ENVIRONMENTALLY INDUCED EPIMUTATIONS, THEIR PERSISTENCE, AND POTENTIAL CAUSALITY IN THE DEVELOPMENT OF DISEASE IN THE OFFSPRING OF EXPOSED INDIVIDUALS

A Dissertation

by

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ABSTRACT

In recent years, there has been increased interest into better understanding how environmental exposures influence the long-term health of an organism. Chemical pollutants, dietary deficiencies, embryonic stress and multiple other external factors have all demonstrated long-lasting effects upon development, metabolism, and health even after transient exposures. The mechanisms by which these exposures can impact development far beyond the period of exposure remain largely unknown. To gain better insight into the developmental origins of both birth defects and disease, we must better understand how environmental exposures alter development.

In this work, we will examine the capacity of the environment to impact chromatin states, and then determine whether these changes are heritable; and are thus potentially causal in the development of disease. This is an important question due to the recent recognition that aberrant chromatin states can lead to pathological patterns of gene expression, a circumstance commonly referred to as “epimutations”. Dysregulation of gene expression patterns during development have been shown to cause a multitude of irregular phenotypes in offspring and lifelong disorders in mature organisms. This altered chromatin state, coined an epimutation by Dr. Emma Whitelaw, is important due to the mutation not being in the genetic code itself, but in the way DNA regulatory regions are packaged within the chromatin template, and thus accessed by the protein factors directing gene transcription. The body of work presented here will examine the ability of common environmental exposures to modulate chromatin structure. We will examine these changes

over time in an effort to better understand the inheritance of epigenetic change. Secondly, we will measure whether environmentally induced alterations in chromatin structure within the germline persist, and are heritable. These questions are all relevant to better understanding the developmental origins of disease.

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NOMENCLATURE

ART	Assisted Reproductive Techniques
BER	Base-Excision Repair
BWS	Beckwith-Weidemann Syndrome
DMR	Differentially Methylated Regions
DNA	Deoxyribonucleic Acid
DOHaD	Developmental Origins of Health and Disease
EtOH	Ethanol
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
IAP	Intra-cisteral A Particle
ICR	Imprinting Control Region
LTR	Long Terminal Repeat
MZT	Maternal-Zygotic Transition
PCNA	Proliferating Cell Nuclear Antigen
PTM	Post-Translational Modification
RFTS	Replication Focus Targeting Sequence
RRBS	Reduced Representation Bisulfite Sequencing
SCNT	Somatic Cell Nuclear Transfer
SRA	SET and RING finger associated
UPD	Uni-Parental Disomy

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW*

In the past decade, there has been considerable interest in better understanding the impact environmental exposures have on long-term health and the incidence of birth defects. Chemical pollutants, dietary deficiencies, embryonic stress and multiple other external factors have all demonstrated long-lasting effects upon development, metabolism, and health (Barker 1992; Barker 1995; Weaver 2004; Christian & Stewart, 2010; Kinsella & Monk, 2009; CastroJiménez 2011; Sebert, Sharkey, Budge, & Symonds, 2011). However, the underlying mechanisms by which these exposures, some of which are transient, can impact development far beyond the period of exposure remain largely unknown. In order to gain better insight into the developmental origins of both birth defects and disease, we must better understand how environmental exposures alter development.

Epigenetics & Epimutations

"Epigenetics" refers to a series of biochemical processes through which heritable changes in gene transcription are achieved throughout the lifecycle of an organism, without a change in DNA sequence (Felsenfeld 2014). Although the term has been around

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for quite some time, the definition has taken on variable meanings. Conrad Waddington, in the 1950's, was widely given credit for establishing the classical definition of epigenetics by describing the "epigenetic landscape", which has evolved to be the basis of modern day developmental genetics (Berger, Kouzarides, Shiekhattar, & Shilatifard, 2009; Waddington 1953). Three decades later, Robin Holliday established a more contemporary view, which was more concerned with elucidating the molecular heritability of DNA methylation and the ability of a cell to retain epigenetic "memory" (Berger et al., 2009; Holliday & Pugh, 1975). To be epigenetic, a phenomenon needs to influence gene expression without changing the underlying genetic code and be heritable. A symposia held at Cold Spring Harbor in 2008, established an operational definition of epigenetics, which described a "stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" (Berger et al., 2009). This, more functional description, has helped curb some of the misconceptions surrounding the definition of epigenetics.

Cellular memory has become a hot topic in research as scientists attempt to understand how cells pass along information not coded in the transmitted DNA. Here, cellular memory refers to non-genetic information being passed from parents to offspring through meiosis (Soubry, Hoyo, Jirtle, & Murphy, 2014; Waterland & Jirtle, 2003) or parent-cell to daughter-cell through mitosis (Gialitakis, Arampatzi, Makatounakis, & Papamatheakis, 2010). Recently, scientists have looked to noncoding/micro RNAs, histone post translational modifications or DNA methylation in an attempt to explain cellular memory. Cells can retain the memory of an activity state of a gene, or retain the

tissue lineage from which it originated through seemingly countless cellular divisions (Henikoff & Greally, 2016). Without cellular memory, we would have no higher order differentiation of tissues. Understanding the basis of the maintenance of the cellular programs through division is at the core in the study of epigenetics.

Dr. Emma Whitelaw, molecular biologist and NHMRC Australia Fellow at the Queensland Institute of Medical Research, was the first to show evidence that inherited chromatin modifications could associate with disease in mammals. Her laboratory showed that the insertion of the intra-cisternal A particle (IAP) retrotransposon upstream of the agouti gene causes ectopic expression of that gene. This insertion resulted in many unfavorable outcomes such as diabetes, obesity and an easily observed yellow fur coat. Researchers found that offspring conceived to agouti mothers failed to silence the IAP retrotransposon during early development resulting in the agouti phenotype (Morgan, Sutherland, Martin, & Whitelaw, 1999). Importantly, the agouti mouse model has led to many advances in our understanding of how the environment influences DNA methylation. The degree of IAP methylation directly corresponds to a fur coat color in mice. Randy Jirtle refers to these mice as an epigenome biosensor (Jirtle 2014). Different exposures can be phenotypically assessed by the color of the mouse coat. To date, experiments have been performed showing the maternal diet can significantly influence the DNA methylation of IAP elements and importantly, the emergence of the agouti phenotype. As examples, methyl donors and genistein (phytoestrogen in soy) were shown to hypermethylate IAP elements (Dolinoy, Weidman, Waterland, & Jirtle, 2006; Waterland & Jirtle, 2003). Exposures to ethanol were able to hypermethylate IAP elements

while exposure to bisphenol A (BPA) and in vitro embryo culture were able to hypomethylate IAP elements (Dolinoy, Huang, & Jirtle, 2007; Kaminen-Ahola et al., 2010; Morgan, Jin, Li, Whitelaw, & O'Neill, 2008). Further, physical agents such as low-dose ionizing radiation were found to increase the hypermethylated the status of IAP elements as well (Bernal et al., 2013). Experiments like these reveal the intricate connection between environmental exposures and the responsive state of the epigenome. Robin Holliday had formally proposed these epigenetic-inherited modifications 1987 but the term "epimutation" would not be coined until later by Dr. Emma Whitelaw (Holliday 1987).

Genomic Imprinting

Through pronuclear injection embryo work, McGrath, Solter and Surani demonstrated that the maternal and paternal genomes are not equal with respect to their contributions to the developmental program of offspring. These experiments transferred either two female or two male pronuclei into enucleated oocytes. Both situations failed to develop past the mid gestation stages and displayed unique phenotypes (McGrath & Solter, 1984). For example, gynogenetic or "female-only" pronuclei developed normal embryos but failed to properly differentiate the placenta, while androgenetic or "male-only" pronuclei developed proper placentation but failed to properly organize embryonic development. Based on these findings, researchers set off to elucidate why both parental genomes were required for proper embryogenesis. Research by McGrath, Solter and Surani shifted the scientific needle in a new direction, which would eventually unveil

imprinted genes (Monk 1988). Imprinted genes are "marked" in a parent-of-origin way (Monk 1988), meaning, alleles from either the mother or father are silenced through DNA methylation. This phenomenon gives rise to UPD (uni-parental disomy), which can be thought of as a dosage compensation mechanism (Bartolomei & Tilghman, 1997). Many imprinted genes are thought to control growth and proliferation. It is also worth mentioning that these growth genes are typically expressed from the paternal allele. This has led researchers to speculate that a battle between the male and female genomes is in large part controlling early cellular proliferation This hypothesized concept is known as the "parental conflict hypothesis" or "kinship theory of genomic imprinting" (Haig & Graham, 1991). Paternal genes generally promote growth and proliferation while the maternal genes are more conservative and promote smaller offspring, but it is worth noting that some imprinted genes to not behave in the fashion predicted by the theory.

DNA methylation is the most recognized epigenetic mechanism involved in the establishment of genomic imprints, with some newer studies also implicating tri-methylation on the 27^{th} lysine on the N-terminus tail of histone 3 (H3K27me3) on the maternal inherited chromosomes as an epigenetic mark controlling imprinting in a DNA methylation-independent manner (Inoue, Jiang, Lu, Suzuki, & Zhang, 2017). Clusters of imprinted genes are often controlled through DMRs (differentially methylated regions), which seem to escape the global demethylation wave that occurs during preimplantation development (Santos, Hendrich, Reik, & Dean, 2002). In fact, more recent research has revealed that these DMRs do not entirely escape demethylation and are quite fluid,

exhibiting periods of expansion and contraction between the gametic stages (Tomizawa et al., 2011).

The most well studied imprinted gene cluster is the H19/Igf2 locus. H19 is expressed solely from the maternal-derived allele, while the Igf2 gene is solely expressed from the paternally-derived allele (Bartolomei, Zemel, & Tilghman, 1991; Bell & Felsenfeld, 2000). Errors in the establishment or maintenance of this imprinted region can lead to a multitude of developmental disorders such as Beckwith-Wiedemann syndrome (BWS), an overgrowth disorder. H19 and Igf2 share a common imprinting control region (ICR). Classically, this ICR is only methylated on the paternal allele. A methylated ICR inhibits the protein insulator, CTCF, also known CCCTC-binding factor, from binding (2000). CTCF has been shown to regulate transcription, participate in insulator activities and regulate chromatin architecture (Bell & Felsenfeld, 2000; Hark et al., 2000). A lack of CTCF binding allows a looping structure with a downstream enhancer to make contact with the Igf2 promoter. This leads to transcription of the paternal *Igf2* allele. In contrast, the maternal allele lacks DNA methylation in the ICR leaving a binding site available for CTCF to attach. CTCF, an insulator by nature, bind the ICR site preventing the looping structure needed to promote the expression of Igf2 resulting in expression of the H19 transcript from the maternal allele (Bell & Felsenfeld, 2000; Kaffer, Grinberg, & Pfeifer, 2001).

DNA Methylation

DNA methylation is the process of adding a methyl group to the fifth carbon on the cystosine base. While cytosines can be methylated in a CHG or CHH (H can be A, C or T) context, generally they are methylated when directly next to a guanine base residue (CpG). In the human genome, it is estimated that there are 3 million CpG dinucleotides in either a methylated or unmethylated state (Edwards et al., 2010). These methylated CpG dinucleotides have been associated with gene silencing through promoter methylation, transcriptional repression of retrotransposons, establishment of imprinted patterns of gene expression and the compaction of the inactivated X-chromosome in females (Edwards, Yarychkivska, Boulard, & Bestor, 2017; Holliday & Pugh, 1975). In addition, DNA methylation has also been associated with the increased incorporation of specific exons within in mRNA transcripts (Chodavarapu et al., 2010) where intragenic DNA methylation helps regulate alternative splicing events (Lev Maor, Yearim, & Ast, 2015). Finally, research indicates that DNA methylation is a crucial component that helps maintains the genomic structure of enhancer binding sites (Charlet et al., 2016). Here, DNA methylation associates with increased transcriptional output. Collectively, these examples demonstrate the dynamic roles DNA methylation exhibit. Importantly, with the advancement of sequencing technology and whole genome resolution, it has become clear that the dynamics of DNA methylation that associate with one biological phenomena do not necessarily apply in every biological situation (Edwards et al., 2017). Thus, a one size fits all description of the cellular role of DNA methylation is not appropriate.

The mammalian genome encodes four proteins with functional DNA methytransferase domains (DNMT1, DNMT2, DNMT3a and DNMT3b) and one methytransferase like protein (DNMT3L). Experiments employing targeted gene mutations in the early 1990's by Timothy Bestor highlighted the critical importance of DNA methylation during early embryonic development by targeting DNMT1. Homozygous mutations in DNA methyltransferase 1 led to global reduction in methyl-cytosines and embryos did not survive past mid-gestation (Li, Bestor, & Jaenisch, 1992). Subsequent experiments by Doherty et al revealed a homozygous mutation in Dnmt1, which in mice, resulted in embryonic lethality by approximately 11 days into gestation (Doherty, Mann, Tremblay, Bartolomei, & Schultz, 2000). Through studies of somatic cell nuclear transfer (SCNT), Golding and colleagues revealed the importance of Dnmt1 in early bovine development. Cytoplasmic injections of short-interfering RNAs targeting embryonic/maternal Dnmt1 transcripts within IVF embryos resulted in developmental arrest of the embryos at the 8/16-cell stage. Through SCNT of a Dnmt1-depleted donor cell line that stably expressed a short hairpin RNA that targeted the bovine Dnmt1 transcript, embryo development made it to the blastocyst stage, but failed to carry to term (Golding, Williamson, Stroud, Westhusin, & Long, 2011). Collectively, these studies clearly reveal the importance methyltransferases hold in embryonic development.

Through targeted gene disruption studies, researchers provided evidence that Dnmt3a and 3b are both essential for de novo methylation and mouse development (Okano, Bell, Haber, & Li, 1999). Heterozygous mice carrying mutations in either Dnmt3a or 3b were both phenotypically normal and fertile. Mice carrying a homozygous deletion

of Dnmt3a developed to term and appeared phenotypically normal yet failed to thrive and died at about 4 weeks of age (Okano, Bell, Haber, & Li, 1999). In contrast, Dnmt3b knockout mice were dead at birth and had major developmental defects such as growth impairment and rostral neural tube defects (Okano, Bell, Haber, & Li, 1999). Interestingly, both knockouts were phenotypically normal until around embryonic day 9.5 indicating an important role for de novo methylation in later stages of development in utero (Okano, Bell, Haber, & Li, 1999). Another DNA methyltransferase, Dnmt2, contains all the conserved motifs shared by known prokaryotic and eukaryotic DNA cytosine methyltransferases, but through gene targeting that results in inactivation of Dnmt2, no disruptions in de novo methylation or maintenance are observed (Okano, Xie, & Li, 1998). Furthermore, homozygous mutations in Dnmt3L resulted in phenotypically normal offspring but both sexes were sterile (Bourc'his, Xu, Lin, Bollman, & Bestor, 2001).

DNA methyltransferases typically work in concert with a team of proteins to control the temporal and spatial activity of DNA methylation within the genome. During replication, the proliferating cell nuclear antigen (PCNA) localizes at the replication fork. This protein has several functions, but one of them is to recruit DNMT1 to the replication fork to facilitate the methylation of newly replicated hemimethylated DNA (Iida et al., 2002). Similarly, DNMT1 and the protein UHRF1 form a complex that facilitates the preference and ability to methylate hemimethylated DNA. UHRF1 contains a SRA (SET and RING finger associated) domain, which binds hemimethylated CpG dinucleotides (Nishiyama et al., 2013). In what is hypothesized to be a classic handoff reaction, UHRF1 displaces the inhibitory RFTS (replication focus targeting sequence) domain of DNMT1,

which in turn releases the hemimethylated residue to the active site of DNMT1, resulting in the addition of a methyl group to the cytosine (Bostick et al., 2007). Disruption of the DNMT1/PCNA/UHRF1 complex leads to a global loss of DNA methylation (Hervouet et al., 2010). Depletion of either UHRF1 or DNMT1 in cultured embryonic stem cells led to an inability to differentiate, with differentiating cells undergoing apoptosis (Bostick et al., 2007). It has been hypothesized that during early developmental stages, methylation only modestly influences transcriptional regulation while once cellular differentiation begins, the role DNA methylation in maintaining transcriptional profiles and cellular identity becomes highly important (Bintu et al., 2016). Perturbations in DNA methylation during differentiation tend to induce apoptosis resulting in lethality (Bostick et al., 2007).

DNA Methylation in Regulation of Transposable Elements

Nobel Prize recipient in Physiology or Medicine (1983), Barbara McClintock, is best known for her discovery of transposable elements or "jumping genes" in maize (McClintock 1950). McClintock is a pioneer in of epigenetics and through her research, was the first person to correctly articulate the nature of epigenetics. Her observations that genes appeared to be differentially expressed or silenced in genetically identical cells through mitosis, led her to describe the existence of some "heritable mechanism" controlling gene expression that does not rely on the underlying genetic code (Felsenfeld 2014). These transposable elements, as described by McClintock, were able to disrupt the expression of genes when inserted nearby leading to a change in corn kernel color. Later, research revealed that when a retrotransposon, intra-cisternal A particle (IAP) was inserted

upstream of the agouti gene it causes ectopic expression of the agouti protein, which results in a distinctive yellow coat color in mice (Morgan et al., 1999). Genomes of most species, including humans and mice, are heavily occupied by parasitic sequences from their genetic history. Viruses, by nature, incorporate their genetic code into somatic cells in order to propagate their lineage. Unchecked, these sequences would transcribe their cellular machinery and hijack the cells. Fortunately, cells have found a way to control these foreign sequences, also known as transposons or endogenous retroviruses. Through extensive DNA methylation of these sequences, the cell is able to silence the expression of these transcripts (Kuff et al., 1983; Qin et al., 2010; Walsh, Chaillet, & Bestor, 1998). In support of this statement, depletion of DNA methyltransferase 1 (Dnmt1) in mouse embryos leads to a 50-100 fold increase the IAP transcript levels (Walsh, Chaillet, & Bestor, 1998). It has been suggested that DNA methylation might not be as critical in the silencing of these ancient viral elements due to the "global" erasure of these methyl post-translational modifications during preimplantation development with no concurrent upregulation of IAP transcripts, but as it turns out, most of the IAP long terminal repeat (LTR) regions maintain their methylation levels even through the epigenome reset (Walsh, Chaillet, & Bestor, 1998).

DNA Methylation in Regulation of X-Chromosome Inactivation

It was not until the mid 1970's when work by Arthur Riggs began to unravel a potential mechanism or candidate for an epigenetic controller of gene expression that a role for DNA methylation in transcriptional control was hypothesized (Riggs 1975). Riggs

proposed a role for DNA methylation in context of silencing gene expression in the process of X chromosome inactivation. Building off the observation made in 1961 by Mary Lyon, which described the inactivation of one of the two X chromosomes in somatic cells (Lyon 1961), Riggs proposed that DNA methylation played a role in the silencing of genes (Lindsay et al., 1985; Russo, Martienssen, & Riggs, 1996). This was very novel because it supported the idea of a method of gene expression control that did not cause a change to the underlying genetic code. X chromosome inactivation is a process in which a single X chromosome from each cell is systematically silenced through extensive DNA methylation. Silencing of an X chromosome is believed to serve as a mechanism of dosage compensation that arises where females contain two X chromosomes to a male's one (Chow, Yen, Ziesche, & Brown, 2005; Monk 1986). The gene Xist is a major player in the organization of this task through the production of the noncoding RNA Xist from the future inactive X-chromosome. The Xist RNA is proposed to recruit protein complexes, specifically polycomb repressive complex 2 (PRC2), that facilitate the facultative heterochromatization of the X chromosome. Heterochromatization through DNA and histone methylation, is stably transmitted through mitotic cellular division. Interestingly, although methylation of the Xist gene on the active X chromosome is required to keep it the gene silenced, knockout of DNMT1, DNMT3B or both did not lead to aberrant expression of Xist on the active X-chromosome despite global demethylation (Vasques et al., 2005). Similarly, on the silenced X-chromosome, even though X-inactivation will occur in the absence of this gene, Xist is required for the faithful maintenance of the silenced chromosome (Kalantry, Purushothaman, Bowen, Starmer, & Magnuson, 2009).

Chromatin & Epigenetics

Deoxyribonucleic acid (DNA) is composed of four nucleic bases wound in a double helix that comprises the blueprint for every living organism (Watson & Crick, 1953). These instructions are contained in each cell and need to be efficiently stored, regulated, transcribed and replicated through cellular divisions. In order to accomplish these tasks, eukaryotic DNA is wound around protein cores collectively called nucleosomes. First described in 1974 as "spheroid chromatin units", each nucleosome is in contact with approximately 147 bases of DNA (Olins & Olins, 1974). Nucleosomes are comprised of a protein octomer, consisting of 4 sets of histone dimers, H2A, H2B, H3 and H4 (Kornberg 1974). These nucleosomes are connected through a "linker" protein H1 (Zhou, Gerchman, Ramakrishnan, Travers, & Muyldermans, 1998). Nucleosomes bundle up to form chromatin fiber, and this fiber further coils to become the larger structure known as a chromosome. "Chromatin" denotes a core of proteins around which the DNA template winds within the nucleus. The term "chromatin state" refers to both the topological organization and relative attraction the histone core and DNA have towards each other. DNA is contained in most every cell and is a blueprint of all the information needed to create and develop every known organism and many viruses. The transfer of this information to functional proteins is known as the "central dogma of molecular biology" as proposed by Francis Crick (Crick 1970). In brief, DNA transfers information to RNA, which transfers the information to proteins (Crick 1970).

Since as early as 1964, it has been shown that post-translational modification (PTMs) of histones can alter RNA synthesis (Allfrey, Faulkner, & Mirsky, 1964). Specific

patterns of histone post-translational modifications and DNA methylation can lead to a chromatin state which has been associated with transcriptional silencing (heterochromatin) (Fuks 2005), or correlated with transcriptional accessibility (euchromatin) (Gifford et al., 2013). The general idea being that DNA loosely wound around the histone core can be easily accessed by the transcriptional machinery, whereas tightly wound DNA is structurally unavailable. This specific case is only generally applicable when referring to X-chromosome inactivation. In reality, transcriptional regulation is far more complex with a plethora of research indicating situations where compact chromatin can maintain high expression of its contained genes or loosely associated chromatin containing silenced genes (Jiang et al., 2017).

Histone octomers have modifiable globular and N-terminus tail domains. The better-studied aspects of the histones are these N-terminus tails in which modifications have been identified that correlate with chromatin structure and the expression of genes. While these histone post-translational modifications were initially believed to simply affect the stabilization between the DNA and histone core, research has now revealed a more dynamic role of these post-translational modifications. Currently, the histone code hypothesis refers to these histone post-translational modifications as recruiters of specific protein complexes with special functions that serve to stabilize or destabilize the histone/DNA interaction, recruit histone-remodeling complexes, and promote transcription or silencing of genes (Jenuwein & Allis, 2001). The “histone code” is a hypothesis that the transcription of genetic information encoded in DNA is in part regulated by chemical modifications to histone proteins, primarily on their unstructured

ends. The basic premise of the hypothesis is that particular patterns of histone modifications correlate with specific biological events such as transcription. Together with DNA methylation, it is part of the epigenetic code (Jenuwein & Allis, 2001). However, genome wide studies have failed to find evidence that broadly supports this hypothesis, suggesting that transcriptional regulation is much more complex than originally thought.

Epigenetic programming in the early mammalian embryo

DNA methylation is thought to be established during both gametogenesis and early development (Bergman & Cedar, 2013; Santos et al., 2002). Since 1987, it has been accepted that there are two waves of global demethylation (Monk, Boubelik, & Lehnert, 1987). The first wave occurring during the proliferation of primordial germ cells while the re-methylation of these cells occurs in the post migratory germ cells (Dean, Lucifero, & Santos, 2005; Monk et al., 1987; Santos et al., 2002). The second wave takes place during the cleavage stage of a rapidly dividing embryo with global minimal levels at the blastocyst stage in mice **(Figure 1)**. Research has shown that this "double dip" model is greatly skewed towards a small fraction of the genome (Edwards et al., 2017). In fact, the majority of sequences that experience this double demethylation are old and inactive transposon artifacts. Despite these observations, it is classically understood that during these demethylation events, the genome trends towards becoming a blank slate. Hypothetically, if this were the case, you would expect the vast majority of the genome, which contains silenced transposable elements, to greatly increase their transcriptional output. However, this is not the case, as IAP LTRs retain much of their methylation (Walsh

et al., 1998). Also, it is worth noting that an estimated 75% of gene promoters are perpetually demethylated and therefore never cycle between methylated and demethylated states (Bestor, Edwards, & Boulard, 2015; Edwards et al., 2010; Ioshikhes & Zhang, 2000; Lister et al., 2009). The other oddity is in the dynamics of DNA methylation that occur in imprinting control regions. Instead of two distinct periods of demethylation, their methylation patterns vary depending on the cells stage of development (Monk et al., 1987; Tomizawa et al., 2011). Additionally, during post implantation development, the CpG residues that are demethylated during early zygotic development see their patterns begin to be slowly reestablished in the post-implantation embryo (Reik, Dean, & Walter, 2001). The majority of this early re-methylation is focused on reestablishing the methyl marks on LTR retrotransposons in order to maintain their silent state (Dean et al., 2005). Although not a huge percentage of overall methylation, as development proceeds, methylation on gene promotors and production of heterochromatin advances as differentiation of specific tissue lineages become established (Reik et al., 2001; Riggs 1975).

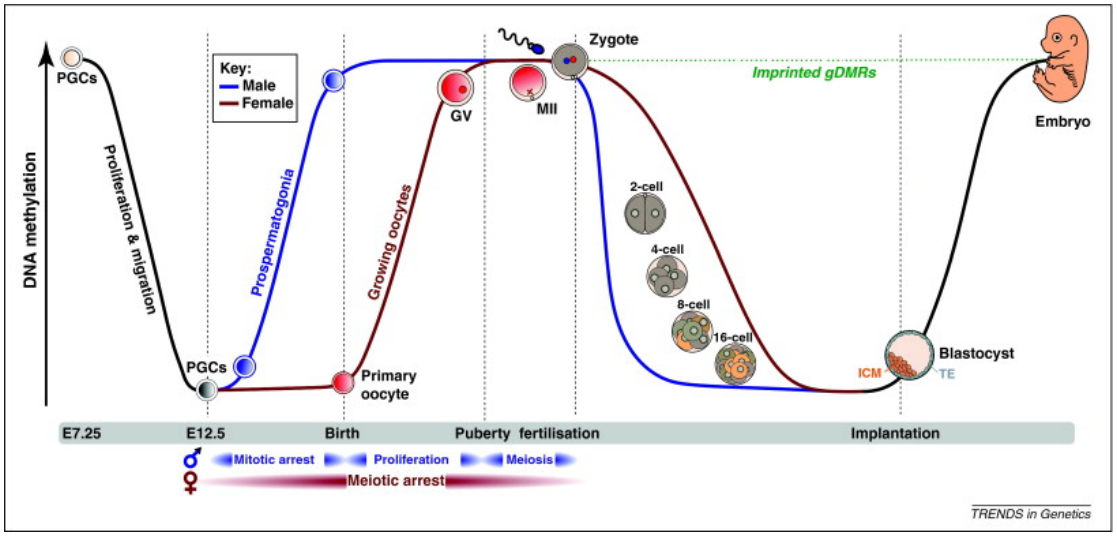


Figure 1.

