to statistically correct for genetic background as the children with FASD and controls had a different distribution of ethnicity. Their analysis revealed 658 differentially methylated CpGs with 41 of those showing a >5% change in methylation. They observed an enrichment for genes with functions related to neurodevelopment. Furthermore, there was an increase of methylation in multiple probes from the clustered protocadherins and also alterations to genomically imprinted regions, which included *H19*. Overall, their observations support key findings from both our mouse and human results and expand them to a more diverse cohort. However, many of the specific CpGs identified are not identical with only one overlapping CpG from the clustered protocadherins. Thus, in addition to the possibility that the methylation profile represents remaining genetic background and environmental differences, it could also represent the stochastic nature of the developmentally established *de novo* methylation profile at the clustered protocadherins (Toyoda et al., 2014).

While the methylation profile of the clustered protocadherins is complex, and will require innovative technologies for detailed examination, they have functions critical to neurodevelopment. These include neurite self-avoidance (Lefebvre, Kostadinov, Chen, Maniatis, & Sanes, 2012), dendritic patterning (Suo, Lu, Ying, Capecchi, & Wu, 2012), and axonal projection (Katori et al., 2009). These functions and the structure of the clustered protocadherins have been expertly reviewed (Chen & Maniatis, 2013; Hayashi & Takeichi, 2015; Hirano & Takeichi, 2012). The DNA methylation profile of the clustered protocadherins is known to be responsive to other experiences. A broad profile of increases and decreases in DNA methylation, histone H3 lysine 9 acetylation (H3K9ac), and gene expression in the hippocampus of the rat revealed that the clustered protocadherins show the largest differential response in adult offspring that received altered maternal care (McGowan et al., 2011). A further comparison to the hippocampus of humans that were abused as children revealed increased methylation in promoters of the clustered protocadherin locus (Suderman et al., 2012). However, in our mouse model there were no observable differences in maternal care (Kleiber, Wright, & Singh, 2011). Therefore, it appears that distinct experiences result in complex methylation profiles at the clustered protocadherins.

The clustered protocadherins have also been implicated in other diseases and disorders. These include schizophrenia and bipolar disorder (Pedrosa et al., 2008) as well as autism (Anitha et al., 2013) and cancer (Dallosso et al., 2009; Wang et al., 2015). The clustered protocadherins are also influenced by a number of transcriptional regulators, which include CTCF (Golan-Mashiach et al., 2012; Guo et al., 2012, 2015), WIZ (Isbel et al., 2016), and SMCHD1 (Mould et al., 2013). Finally, gene dosage is known to affect clustered protocadherin methylation. Increased methylation and decreased

expression of the γ protocadherins has been observed in fetal Down syndrome (trisomy 21) cortex (Hajj et al., 2016). Deletion (Williams syndrome) or duplication of 7q11.23 produces symmetrical dose-dependent DNA methylation profiles in blood cells, with deletion producing hypermethylation and duplication producing hypomethylation (Strong et al., 2015). Thus, the clustered protocadherins contain a large-scale and complex signature related to genetic and environmental differences in neurodevelopment. While responsive to a number of conditions, intensive investigation of the methylation profile present may reveal unique patterns specific to disorders that can be analyzed in consideration with other biomarkers.

5. Considerations for biomarker development

Although the 450K array is suited for discovery, individual sequencing is a more practical approach for clinical use as a substantial change in methylation level at a biomarker locus would allow for the detection of exposure without the need for carefully matched controls. However, individual sequencing does not have the advantage of normalization of signal across matched samples and reference genes. Therefore obtaining an absolute level of methylation that is both precise and accurate remains as the major obstacle for the development of DNA methylation diagnostics.

Our observations in children also highlight the difficulty of expanding from the homogeneity of a small carefully matched group to that of the heterogeneous general population. In the follow-up pyrosequencing and NGS panel, no single CpG site examined could consistently distinguish children with FASD from controls. This may be due to the fact that the children with FASD from the general population were more variable across a number of factors including age, sex, drug exposure, genetic background, socioeconomic status, as well as family history of mental health disorders and substance use (Laufer, 2016). The control patients from the general population were also in the clinic for a more heterogeneous mixture of acute and chronic medical problems, which involved medication in some cases. Ultimately, given the potential for variation across a number of factors, it appears that each case of FASD may be represented in the epigenome as a complex signature that is altered by other environmental factors. This large amount of heterogeneity would benefit from future experimentation with much larger sample sizes, which would provide added statistical power. Furthermore, with a larger sample size, a regression could also be used to dissect the complex environmental interactions that shape the general population.