DNA Methylation pattern during early development. (Smallwood & Kelsey, 2012)

DNA methylation changes during developmental epigenetic reprogramming. Primordial germ cells (PGCs) emerge in embryos at E7.5 and, concomitant with their proliferation and migration towards the genital ridge, DNA methylation is globally erased (black line). Following sex-determination, new DNA-methylation landscapes are established in germ-cell precursors in an asymmetrical fashion in male and female embryos. In the male embryo (blue line), de novo methylation takes place before meiosis in mitotically arrested cells (G1-phase; prospermatogonia) and is completed before birth. In the female embryo (red line), primary oocytes enter meiosis and arrest in prophase-I (diplotene stage); DNA methylation is established after birth during the follicular/oocyte growth phase. At puberty, under specific endocrine triggers, fully-grown germinal vesicle (GV) oocytes resume the first meiotic division. After extrusion of the first polar body, oocytes arrest in metaphase of the second meiotic division (MII oocytes) and meiosis is completed only upon fertilization. Following fertilization, a new wave of DNA demethylation takes place that is distinct on the parental genomes. In the zygote, DNA methylation of the paternal genome is rapidly erased by an active mechanism (blue line). Demethylation of the maternal genome is slower (red line) and is dependent on DNA replication (passive demethylation). These post-fertilization demethylation events do not include imprinted gDMRs (green dotted line), resulting in parental-allele-specific methylation of these elements in early embryos and consequent parental-allele-specific expression of associated imprinted genes. Concomitant with blastocyst implantation and cell-lineage determination, new methylation landscapes become established, associated with cellular differentiation.

Active & Passive Demethylation

Following the transmission of the male genome to the egg, the male pronuclei undergoes a massive exchange of protamines for histones (Hecht 1990). In addition, the male pronuclei undergoes rapid demethylation relative to the female pronuclei (Santos et al., 2002). Research has suggested that this rapid demethylation is due to the enzyme, TET3, in the male pronuclei, which oxidizes the methyl group on the fifth carbon in cytosine (5meC) to a hydroxymethyl group (5hmc) (Zhang et al., 2014). This is the first step in an oxidation pathway, which subsequently formylates then carboxylates the 5-methyl cytosine. Carboxylated cytosines are removed through the cell's base-excision repair (BER) mechanisms, which return the cytosine to an unmethylated state. It is said that the female pronuclei is "protected" from this active DNA demethylation by proteins such as STELLA as well as lower levels of TET3 (Kawasaki et al., 2014). With that said, recent research has shown that TET3 is active in the female pronuclei and active demethylation is also a characteristic of the female pronucleus (Guo et al., 2014). The established thought was that while the male pronucleus is actively demethylated, the female pronucleus is passively demethylated through cellular division (Dean et al., 2005; Santos et al., 2002). This model has been challenged by current research, which shows that active demethylation occurs in both the male and female pronuclei and that re-methylation begins around the same time as MZT (maternal-zygotic transition) in mice (Edwards et al., 2017). None-the-less, this period of chromatin reorganization is an essential component of early embryonic development.

In clones, or animals produced via somatic cell nuclear transfer (SCNT), it had been shown that the nuclei of these somatic cells undergo deficient epigenetic reprogramming after insertion of the donor nucleus into the enucleated oocyte. Mammalian cloning was first achieved in sheep in the late nineties by Dr. Ian Wilmut (Campbell, McWhir, Ritchie, & Wilmut, 1996). While amazingly novel and exciting, phenotypic defects in the offspring produced via cloning began to be published (Murphey et al., 2009; Smith & Murphy, 2004; Tamashiro et al., 2003). These abnormalities were correlated with alterations in the profile of DNA and histone methylation that suggested that these epigenetic modifications were found to insufficiently reset compared to naturally derived embryos (Santos et al., 2003). This is not totally shocking given the somatic cell nucleus circumvents the reprogramming events during gametogenesis that are in place to successfully prepare the genomes for development after fertilization (Murphey et al., 2009). These cloning experiments are an important observation that gives strength to the idea that DNA and histone post-translational modifications are associated with the developmental potential of an organism (Santos et al., 2003).

Histone Post-Translational Modifications

The N-terminal tails of histones H2A, H2B, H3 and H4 is flexible and subject to many distinct chemical modifications, which associate with specific chromatin states and gene expression states. The best studies posttranslational modifications (PTMs) to histone tail amino acids include methylation, acetylation, phosphorylation and ubiquitination. While the PTMs on histone tails are well studied, it is worth mentioning that modifications

to the histone's globular core have also been associated with protein recruitment, and signaling, as well as structural changes (Tropberger & Schneider, 2013). Histone methylation can be both associated with active and repressed gene expression as well as hetero- and euchromatic states. The state achieved is reliant upon which specific amino acid is methylated (Fuks 2005). These modifications typically occur on lysine residues. Trimethylation of lysine 4 on histone 3 (H3K4me3) is one of the most robust epigenetic post-translational modifications known. This specific mark is associated with actively transcribed regions in the genome and correlate with expressed genes in early development (Benevolenskaya 2007). The Trithorax family of proteins establish these post-translational modifications in early development (Petruk et al., 2012). The Trithorax family maintains the expression of Hox genes in early development which are responsible for body plan segmentation. They achieve this by maintaining the H3K4me3 mark around these genes (Andrey et al., 2013). Another epigenetic methylation mark discovered was H3K27me3. This epigenetic modification first appeared to work in opposition to the activating H3K4me3 mark to reduce expression of genes. Interestingly, these two opposing post-translational modifications could be observed in the same chromatin space in a proposed a state of "bivalency" during development, which was hypothesized to prime certain genes awaiting instruction (Bernstein et al., 2006). While an interesting observation, this goes to show the complexity of the histone code to be far more than previously believed.

Histone lysine acetylation is a mark highly associated with open chromatin structure and active gene expression (Eberharter & Becker, 2002). Acetylation of lysines are established by histone acetyltransferases (HATs) and regulated by proteins such as

Gcn5, which specifically acetylates the fourth lysine on the N-terminal tail of histone 3 (H3K4) (Govind, Zhang, Qiu, Hofmeyer, & Hinnebusch, 2007). These acetyl post-translational modifications can be subsequently removed by the histone deacetylase (HDAC) family of proteins. Acetylation of lysine 27 on histone 3 (H3K27ac) associates with transcription factor binding sites of enhancers (Charlet et al., 2016). Enhancers function to increase activators at the site of transcription in order to increase transcription rates. They "enhance" transcription rates through chromatin looping to the site of promoters upstream of genes. Therefore, evidence has shown that this mark maintains the binding sites of enhancers, which promote transcription.

Environmental Exposures & Epigenetics

Over the past 20 years, evidence has emerged indicating that epigenetic modifications to chromatin structure provide a plausible link between environmental exposures and alterations in cellular function leading to the development of birth defects (Feil & Fraga, 2011). It has been known for quite some time that *in utero* exposures to maternal malnutrition, toxins, infection, disease, alcohol or other stressors can lead to a plethora of congenital abnormalities later in the life of the offspring (Hales & Barker, 1992). Collectively, this has become known as the "Developmental Origins of Health and Disease" (DOHaD) hypothesis (Gillman 2005). However, the specific biochemical mechanisms by which environmental exposures alter chromatin structure, and whether epigenetic changes are truly causal in imparting dysgenesis remains to be determined. Previous work has shown alterations in numerous environmental conditions, good or bad,

during early embryonic development can adversely affect heritable patterns of DNA methylation and post-translational histone modifications, which lead to altered patterns of gene transcription (Blondin, Farin, Crosier, Alexander, & Farin, 2000; Dindot et al., 2004; Mann et al., 2004). These aberrant patterns of DNA methylation and histone structure directly affect the chromatin state of the organism (Kouzarides 2007; Zhu et al., 2013). In recent years, the effects of oxidative stress on the epigenome has received more attention. It was shown that proteins involved in DNA and histone demethylation, such as the ten-eleven translocation (TET) and Jumonji-family histone demethylases (KDM), require alpha ketoglutarate (α-KG) as a cofactor for their activation Wang 2011. NADH and NADPH provide reducing capacity to combat oxidative stress in the cell. Reduction in these cofactors can drive the citric acid cycle and alter the levels of α-KG thus linking cellular metabolism with regulation of DNA and histone methylation (Wang 2011). Additionally, studies have shown that chronic alcohol consumption has been shown to alter DNA methylation, histone modifications, and miRNA components (Mahnke 2017). Interestingly, alcohol consumption has been found to induce cellular oxidative stress through the byproducts of ethanol degradation (Brocardo 2011). Although the mechanisms underlying alcohol's ability to induce an altered cellular redox state are not completely understood, oxidative stress might provide a bridge between alcohol metabolism and epigenetic dysregulation (Brocardo 2011).

Overview & Hypotheses

The overall goal of this study is twofold. First, we will evaluate how atmospheric-derived oxidative stress can influence the expression of chromatin modifying genes and dysregulate epigenetic regulatory mechanisms in both in vivo bovine embryo culture conditions as well as in mouse embryonic stem cells. We will focus on oxidative stress due to the association with altered epigenetic programming within in vitro produced embryos and the resulting growth abnormalities linked to alterations in imprinted gene expression. These studies will help establish the environment as a powerful modulator of the epigenome and allow mechanistic studies into which families of chromatin modifying enzymes are predominantly impacted. *Our hypothesis is that deviating from physiological uterine oxygen concentrations (3-5% O_2) during the preimplantation phase of embryo development results in the abnormal transcriptional regulation of genes controlling chromatin structure, which impact the establishment and maintenance of patterns of imprinted gene expression.*

Next, we will evaluate whether environmentally induced epimutations can be inherited through the male germline. Specifically, we will assess the capacity of preconception paternal alcohol exposures to transmit epimutations to the offspring. Paternally inherited alterations in developmental programming have been correlated with changes in the DNA methylation profiles of paternal sperm, although an increasing number of cases question the heritability of this epigenetic modification. Thus, the universal dependence of these phenotypes on DNA methylation-based mechanisms of epigenetic inheritance remains an unanswered question central to understanding the

paternal transmission of environmentally induced traits. *We hypothesize that epimutation(s) induced by chronic preconception male ethanol exposure are carried by sperm through aberrant DNA methylation, persist through preimplantation development and alter the health and development of offspring.*

The ability to adapt to the environment is critical for survival in a fully developed, mature organism. Upon exposure to an environmental stress, an organism's chromatin structure can be altered and these changes persist on into later developmental stages to impact health and development. Collectively, these studies will determine whether atmospheric-derived oxidative stress can influence the expression of chromatin modifying genes and dysregulate epigenetic regulatory mechanisms in both in vitro derived bovine embryos, as well as in mouse embryonic stem cells. This is an important question based on current ART industry practices commonly exposing mature gametes and early embryos to transient levels of atmospheric oxygen. Additionally, these studies will help elucidate whether environmentally induced epimutations can be inherited through the male germline through DNA methylation-based mechanisms of epigenetic inheritance which remains an unanswered question central to understanding the paternal transmission of environmentally induced traits. Based on the largely ignored social issue of male alcohol abuse and the general dismissal of paternal contributions to the health of their offspring, these studies will potentially reveal an inconvenient truth regarding preconception alcohol consumption and the potential for alcohol-induced epimutations lasting into the subsequent generation. *Our overarching hypothesis is that environmental exposures can*

induce epimutations in an organism, that these changes have the capacity to persist, and correlate with diseases or disorders in the offspring of exposed individuals.

CHAPTER II

ATMOSPHERIC-MEDIATED OXIDATIVE STRESS DURING BOVINE IN VITRO EMBRYO CULTURE LEADS TO ALTERATIONS IN THE EXPRESSION OF CHROMATIN STRUCTURE REGULATING GENES.

From "Oxygen-induced alterations in the expression of chromatin modifying enzymes and the transcriptional regulation of imprinted genes" submitted for publication in Gene Expression Patterns (August 25, 2017)

Introduction

Some of the first evidence that environmental exposures could influence the long-term health of offspring came from studies employing mammalian embryology. Here, researchers noted an overgrowth phenotype in livestock that was induced by exposure of the embryo to conditions associated with assisted reproductive technologies (ART), and not spontaneous mutations or experimental genetic manipulation (Wilson 1995; Farin, Piedrahita, & Farin, 2006; Young, Sinclair, & Wilmut, 1998). These pioneering studies were later followed up by work examining altered DNA methylation, and abnormal patterns of imprinted gene expression arising as a consequence of *in vitro* culture conditions and somatic cell nuclear transfer (Doherty et al., 2000). Collectively, these works demonstrate that the environment has the capacity to alter both chromatin structure and long-term patterns of gene expression crucial in patterning the early embryo. However, the mechanisms by which embryo culture heritably alters phenotype, and the

extent to which these changes help explain the wider development of environmentally induced birth defects, remains to be demonstrated.

The use of ART as a method of conception has increased steadily in the United States over the past two decades to over double the former levels (Control & Prevention, 2012). Increasing evidence suggests a higher incidence of congenital malformations, intrauterine growth retardation, premature birth and low birth weights in children conceived via ARTs (Arnaud & Feil, 2005; Dean et al., 2005; Hansen, Bower, Milne, de Klerk, & Kurinczuk, 2005; Hansen, Kurinczuk, Milne, de Klerk, & Bower, 2013; Kurinczuk, Hansen, & Bower, 2004; Niemitz & Feinberg, 2004; Wrenzycki et al., 2005). Interestingly, a high incidence of these diseases had origins in abnormal epigenetic regulation of imprinted genes and result in imprinting disorders such as Angelman, Prader-Willi and Beckwith-Weidemann syndrome (DeBaun, Niemitz, & Feinberg, 2003; Gicquel et al., 2003; Gosden, Trasler, Lucifero, & Faddy, 2003; Niemitz & Feinberg, 2004; Powell 2003). Although there is still much to discover regarding how the embryo reprograms itself during this dynamic epigenomic period, what we do know is that the environment can detrimentally impact this period resulting in improper establishment of imprinted patterns of gene expression.

Potential errors resulting from assisted reproductive technologies were first reported as a fetal overgrowth phenotype in cloned cattle (Wilson 1995). Soon after, large offspring syndrome was characterized in cattle and sheep conceived via ART methods such as in-vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI) and cloning (Young et al, 1998; Kurinczuk 2003; Kurinczuk 2004). Since these studies, meta-analysis

has shown that human assisted reproductive technologies yield a ~4.8% chance of producing offspring with Beckwith-Weidemann syndrome when the background rate in the US is 0.8% (Hansen et al., 2013; Kurinczuk et al., 2004). Not all incidences of BWS are due to abnormal imprint establishment in the general population but through ART, approximately 80% of BWS are a result of imprinting related disorders in the Cdkn1c or Igf2 imprinting control regions (Hoeijmakers, Kempe, & Verschure, 2016). In the H19/Igf2 locus, improper methylation of the maternal allele leads to expression of IGF2 in a bi-allelic manner, which results in over expression of IGF2. In contrast, loss of DNA methylation at the Kcnq1ot1 locus leads to the over expression of Cdkn1c. Both of these genes are growth promoting which help explain the overgrowth phenotype seen in BWS (Algar, Deeble, & Smith, 1999).

Studies in multiple species have shown that embryos cultured under atmospheric oxygen concentrations result in a reduced rate of embryo development to the blastocyst stage (Harvey, Kind, & Thompson, 2007; Preis, Seidel, & Gardner, 2007). Fischer and Bavister demonstrated that intrauterine and oviductal oxygen concentrations range between 2-7% oxygen (Fischer & Bavister, 1993). This implies that culture of embryos under low atmospheric oxygen concentrations represents an environment more similar to the *in vivo* condition. However, embryo handling and best *in vitro* practices still necessitate transient exposures to atmospheric (20.9%) oxygen. Recently, a link between components of the oxidative stress pathways and enzymes controlling chromatin structure has been identified (Delatte et al., 2015; Wang, Chia, Lu, & Ruden, 2011). For instance, the TET enzymes that catalyze the first step of DNA demethylation require the cofactor

α-KG for activation which is influenced by the redox state of the cell (Delatte 2015). Similarly, the Jumanji-family histone demethylases were found to require α-KG for activation linking their activity to the redox state of the cell as well (Wang 2011). To gain a better understanding of the potential impact of oxygen exposures during *in vitro* embryo culture on the regulation of chromatin structure, we evaluated the capacity of differing oxygen concentrations to affect the transcriptional control of chromatin modifying genes and the regulation of gene expression. *We hypothesized that deviating from physiological uterine oxygen concentrations (i.e. 5% O_2) during the preimplantation phase of embryo development would induce alterations in the expression of genes regulating chromatin structure.*

Results

High oxygen concentrations negatively impact embryo development and alter the expression of genes regulating pluripotency

Embryos are commonly cultured in an atmospheric environment composed of premixed gases (5% CO_2, 5% O_2, 90% N_2) or incubators with can regulate the internal conditions to preset levels (5% CO_2, 5-6% O_2). Although atmospheric oxygen levels average to roughly 20.9%, we will refer to atmospheric levels of oxygen as 20%. Culturing bovine embryos under atmospheric oxygen concentrations (20%) as compared to the industry-standard 5%, resulted in reduced cleavage and blastocyst rates (**Figure 2A)**. These results are consistent with previous studies examining the effects of high oxygen levels on preimplantation bovine embryo development (Harvey 2007; Harvey et al., 2007;

Li et al., 2014). While standard industry practices do not employ atmospheric oxygen concentrations, cultured embryos are frequently exposed to this condition anytime they are removed from the incubators for manipulation. Therefore, we employed a model of constant exposure in an effort to determine the extreme consequence oxygen exposure has on the genetic pathways regulating chromatin structure. To validate our model, we first examined the levels of transcripts encoding genes involved in pathways regulating cellular hypoxia and oxidative stress. Blastocyst stage embryos from the 20% culture group displayed decreased expression of two hypoxia-inducible factors, while increased abundance of transcripts encoding components of the antioxidant response **(Figure 2B)**. Specifically, the superoxide dismutase family was significantly up regulated in the 20% oxygen group, with an almost three-fold increase for SOD1, while SOD2 displayed more modest changes in transcript abundance **(Figure 2B)**. We next examined transcript levels of genes involved in the maintenance of pluripotency and found a down-regulation of NANOG and SOX2 in the high oxygen (20%) treatment group **(Figure 2C)**. These data indicate that exposure to atmospheric oxygen is associated with alterations in the transcriptional control of genes involved in the oxidative stress response and with a reduction in the level of transcripts encoding major factors driving embryonic pluripotency.

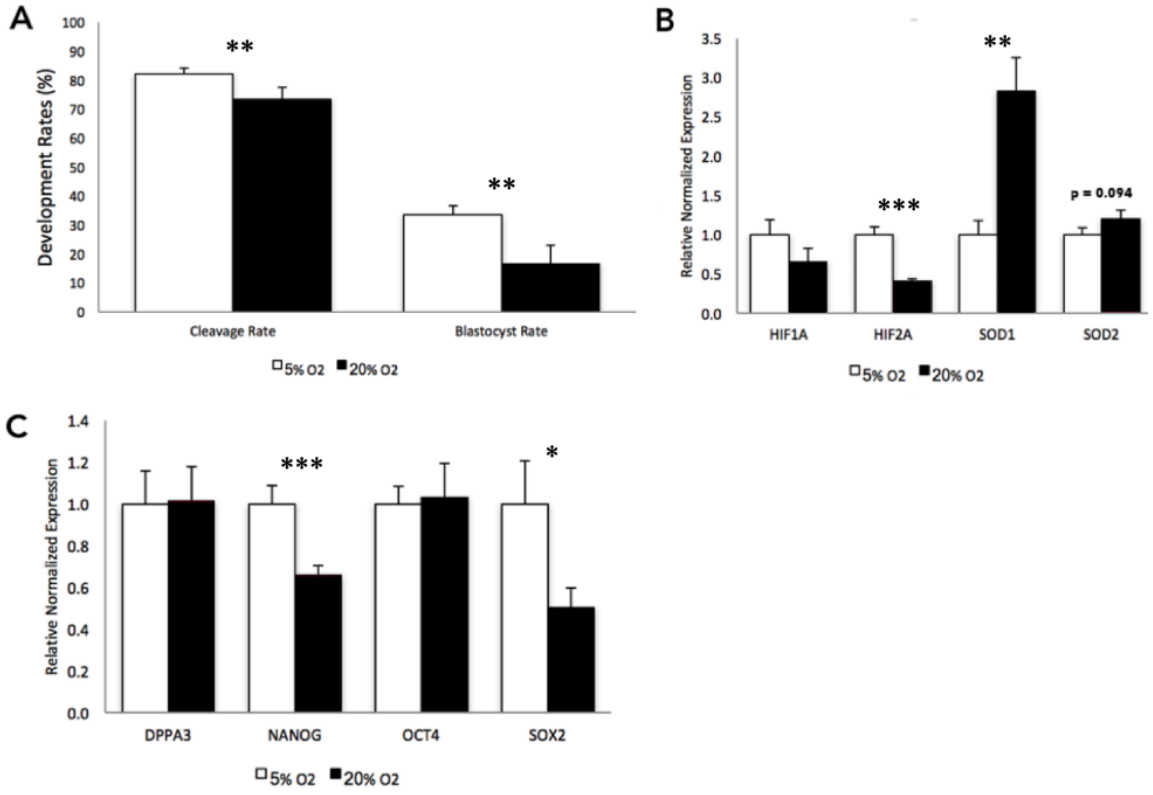


Figure 2. The impact of oxygen concentrations on bovine preimplantation development and the transcriptional regulation of select genes controlling the oxidative stress response.
A. Cleavage and blastocyst rates of bovine embryos cultured under low (5%) and high (20% - atmospheric) oxygen concentrations (5% oxygen n = 682, 20% oxygen n= 890, from 4 independent replicates). **B.** Measurement of transcripts encoding genes involved in oxygen sensing and the oxidative stress response using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). **C.** RT-qPCR measurement of transcripts encoding key bovine pluripotency genes. For experiments employing RT-qPCR, measurements were normalized to the geometric mean of GAPDH, SDHA and YWHAZ. Graphs represent 3 independent biological replicates with 2 independent RT reactions per replicate. (*P*-value: $* \leq 0.05$; $** \leq 0.01$; $*** \leq 0.001$; $**** \leq 0.0001$).

Atmospheric oxygen levels alter the transcription of the Ten-eleven translocation (TET) family and genes regulating DNA methylation

To determine if genes involved in the establishment, maintenance and/or regulation of the epigenome are affected by exposure to atmospheric oxygen during preimplantation development, we examined the levels of transcripts encoding a family of genes found to catalyze the first step in DNA demethylation (Delatte, Deplus, & Fuks, 2014). The ten-eleven translocation (TET) family of genes hydroxylate methylated cystosines in a series of reactions that end with the base-excision repair (BER) machinery removing a 5'-formyl/carboxycystosine, generating a standard, unmethylated cytosine (Kohli & Zhang, 2013). This family of genes, while its precise role remains unclear, has been implicated in the cellular response to oxidative stress (Delatte et al., 2015). During bovine preimplantation development, the TET family of genes are thought to play a role in active genomic demethylation and resetting of the epigenome (Gu et al., 2011). In our experiments, the high-oxygen treatment group exhibited a significant down-regulation of TET1 and TET3 at the blastocyst stage of development, compared to their low oxygen counterparts **(Figure 3A)**. We next investigated the capacity of atmospheric oxygen to influence the expression of genes regulating DNA methylation. No difference in the levels of transcripts encoding DNMT1 were observed, while the *de novo* methyltransferase DNMT3A, as well as a gene responsible for the localization of DNMTs to specific loci, HELLS, were both significantly ($P<0.05$) downregulated in the high atmospheric culture treatment group **(Figure 3B)**.

Oxygen-induced down regulation of multiple histone methyltransferase enzymes in cultured bovine blastocysts

Various post-translational histone modifications correlate with distinct transcriptional states and have been associated with the nucleation of repressed or relaxed chromatin structure. These modifications are hypothesized to act in a coordinated context to regulate DNA accessibility (Zhang & Reinberg, 2001). We therefore examined the levels of transcripts encoding lysine-specific histone demethylase (KDMs) proteins. KDM5 of the KDM family has been implicated in the regulation of the cellular response to oxidative stress (Liu, Greer, & Secombe, 2014). Almost all the candidate genes examined were reduced in the high oxygen treatment group **(Figure 3C).** Transcripts encoding KDM1A, 4B and 4C were all significantly reduced while KDM3A tended to be lower (P=0.051). The polycomb-repressive complexes (PRCs) are important in establishing and maintaining the transcriptional memory of differentiated cells as well as in the long-term silencing of chromatin (Margueron et al., 2009). Therefore, we examined the abundance of transcripts encoding the core polycomb-repressive complex 1 (PRC1) and polycomb-repressive complex 2 (PRC2) members. EED and EZH2 both displayed a reduction in transcript levels in the 20% oxygen treatment group, while in contrast BMI1 and RING1, members of the PRC1 complex, showed no signs of altered gene expression **(Figure 3D)**. We next examined the abundance of transcripts encoding the histone methyltransferases EHMT1 (G9a) and SETDB1. Of these two, only SETDB1 displayed a significant decrease in the high-oxygen group **(Figure 3D)**.

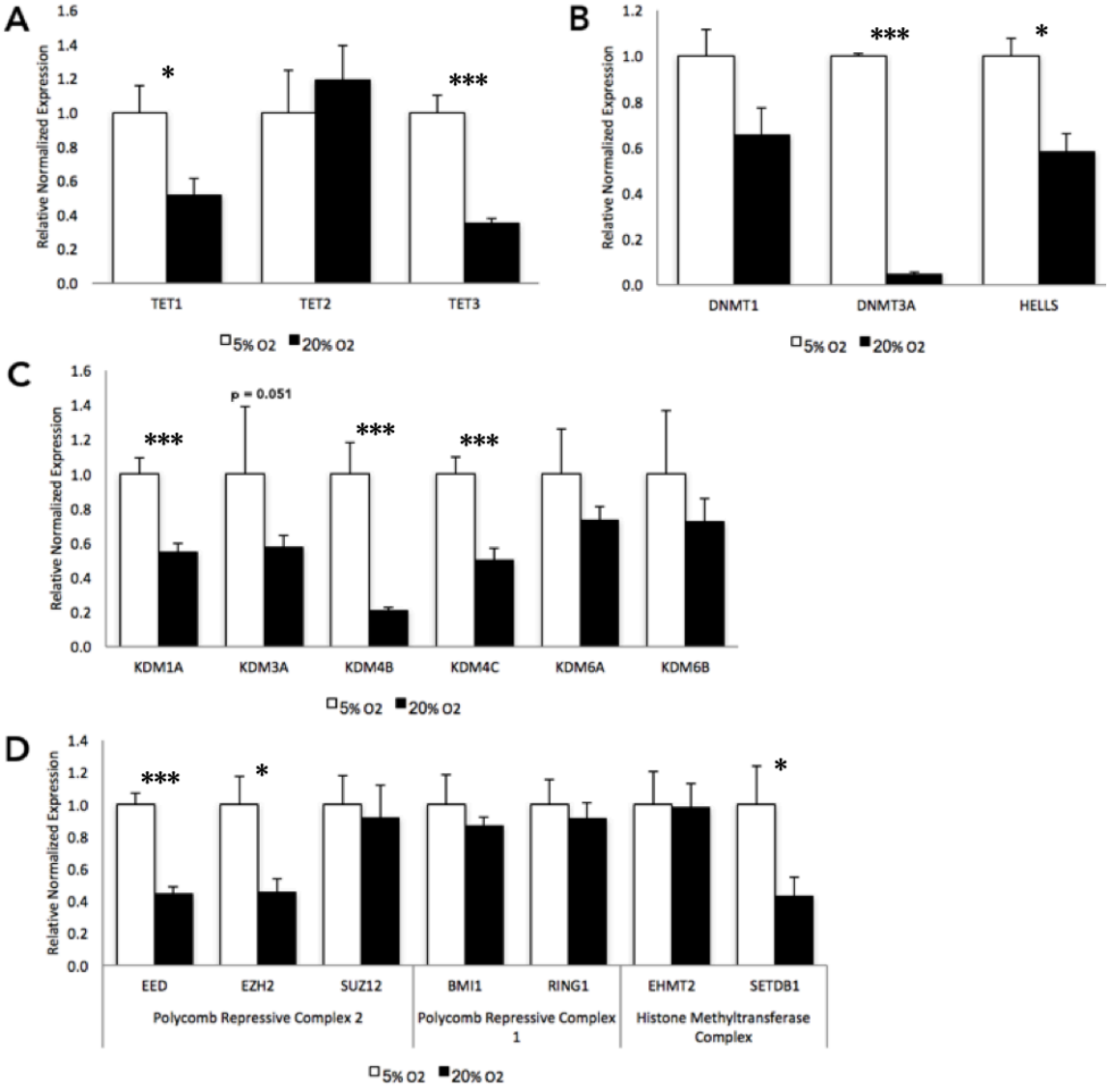


Figure 3. Oxygen-induced changes in the transcriptional regulation of genes controlling DNA methylation during preimplantation bovine development.