One possible approach to dissect the heterogeneity of FASD would be to mirror an array discovery approach and machine learning strategy used to detect prenatal exposure to smoking. In this example, the cord blood from 1062 newborn Norwegian children prenatally exposed to maternal smoking during the second trimester was analyzed on the 450K array, revealing 26 significantly altered CpGs that mapped to 10 genes (Joubert et al., 2012). The genes distinctly altered had functions related to detoxification and development. A separate follow-up study utilized whole blood from an American cohort (of 531 children) that were 3–5 years old and analyzed the same 26 CpGs using machine learning, where they found that 81% of the time they could detect prenatal exposure to smoking (Ladd-Acosta et al., 2016). The above examples suggest that larger sample sizes from interdisciplinary collaborative efforts increase statistical power. However, given that 19% of cases were not identified, there are still limitations to the approach of epigenetic epidemiology and the statistical approaches available need further improvement, in terms of both loci examined and computational analysis, if they are to properly model the heterogeneity of the human population and be used for public health decisions (Ramsay, 2015). To further expand on this point, a massive meta-analysis of prenatal exposure to maternal smoking found that even a sustained and heavy exposure results in a 450K profile with only nominal significance when applied to different cohorts of older children with the same heavy prenatal exposure (Joubert et al., 2016). In the same study, moderate exposure to any level of smoking, which made up a larger amount of children, produced a much less distinct profile that was not fully explored.

It is also noteworthy that while we have primarily observed alterations to CpG islands, CpG shores, and CpG shelves, other regions of the genome are also important. For instance, DMRs in the gene body and untranslated regions (UTRs) have been identified in other studies of prenatal exposures (Joubert et al., 2016; Portales-Casamar et al., 2016). Interestingly, differential methylation in the gene body appears to be an activating mark and non-CpG DNA methylation is also important (Lister et al., 2009). Finally, studies of autism spectrum disorders have also identified DMRs in intergenic regions (Nardone et al., 2014). Additionally, the 450K array offers limited coverage of the genome as it is targeted toward a set of a CpGs that do not fully represent the diversity of regulatory features in the genome. For instance, it has low coverage of enhancers, intergenic regions, non-CpG methylation, and a limited profile of the gene body. Thus, there are likely highly informative DMRs that have not yet been identified. These limitations could begin to be addressed by the use of the Illumina's new EPIC (850K) array or eliminated by the use of whole genome bisulfite sequencing.

Ultimately, our findings suggest that targeted single locus analysis of the regions identified is not suitable for use as a diagnostic of FASD. Methodologies that allow for accurate, precise, rapid, and inexpensive quantification of DNA methylation levels across large regions of multiple loci in a single sample, without the need for matched controls, will soon emerge. These future technologies could be used to further examine the sites identified, as well as those identified by deeper epigenome wide screens, in a larger number of samples that will allow for the dissection of the interaction of other environmental and inherited variables. The above future improvements and a deeper understanding of natural variation in the methylome may one day enable the possibility of diagnosing FASD from buccal swabs. Furthermore, this insight into DNA methylation from buccal swabs would be particularly suited for use with other modern diagnostics (Portales-Casamar et al., 2015; Reynolds et al., 2011), such as eye movement recordings (Paolozza, Rasmussen, et al., 2014a,b; Paolozza, Munn, Munoz, & Reynolds, 2015), diffusion tensor imaging (Paolozza, Treit, Beaulieu, & Reynolds, 2014), and/or a robotic virtual reality platform (Williams et al., 2014).

Furthermore, while systems biology based approaches are suited for discovery, they also require functional confirmation experiments to show causation of a candidate gene in producing an endophenotype. Functional experiments could be carried out by epigenome editing with CRISPR/Cas9 biotechnology (Day, 2014; Laufer & Singh, 2015; Rienecker, Hill, & Isles, 2016). First, mouse models without PAE would be used to establish causation by recreating key marks, which include differential methylation in imprinting control regions and *Colec11* as well as increased methylation at the clustered protocadherins. Then, a series of experiments could be carried out with the opposite effector that targets the same regions in PAE mice. These causative confirmation experiments could be used to verify biomarkers and develop therapeutic interventions for FASD patients that are based on somatic epigenome editing of the brain (once the technology is developed for the clinic).

Finally, there are a number of ethical, legal, and social implications when screening for PAE, as identification of PAE can result in the removal of the child from their mother and also her criminal

prosecution (Zizzo et al., 2013). Given the extent of public misinformation about the safety of PAE and how it results in FASD (Singh et al., 2014), any screening for PAE should be used to only provide the appropriate therapies and educational aids for the child, which are most effective the earlier they are received, and not be used to persecute the past.

Footnote

This review contains sections from the following Ph.D. thesis: Laufer, Benjamin I., "A Long-Term Neuroepigenomic Profile of Prenatal Alcohol Exposure" (2016). Electronic Thesis and Dissertation Repository. Paper 3913. http://ir.lib.uwo.ca/etd/3913.

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