A. RT-qPCR measurements of transcripts encoding the bovine ten-eleven translocation (TET) gene family. **B**. Transcript levels of the bovine DNA methyltransferase genes and an associated recruiter, HELLS. **C**. Measurement of transcripts encoding the bovine histone lysine demethylase family of genes. **D.** Gene transcript levels of the histone modifying complexes PRC1, PRC2 and histone methyltransferases. RT-qPCR measures were normalized to the geometric mean of GAPDH, SDHA and YWHAZ. Graphs represent 3 independent biological replicates with 2 independent RT reactions per replicate. (*P*-value: $* \leq 0.05$; $** \leq 0.01$; $*** \leq 0.001$; $**** \leq 0.0001$).

Discussion

Prior research has shown uterine and oviductal oxygen concentrations to range between 2-7% oxygen, but embryo handling and best *in vitro* practices still necessitate transient exposures to atmospheric (20%) O_2 during assisted reproductive techniques. Due to the suggestion that ARTs result in an increased percentage of offspring exhibiting imprinting-related disorders, we wanted to examine whether exposure to atmospheric O_2 could act as a stressor during the vulnerable period during preimplantation embryonic development and influence the transcription of genes that play a role in either the establishment, maintenance or regulation of the epigenome. In order to test these effects, we investigated the capacity of distinct levels of atmospheric oxygen to impact embryonic development, the transcriptional control of chromatin modifying genes and the regulation of pluripotency associated genes. Our hypothesis was that deviating from physiological uterine oxygen concentrations (5% O_2) during the preimplantation phase of embryo development will induce alterations in the expression of genes regulating chromatin structure.

Bovine embryos cultured under atmospheric oxygen concentrations (20%), as compared to the industry-standard 5% oxygen, resulted in reduced cleavage and blastocyst rates as seen in **Figure 2A**. This observation is consistent with previous studies examining the effects of high oxygen levels on preimplantation bovine embryo development (Harvey 2007; Harvey et al., 2007; Li et al., 2014). In our experiments, we observed an upregulation of the oxidative stress response gene, SOD1, suggesting the mobilization of this cellular response. In addition, we observed a down-regulation of several genes

involved in maintenance of pluripotency. These data indicate that exposure to atmospheric oxygen negatively impacts embryonic development and is broadly associated with a reduction in the level of transcripts encoding the major factors driving embryonic patterning. Conversely, the reduction observed may not be directly related to embryonic patterning, but a result thereof. If NANOG, OCT4 and SOX2 are primarily expressed in the ICM, then any conditions that would stimulate a shift in the trophectoderm to ICM cell ratio would look like a reduction in these transcription factors.

Under high oxygen, transcripts encoding TET1, TET3, DNMT1 and DNMT3a were significantly down regulated. In mice, *TET3* is essential for embryonic development and the demethylation of the paternal methylome (Gu et al., 2011). Without prior time points of development, our data merely offer a snapshot of these epigenetic processes, but do suggest a potential impact on the parental methylome. Further experiments examining patterns of DNA methylation will need to be performed to elucidate the effects of the TET dysregulation during the preimplantation stages.

When we examined the regulation of chromatin modifying genes, we identified alterations in the expression of several candidates with established roles in fetal growth syndromes and the control of cellular differentiation. For example, we found broad downregulation across the KDM family of lysine demethylases under high oxygen conditions. Altering these genes would likely lead to alterations in the histone methylation and hinder the cell's ability to alter these marks. While not directly indicative of errors in the epigenome, the post-translational modifications imparted by these proteins serve as common signals and scaffolding for complexes known to alter the chromatin landscape.

To this point, we observe down-regulation of two important subunits in the *Polycomb* repressive complex 2 (EED and EZH2). This complex is recruited to and silences specific regions of the genome by transferring methyl groups onto lysine 27 of histone H3. This chromatin modification has well-defined roles in the maintenance of the stem cell state and genomic imprinting (Inoue et al., 2017; Juan et al., 2016). Collectively, these observations indicate that oxygen concentrations have a strong influence on the transcriptional control of chromatin modifying enzymes and significantly impact the regulation of two crucial pluripotency-associated genes. Finally, HELLS, which is known to recruit DNMTs to CDKN1C, displayed significantly decreased transcript levels under increased oxygen concentrations. CDKN1C is an imprinted gene that has been shown to be responsible for sporadic cancers and the overgrowth phenotypes associated with Beckwith-Weidemann syndrome (Algar et al., 1999). Additional studies will be required to determine of oxygen-induced changes in HELLS can be linked to the overgrowth phenotypes observed in large offspring syndrome in cattle. In summary, our data indicate that oxygen has the power to influence crucial gene regulatory networks involved in the establishment and maintenance of chromatin structure and the control of stem cell pluripotency.

Materials & Methods

In vitro fertilization and embryo production

Bovine oocytes were collected at a commercial abattoir (DeSoto Biosciences, Seymour, TN, USA] and shipped in an metal bead incubator (MOFA Global, Verona, WI, USA) at 38.5°C overnight in sealed sterile vials containing 5% CO_2 and air-equilibrated maturation medium (Medium 199 with Earle's salts (Invitrogen, Life Technologies Inc., Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 1% penicillin–streptomycin (Invitrogen), 0.2-mM sodium pyruvate, 2-mM L-glutamine (Sigma Chemical Co., St. Louis, MO, USA), and 5.0 mg/mL of Folltropin (Vetoquinol, Pullman, WA, USA). The oocytes were matured in this medium for 22 to 24 hours. Matured oocytes were washed twice in warm Tyrode lactate (TL) HEPES (Vetoquinol) supplemented with 0.05 μg/μL of gentamicin (Invitrogen) while being handled on a 38.5C stage warmer (Minitube). *In vitro* fertilization was conducted using a 2-hour pre-equilibrated modified TL medium supplemented with 250-mM sodium pyruvate, 1% penicillin–streptomycin, 6 mg/mL of fatty acid–free BSA (Sigma), 20-mM penicillamine, 10-mM hypotaurine, and 10 μg/mL of heparin (Sigma) at 38.5°C, 5% CO_2 in a humidified air incubator. Frozen semen was thawed at 35°C for 1 minute, then separated by centrifugation at 200g for 20 minutes in a density gradient medium (Isolate; Irvine Scientific, Santa Ana, CA, USA) 50% upper and 90% lower. Supernatant was removed; the sperm pellet was resuspended in 2-mL modified Tyrode's medium and centrifuged at 200 g for 10 minutes to wash. The sperm pellet was then removed and placed into a warm 0.65-mL microtube (VWR Scientific, Pittsburg, PA, USA) before

mixing in Nunc four-well multidishes (VWR)) containing up to 50 matured oocytes per well at a concentration of $1.0x10^6$ sperm/mL. Sixteen to 18 hours after insemination, oocytes were cleaned of cumulus cells by a 2-minute vortex in 0.45-mL TL HEPES in a 0.65-mL microtube (VWR), washed in TL HEPES, and then randomly assigned to the different treatment groups: 5% oxygen controls and 20% atmospheric oxygen treatment and cultured until the blastocyst stage. Specifically, the "5%" treatment group is cultured in a hypoxia chamber perfused with a premixed gas (5% CO_2, 5% O_2, 90% N_2). The "20%" treatment group is cultured in an incubator regulating CO_2 to 5% with the rest composed of air (~20.9% O_2). Cleavage rates were recorded on Day 2, at which point viable embryos were separated from nonviable embryos. Embryos were monitored daily for morphologic progression, and blastocyst rates were recorded on Day 8 after IVF.

RNA isolation and RT-qPCR.

Bovine blastocysts were pooled within each treatment in groups of 10 and RNA extracted using the miRNeasy Micro kit (Qiagen), according to the manufacturer's instructions. cDNA was generated and pre-amplified with the RT^2 PreAMP cDNA Synthesis kit (Qiagen). cDNA was quantified on a custom RT^2 Profiler PCR Array: CAPB11472 (Qiagen). The plate was run on the CFX384 Real-Time System (Bio-Rad). Reference genes were GAPDH (NM_001034034) (Fwd 5-CTGCCCGTTCGACAGATAG-3, Rev 5-CTCCGACCTTCACCATCTTG-3), SDHA (NM_174178) (Fwd 5-ACCTGATGCTTTGTGCTCTG-3 Rev 5-

TCGTACTCGTCAACCCTCTC-3), YWHAZ (NM_174814) (Fwd 5-CTGAACTCCCCTGAGAAAGC-3 Rev 5-CCTTCTCCTGCTTCAGCTTC-3).

Statistical analysis

For all experiments, statistical significance was set at alpha = 0.05. For analysis of gene expression, the replicate cycle threshold (Ct) values for each transcript were compiled and normalized to the geometric mean of three validated reference genes Golding 2011a. Normalized expression levels were calculated using the ddCt method described previously (Schmittgen & Livak, 2008). Relative fold change values from a minimum of three biological replicates were transferred into the statistical analysis program GraphPad (GraphPad Software, Inc., La Jolla, CA) where datasets were first verified for normality using the Brown-Forsythe test. For single comparisons, an unpaired student's t-test was applied. In all instances, we have marked statistically significant differences with an asterisk (∗).

CHAPTER III

ATMOSPHERIC-DERIVED OXIDATIVE STRESS INDUCES DYSREGULATION OF CHROMATIN MODIFIERS AND EPIGENETIC GENE REGULATORY MECHANISMS IN A MOUSE EMBRYONIC STEM CELL MODEL.

From "Oxygen-induced alterations in the expression of chromatin modifying enzymes and the transcriptional regulation of imprinted genes" submitted for publication in Gene Expression Patterns (August 25, 2017)

Introduction

In order to better understand the interactions between oxygen exposure and the transcriptional regulation of factors controlling chromatin structure, we shifted our studies into a murine embryonic stem cell (mESC) model. Although not directly equivalent to cultured embryos, mESCs are an embryonic cell type, which can be cultured with high uniformity in terms of cellular phenotype, chromatin profiles and transcriptional output (Galonska, Ziller, Karnik, & Meissner, 2015). Therefore, this model allowed us to observe the time course of transcriptional effects that differential concentrations of oxygen exert, as well as determine the impact of extended exposures to differing oxygen concentrations. Mouse ESCs are routinely cultured at 20% oxygen due to lack of significant benefits of maintaining the cell lines at reduced oxygen levels (Chen et al., 2009). In these experiments, cells were split into two groups: one cultured at 20% oxygen and the other under 5% oxygen, for a duration of 8 passages **(Figure 4A).** Specifically, the 20% treatment group was cultured in an incubator with a gas mixture of 5% CO_2 and the rest

consisting of air composed of O_2 (20.9%). The 5% treatment group was cultured in a hypoxia chamber perfused with a gas mixture of 5% CO_2, 5% O_2, and 90% N_2. The mESC line that we employed was of a C57BL/6 (Black6) by castaneus (CAST) cross (Golding et al., 2011). These distinctive genetic backgrounds allow for allele-specific differences in gene expression to be identified through single nucleotide polymorphisms (SNPs).

We hypothesized that atmospheric-derived oxidative stress will induce dysregulation of chromatin modifiers and epigenetic gene regulatory mechanisms in our mouse embryonic stem cell mode. In these studies, we investigated atmospheric-derived oxidative stress mediated dysregulation of chromatin modifiers and epigenetic gene regulatory mechanisms in a mouse embryonic stem cell model. In these experiments, we utilized a modular incubation chamber, which allows for the maintenance of specific atmospheric conditions during long-term cell culture. This system allowed us to observe both the acute impacts and prolonged effects of differing oxygen concentrations.

Results

Oxygen-induced transcriptional changes in a murine embryonic stem cell (mESC) model of development.

At 24 hours after exposure to the different oxygen tensions, we noted a smaller cell size in the 5% O_2 treatment group as compared to the cells cultured under 20% O_2 **(Figure 4B)**. This change was consistent throughout the experiment at passage 8 **(Figure 4C)**. To measure the impact of oxygen concentrations on patterns of gene expression, we examined the expression of genes associated with the oxidative stress response. Here, RT-

qPCR was employed to analyze a panel of 12 well-established oxidative stress response genes (Veazey, Parnell, Miranda, & Golding, 2015). After only a single cellular passage, we observed differential expression of *Gstm3* and *Nqo1*, which were significantly reduced in the low oxygen treatment group **(Figure 4D)**. *Gstm3* is known to detoxify byproducts of oxidative stress in conjunction with glutathione. After 8 passages, *Gpx2*, *Gpx3* and *Sod2*, which are all involved in cellular detoxification processes, were significantly down regulated in the low oxygen treatment **(Figure 4E)**. These results indicate that cultured mESCs under lower oxygen concentrations reduce the expression of a core set of genes involved in combating cellular oxidative stress.

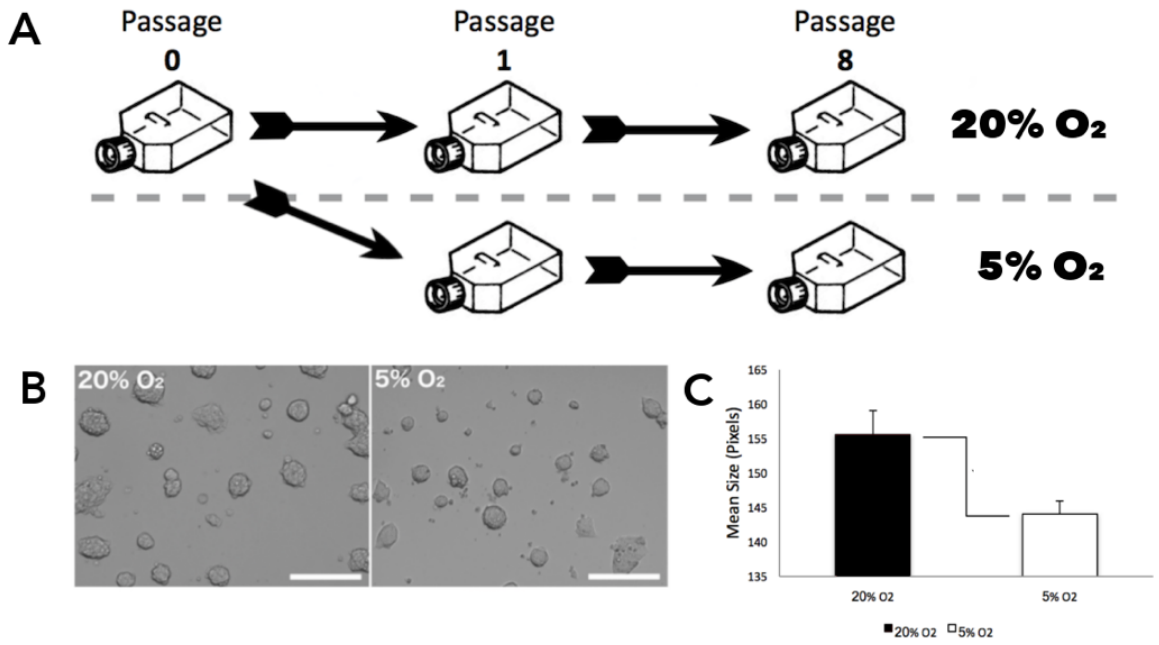


Figure 4. Experimental design and transcriptional response in oxidative stress response genes in a mouse embryonic stem cell model.
A. Schematic representation of experimental workflow. Cells split from a common flask (Passage 0) were separated into high and low treatment groups. Low (5%) O_2 cells were cultured in a "hypoxia chamber". **B**. Representative image of murine embryonic stem cells at mid-confluency during experimental treatments. **C.** Mean surface area (as calculated using ImageJ software) of representative stem cells between the treatment groups. **D-E**. Measurement of transcripts encoding genes involved in the oxidative stress response at cellular passage 1 and 8. RT-qPCR measures were normalized to the geometric mean of validated reference genes, *Hprt*, *Mrpl*, *Ppia* and *Ywhaz Veazey 2011*. n = 3 biological replicates. (*P*-value: $* \leq 0.05$; $** \leq 0.01$; $*** \leq 0.001$; $**** \leq 0.0001$).

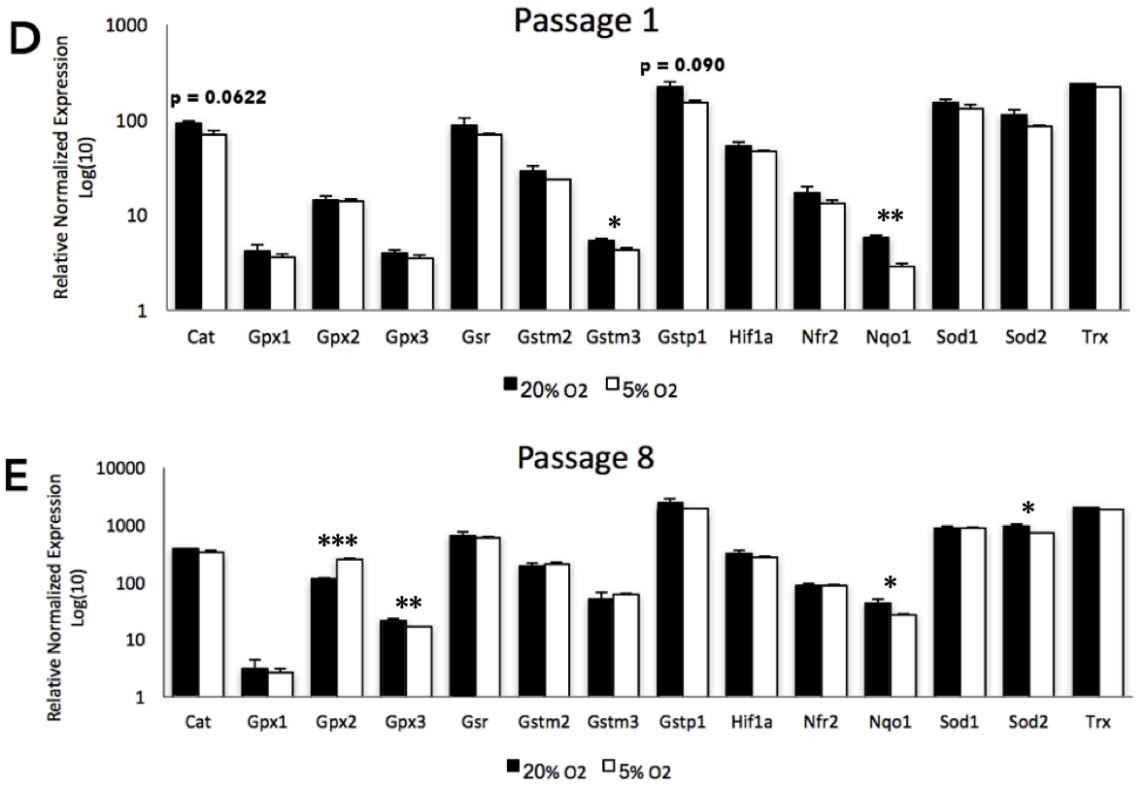


Figure 4. Continued.

Distinct acute and prolonged transcriptional responses of the DNMT and TET families of genes.

In our studies examining the effects of oxygen concentrations on bovine embryonic development, we noted that select TET genes and DNA methyltransferases were responsive to oxygen concentrations. After a single passage under low oxygen conditions, transcripts encoding *Tet2* were significantly downregulated in the 5% O_2 group **(Figure 5A)**. By the 8th passage, *Tet2* was identical to the 20% group, while transcripts encoding Tet1 had become significantly decreased in the 5% O_2 group **(Figure 5B)**.

Transcript levels of *Dnmt3a* were significantly reduced after a single passage **(Figure 5C)** while by the 8th passage, transcripts encoding *Dnmt1* had become significantly reduced in the low oxygen group **(Figure 5D)**. This data suggests that the *Tet* and *Dnmt* families are not only responsive to oxygen concentrations, but display distinct acute and prolonged responses.

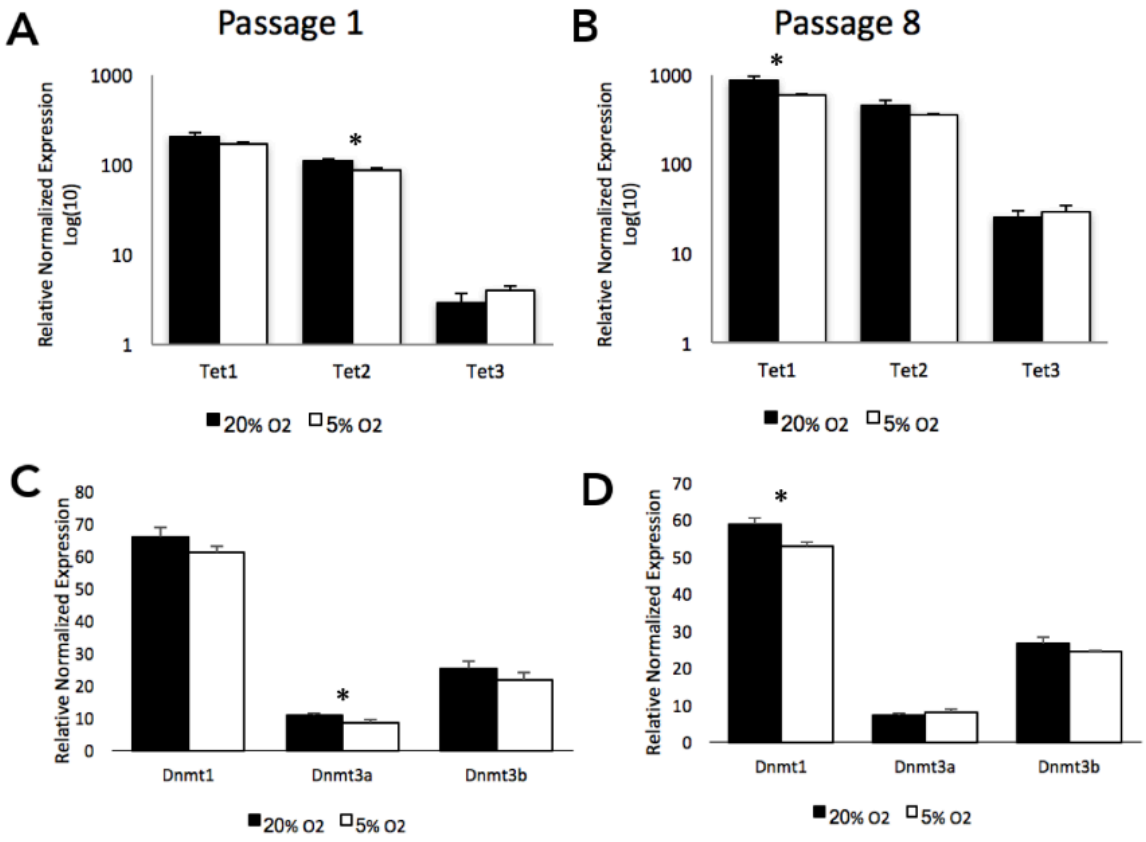


Figure 5. Oxygen-induced changes in the transcriptional regulation of genes controlling DNA methylation in mouse embryonic stem cells.
A-B. Transcript levels of the Ten-eleven translocation (TET) family of genes at passage 1 and 8. **C-D.** Transcript levels of the DNA methyltransferase [DNMT] family of genes at passage 1 and 8. RT-qPCR measures were normalized to the geometric mean of validated reference genes, *Hprt*, *Mrpl*, *Ppia* and *Ywhaz Veazey 2011*. N = 3 biological replicates. (*P*-value: ∗ ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001; **** ≤ 0.0001).

Transcriptome sequencing analysis does not reveal major global differences between high and low oxygen culture conditions.

We next examined the transcriptome of cells cultured under either high and low oxygen using high-throughput RNA sequencing. Samples clustered based on passage number rather than based on treatment, which implies a compounding variable when assessing multiple passage comparisons (**Figure 6A**). We assessed all quality parameters, which indicated good quality reads and little variation in the distribution of the reads (FPKM) globally (**Figure 6B**). Pairwise scatterplots indicated high correlation between all the samples (**Figure 6C**) and through a volcano plot analysis, it was revealed genes we had not assessed were significantly different (**Figure 6D**). In order to follow up on some of these candidates, we selected the top 6 differentially expressed genes and verified their expression differences through RT-qPCR (**Figure 7**). We determined if the top 6 genes differentially expressed in our RNAseq data in the mESCs were also different in RNA extracts derived from the bovine embryos described in Chapter II. All 6 of the candidate genes were either not significant or not expressed in the bovine embryo. We did not pursue investigating these gene candidates any further.

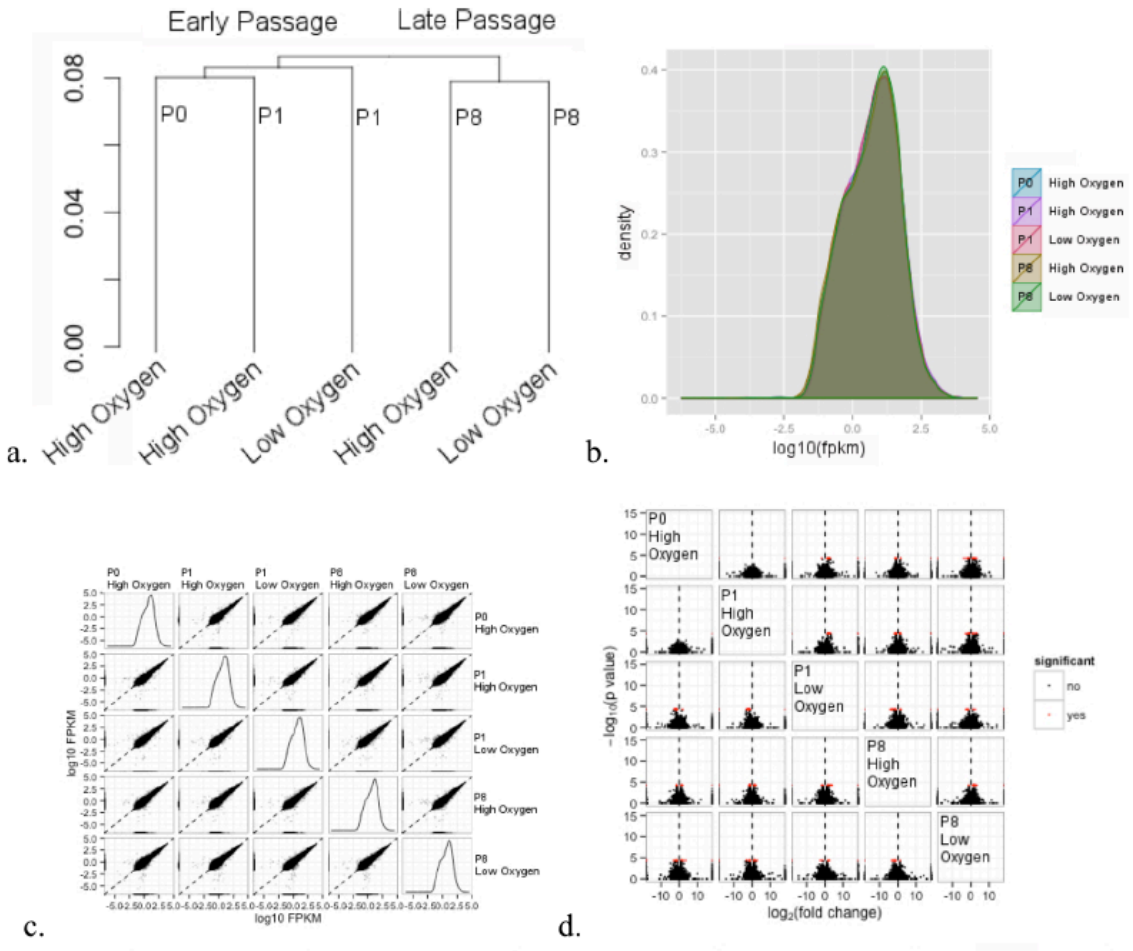
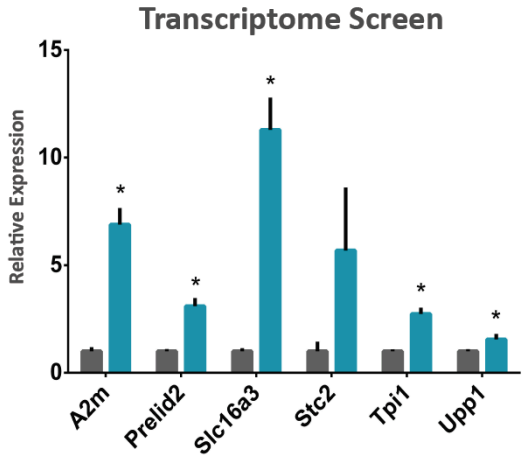


Figure 6. Murine embryonic stem cell transcriptome sequencing.
A. Clustering dendro diagram based on differential expression data. **B.** Density plot. Distribution of FPKM scores across samples. **C.** Matrix of pairwise scatterplots. **D**. Volcano plots assessing significant vs non-significant expression differences of all genes. All images in this figure were produced through CummeRbund.



Gene	Name	Description
A2m	alpha-2-macroglobulin	Inhibits all four classes of proteinases by a unique "trapping" mechanism
Prelid2	PRELI domain containing 2	**Prelid2 Unknown** ***Prelid1*** modulation of the mitochondrial apoptotic pathway by ensuring the accumulation of cardiolipin (CL) in mitochondrial membranes.
Slc16a3	solute carrier family 16 (monocarboxylic acid transporters), member 3	Lactic acid and pyruvate transport across plasma membranes
Stc2	stanniocalcin 2	Homodimeric glycoprotein that may have autocrine or paracrine functions
Tpi1	triosephosphate isomerase 1	Catalyzes the isomerization of glyceraldehydes 3-phosphate (G3P) and dihydroxy-acetone phosphate (DHAP) in glycolysis and gluconeogenesis
Upp1	uridine phosphorylase 1	Pyrimidine nucleoside phosphorylases

Figure 7. RT-qPCR verification of gene candidates identified by RNA sequencing in the mESC.
Measurements were normalized to the geometric mean of the reference genes *Gapdh*, *Hprt*, *Mrpl* and *Sdha* and samples graphed relative to High Oxygen group (20% oxygen).
(*P*-value: $* \leq 0.05$)

H3K4 acetylation, in a subset of imprinted genes, was altered based on oxygen concentration.

Due to the apparent responsive nature of Dnmt3a to oxygen concentrations and its role in the establishment and maintenance of imprinted genes, we assessed if other post translational modifications at the imprinting control regions (ICR) of select imprinted genes (Copg2, Dlk1, H19, Mest, Zac1) were affected by changes in oxygen concentration. Utilizing chromatin immunoprecipitation (ChIP), we assessed 4 histone post-translational modifications (PTMs) at these ICRs. Of the 4 PTMs analyzed, only histone 3 lysine 9 acetylation (H3K9ac) indicated any significant changes between the treatment groups. Interestingly, there was a significant increase in the low oxygen groups for the H3K9ac PTM in all 5 ICRs evaluated (**Figure 8A-E**).

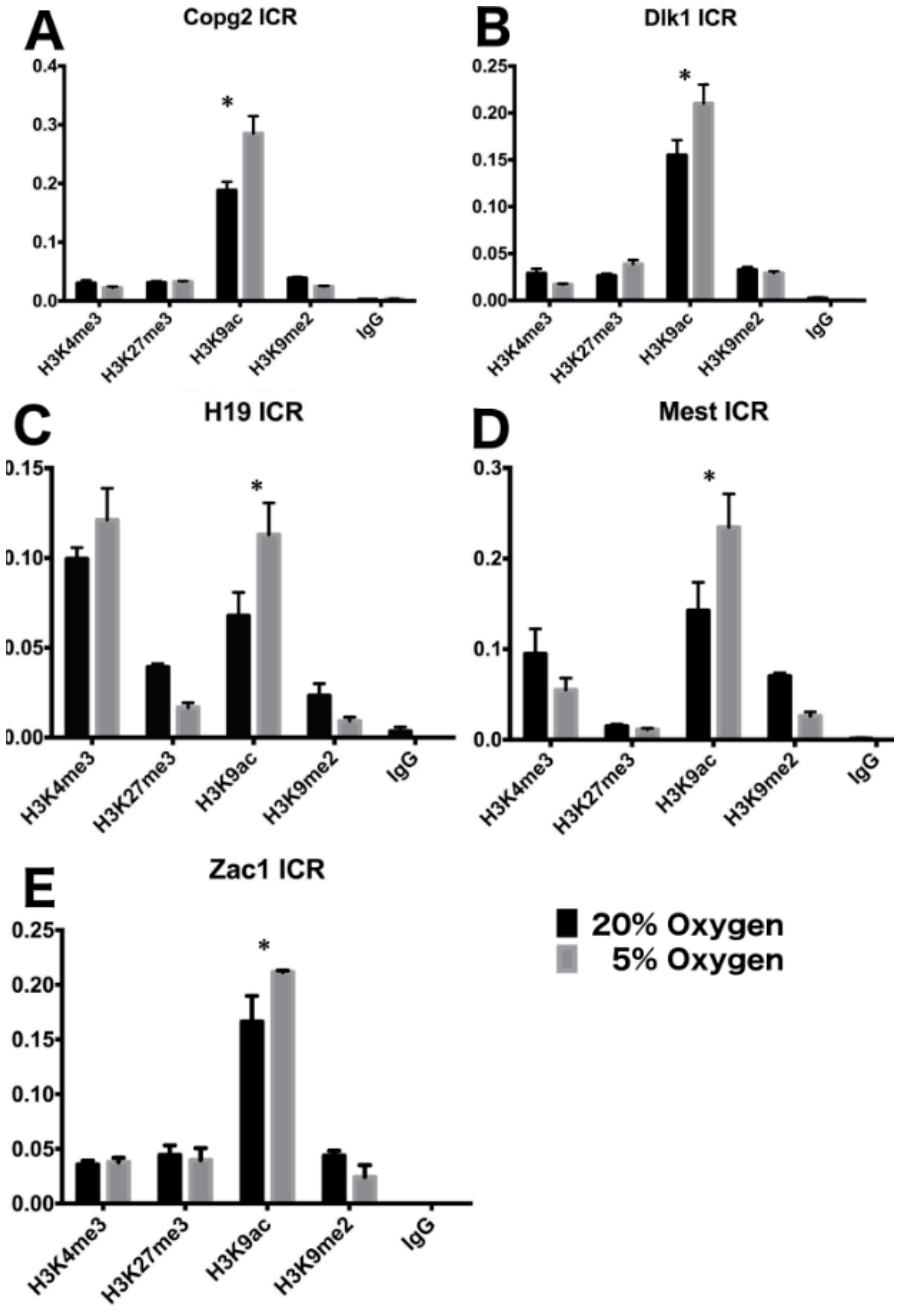


Figure 8. Chromatin Immunoprecipitation of Imprinting Control Regions in embryonic stem cells under high and low oxygen culture conditions.
Levels in the relative enrichment of Histone 3 Lysine 4 Trimethylation (H3K4me3), Histone 3 Lysine 27 Trimethylation (H3K27me3), Histone 3 Lysine 9 Acetylation (H3K9ac) and Histone 3 Lysine 9 Dimethylation (H3K9me2) were measure using ChIP for **(A)** Copg2 Imprinting Control Region **(B)** Dlk1 Imprinting Control Region **(C)** H19 Imprinting Control Region **(D)** Mest Imprinting Control Region and **(E)** Zac1 Imprinting Control Region. Murine embryonic stem cells were cultured under high (20%) or low (5%) oxygen for the duration of four passages and extracts

examined as outlined in the materials and methods. Statistical significance as measured using a paired students t-test at $p \leq 0.05$ and significant differences designated by a single ∗. For these experiments, three independent cell culture experiments were conducted (n = 3). For each sample, background levels are represented by the IgG control as previously described Golding 2010.

Oxygen-induced changes in the regulation of Igf2 / Igf2r and the allelic expression of multiple imprinted genes

An analysis of genes known to be regulated through genomic imprinting was conducted. The largest changes were observed for transcripts encoding *Igf2* and *Igf2r*. *Igf2* is part of the imprinting locus on mouse chromosome 7 that contains the *H19/Igf2* cluster. *H19* and *Igf2* are reciprocally expressed with *H19* being expressed from the maternal allele and *Igf2* expressed from the paternal allele (Kaffer et al., 2001). Consistent with this paradigm, *Igf2* is up-regulated while transcripts encoding *H19* are significantly down-regulated in the 5% oxygen group **(Figure 9).** Concurrent changes for both *Igf2* and *Igf2r* in the low oxygen group were observed. RNA-sequencing was employed to assess single-nucleotide polymorphisms in a panel of known imprinted genes to determine if oxygen concentration could impact allele-specific patterns of gene expression between the treatment groups. In mESCs, imprinted genes often display leaky expression, where monoallelic expression is defined as ≥90% expression from one parental allele (Golding et al., 2011). After a single passage in either 5% or 20% oxygen, we observed significant shifts between maternal and paternally derived transcripts for *H19*, and *Peg3*. *H19*, which is predominantly expressed from the maternal allele, exhibited a decreased contribution

from the paternal allele under low oxygen conditions **(Figure 10C).** However, *Peg3,* a paternally expressed gene, displayed the reverse trend, going from predominantly paternally derived transcripts in the high oxygen treatment to a maternally dominated transcript in the low oxygen group **(Figure 9C)**. No other imprinted genes exhibited significant shifts in allelic expression.

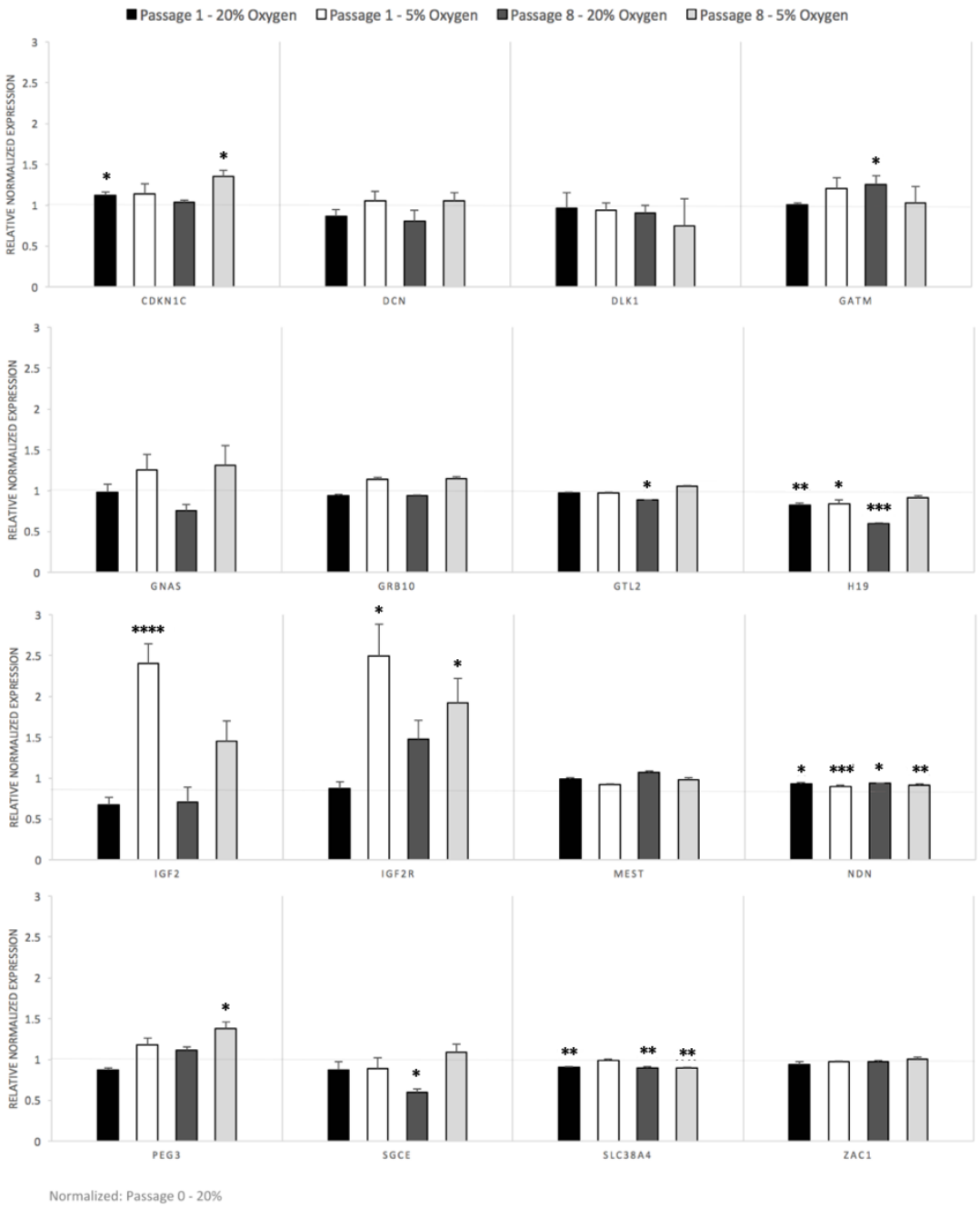


Figure 9. Oxygen-induced changes in the transcriptional regulation of imprinted genes within mouse embryonic stem cells.

Analysis of imprinted gene transcript levels at passage 1 and 8, as determined by RT-qPCR. Measurements were normalized to the geometric mean of the reference genes *Gapdh*, *Hprt*, *Mrpl* and *Sdha,* and samples graphed relative to Passage 0 (20% oxygen). n = 5 (*P*-value: * ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001; **** ≤ 0.0001)

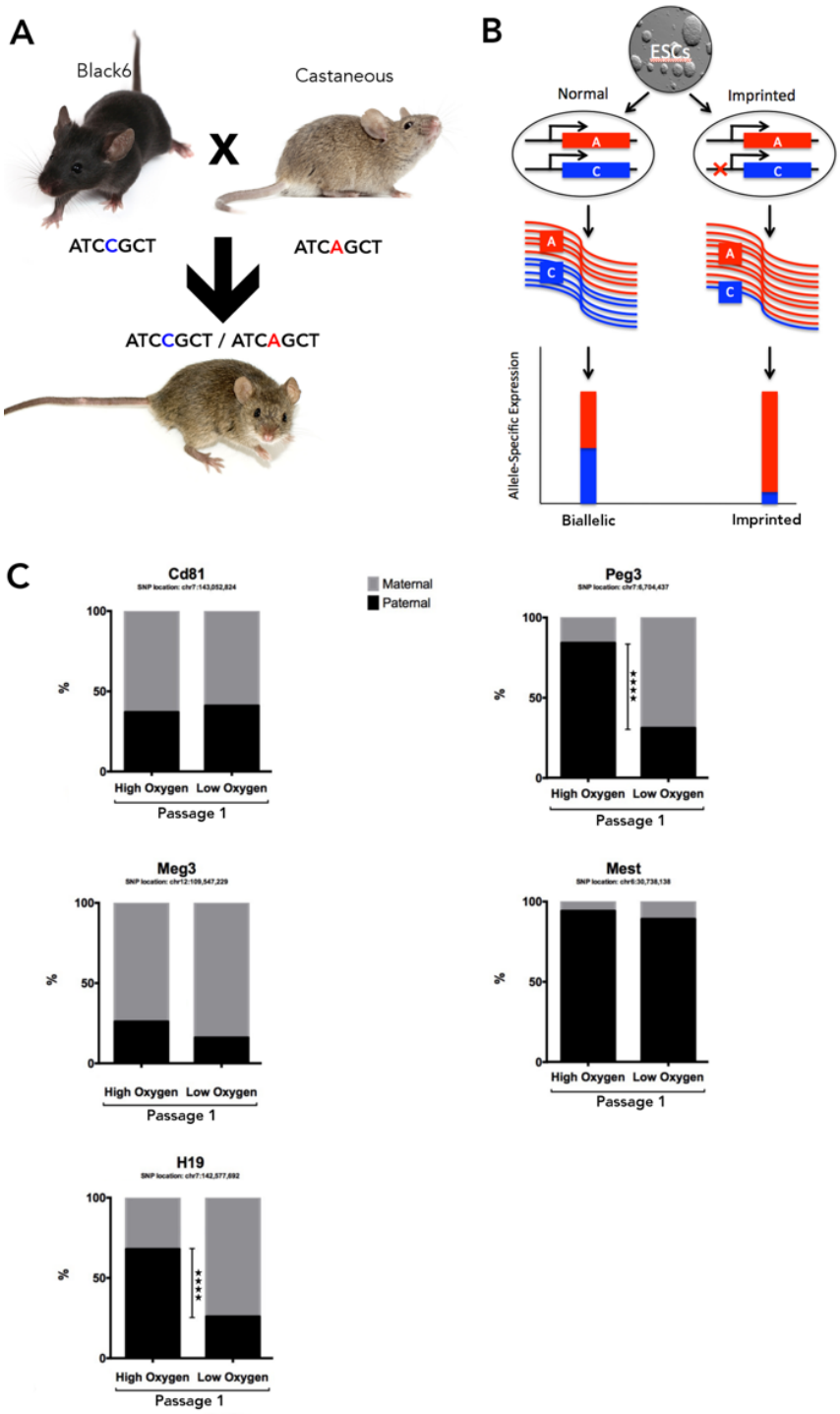


Figure 10. Analysis of oxygen-induced changes in parent of origin expression patterns for select imprinted loci.

A. Visual representation of allelic inheritance from the distinct genetic backgrounds used in this study. This strategy allows tracking of the parental alleles by using single nucleotide polymorphisms. **B.** Visual representation of balanced, bi-allelically expressed transcripts as well

as imbalanced, imprinted genes. **C.** Allele-specific transcript analysis of a select panel of known imprinted genes, as determined using RNA-sequencing. 101 bp paired-end reads with an average of 21.6 million reads per sample. (*P*-value: ∗ ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001; **** ≤ 0.0001).

Discussion

To further our understanding regarding the effects of differing oxygen concentrations on the epigenome, we shifted to a model employing a murine embryonic stem cell line. This system allowed us to observe both the acute impacts and prolonged effects of differing oxygen concentrations. While it is known that atmospheric levels of oxygen (20%) during culture negatively affect the ability of the mouse embryo to implant, the mechanisms behind this observation are not well understood (Wale & Gardner, 2013). Culturing these mESCs under differing oxygen levels revealed a restricted growth phenotype in the lower oxygen group. Similar to previous studies, cell size was significantly smaller in size than the low-oxygen group, albeit morphologically normal (Kurosawa, Kimura, Noda, & Amano, 2006). During the transition from atmospheric to low oxygen, we observed a down regulation of genes (Gstm3 and Nqo1), which are known to be involved in the cellular response to oxidative stress. Similarly, after 8 passages, Gpx2 and Gpx3, Sod2 and Nqo1 were all significantly different. The general trend of lower transcript levels under the 5% oxygen treatment suggests this condition is associated with a reduced oxidative stress response, with the notable exception of Gpx2. These results, along with previous studies, suggest that transferring mESCs into a low oxygen environment may be more favorable, although the limited experiments done here only suggest this possibility (Kurosawa, Kimura, Noda, & Amano, 2006).

Similar to our work in bovine embryos, studies in mESCs identified alterations in transcripts encoding the Tet family of demethylases. Here, low oxygen conditions were associated with a reduction in transcripts encoding *Tet2* but only as an acute response. After 8 passages, no significant differences in *Tet2* transcript levels could be detected, while by this point, *Tet1* had become significantly downregulated in the low oxygen group. In mice, *Tet1* and *Tet2* are hypothesized to have partially overlapping functions, and genetic deletion of both genes is still compatible with life, albeit with a 60% increase in developmental failure (Dawlaty et al., 2013). Therefore, the consequences of oxygen-induced alterations to *Tet1* / *Tet2* expression are difficult to anticipate. Unlike our studies in bovine embryos, we did not detect any impact on *Tet3* in mESCs under different oxygen atmospheres.

We turned our attention to post translational modifications at the imprinting control regions (ICR) of select imprinted genes (Copg2, Dlk1, H19, Mest, Zac1) to observe if post-translational modifications were affected by changes in oxygen concentration. The histone post-translational modification H3K9ac was significantly increased in the 5% treatment group in all 5 ICRs evaluated. H3K9ac is associated with a transcriptionally permissive state, but without supportive evidence like DNA methylation associated with these ICRs or allele-specific analysis, it is difficult to make any assumptions based off this ChIP data on the role H3K9ac plays in the bigger picture. However, based on the uniformity of the results, these data suggest that the chromatin structure of ICRs are susceptible to alterations in oxygen concentration

To evaluate the downstream effects of these transcriptional changes, we turned our attention to a cohort of genes that are known to be regulated through the processes of genomic imprinting. Our data indicated that several imprinted genes from our panel were dysregulated between oxygen conditions tested. One of the more interesting findings was that *Igf2* and *H19* were dysregulated in contrasting manner. These two genes are members of an imprinting locus in which *Igf2* is paternally expressed while *H19* is maternally expressed. In our experiments, *Igf2* displayed a 2.5-fold increase in the low oxygen group with an accompanying downregulation of *H19*. Further investigation is needed to understand the mechanisms by which this occurs and the potential role oxygen concentrations have in modulating the transcriptional regulation of this locus. Another interesting observation to emerge from these studies was the correlative changes in *Igf2* and *Igf2r*. *Igf2r* binds and sequesters *Igf2*, leading to a decrease in the amount of Igf2 available to interact with its receptors. Thus, while over-expression of *Igf2* has been associated with an overgrowth phenotype (Morison & Reeve, 1998) excessive Igf2r is associated with growth suppression (Rezgui et al., 2008). Thus, due to *Igf2r's* affinity for *Igf2*, it has also been found to regulate growth indirectly by reducing *Igf2*. In our studies, we observed oxygen-dependent upregulation of both *Igf2* and *Igf2r*. Here, the increased *Igf2r* transcript levels could be a compensatory mechanism to combat the elevated levels of *Igf2*. However, given the established relationship between these proteins and the development of large-offspring syndrome in cattle, further studies into the capacity of oxygen to influence IGF expression are warranted.

Finally, we examined single nucleotide polymorphisms (SNPs) within a cohort of imprinted genes to evaluate the acute effects of reduced oxygen concentrations on allele-specific patterns of gene expression. Patterns of imprinted gene expression are established and maintained though epigenetic mechanisms and are often inferred to be good indicators of the "health" of the epigenome (Tang & Ho, 2007). We utilized an established mouse model (Golding et al., 2011) which allowed the identification of parental alleles using single nucleotide polymorphisms, and therefore the capacity to trace the parental origins of mRNA transcripts. Culture under low oxygen conditions induced the H19 transcript pool to exhibit a maternally dominated pattern. However, the opposite trend occurred for Peg3, indicating the 5% treatment was not necessarily more stable, with respect to the regulation of imprinted genes. Collectively, our data suggest that oxygen exposure has the power to influence the transcriptional control of crucial gene networks involved in the establishment and maintenance of chromatin structure and the control of imprinted genes.

It has recently been shown that an imprinted gene, Zac1, is a part of a larger imprinted gene network and knockdown of Zac1 alone has been shown to dysregulate a host of other imprinted genes (Varrault et al., 2006). Furthermore, knockdown of a single allele leading to a heterozygous Zac1 locus leads to a distinct growth restricted phenotype as seen in many imprinting-related disorders (2006). Zac1 is a zinc-finger protein that has been shown to directly regulate the H19/Igf2 locus through binding to a shared enhancer (Valente & Auladell, 2001). Based on our prior results looking at imprinted genes, particularly the H19/Igf2 locus, this gene became of great interest. Since it is known that Zac1 has a direct regulatory function over the H19/Igf2 locus (Varrault et al., 2006), it

would be valuable to elucidate whether oxygen-induced changes in Zac1 activity might be responsible for our observations.

Mining data acquired from our murine embryonic stem cells, we found that Zac1 exhibited altered parental allelic expression under low oxygen conditions as determined through a SNP analysis. The pattern of altered parental allelic expression suggested alternative splicing events at the 3' end of the gene. The region of the Zac1 gene corresponds to the zinc-finger domain (**Figure 11A, gray boxes**). Our analysis suggested an apparent loss of expression from one of the parental alleles in the low oxygen group (**Figure 11A, red arrow heads**). Looking individually at the SNPs that are within the altered transcriptome landscape, we observed significant changes in several SNPs in either the paternal or maternal alleles (**Figure 11C**). Based off of the changes in SNPs, along with the alterations seen in the transcriptome landscape, we assessed differences in alternative splicing events in the 3' end of the zinc-finger potentially leading to a different Zac1 isoform. Through alternative splicing analysis performed by MISO, it was found that the low oxygen group (**blue**) did in fact have a dramatic deviation from the high oxygen groups regarding splicing events (**Figure 11B**). Taken collectively, changes in the oxygen concentration during culture of mESCs may lead to alterations in the zinc-finger domain of a major imprinted gene regulator. It is possible that Zac1 is a master regulator for imprinted genes and the alternative splicing events are resulting in the dysregulation we see in our imprinted gene panel. Further research is needed to elucidate these findings.

In summary, these experiments, sought to determine if murine embryonic stem cells, which are cultured at standard 20% oxygen, experienced atmospheric-derived

oxidative stress, and if this stress could induce dysregulation of chromatin modifiers and epigenetic gene regulatory mechanisms. Our data suggest that oxidative stress has the power to dysregulate crucial gene networks involved in the establishment and maintenance of histone post-translational modifications and the control of imprinted gene expression.

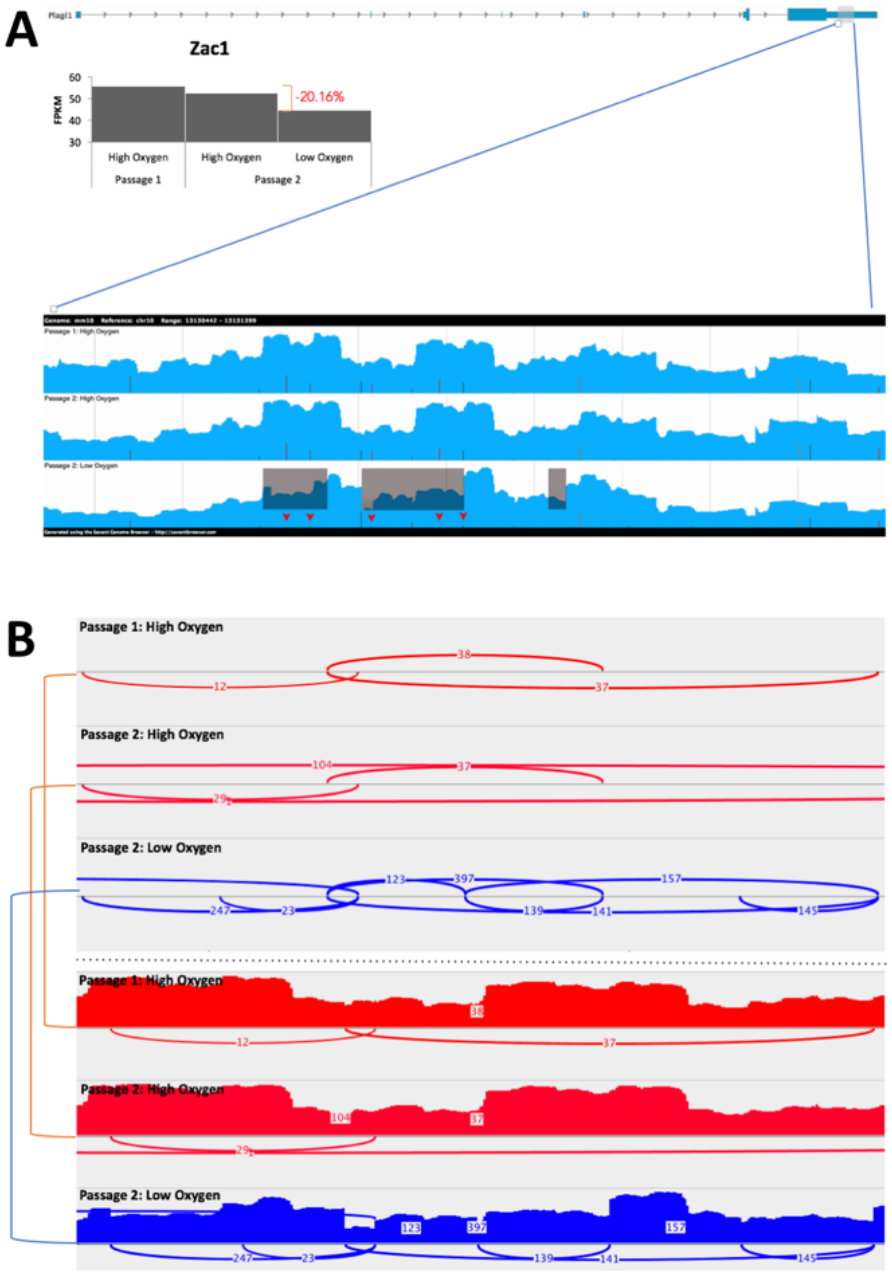


Figure 11. Zac1 expresses altered SNP patterns under low oxygen rescue corresponding with different alternative splicing events.
A. SNP analysis of 3' end of Zac1 gene. Gray bars indicate areas of transcriptome landscape alterations under low oxygen cellular culture. Within the gray boxes, red arrow heads indicate altered allele-specific expression under low oxygen conditions. **B.** Analysis of alternative splicing events covering the area of differing SNP allele-specific representation in the 3' end of Zac1. Splicing events were determined through MISO (mixture-of-isoforms model). **C.** Analysis of specific SNPs in the 3' end of Zac1.

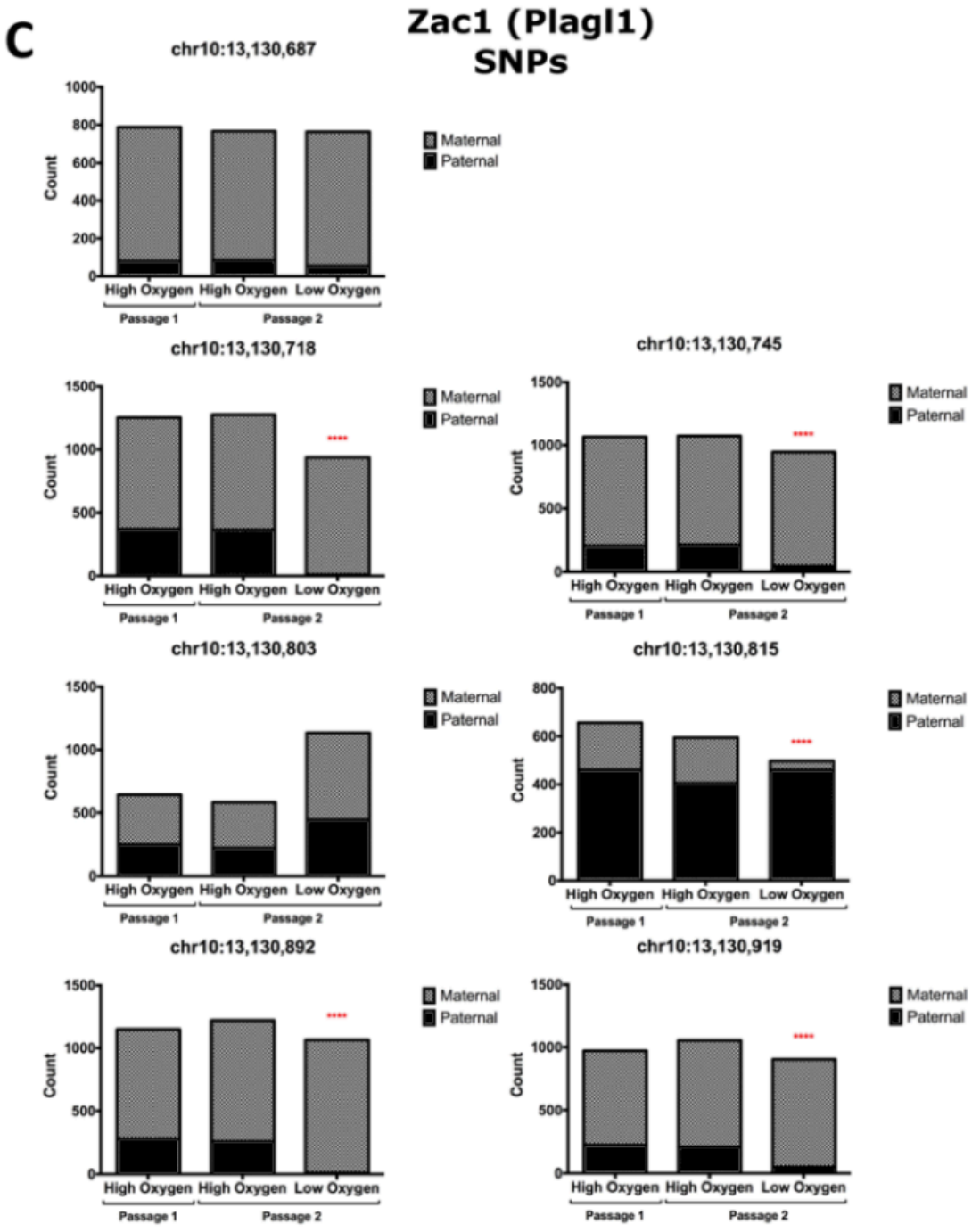


Figure 11. Continued.

Materials & Methods

Cell culture

Primary mESC cells were derived from B6XCAST F1 embryos as previously described (Golding, Zhang, & Mann, 2010). In brief, ESC cultures were maintained using 2i medium (Galonska et al., 2015) composed of: DMEM (Sigma D5671) supplemented with 10 µg/mL LIF (Sigma L5158), BIO (Sigma B1686), 1 µM PD0325901 (Sigma PZ0162), 50 mg/ml penicillin/streptomycin (Sigma P4333-100), 100 µM b-mercaptoethanol (Sigma M3148), 2 mM L-Glutamine (Sigma G7513-100), MEM nonessential amino acids (Invitrogen 11140-050), and 15% Hyclone ESC grade fetal bovine serum (Fisher SH30070.03E) under high (20%) and low (5%) oxygen conditions for duration of 8 passages. Low oxygen culture conditions were maintained by using a modular incubation chamber perfused with a premixed gas of 5% O_2, 5% CO_2 and 90% N_2. Images of cell cultures were taken with an inverted Nikon microscope (Eclipse TE300) running NIS Elements and mESC size was measured using ImageJ software (Schneider, Rasband, & Eliceiri, 2012). DNA and RNA were isolated during each passage with TRIzol (Life Technologies).

RNA isolation and qRT-PCR.

Total RNAs from murine embryonic stem cell samples were isolated with TRizol (Life technologies), and cDNAs were generated with random primer sets and Superscript II first-strand synthesis system (Invitrogen). Real-time qPCR reactions were performed on a CFX384 Real-Time System (Bio-Rad) using SYBR green (Applied Biosystems).

Relative gene expression levels were calculated using comparative Ct values, in which Ct is the cycle threshold number, and normalized to the geomean of Hprt, Mrpl, Ppia and Ywhaz.

Chromatin Immunoprecipitation (ChIP) Analysis

Cultured embryonic stem cells were grown to 80% confluence, washed twice in warm PBS, trypsinized then resuspended in warm growth medium containing 0.1 volume of crosslinking solution (Kondo, Shen, Yan, Huang, & Issa, 2004). Subsequently, Chromatin Immunoprecipitation *(*ChIP) reactions were performed as described previously (Golding et al., 2010), which was followed by DNA purification using a Qiagen PCR Cleanup kit. Antibodies used in the immunoprecipitation of modified histones were anti-Rabbit IGG (Santa Cruz SC-2027), anti-Trimethyl Histone H3 Lysine 4 (Millipore 04-745), anti-Trimethyl Histone H3 Lysine 27 (Millipore 17-622), anti-Acetylation Histone 3 Lysine 9 (Millipore 07-352), and anti-Dimethylation Histone 3 Lysine 9 (Millipore 22-004). Antibodies for modified histones were used at 1 μg/ChIP. The concentration of IgG was adjusted from 1 μg to 2 μg as appropriate. For these experiments, a minimum of three independent experimental replicates were conducted. Cellular extracts from each of these replicates were subjected to 2 independent chromatin immunoprecipitations for each of the post-translational histone modifications examined. DNA precipitated from these ChIP reactions were analyzed by qPCR and results normalized to 1% of the total input. Analysis of candidate imprinting control regions was carried out using the DyNAmo Flash SYBR Green qPCR Mastermix (Fisher Scientific, Pittsburgh PA. Cat # F-415L) following the

manufacturer's instructions. Reactions were performed on a CFX384 Real Time System (BioRad Hercules CA).

RNA-seq library preparation and data analysis

Total RNA was purified with an RNeasy Mini kit (Qiagen). RNA samples were analyzed for quality with a BioAnalyzer 2100 (Agilent) and shipped to Global Biologics LLC (Columbia, Missouri) for library preparation and sequencing (1 µg RNA in 20 µL nuclease free water). Libraries were pooled and sequenced on an Illumina HISEQ 2500 (paired-end, 2x101 bp) with an average of 21.6 million reads per sample. The RNAseq reads were assessed for quality with FastQC. Adapters were removed with Cutadapt (Martin 2011) and processed for quality with FastX-toolkit. Mapping was performed with Tophat2 and assembly by Cufflinks using the UCSC mm10 annotation (Trapnell, Pachter, & Salzberg, 2009; Trapnell et al., 2012). RNA expression levels were determined with Cuffmerge and Cuffdiff (Trapnell et al., 2013). Statistical analysis and visualization was performed with R, using RStudio running cummeRbund.

Alternative splicing

Tophat BAM output files created through our RNA-seq pipeline were used to determine alternative splicing events through MISO (mixture-of-isoform model) (Katz, Wang, Airoldi, & Burge, 2010). We assessed alternative splicing events through the "Skipped exons (SE)" or "Exon-centric" analysis method. Scripts:

index_gff --index /path/to/SE.mm10.gff3 /path/to/output/directory/indexed/ ;

miso --run /path/to/output/directory/indexed/ /path/to/filename.sorted.bam --output-dir /path/to/output/directory/MISO_OUT/ --read-len 28-p 24 ;

summarize_miso --summarize-samples /path/to/output/directory/MISO_OUT/ /path/to/summary_output/ ;

compare_miso --compare-samples /path/to/sample/one/directory/ /path/to/sample/two/directory/ /path/to/output/directory/ ;

filter_events --filter /path/to/bayes-factors/file.miso_bf --num-inc 1 --num-exc 1 --num-sum-inc-exc 10 --delta-psi 0.20 -bayes-factor 10 --output-dir /path/to/output/directory/

Statistical analysis

For all experiments, statistical significance was set at alpha = 0.05. For analysis of gene expression, the replicate cycle threshold (Ct) values for each transcript were compiled and normalized to the geometric mean of three validated reference genes Gapdh, Hprt, Mrpl and Sdha. Normalized expression levels were calculated using the ddCt method described previously (Schmittgen & Livak, 2008). Relative fold change values from a minimum of three biological replicates were transferred into the statistical analysis program GraphPad (GraphPad Software, Inc., La Jolla, CA) where datasets were first verified for normality using the Brown-Forsythe test. For single comparisons, an unpaired student's t-test was applied. In all instances, we have marked statistically significant differences with an asterisk (*). In our mouse model, distinct single nucleotide polymorphisms between the maternal (C57BL/6J) and paternal (C57BL/6(Cast7) - *Mus musculus castaneus* bred onto a C57BL/6J (B6) background) strains (Mann et al., 2003)

allowed us to track allelic patterns of gene transcription for multiple imprinted genes. For RNA sequence-based comparisons of allelic patterns of imprinted gene expression, the proportion of identified single nucleotide polymorphisms were analyzed using either Chi-Square or, if read counts were less than 5, a Fisher's Exact test in order to test for significant differences in allelic expression.

CHAPTER IV

CHRONIC MALE PRECONCEPTION ALCOHOL CONSUMPTION ASSOCIATES WITH ABNORMALITIES IN CHOLESTEROL TRAFFICKING GENES, SEX-SPECIFIC ALTERATION IN THE GENETIC PATHWAYS REGULATING HEPATIC FIBROSIS AND DISRUPTIONS IN THE REGULATION OF IMPRINTED GENES[*]

Introduction

Epidemiologic studies demonstrate that alcohol is the most prevalent teratogen to which humans are exposed, with 6 to 17 children per 1000 live births diagnosed with some degree of fetal alcohol spectrum disorder (FASD) (Roozen et al., 2016). This condition is characterized by a spectrum of structural defects, central nervous system disorders and growth deficits that persist well into postnatal life (Carter et al., 2016; Roozen et al., 2016). Studies have shown that following prenatal alcohol exposure, the DNA methylation profiles of the Igf2 locus are altered in offspring with a partial recovery being observed when dams were supplemented with a methyl-rich diet (Downing et al., 2011). Other studies have shown that prenatal environmental exposures including air pollution, tobacco

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smoke or alcohol can affect birth weight and the DNA methylation levels of repetitive elements in the offspring (Wilhelm-Benartzi et al., 2012). These studies reveal a small glimpse into just how susceptible the *in-utero* environment is to epigenetic perturbation (Nelissen, van Montfoort, Dumoulin, & Evers, 2011).

In addition to the uterine environment, the preconception environment is a significant modifier of dysgenesis and the development of environmentally induced disease. To date, Fetal Alcohol Spectrum Disorders (FASDs) have been exclusively associated with maternal exposures, yet emerging evidence suggests male-inherited alterations in the developmental program of sperm may be relevant to the growth-restriction phenotypes of this condition. The long-term persistence of FASD growth defects and emerging association with the development of chronic disease later in life, suggest alcohol has the capacity to heritably disrupt fundamental aspects of epigenetic programming (Basavarajappa & Subbanna, 2016; Chastain & Sarkar, 2017; Chater-Diehl, Laufer, & Singh, 2017; Mahnke, Miranda, & Homanics, 2017). These observations suggest a potential association between preconception male alcohol exposure, altered epigenetic programming in sperm, and the development of FASD-associated growth defects in the offspring. In support of this assertion, animal models of paternal alcohol exposure report alterations in the control of key enzymes regulating chromatin structure as well as changes in the DNA methylation profiles of alcohol-exposed sperm (Bielawski, Zaher, Svinarich, & Abel, 2002; Finegersh & Homanics, 2014; Knezovich & Ramsay, 2012; Liang et al., 2014).

While alcohol exposure *in utero* is, undoubtedly, a significant element in the origins

of FASD-associated growth defects, several independent studies have also emerged indicating a link between paternal alcohol consumption prior to conception and growth deficits consistent with those of FASDs (Day, Savani, Krempley, Nguyen, & Kitlinska, 2016; Finegersh, Rompala, Martin, & Homanics, 2015). A comparison of relevant preconception paternal alcohol exposure studies is presented in **Table 1**. Many different methods were utilized to study male preconception exposures on offspring with many different doses and routes of exposure making consolidation of this information difficult (Bielawski & Abel, 1997; Bielawski et al., 2002; Jamerson, Wulser, & Kimler, 2004; Knezovich & Ramsay, 2012; Ledig et al., 1998; Liang et al., 2014; Meek, Myren, Sturm, & Burau, 2007; Ouko et al., 2009; Stouder, Somm, & Paoloni-Giacobino, 2011).

Using a mouse model of voluntary consumption, we sought to determine if chronic preconception male ethanol exposure results in heritable epimutations carried through the male germ line that influence the health and development of subsequent generations. *We hypothesized that epimutation(s) induced by chronic preconception male ethanol exposure are carried by sperm, persist through preimplantation development and alter the health and development of offspring.*

PMID	Species	Male Age	Alcohol Dose(s)	Delivery Method	Frequency	Duration
9209556	Sprague-Dawley Rat	76-82 days old	6,4,2 or 0 g/kg	Oral Gavage Intubation	Once	Acute
9680259	Italian Wistar Rat	60 days old	10% w/v for first week and 20% w/v for three months	Ad libitum Drinking	Daily	3 months
11923587	Sprague-Dawley Rat	90 days old	6g/kg (20% w/v)	Oral Gavage Intubation	Every 3rd day	9 weeks
15063090	Sprague-Dawley Rat	84-112 days old	6g/kg (20% w/v) (from 2.5% gradually increase to 20%)	Ad libitum	Daily	34 or 48 days
17433387	Swiss Webster Mice	90 days old	5g/kg ([20% ethanol])	Intraperitoneal (I.P.)	Once, 12hr before mating	Acute
19519716	Human	19-40 years old	Variable survey responses	Ad libitum Drinking	N.A.	Variable
22371710	C57BL/6 Mice	Unknown	5.917g/kg (7.5ul/g of 50% EtOH)	Oral Gavage intubation	every second day for week 1, five days for week 2, daily 30 days, weekly afterwards	5 weeks
21382472	FVB/N Mice	60 days old	0.5g/kg	Oral Gavage Intubation in pregnant FEMALES	Daily	From GD10-18 (9 days)
24486713	Kunming Mice	35-42 days old	3.3,1.1,0 g/kg	Oral Gavage Intubation	Every 2nd day	4 weeks

Table 1: Comparison of relevant male preconception alcohol exposure methods from the current literature.

Results

Preconception alcohol exposure does not impact male fertility or sire weight

Previous studies examining alterations in the DNA methylation profiles of alcohol-exposed sperm have utilized rodent models of alcohol exposure employing oral gavage (Bielawski et al., 2002; Knezovich & Ramsay, 2012; Liang et al., 2014). This technique

is known to induce activation of the stress response (Brown, Dinger, & Levine, 2000), which has previously been shown to modulate epigenetic programming in sperm (Gapp et al., 2014). To avoid this confounding factor, we elected to use a voluntary model of consumption (Drinking in the Dark), where males were provided limited access to ethanol (EtOH) during a four-hour window immediately after their sleep cycle (Brady, Allan, & Caldwell, 2012). Here, postnatal day 90, adult C57BL/6(Cast7) males were maintained on a 12-hour light/dark cycle and provided access to either a solution of 10% (w⁄v) EtOH plus 0.066% (w⁄v) Sweet'N Low (experimental) or 0.066% (w⁄v) Sweet'N Low alone (control) for four hours a day. Once consistent patterns of drinking were established (one week), males were maintained on this protocol for a period of 70 days, which corresponds to the length of approximately two complete spermatogenic cycles, thereby ensuring that both pre-meiotic and post-meiotic sperm were exposed to EtOH (Adler 1996; Braun, Lee, & Schumacher, 2013). Using this model of exposure, experimental males consistently achieved blood EtOH concentrations of 210 mg/dL (Chang et al., 2017). As a reference, this concentration is approximately 2.5 times the legal limit, and reflects a range frequently obtained by binge drinkers and alcoholics (White, Kraus, & Swartzwelder, 2006). As previous studies have shown increased paternal weight can influence both epigenetic programming in sperm and fertility, we assayed these parameters at the conclusion of the exposure window (Fullston et al., 2013; White et al., 2006) . Chronic EtOH consumption did not influence paternal body weight, fertility, or the sex distribution of the offspring (**Figure 12A-C**).

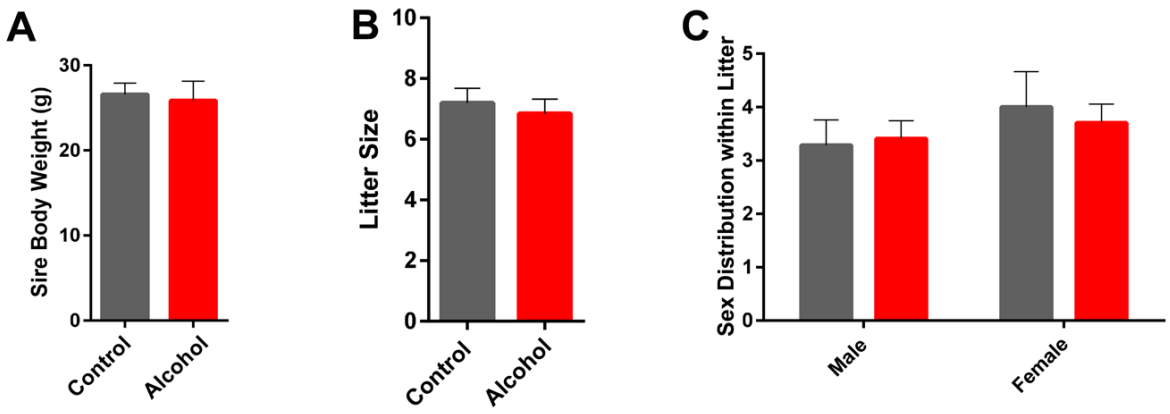

Figure 12. Chronic male alcohol exposure does not impact fertility or sire weight.

A. Average body weight of sires at the end of the 70-day EtOH exposure period (n=8 control, 10 alcohol). **B.** Average litter size and **C.** distribution of offspring sex between litters sired by preconception EtOH-exposed and control males (n=15 litters control, 20 litters alcohol). Error bars represent SEM $*P < 0.05$ and $***P < 0.001$ (comparisons between 10% (w⁄v) EtOH plus 0.066% (w⁄v) Sweet'N Low (alcohol) versus 0.066% (w⁄v) Sweet'N Low alone (control) preconception treatments). Data analyzed using either an unpaired t-test or a one-way ANOVA followed by Sidak post hoc analysis.

Alterations in the regulation of imprinted genes.

Given the link between imprinted genes, hepatic dysfunction and fetal-placental growth (Coan, Burton, & Ferguson-Smith, 2005; He et al., 2014; Piedrahita 2011), we assayed the parent of origin-specific expression of 16 genes regulated through genomic imprinting within the placenta and fetal liver. These analyses identified decreased expression of *Gnas* and *Grb10* within the placentas of male offspring and *Sgce* in the female offspring of EtOH-exposed males (**Figure 13A-B**). In the fetal liver, we observed significant reductions in transcripts encoding *Cdkn1c, Dcn* and *Gnas* in male offspring and *Meg3* in female offspring (**Figure 13C-D**). However, none of the examined imprinted genes displayed significant differences in the contribution from the normally silenced

allele (Appendix: **Table 1**). These observations associate preconception paternal EtOH exposure with decreased expression of select imprinted genes that is independent of compromised genomic imprinting.

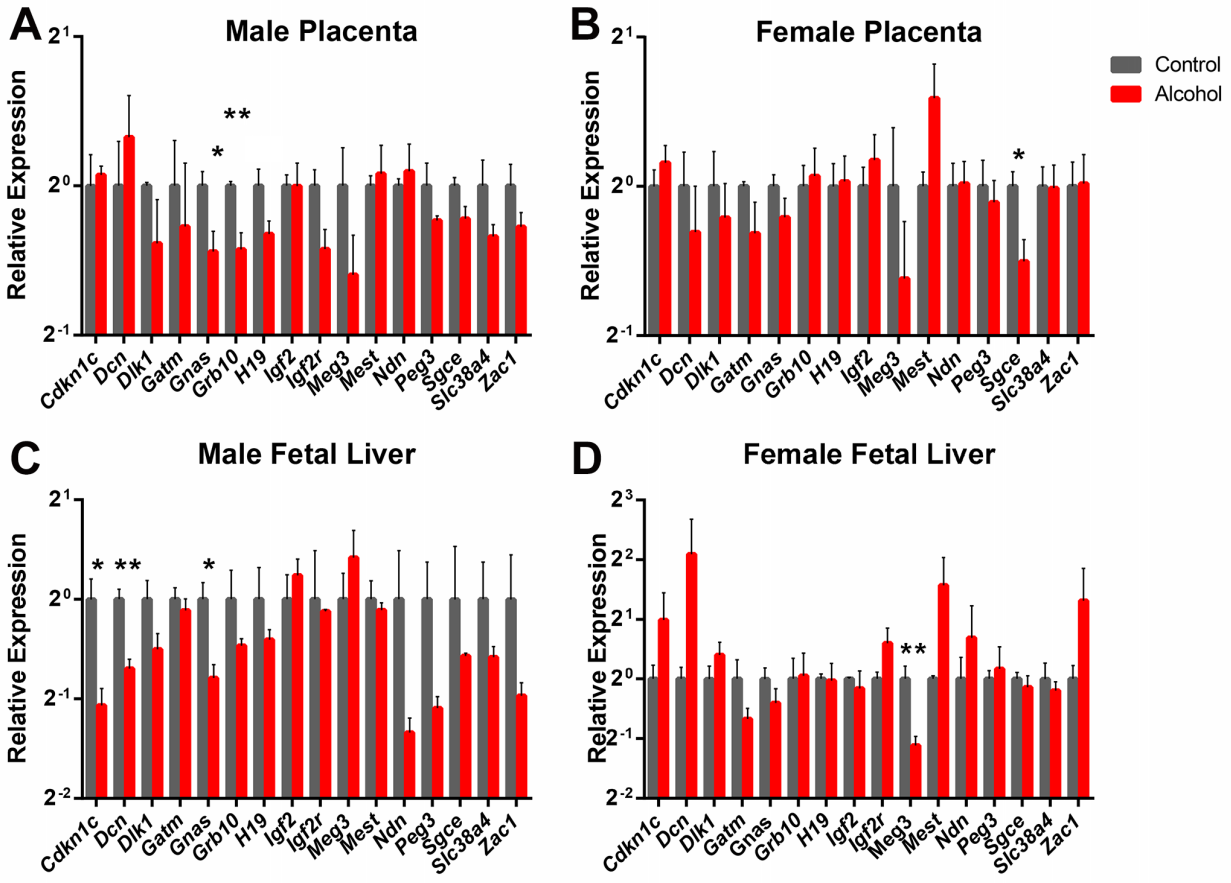

Figure 13. Altered patterns of imprinted gene expression within the offspring of alcohol-exposed males.

A. RT-qPCR analysis of imprinted genes in the placentas of male and **B.** female offspring sired by EtOH-exposed and control sires. **C.** qRT-PCR analysis of imprinted genes in the male and **D.** female fetal liver. Graphs represent independent replicates (n=8 male, 8 female), with two independent RT reactions and three RT-qPCR measurements for each RT. Data analyzed using an unpaired t-test. Error bars represent SEM $*P < 0.05$ and $**P < 0.01$ (comparisons between alcohol and control preconception treatments).

Analysis of the fetal liver transcriptome in the offspring of control and alcohol-exposed males.

When analyzing the RNAseq data in the fetal livers, we identified a small set of differentially expressed genes (~350 in males, ~500 in females), with a large proportion of the candidate genes participating in the genetic pathways regulating hepatic fibrosis and stellate cell activation (Ding et al., 2013). Interestingly, of the 153 genes common between both sexes, 147 (96%) displayed alterations in transcription that were diametrically opposed among males and females. Of all the diametrically opposed genes identified, a family of collagenase genes stood out due to their increased representation (**Figure 14A**). Verification of this sequencing data was obtained through RT-qPCR (**Figure 14B-C**). The major component of the extracellular matrix in the fibrotic tissue is composed of a heterotrimer composed of 2 α1 chains and 1 α2 chain referred to as type I collagen (Inagaki et al., 2005). This collagen is encoded by 2 distinct genes, COL1A1 and COL1A2, both of which were identified by our sequencing data as being dysregulated. Increased collagen expression during the development of organ fibrosis is exerted mainly at the level of transcription of these genes (2005). Further research into these findings is necessary, given hepatic fibrosis has been a long time clinical manifestation of alcoholic liver disease in adults (O'Shea, Dasarathy, McCullough, & Practice Parameters Committee of the American College of Gastroenterology, 2010).

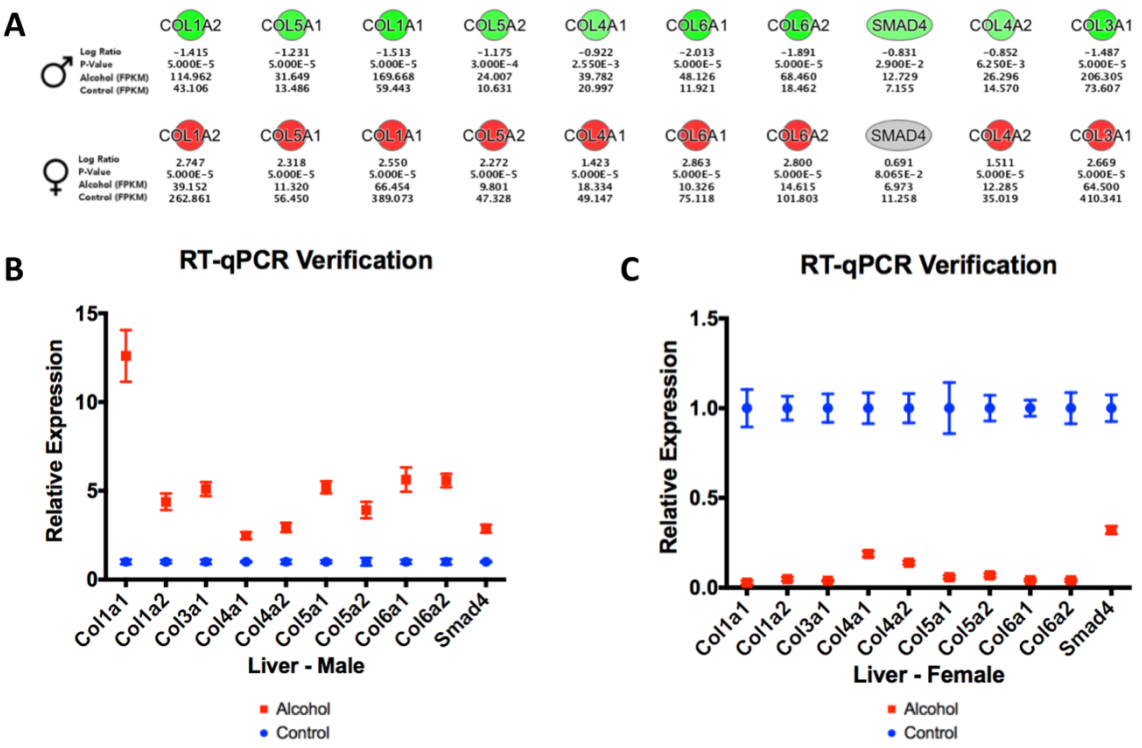

Figure 14. RNA sequencing reveals transcriptional differences in family of collagenase genes in the liver.

A. RNA sequencing. FPKM (Fragments Per Kilobase of transcript per Million mapped reads) the relative expression of a transcript is proportional to the number of cDNA fragments that originate from it. 40 bp paired-end reads with an average of 35.9 million reads per sample. Visualization of RNAseq data as well as statistical analysis produced through Ingenuity Pathway Analysis (IPA) (Jiménez-Marín, Collado-Romero, Ramirez-Boo, Arce, & Garrido, 2009). **B.** RT-qPCR verification of gene expression in the liver of male offspring. **C.** RT-qPCR verification of gene expression in the liver of female offspring. Measurements were normalized to the geometric mean of the reference genes *Gapdh*, *Hprt*, and *Ppia,* and samples graphed relative to Control. n = 4. Statistical significance determined using unpaired student's t-test. (All genes shown in B and C have a P-value ≤ 0.05).

Methylation analysis of liver from offspring conceived from control and alcoholic fathers.

Due to the observed reductions in the transcripts encoding the imprinted genes Cdnkn1c, Dcn and Gnas in the fetal liver of male offspring and Meg3 in the fetal liver of female offspring, we assayed the global DNA methylation profile using high through put bisulfite sequencing. Analysis of the methylation heat map did not reveal any obvious global differences in methylation between treatment groups in any of the CpG, CHG or CHH contexts (**Figure 15A**). Interestingly, in the CpG context, the global methylation levels between the control and alcohol treatments were nearing significance ($p = 0.0912$) with a trend towards a reduction in methylation in the alcohol group (**Figure 15B**). This led us to separately analyze the treatment groups by sex. In this analysis, we observed a significant 5.6% reduction in the global DNA methylation profiles of the male offspring, while females were identical between treatments (**Figure 15C-D**). While interesting, these sex differences in response to preconception alcohol treatment require further investigation to elucidate their importance. Lastly, differential methylation analysis looking at both the individual CpG residue and 1kb non-overlapping tile level detected no significant changes in methylation status in any imprinted genes loci between our treatment groups (Appendix: **Table 2**).

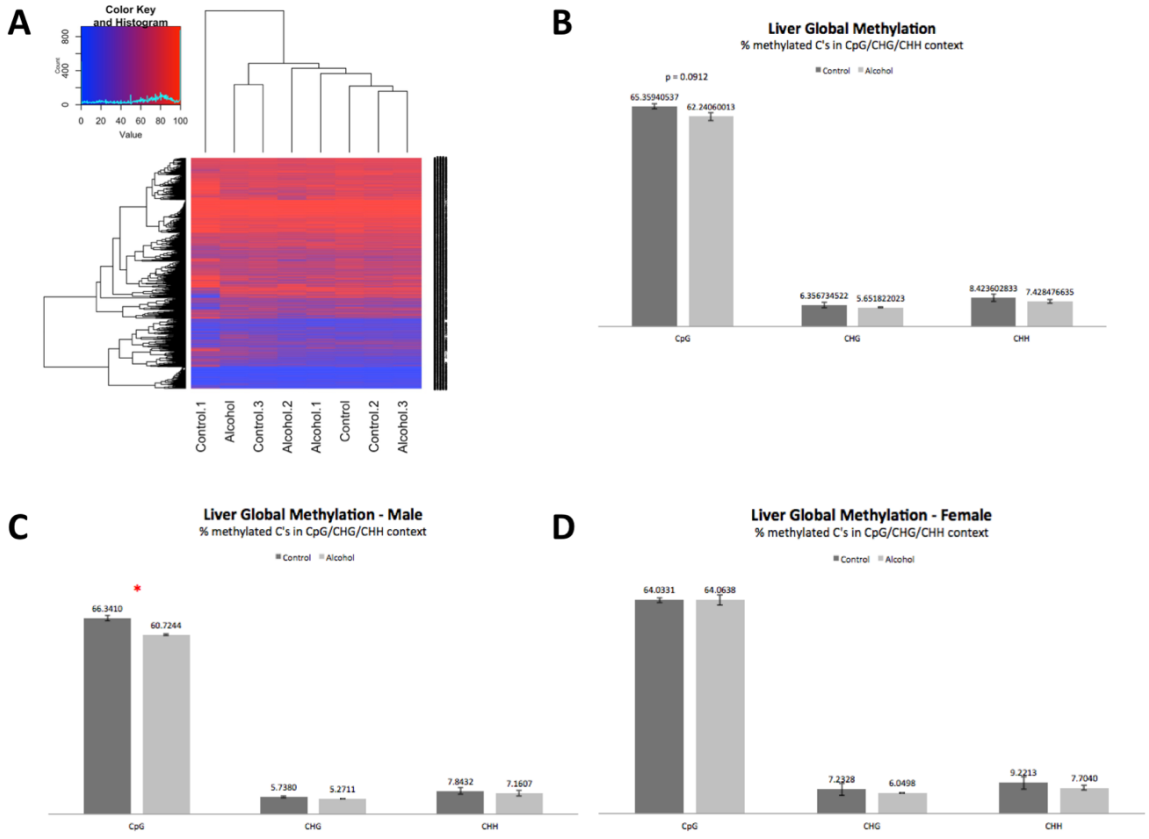

Figure 15. Comparison of methylation profiles and global methylation analysis in the liver of offspring.

A. Liver global methylation heat map of differentially methylated bases in each of the control or alcohol treatments. **B.** Liver global methylation comparison. % Methylated C's in CpG/CHG/CHH context. **C.** Male liver global methylation comparison. % Methylated C's in CpG/CHG/CHH context **D.** Female liver global methylation comparison. % Methylated C's in CpG/CHG/CHH context. For each individual methylation context (CpG/CHG/CHH), data was analyzed using an unpaired t-test. Error bars represent SEM $*P < 0.05$ and $**P < 0.01$ (comparisons between alcohol and control preconception treatments).

Analysis of the placental transcriptome in the offspring of control and alcohol-exposed males.

Sequencing analysis of the placental transcriptome identified ~1000 differentially expressed genes between the placentas of offspring sired by EtOH-exposed and control

males. Analysis of these data sets identified disruptions in genetic pathways related to both lipid transport and cellular proliferation (Chang 2017). Of particular interest, the reported "disruption of lipid transport" revealed by our pathway analysis software identified dysregulation of a family of apolipoproteins in the placenta. These reported changes in gene transcription were again, polarized in a sex-specific manner. Apolipoproteins are a family of proteins that bind insoluble lipids in order to transport them through the lymphatic or circulatory systems (Bolanos-Garcia & Miguel, 2003). They are known to transport lipids through the placenta in both a receptor-dependent and receptor-independent processes (Wyne & Woollett, 1998). Our experiments revealed the male offspring's placenta displayed a dramatic increase in the apolipoprotein family of genes, while the female offspring displayed an opposing trend, which resulted in dramatic decreases in the expression of these genes (**Figure 16A**). This RNAseq data was independently verified through RT-qPCR in both the male and females (**Figure 16B-C**). Another transport gene, RBP4, displayed similar sex-specific changes in the placenta. RBP4, retinol-binding protein 4, is a member of the lipocalin family which specializes in the transport of small hydrophobic molecules such as steroids, bilins, retinoids, and lipids (1998). Specifically, RBP4 binds and transports retinol (vitamin A alcohol) (Newcomer & Ong, 2000). Interestingly, RBP4 has been found to be upregulated in metabolically challenged mice (Yang et al., 2005). Due to the unique nature of the sex-specific response of this family of genes to preconception male alcohol exposure, as well as the reported fetal growth restriction phenotype, further investigation into these findings is necessary.

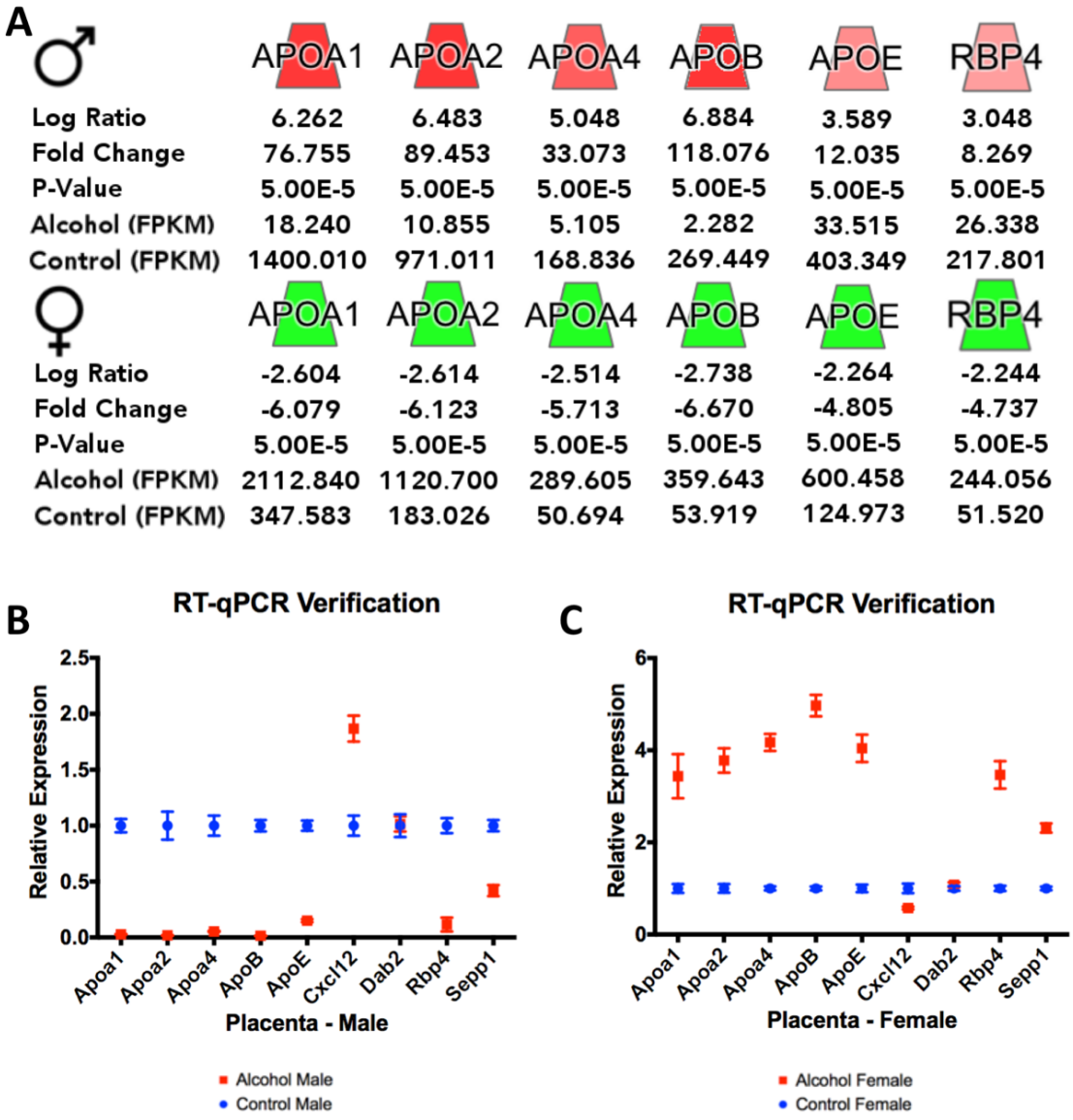

Figure 16. RNA sequencing reveals transcriptional differences in family of apolipoproteins and transport genes in the placenta.

A. RNA sequencing. FPKM (Fragments Per Kilobase of transcript per Million mapped reads) the relative expression of a transcript is proportional to the number of cDNA fragments that originate from it. 40 bp paired-end reads with an average of 34.2 million reads per sample. Visualization of RNAseq data as well as statistical analysis produced through Ingenuity Pathway Analysis (IPA) (Jiménez-Marín et al., 2009). **B.** RT-qPCR verification of gene expression in the placenta of male offspring. **C.** RT-qPCR verification of gene expression in the placenta of female offspring. Measurements were normalized to the geometric mean of the reference genes *Sdha*, *Hprt*, and *Mrpl,* and samples graphed relative to Control. n = 4. Statistical significance determined using unpaired student's t-test. (All genes shown in B and C (with the exception of Dab2) have a *P*-value ≤ 0.05).

Methylation analysis of placenta from offspring conceived from control and alcoholic fathers.

RT-qPCR analyses identified decreased expression of the imprinted genes *Gnas* and *Grb10* within the placentas of male offspring and *Sgce* in the female offspring of EtOH-exposed males (**Figure 13A-B**). Deep-methylome analysis did not reveal any global differences in the status of methylation under any context (CpG, CHG, CHH) in the placenta (**Figure 17A**). Separation by sex did not reveal any global differences in methylation between alcohol and control preconception treatments in the placenta (**Figure 17B-C**). Statistical analysis did not reveal treatment-specific differences within the control or alcohol groups. Using a Pearson correlation analysis, which compares the strength and direction of a linear relationship between two variables on a scatterplot, we found high similarities between all of our samples regardless of sex or treatment condition ($r \geq 0.91$) (**Figure 18A).** Clustering analysis for all samples did not reveal any trends or groupings beyond what appears to be random arrangements (**Figure 18B**). Next, we subdivided our samples down by sex and repeated the analysis. Again, in the males, high similarities across treatments were observed in our Pearson correlation analysis ($r \geq 0.91$) and random clustering, irrespective of treatment (**Figure 18C-D**). The same observations were made in the female groups ($r \geq 0.92$) **(Figure 18E-F**). Lastly, differential methylation analysis looking at both the individual CpG residue and 1kb non-overlapping tiles detected no significant changes in methylation status in any imprinted genes loci between our treatment groups in either the placenta (Appendix: **Table 2**). Therefore, from these

studies, we conclude that DNA Methylation in the placenta does not appear to be affected by paternal preconception alcohol consumption.

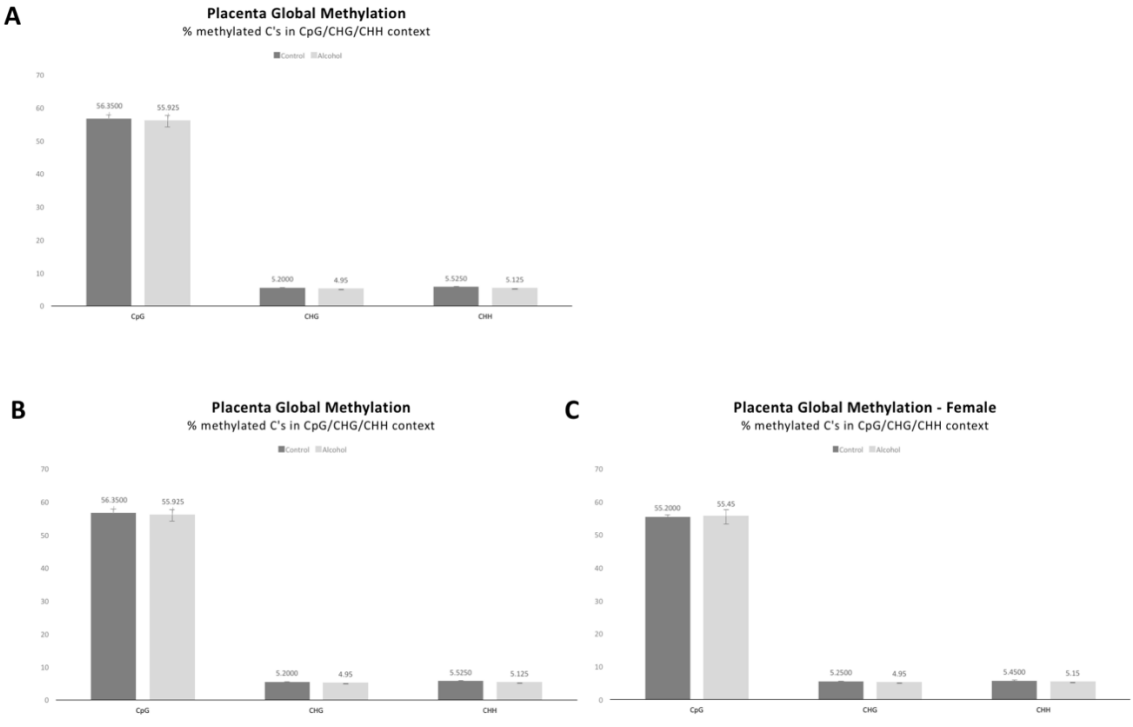

Figure 17. Comparison of methylation profiles and global methylation analysis in the placenta of offspring.

A. Placenta global methylation comparison. % Methylated C's in CpG/CHG/CHH context. **B.** Male placenta global methylation comparison. % Methylated C's in CpG/CHG/CHH context **D.** Female placenta global methylation comparison. % Methylated C's in CpG/CHG/CHH context. For each individual methylation context (CpG/CHG/CHH) data was analyzed using an unpaired t-test. Error bars represent SEM $*P < 0.05$ and $**P < 0.01$ (comparisons between alcohol and control preconception treatments).

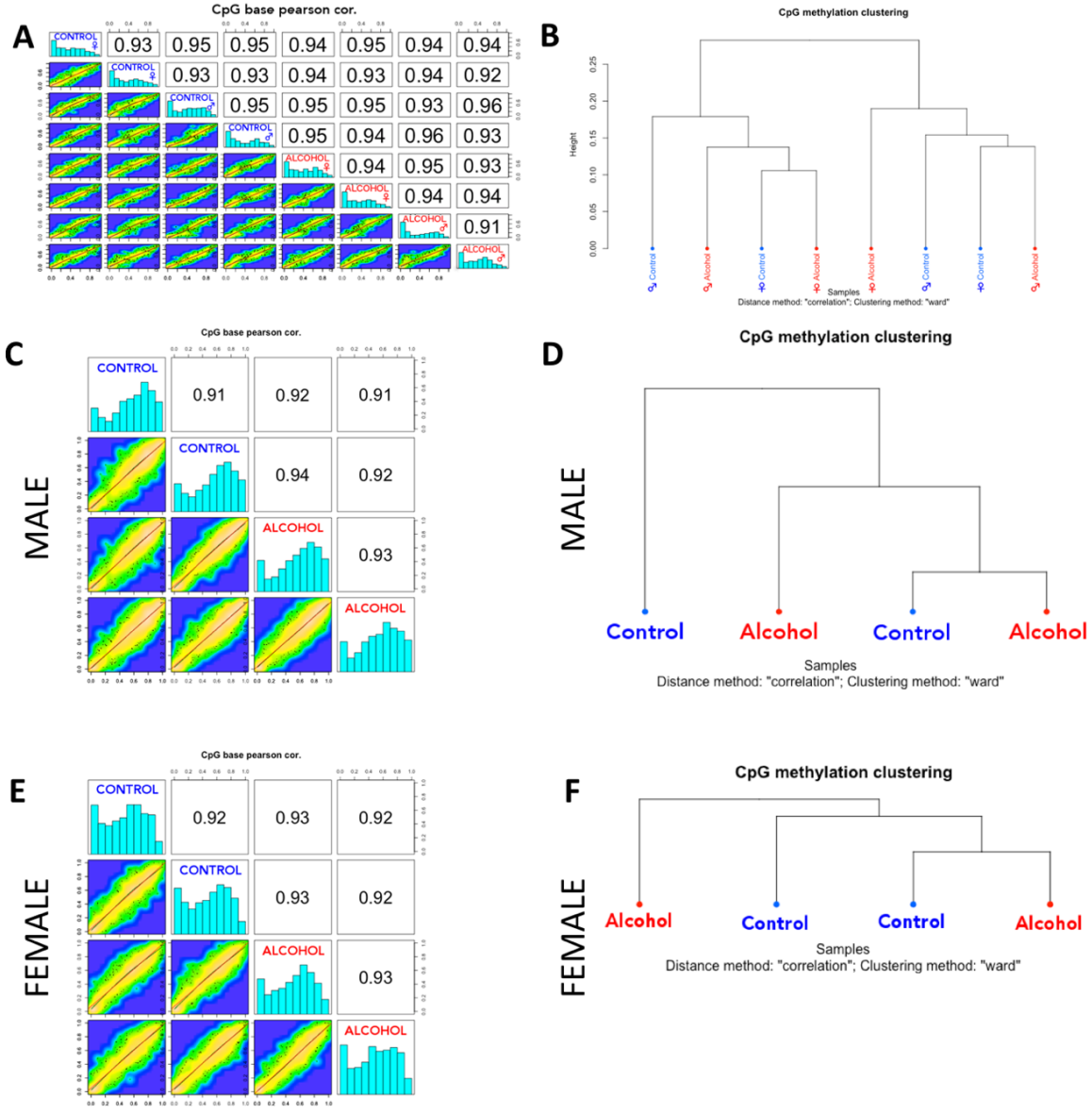

Figure 18. Comparison of methylation profiles and global methylation analysis in the placenta of offspring.

A. Placental global methylation comparison. % Methylated C's in CpG/CHG/CHH context. **B.** Global pearson correlation analysis comparing individual treatments and sex. **C.** Global clustering diagram of CpG methylation analysis broken down by treatment and sex. **D.** Male-only pearson correlation analysis comparing individual treatments. **E.** Male-only clustering diagram of CpG methylation analysis by treatment. **F.** Female-only pearson correlation analysis comparing individual treatments. **G.** Female-only clustering diagram of CpG methylation analysis by treatment.

Discussion

Given the recent association between paternal environmental exposures and long-term metabolic dysfunction (Rando & Simmons, 2015), we sought to examine the capacity of preconception male ethanol exposure to impact developmental programming in the offspring and determine if these prenatal growth deficits were the result of alterations in the DNA methylation profiles of the fetal liver and placenta. To this end, we employed an established mouse model of epigenetic programming, in which distinct single nucleotide polymorphisms between the maternal (C57BL/6J) and paternal (C57BL/6(Cast7)) strains allowed us to track allelic patterns of gene transcription within select imprinted loci (Mann et al., 2003). Our studies associate preconception male ethanol exposure with disruptions in the genetic pathways controlling both lipid metabolism and hepatic fibrosis and the abnormal expression of imprinted genes. In the adult males, preconception alcohol exposure did not impact fertility or sire weight even with consistently recorded blood alcohol levels above 200 mg/dL (Chang 2017). Sire body weight, liter size and liter sex distribution were all unaffected by alcohol consumption in the males (Chang 2017). Interestingly, even with very limited quantifiable changes in the fathers, chronic paternal EtOH exposure was determined to be significantly associated with a reduction in the weight of the gestational sac, a reduction in crown rump length, and in female offspring only, a reduction in fetal weight in the offspring (Chang 2017). These data indicate that male preconception alcohol exposure is associated with fetal growth restriction, similar in magnitude to those observed in maternal *in utero* models of EtOH exposure (Gundogan et al., 2008).

Following up on the growth restriction phenotype, analysis of the transcriptome using deep-sequencing analysis identified 979 differentially expressed genes between the placentas of offspring sired by EtOH-exposed and control males. These data sets identified disruptions in genetic pathways related to both lipid transport and cellular proliferation. Furthermore, while a handful of imprinted genes showed alterations in gene expression, none of them displayed significant differences in the contribution from the normally silenced allele. These observations associate preconception paternal EtOH exposure with decreased expression of select imprinted genes that is independent of compromised genomic imprinting.

DNA methylation analysis in both the fetal liver and placenta did not reveal any detectable differences in the global levels of methylation between any of the preconception treatments. Breaking down the samples by sex did reveal a global difference in the CpG context of methylation in the males only. While statistically significant, the biological significance of this modest change in methylation could not be assessed. Our analysis failed to detect any differences between the methylation status of a select group of imprinted genes at either the base or 1kb tile level. Though DNA methylation has established roles in the epigenetic transcriptional regulation and establishment of imprinted gene loci, these results question their role in the establishment of the growth restriction and metabolic phenotypes observed in the offspring. Our data indicate another epigenetic mechanism independent of DNA methylation as being responsible.

We hypothesized that chronic preconception male ethanol exposure could result in heritable epimutations carried through the male germ line that influence the health and

development of subsequent generations. Based on our observations that associate preconception paternal EtOH exposure with sex-specific alterations in the genetic pathways regulating cholesterol trafficking in the placenta, hepatic fibrosis in the liver and disruptions in the regulation of imprinted genes, it appears that epimutations can be carried by sperm and persist through preimplantation development and alter the health and development of offspring. Interestingly, these epimutations do not appear to manifest through DNA methylation-based epigenetic mechanisms.

Materials & Methods

Animal work

All experiments were conducted under AUP 2014-0087 and approved by Texas A&M University IACUC. The C57BL/6(Cast7) strain of mice were generated in the Bartolomei laboratory and were selected to possess portions of a *Mus musculus castaneus* (CAST) chromosome 7 and chromosome 12 (where at least 5 imprinting domains and more than 30 imprinted genes reside) bred onto a C57BL/6J background (Mann et al., 2003). When using F1 hybrid crosses between the B6(CAST7) strain and a C57BL/6J strain, we can distinguish the maternal and paternal alleles of select genes using C57BL/6(Cast7) and C57BL/6J polymorphisms that we have identified by either by primary sequence or database analysis (Golding et al., 2011). C57BL/6(Cast7) males were employed in a chronic, moderate-dose, voluntary alcohol exposure paradigm referred to as 'Drinking in the Dark' (Brady et al., 2012). Here, individually caged, postnatal day 90, adult males were provided limited access to EtOH during a four-hour window immediately

after their sleep cycle (2012). Males were maintained on a 12-hour light/dark cycle and provided access to either a solution of 10% (w⁄v) EtOH (catalog# E7023, Sigma) plus 0.066% (w⁄v) Sweet'N Low (Cumberland Packing Corp, Brooklyn NY.) versus 0.066% (w⁄v) Sweet'N Low alone for four hours a day. Prolonged exposure to a 10% Sweet'N Low solution has been shown to drive the development of glucose intolerance through functional alterations to the intestinal microbiota (Suez et al., 2014). Although the experimental paradigm reported here utilized a 0.066% Sweet'N Low solution, which is 150-fold lower than those cited in these previous experiments (2014), we were careful to ensure that mice in both preconception treatment groups received equivalent exposures. Once the 70-day preconception treatment was achieved, two naturally cycling females were placed into a new cage along with each exposed male. During these matings, males were not provided access to the alcohol/control preconception treatments. The next morning, matings were confirmed by the presence of a vaginal plug and both the male and female mice returned to their original cages. Males were allowed a 24-hour rest period, during which the preconception exposure was resumed and then used in a subsequent mating. This procedure was repeated until a minimum of three confirmed matings had been achieved, at which point sires were sacrificed and their reproductive tracts isolated.

Sex Determination

Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, catalog# 69504) and PCR amplification of the *Zyf* and *Xist* genes conducted. Extracted geomic DNA from the adult male and female mice were used as the positive and negative controls.

RNA analyses

Total RNA was isolated from E14.5 fetal liver and placenta using the RNeasy Plus Mini Kit, (catalog # 74134, Qiagen, Germantown MD, USA) according to manufacturer's instructions. Samples were randomized prior to RNASeq library preparation. Libraries were generated from 10ng of RNA using the TruSeq RNA Sample Preparation kit (Illumina, San Diego, CA) and pooled for sequencing on an Illumina HiSeq 2500 at Whitehead Genomic Services (Cambridge, MA). Sequencing data were demultiplexed, aligned using STAR with default parameters (Dobin et al., 2013) and referenced against the *Mus musculus* genome (UCSC version mm10).

RNA Sequencing Data Analysis, Selection of Candidate mRNAs, and Functional Enrichment

Following sequencing (40x40 paired-end reads), Bowtie and Tophat were used to align the reads into transcripts based on the Mouse Reference Genome. To measure the relative abundance of each transcript, the resulting aligned reads were analyzed using the Cufflinks suite. Expression was quantified as the number of reads mapping to a gene divided by the gene length in kilobases and the total number of mapped reads in millions, and designated as fragments per kilobase of exon per million fragments mapped (FPKM). To select differentially expressed transcriptomes, the volcano plot measuring statistical significance and magnitude of fold-change was generated based on the log2 fold-change (X-axis) and −log10 *P*-value from Cuffdiff analysis within the Cufflinks suite (Y-axis).

Differentially expressed mRNAs were selected on the basis of linear *P*-value cut off of at 0.05, which was considered significant and highlighted by colored dots in the volcano plot. Subsequently, functional clusters were identified by applying Ingenuity Pathway Analysis (IPA, Ingenuity System Inc, USA) (Jiménez-Marín et al., 2009).

Reduced Representation Bisulfite Sequencing (RRBS)

For each sample, 300 ng of DNA was digested for 2 hours with MSP1 enzyme 20U/sample at 37°C followed by 2 hours with TaqαI 20U/sample at 65°C. The digested DNA was size selected for DNA fragments smaller than 300 bp, which represent CGI enriched fragments and subjected to bisulfite treatment using the Methylamp DNA Bisulfite Conversion Kit (Epigentek catalog # P-1001). DNA was verified to be >99% converted prior to moving forward. Library Preparation was performed by DNA end polishing and adaptor ligation followed by library amplification using indexed primers and library purification. Purified library DNA was then eluted in 12 µl of water. Library was verified on a Bioanalyzer and by KAPA Library Quantification. 10 nM of sample libraries were subjected to next generation sequencing on an Illumina HiSeq 2500 (2 x 250bp paired-end reads with 250M reads per lane encompassing 7-8 million unique CpG sites) (EpigenTek - Farmingdale, New York). Fastq files from multiple replicates were merged to a single fastq file, analyzed for quality by Fastqc and reads trimmed by TrimGalore (-q 20, adapter AGATCGGAAGAGC, --length 20, --rrbs). Reads were processed through Babraham Bioinformatic's program, Bismark (www.bioinformatics.babraham.ac.uk/projects/bismark/Bismark_User_Guide.pdf). The

reference bisulfite genome was produced via "bismark_genome_preparation", and "bismark" was run against the converted genome (--non_directional, --bowtie1, -n 1, -q). Resulting BAM files were sorted and indexed by "samtools sort" and "samtools index" programs. Sorted/indexed BAM files were converted to SAM files via "samtools view" program. The rest of the analysis was performed through MethylKit in RStudio, with a minimum 30X coverage, minimum difference of 25%, and both *P*-value and q-value cutoffs of 0.01. Analysis was repeated at the base residue level (1bp) as well as the 1000bp tile window level. Read count and quality report can be found in **Table 3**. The RefGene file can be obtained at (http://genome.ucsc.edu/cgi-bin/hgTables). Select mm10 version from ucsc tables --> genes and genes prediction tracks --> refseq genes --> output format -->bed -->output file: mm10.refseq.genes.bed.

Command Line & RStudio

BAM files are sorted then converted to SAM files through Samtools.

- samtools sort SAMPLE_trimmed.fq_bismark.bam SAMPLE_trimmed.fq_bismark.bam.sorted
- samtools view -h SAMPLE_trimmed.fq_bismark.bam.sorted.bam > SAMPLE_trimmed.fq_bismark.bam.sorted.sam

SAM files were brought into RStudio for processing through the Bioconductor package, MethylKit.

- R version 3.2.1 (2015-06-18) -- "World-Famous Astronaut"
- Platform: x86_64-apple-darwin10.8.0 (64-bit)

- https://bioconductor.org/packages/release/bioc/manuals/methylKit/man/methylKit.pdf

Commands used as follows:

- source("https://bioconductor.org/biocLite.R")
- biocLite("methylKit")
- library(methylKit)
- biocLite("graphics")
- library(graphics)
- setwd("~/path/to/directory/")
- file.list=list("SAMPLE_1.fq_bismark.bam.sorted.sam","SAMPLE_2.fq_bismark.bam.sorted.sam","SAMPLE_n.fq_bismark.bam.sorted.sam")
- my.methRaw=read.bismark(location=file.list,sample.id=list("SAMPLE_1","SAMPLE_2","SAMPLE_N"),assembly="mm10",save.folder=getwd(),save.context=NULL,read.context="CpG",nolap=FALSE,mincov=10,minqual=20,phred64=FALSE,treatment=c(1,1,0,0))

30X Coverage Minimum - Base

- filtered.myobj30 <- filterByCoverage(my.methRaw, lo.count = 30,lo.perc = NULL, hi.count = NULL, hi.perc = 99.9)
- meth30 <- unite(filtered.myobj30, destrand = FALSE)
- myDiff30 <- calculateDiffMeth(meth30)
- myDiff25p30 <- get.methylDiff(myDiff30, difference = 25, qvalue = 0.01)

30X Coverage Minimum - Tile

- tiles30 <- tileMethylCounts(filtered.myobj30, win.size = 1000,step.size = 1000)
- filtered.tiles30 <- filterByCoverage(tiles30, lo.count = 30,lo.perc = NULL, hi.count = NULL, hi.perc = 99.9)
- meth.tiles30 <- unite(filtered.tiles30, destrand = FALSE)
- myDiff.tiles30 <- calculateDiffMeth(meth.tiles30)
- myDiff25p.tiles30 <- get.methylDiff(myDiff.tiles30, difference = 25, qvalue = 0.01)

- gene.obj <- read.transcript.features("mm10.refseq.genes.bed.txt")

- **diffAnn30** <- annotate.WithGenicParts(**myDiff25p30**, gene.obj)
- getAssociationWithTSS(**diffAnn30**)
- clusterSamples(**meth30**,dist = "correlation",method = "ward",plot = TRUE)
- getCorrelation(**meth30**, plot = T)

Real-time qRT-PCR Analysis of Gene Expression

Total RNA was isolated from isolated E14.5 fetal liver and placenta using the RNeasy Plus Mini Kit, (catalog # 74134, Qiagen, Germantown MD, USA) according to manufacturer's instructions. One microgram of purified total RNA was treated with amplification grade DNase I (catalog # AMPD1; Sigma) according to the manufacturer's recommendations, and 250 ng RNA seeded into a reverse transcription reaction using the

SuperScriptII system (catalog # 18064-071; Invitrogen) by combining 1 μL random hexamer oligonucleotides (catalog # 48190011; Invitrogen), 1 μL 10 mM dNTP (catalog # 18427- 013; Invitrogen), and 11 μL RNA plus water. This mixture was brought to 70°C for 5 minutes and then cooled to room temperature. SuperScriptII reaction buffer, DTT, and SuperScriptII were then added according to manufacturer's protocol, and the mixture brought to 25°C for 5 minutes, 42°C for 50 minutes, 45°C for 20 minutes, 50°C for 15 minutes, and then 70°C for 5 minutes. Relative levels of candidate gene transcripts were analyzed using the Dynamo Flash mastermix (catalog # F-415XL; Thermo Scientific) according to the recommended protocol. Reactions were performed on a Bio-Rad CFX384.

Statistical analysis

For all experiments, statistical significance was set at alpha = 0.05. For analysis of gene expression, the replicate cycle threshold (Ct) values for each transcript were compiled and normalized to the geometric mean of three validated reference genes. Liver: Gapdh, Hprt, and Ppia. Placenta: Sdha, Hprt, and Mrpl. Normalized expression levels were calculated using the ddCt method described previously (Schmittgen & Livak, 2008). Relative fold change values from a minimum of three biological replicates were transferred into the statistical analysis program GraphPad (GraphPad Software, Inc., La Jolla, CA) where datasets were first verified for normality using the Brown-Forsythe test. For single comparisons, an unpaired student's t-test was applied. In all instances, we have marked statistically significant differences with an asterisk (∗). For RNA sequence-based

comparisons of allelic patterns of imprinted gene expression, the proportion of identified single nucleotide polymorphisms were analyzed using either Chi-Square or, if read counts were less than 5, a Fisher's Exact test.

CHAPTER V

PRECONCEPTION ETOH EXPOSURE DOES NOT INFLUENCE THE GLOBAL DNA METHYLATION PROFILE OF SPERM.*

Introduction

It has recently become clear that through epigenetic means, environmental exposures prior to conception exert a significant impact on offspring health and development (Lane, Robker, & Robertson, 2014). In particular, preconception male exposures to a range of environmental factors induce alterations to the developmental program of sperm, which can be correlated with increased rates of structural and metabolic defects in the next generation (Anway, Cupp, Uzumcu, & Skinner, 2005; Carone et al., 2010; Dias & Ressler, 2014; Donkin et al., 2016; Esakky et al., 2016; Fullston et al., 2013; Gapp et al., 2014; Lambrot et al., 2013; Ng et al., 2010; Radford et al., 2012, 2014; Shea et al., 2015; Terashima et al., 2015; Wei et al., 2014; Zeybel et al., 2012). These studies challenge the singular importance of maternal *in utero* environmental exposures and implicate paternal exposure history as an additional and important mediator of both environmentally induced disease and dysgenesis (Day et al., 2016; Rando & Simmons, 2015). However, the capacity of preconception paternal exposures to broadly contribute

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to the development of environmentally induced disease has not been rigorously explored, largely due to the common misconception that sperm do not transmit heritable information beyond the genetic code.

The majority of these studies have concentrated on alterations in DNA methylation occurring within the regulatory regions of imprinted genes, suggesting altered placentation may be at the root of the observed fetal growth restriction (Finegersh & Homanics, 2014; Knezovich & Ramsay, 2012; Liang et al., 2014). Indeed, disruptions in genomic imprinting have well-characterized impacts on the development of the placenta and the faithful maintenance of these imprints through early development is crucial for both fetal and placental growth (Coan et al., 2005; Piedrahita 2011). Importantly, similar alterations in the DNA methylation profiles of alcohol-exposed sperm can be identified in humans, suggesting the legacy of paternal drinking may transmit to the offspring *via* heritable disruptions in the regulation of imprinted genes (Ouko et al., 2009). However, while several instances of paternally-inherited alterations in developmental programming have been correlated with changes in the DNA methylation profiles of paternal sperm, an increasing number of cases suggest these heritable phenotypes transmit independent of this epigenetic modification (Carone et al., 2010; Shea et al., 2015; Terashima et al., 2015). Thus, the universal dependence of these phenotypes on DNA methylation-based mechanisms of epigenetic inheritance remains an unanswered question central to understanding the paternal transmission of environmentally induced traits.

Through these series of experiments, we wanted to determine if chronic male alcohol consumption induced epimutations in sperm that are transmitted through DNA

methylation-based mechanisms. We hypothesized that alcohol consumption in males can cause aberrant changes to DNA methylation in sperm resulting in transmissible epimutations passed along to the offspring.

Results

Preconception EtOH exposure does not influence male fertility

To determine if chronic EtOH exposure could influence the male-inherited epigenetic program, breeder males were sacrificed and both reproductive and epigenetic parameters examined. No significant differences in sperm concentration or the percentage of live sperm were observed between treatment groups (**Figure 19A-B**). No treatment effects were observed on the weights of the paternal liver or epididymal fat (Chang 2017).

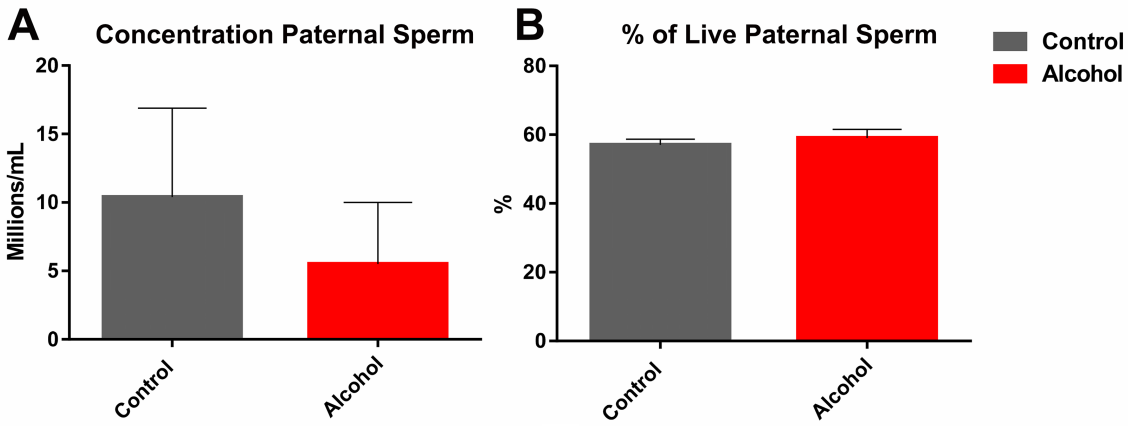


Figure 19. Preconception male alcohol exposure does not impact base fertility.

A. Concentration of sperm between EtOH-exposed and control males. **B.** percentage of live sperm between EtOH-exposed and control males (n=8).

Preconception EtOH exposure does not influence the DNA methylation profile of sperm.

We quantified the DNA methylation profiles of alcohol-exposed and control sperm using bisulfite mutagenesis and second-generation deep sequencing. In these analyses, we could not detect any differences in the global levels of DNA methylation between any of the preconception treatments, in any of the CpG, CHG or CHH contexts (**Figure 20A**). Informatic analysis performed using the RStudio Bioconductor package MethylKit identified extremely strong correlation between treatment groups and clustering analysis failed to separate sperm samples by preconception treatment (**Figure 20B-C**). Setting a cutoff of 30x coverage, a minimal change in DNA methylation of 25%, a q-value of 0.01 and *P*-value of less than 0.01 (Ziller, Hansen, Meissner, & Aryee, 2015), we were only able to identify two high-confidence differentially methylated regions (*Filip1l* and *Rn45s*) between the EtOH-exposed and control males. When we relaxed these criteria to include regions with only 5x coverage (remaining parameters kept the same), we could expand this list to 18 candidate CpGs and 124 tiled loci (Appendix: **Table 3**). Unexpectedly, 68% of these differentially methylated regions mapped to gene bodies (37% exonic and 31% intronic), while only 3% could be mapped to gene promoters (**Figure 20D**). When we randomly selected four of these lower confidence candidate loci to contrast measures between Methyl-seq and PCR-based bisulfite sequencing approaches, only two displayed significant alterations in DNA methylation using both methods (**Figure 20E**). Using PCR-based bisulfite sequencing, the two significant regions identified both displayed less than a 25% change.

To determine if these significant changes in methylation correlated to changes in gene expression in the placenta and fetal liver, we assessed the transcript levels through RT-qPCR. These results failed to identify any correlative changes in gene expression (**Placenta**: **Figure 21A-B, Liver: Figure 21C-D**). For example, the methylation tile associated with Slc43a2 spanned the exon and intronic regions of the gene, while the tile associated with 6820431F20RiK had methylation changes spanning across all of the annotated features including the promoter. Neither of these genes displayed alterations in gene expression within the placenta (**Figure 21E**). Importantly, no differences in the DNA methylation profiles of a select group of imprinted genes were observed in the sperm (**Figure 22A-D**).

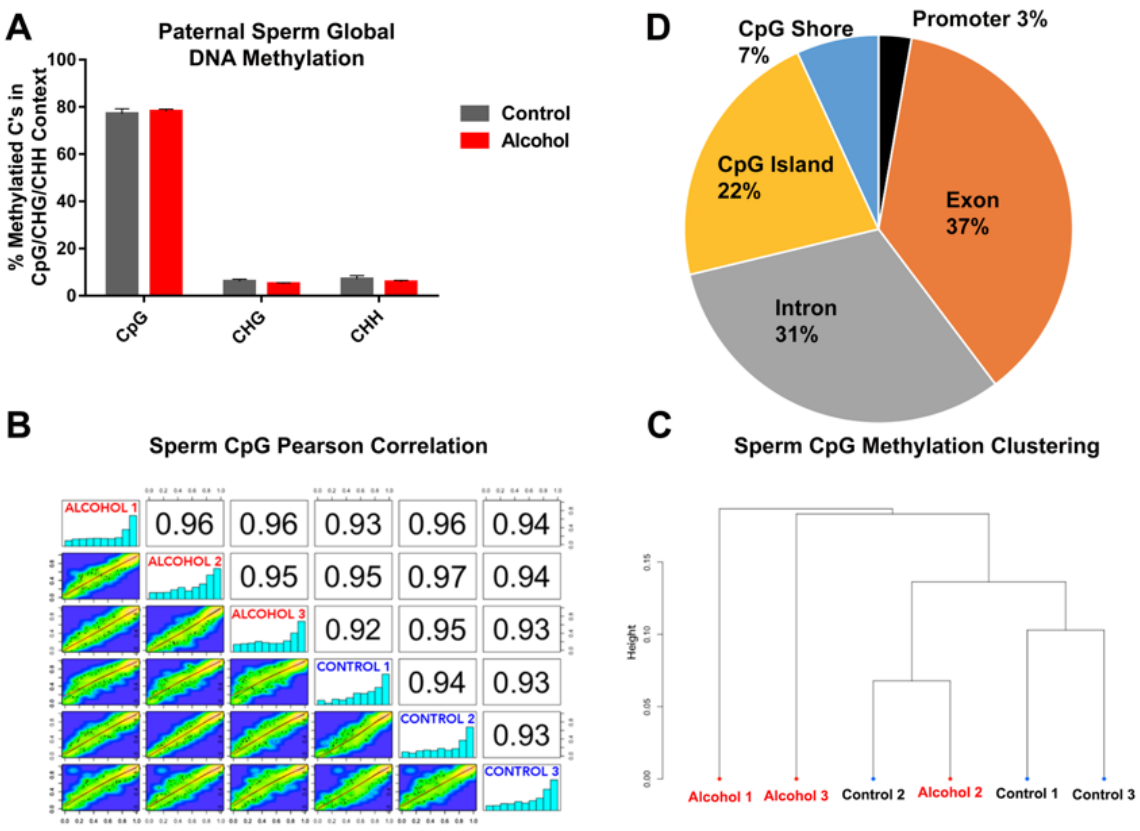


Figure 20. Preconception male alcohol exposure does not impact the DNA methylation profiles of paternal sperm.

A. Global DNA methylation profiles of paternal sperm (n = 3) across CpG, CHG and CHH contexts. **B.** Pearson correlation analysis between genomic DNA methylation profiles of paternal sperm (n=3). **C.** Clustering analysis between EtOH-exposed and control DNA methylation profiles of paternal sperm. **D.** Association of differentially methylated loci with genomic features. **E.** Comparison of low confidence differentially methylated loci between Methyl-Seq data sets and PCR-based Bisulfite sequencing. Bisulfite sequencing data were compared using an unpaired t-test. Error bars represent SEM *P < 0.05, **P < 0.01 and **** P < 0.0001.

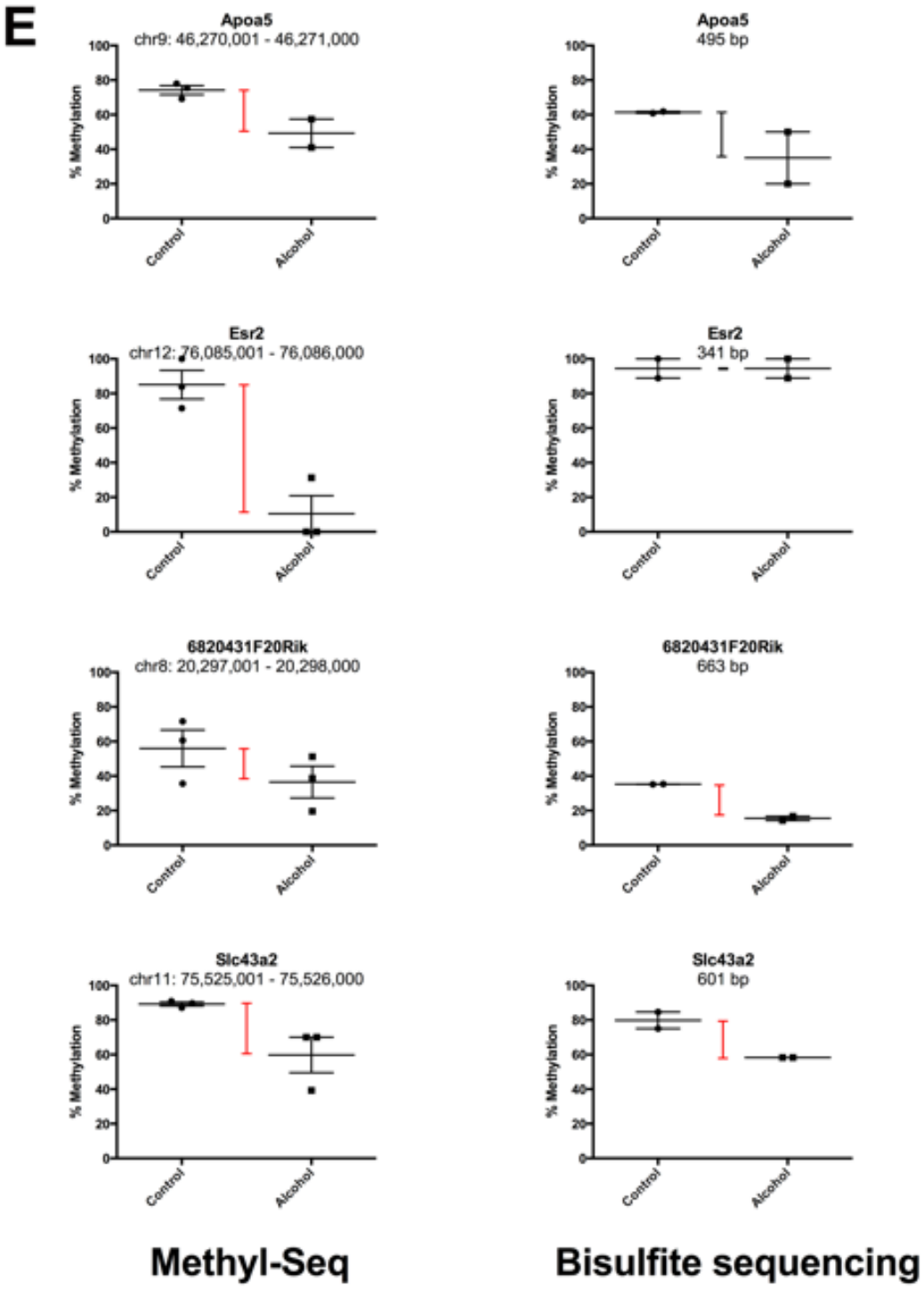
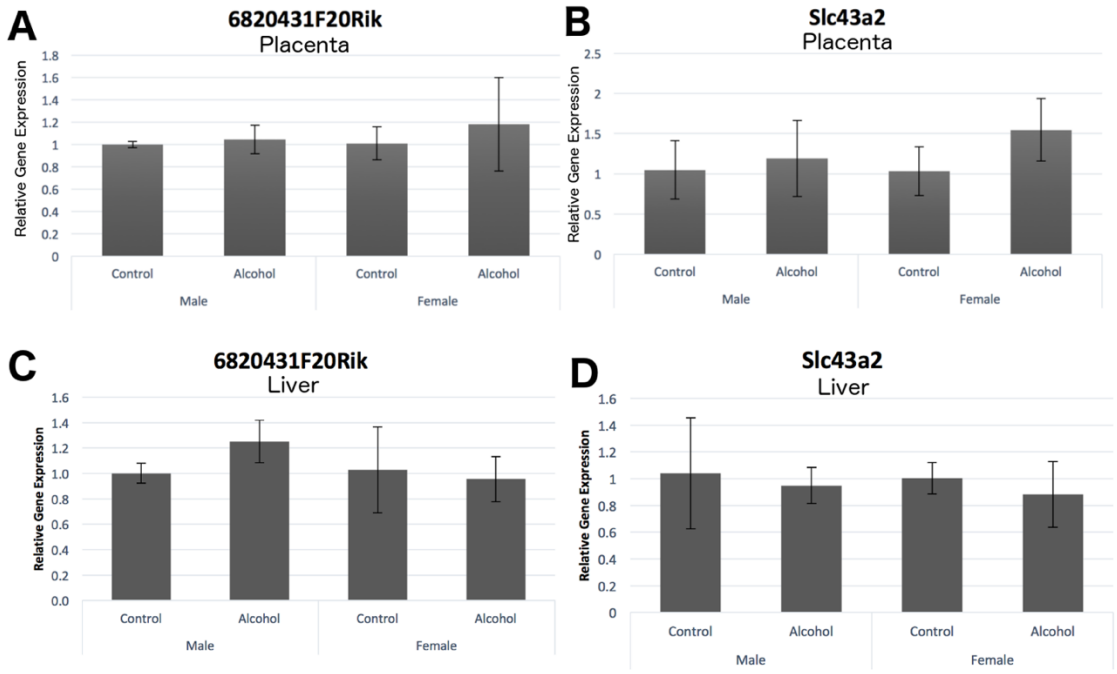


Figure 20. Continued.



E

Gene	Tile	Promoter	Exon	Intron	CpGi	Shores
Apoa5	chr9:46270001-46271000	0	0	**1**	0	0
Esr2	chr12: 76085001-76086000	0	**1**	0	**1**	**1**
Slc43a2	chr11: 75525001-75526000	0	**1**	**1**	0	0
6820431F20Rik	chr8: 20297001-20298000	**1**	**1**	**1**	**1**	**1**

Figure 21. RT-qPCR Gene Expression Verification in the Placenta and Liver.

A. RT-qPCR measurements of transcripts encoding the murine 6820431F20Rik gene in the placenta. **B**. RT-qPCR measurements of transcripts encoding the murine Slc43a2 (Lat4) gene in the placenta. **C**. RT-qPCR measurements of transcripts encoding the murine 6820431F20Rik gene in the liver. **D**. RT-qPCR measurements of transcripts encoding the murine Slc43a2 (Lat4) gene in the liver. **E**. Association of differentially methylated loci of select genes with genomic features. RT-qPCR measures were normalized to the geometric mean of Gapdh, Hprt and Sdha. n =2. (*P*-value: * ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001; **** ≤ 0.0001)

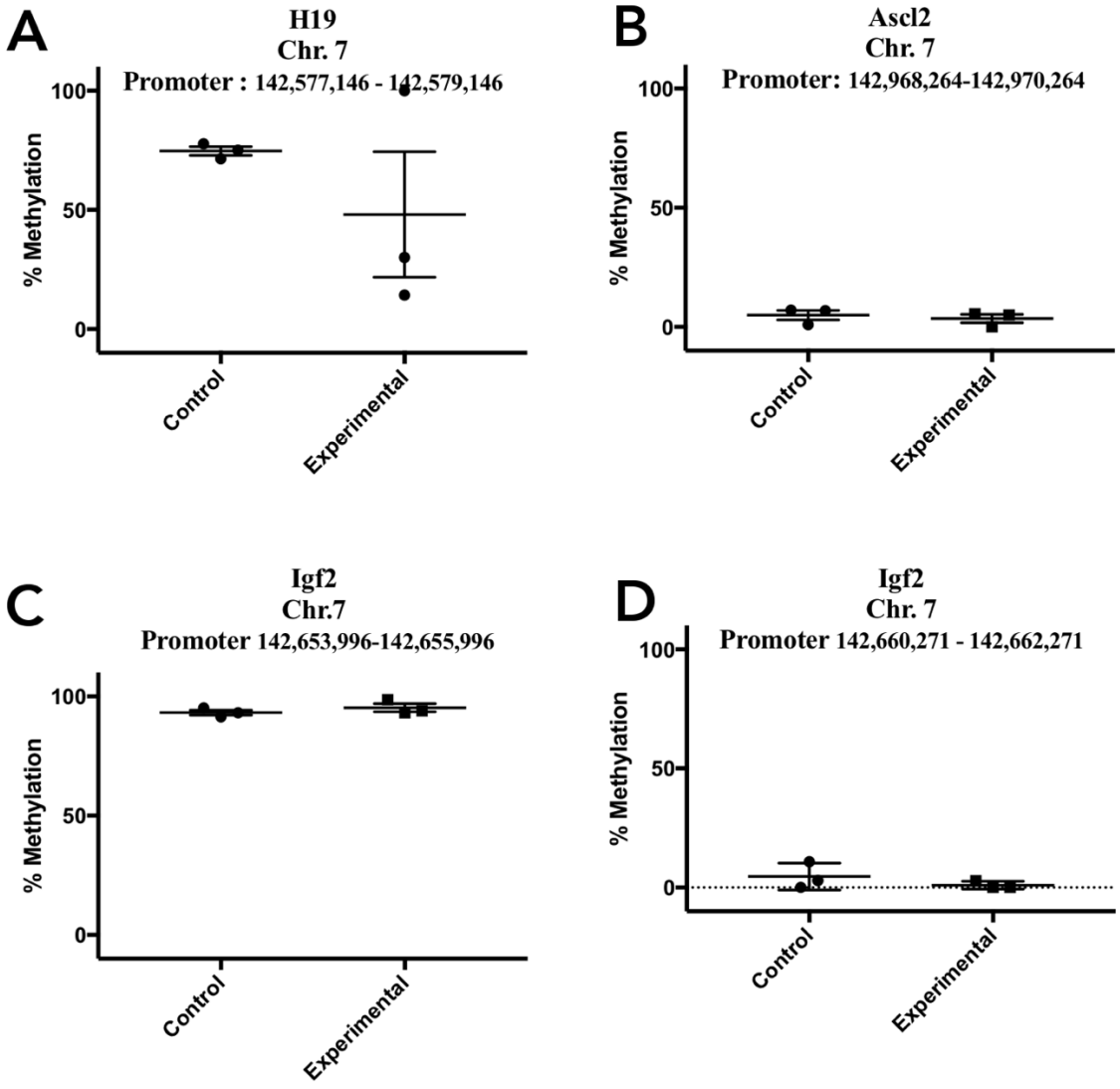


Figure 22: Tile-based assessment of DNA methylation at select imprinted genes in sperm. **A-D.** Differentially methylated tiled loci from Methyl-Seq data. Data were compared using an unpaired t-test. Error bars represent SEM. Analysis compared the average methylation in the CpG context of a 1000bp region encompassing the promoter region of listed gene.

Analysis of Rn45S

In our analysis, the common ribosomal precursor Rn45s was differentially methylated in the sperm, placenta and fetal liver across all of our samples (**Table 2**). Rn45s is a precursor transcript to the major ribosomal complexes. Ribosomal complexes, specifically the gene bodies of rDNA, have been found to regulated through RNA-guided mechanisms directly impacting chromatin structure (Grummt 2007). Specific ribosomal deficiencies, coined ribosomopathies, have been linked with growth retardation and skeletal defects in affected individuals providing a potential link to the observed fetal growth restriction in our model (Robson, Owens, Baserga, Khokha, & Griffin, 2016). Dysregulation of this precursor could lead to structural dysfunction of the ribosomal complex leading to aberrant protein synthesis. In follow-up to these experiments, future studies will determine if these environmentally-induced methylation changes to the Rn45s locus have the capacity to alter Rn45s transcript levels, to heritably impact chromatin structure at this locus and potentially impact development of offspring.

Sperm - Differential Methylation Annotation: CpG - Base (1bp)
Genes with nearest TSS

SPERM		PLACENTA				LIVER			
		MALE		FEMALE		MALE		FEMALE	
ACCESSION	GENE ID	ACCESSION	GENE ID	ACCESSION	GENE ID	ACCESSION	GENE ID	ACCESSION	GENE ID
NR_046233	Rn45s	NR_046233	Rn45s	NR_046233	Rn45s	NR_046233	Rn45s	NR_046233	Rn45s
		NM_030207	Sfi1	NM_030207	Sfi1			NM_030207	Sfi1
NM_001024706	Gm5458			NM_001024706	Gm5458			NM_001024706	Gm5458
NM_009970	Csf2ra							NM_009970	Csf2ra
				NM_008739	Nsd1			NM_008739	Nsd1

Table 2. Differential methylation analysis of sperm, placenta, and liver tissues at a 1bp resolution as determined by the MethylKit package through R.
Genes with nearest transcriptional start site (TSS) to the differentially methylated CpG residue were identified and cross-referenced across the tissues. MethylKit (R) filter parameters: 5X coverage minimum, Pvalue cutoff 0.05, Qvalue cutoff 0.01, methylation difference > 25% between groups.

Discussion

It is evident that the environment can have an influence on the epigenome resulting in impacts on the health and development of offspring. We have shown that male germ line exposure to alcohol prior to conception can transmit epimutations resulting in fetal growth restriction, decreased placental efficiency, abnormalities in cholesterol trafficking, sex-specific alterations in the genetic pathways regulating hepatic fibrosis and disruptions in the regulation of imprinted genes (Chang 2017).

While we hypothesized that transmissible epimutations carried by sperm were a result of aberrant DNA methylation, using bisulfite mutagenesis and second-generation RNA sequencing, we find no evidence to suggest that any of the associated transcriptional changes are linked to alterations in the sperm-inherited DNA methylation profile. Alcohol exposure did not alter one-carbon metabolite s-adenosylmethionine or global levels of methylation in sperm compared to our control males (Chang 2017). Of the significant

candidates identified in our analysis, the alterations in methylation status did not translate to altered transcript levels in the placenta. There is a possibility that our treatment levels of alcohol were not high enough to cause changes in DNA methylation in sperm, yet based on the literature, our alcohol dose was a physiologically relevant to the habitual human male drinker and therefore an appropriate dose. These observations are consistent with recent studies examining the male transmission of diet-induced phenotypes and emphasize the importance of epigenetic mechanisms of paternal inheritance beyond DNA methylation. This study challenges the singular importance of maternal alcohol exposures and suggests paternal alcohol abuse is a significant, yet overlooked epidemiological factor complicit in the genesis of alcohol-induced growth defects, and may provide mechanistic insight into the failure of FASD children to thrive postnatally.

Materials & Methods

Sperm Collection and Analysis

After a minimum of three matings, breeder males were sacrificed and sperm collected using a modified swim-up procedure (Zalenskaya, Bradbury, & Zalensky, 2000). Briefly, human tubal fluid (HTF) medium (catalog # GMHT-100, Life Global Group) supplemented with 4mg/ml of BSA fraction V (catalog # A5611, Sigma Aldrich,) and 1µl/ml of Gentamicin (catalog # 15750-060, Invitrogen) was covered with mineral oil, the dish set in an incubator at 37°C in a 5% CO_2, 5% O_2, 90% N_2 atmosphere for three hours before males were to be sacrificed. The male reproductive tract was surgically excised and

placed into a petri dish containing warmed fertilization medium. Forceps were used to force the sperm through cuts in the vas deferens and epididymis. Concentrated sperm were placed in 5mL of HTF medium in a polystyrene round bottom tube (catalog # 353058, BD Falcon) at a 45° angle in the incubator for 1 hour. The top 1.5 mls were removed and sperm counted using a hemocytometer. For all experiments, sperm samples were judged to be >99% pure, as assessed by microscopy. Freshly washed (in PBS) sperm were incubated 1:1 with a lysis buffer containing 20 mM TrisCl (pH 8), 20mM EDTA, 200 mM NaCl and 4% SDS, supplemented prior to use with 100 mM DTT and 250 µg/ml Proteinase K. Incubation was performed for 4 hours at 55°C with frequent vortexing. Samples were combined with 200 µL of 100% ethanol and 200 µL of DNeasy lysis buffer (DNeasy Blood and Tissue Kit, Qiagen catalog # 69504), then this mixture added to the DNA isolation columns. The remaining purification steps were performed according to the Qiagen DNeasy Blood & Tissue Kit instructions.

Reduced Representation Bisulfite Sequencing (RRBS)

For each sample, 300 ng of DNA was digested for 2 hours with MSP1 enzyme 20U/sample at 37°C followed by 2 hours with TaqαI 20U/sample at 65°C. The digested DNA was size selected for DNA fragments smaller than 300 bp, which represent CGI enriched fragments and subjected to bisulfite treatment using the Methylamp DNA Bisulfite Conversion Kit (Epigentek catalog # P-1001). DNA was verified to be >99% converted prior to moving forward. Library Preparation was performed by DNA end polishing and adaptor ligation followed by library amplification using indexed primers

and library purification. Purified library DNA was then eluted in 12 µl of water. Library was verified on a Bioanalyzer and by KAPA Library Quantification. 10 nM of sample libraries were subjected to next generation sequencing on an Illumina HiSeq 2500 (2 x 50bp paired-end reads with 250M reads per lane encompassing 7-8 million unique CpG sites) (EpigenTek - Farmingdale, New York). Fastq files from multiple replicates were merged to a single fastq file, analyzed for quality by Fastqc and reads trimmed by TrimGalore (-q 20, adapter AGATCGGAAGAGC, --length 20, --rrbs). Reads were processed through Babraham Bioinformatic's program, Bismark (www.bioinformatics.babraham.ac.uk/projects/bismark/Bismark_User_Guide.pdf). The reference bisulfite genome was produced via "bismark_genome_preparation", and "bismark" was run against the converted genome (--non_directional, --bowtie1, -n 1, -q). Resulting BAM files were sorted and indexed by "samtools sort" and "samtools index" programs. Sorted/indexed BAM files were converted to SAM files via "samtools view" program. The rest of the analysis was performed through MethylKit in RStudio, with a minimum 30X coverage, minimum difference of 25%, and both *P*-value and q-value cutoffs of 0.01. 30X coverage was set as our threshold, but 15X and 5X coverage were evaluated as well to ensure we were not alienating potential loci that might not meet coverage depth requirements in underrepresented samples. Read count and quality report can be found in **Table 7**. The RefGene file can be obtained at (http://genome.ucsc.edu/cgi-bin/hgTables). Select mm10 version from ucsc tables --> genes and genes prediction tracks --> refseq genes --> output format -->bed -->output file: mm10.refseq.genes.bed.

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- R version 3.2.1 (2015-06-18) -- "World-Famous Astronaut"
- Platform: x86_64-apple-darwin10.8.0 (64-bit)
- https://bioconductor.org/packages/release/bioc/manuals/methylKit/man/methylKit.pdf

Commands used as follows:

- source("https://bioconductor.org/biocLite.R")
- biocLite("methylKit")
- library(methylKit)
- biocLite("graphics")
- library(graphics)
- setwd("~/path/to/directory/")
- file.list=list("SAMPLE_1.fq_bismark.bam.sorted.sam","SAMPLE_2.fq_bismark.bam.sorted.sam","SAMPLE_n.fq_bismark.bam.sorted.sam")

- my.methRaw=read.bismark(location=file.list,sample.id=list("SAMPLE_1","SAMPLE_2","SAMPLE_N"),assembly="mm10",save.folder=getwd(),save.context=NULL,read.context="CpG",nolap=FALSE,mincov=10,minqual=20,phred64=FALSE,treatment=c(1,1,0,0))

30X Coverage Minimum - Base

- filtered.myobj30 <- filterByCoverage(my.methRaw, lo.count = 30,lo.perc = NULL, hi.count = NULL, hi.perc = 99.9)
- meth30 <- unite(filtered.myobj30, destrand = FALSE)
- myDiff30 <- calculateDiffMeth(meth30)
- myDiff25p30 <- get.methylDiff(myDiff30, difference = 25, qvalue = 0.01)

30X Coverage Minimum - Tile

- tiles30 <- tileMethylCounts(filtered.myobj30, win.size = 1000,step.size = 1000)
- filtered.tiles30 <- filterByCoverage(tiles30, lo.count = 30,lo.perc = NULL, hi.count = NULL, hi.perc = 99.9)
- meth.tiles30 <- unite(filtered.tiles30, destrand = FALSE)
- myDiff.tiles30 <- calculateDiffMeth(meth.tiles30)
- myDiff25p.tiles30 <- get.methylDiff(myDiff.tiles30, difference = 25, qvalue = 0.01)

- gene.obj <- read.transcript.features("mm10.refseq.genes.bed.txt")

- **diffAnn30** <- annotate.WithGenicParts(**myDiff25p30**, gene.obj)
- getAssociationWithTSS(**diffAnn30**)
- clusterSamples(**meth30**,dist = "correlation",method = "ward",plot = TRUE)
- getCorrelation(**meth30**, plot = T)

	Sex	Group	# Reads	% of >= Q30 Bases (PF)	Mean Quality Score (PF)
Placenta	Male	Control	111,560,067	92.01	35.76
		Control	132,395,934	91.73	35.69
	Female	Control	77,650,165	92.23	35.81
		Control	115,126,209	92.48	35.88
	Male	Alcohol	151,236,261	90.79	34.61
		Alcohol	116,876,167	90.11	34.45
	Female	Alcohol	98,160,344	90.92	34.63
		Alcohol	95,394,830	90.98	34.64
	Sex	**Group**	**# Reads**	**% of >= Q30 Bases (PF)**	**Mean Quality Score (PF)**
Liver	Male	Control	64,957,556	90.44	34.58
		Control	75,393,871	87.1	33.48
	Female	Control	56,527,793	90.63	34.62
		Control	28,458,781	88.31	33.85
	Male	Alcohol	63,221,954	90.51	34.58
		Alcohol	71,788,570	90.38	34.56
	Female	Alcohol	70,548,758	87.44	33.59
		Alcohol	69,150,295	87.81	33.7
		Group	**# Reads**	**% of >= Q30 Bases (PF)**	**Mean Quality Score (PF)**
Sperm		Control	81,319,464	90.65	34.57
		Control	89,344,904	90.64	34.57
		Control	88,993,402	91.25	34.73
		Alcohol	74,903,705	89.04	34.17
		Alcohol	127,504,972	88.46	34.02
		Alcohol	72,158,176	88.87	34.11

Table 3. Reduced representation bisulfite sequencing (RRBS) Read Quality Report.
"# Reads": The number of trimmed RRBS reads that were mapped to the UCSC *Mus musculus* (mouse) genome sequence (version mm10) using a methylation-aware mapper, bismark, version 0.13.0. 2x250 bp paired-end reads. Bismark utilizes Bowtie, version 1.0.0, with the option "-n 1", which permits only up to one mismatch in the seed region. "% of >= Q30 Based (PF)":

Percent (%) of reads with a Sanger Phred score greater than 30. "Mean Quality Score (PF)": The mean Sanger Phred score across all mapable reads.

Bisulfite PCR

DNA was isolated using a DNeasy Blood & Tissue Kit (Qiagen catalog # 69504) and quantified using a NanoDrop 2000c. 300ng of DNA input was bisulfite converted using the EZ DNA Methylation Kit (Zymo Research catalog # D5001). Primers were designed through the online program "Bisulfite Primer Seeker" available through the Zymo Research website. Gel extraction of PCR product was performed using the Qiagen QIAEX II Gel Extraction Kit (Qiagen catalog # 20021). PCR products were cloned into the pGEM-T Easy Vector System (Promega catalog # A1360) and sequenced at the Texas A&M's Plant Genome Technologies core. Analysis was performed using BiQ Analyzer (http://biq-analyzer.bioinf.mpi-inf.mpg.de/).

Statistical analysis

For all experiments, statistical significance was set at alpha = 0.05. For analysis of gene expression, the replicate cycle threshold (Ct) values for each transcript were compiled and normalized to the geometric mean of three validated reference genes, Gapdh, Hprt, and Sdha. Normalized expression levels were calculated using the ddCt method described previously (Schmittgen & Livak, 2008). Relative fold change values from a minimum of two biological replicates were transferred into the statistical analysis program GraphPad (GraphPad Software, Inc., La Jolla, CA) where datasets were first

verified for normality using the Brown-Forsythe test. For single comparisons, an unpaired student's t-test was applied. In all instances, we have marked statistically significant differences with an asterisk (*). Bisulfite sequencing data were compared using an unpaired t-test. Error bars represent SEM *$P < 0.05$, **P < 0.01 and **** $P < 0.0001$. RRBS bioinformatic analysis performed using the RStudio Bioconductor package MethylKit setting a cutoff of 30x coverage, a minimal change in DNA methylation of 25%, a q-value of 0.01 and *P*-value of less than 0.01 (Ziller et al., 2015).

CHAPTER VI

FUTURE DIRECTIONS: EVALUATION OF SPERM HISTONE POST-TRANSLATIONAL MODIFICATIONS IN RESPONSE TO CHRONIC PRECONCEPTION ALCOHOL EXPOSURE, A PROTEOMIC APPROACH.[**]

Introduction

Largely, sperm had been dismissed as anything more than a passive transport vessel given the almost global replacement of histones and the reprogramming of DNA methylation shortly after fertilization. In conjunction with the known maternal oocyte contributions to the zygote such as histones, cytoplasmic components, maternally deposited RNAs and protein, as wells as mitochondria, the majority of effort in the field has focused its energy almost exclusively on maternal contributions.(Oliva & Dixon, 1991) This view has shifted as new information has been revealed regarding the nature of sperm's retained histones, non-coding RNAs and even role of DNA methylation in the proper establishment of embryo development (Chastain & Sarkar, 2017). In recent years, more and more research has emerged examining the role of epigenetic programming within the male germline with regards to offspring health and development. In mature

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[*] Reprinted with permission from "Analyzing the sperm epigenome: Roles in early embryogenesis and assisted reproduction." by Schagdarsurengin, U., Paradowska, A., & Steger, K., 2012, *Nature Reviews. Urology*, *9*(11), 609-19., Copyright [2017] by Nature Publishing Group.

[*] Reprinted with permission from "Evidence for a dynamic role of the linker histone variant h1x during retinoic acid-induced differentiation of NT2 cells." by Shahhoseini M, Favaedi R, Baharvand H, Sharma V, Stunnenberg HG., 2010, *FEBS Lett*, 584(22):4661-4., Copyright [2017] by John Wiley and Sons.

human sperm, approximately 85% - 95% of histones are replaced with protamines during the late haploid phase of spermatogenesis (Gardiner-Garden, Ballesteros, Gordon, & Tam, 1998; Hecht 1990; Oliva & Ballescà, 2012). In mice, protamine replacement of histones was determined to be 95-99% (Erkek et al., 2013). Protamines are encoded by two separate proteins, protamine 1 (P1) and protamine 2 (P2) which are expressed in equal proportion (Hammoud, Liu, & Carrell, 2009). Deviating from this equal proportion of P1 and P2 has been associated with varying levels of infertility and susceptibility to DNA damage (Dadoune 1995; Grassetti et al., 2012; Hammoud et al., 2009; Hecht 1990). The biological purpose behind histone replacement rest in the compactable properties of protamines as well as the protection afforded to DNA in such a compact state. DNA can be significantly compacted (~6-20-fold reduction) by replacing bulky histone complexes with smaller protamines and forming a compact structure known as a toroid (**Figure 23A**) (Schagdarsurengin, Paradowska, & Steger, 2012). This allows the sperm to become compact and highly protected from DNA damage producing a highly efficient DNA transporting vehicle. Following the transmission of the male genome to the egg, the male pronucleus is decondensed and protamines replaced by maternally-derived histones (van der Heijden et al., 2006). Protamines are basic, arginine-rich proteins that do not carry any post-translational modifications and therefore, believed to carry no epigenetic information (Gardiner-Garden et al., 1998). It was believed that since the histones, cytoplasmic components, maternally deposited RNAs and protein, and mitochondria are all provided from the maternal oocyte, there is little epigenetic contribution to the zygote in regards to the paternal involvement outside of DNA methylation (Puri, Dhawan, & Mishra, 2010).

Sperm was more commonly viewed as a passive vessel of the paternal germline (2010). However, recent studies shed new light on the positioning of the retained histones in sperm and indicate an important role for these factors during development (Siklenka et al., 2015). Interestingly, of the estimated 1 – 15% of histones retained in sperm (percent species specific), the locus in which they are maintained was found not to be randomly distributed, rather these regions are enriched at crucial gene promoters, non-coding RNA clusters and imprinted gene loci, suggesting a potential mechanism of paternal epigenetic transmission beyond DNA methylation (Hammoud et al., 2009; Hammoud et al., 2011; Ooi & Henikoff, 2007; Schagdarsurengin et al., 2012). In addition, these histone-bound regions were found to localize to the nuclear envelope and maintained through the non-coding RNAs located there (Jenkins & Carrell, 2011). The H3.3 histone variant as well as extensive H3K4me3 histone post-translational modification of histone H3 are highly enriched at sites of histone retention (Erkek et al., 2013). Other research groups have shown that BORIS, a CTCF analog, is a marker for histone retention in mature spermatozoa (Rivero-Hinojosa, Kang, Lobanenkov, & Zentner, 2017). CTCF & BORIS cooperate with additional TSTRs (testis-specific transcriptional regulators) to regulate gene expression in developing male gametes, potentially priming certain regions of the genome for rapid activation following fertilization (Rivero-Hinojosa, Kang, Lobanenkov, & Zentner, 2017).

Experiments in the late 1990's indicated loci-specific histone retention in a subset of β-Globin domain genes that are critical for embryonic yolk sac development. However, other genes in this protein family that are not required during early development showed no retention of histones in sperm (Gardiner-Garden et al., 1998). While this is a correlative

finding and correlations do not always translate to causation, further experiments were conducted in order to elucidate if retained histones truly influenced the expression of genes at their loci and whether they could be disrupted to alter gene expression levels in the embryo. Disruption of methylation on the histone H3 (H3K4me2) in the retained sperm through targeted overexpression of KDM1A, a histone lysine demethylase that specifically targets demethylation on H3K4, was enough to severely impact development and survivability in offspring that lasted several generations (Siklenka et al., 2015). Other research indicates a comparable role for histone repressive and active methylation marks in spermatozoa as in somatic cells. Even with a broadly reduced representation of histones in sperm, the H3K27me3 modification, associated with gene silencing, was extensively localized around the transcriptional start sites of silenced genes implicating the role of polycomb-repressive complexes in the silencing of genes even in sperm (Brykczynska et al., 2010). Methylation marks on H3K4 were found around genes relevant to spermatogenesis and cellular homeostasis implicating Trithorax protein complexes in maintaining active gene states (Brykczynska et al., 2010). Not only does this suggest specific genes with distinct gene functions with early developmental roles are poised for early activation but most other genes are maintained in a highly silenced state through histone modifications. In other work, utilizing two independent mouse models that display abnormal histone positioning in mature sperm as a result of impaired poly(ADP-ribose) (PAR) metabolism during spermatogenesis, Ihara et al. revealed that when sperm from these mice were used to produce offspring, a small yet significant portion of genes that were differentially expressed compared to controls in the embryos showed aberrant

histone retention in the corresponding gene loci (Ihara et al., 2014). These experiments provide evidence for a linkage between the retention/loss of histones in sperm and altered gene expression of associated loci in the embryo. Based on these observations linking retained histones in sperm with developmental gene promoters and imprinted gene loci, as well as our evidence of a DNA methylation-independent transmission of epigenetic information, assessing the post-translational modifications of retained histones in sperm might provide valuable insight into the heritable nature of epimutations through the male germline. Our data indicates that the male germline exposed to alcohol before conception possess the ability to transfer epimutations, or aberrant gene expression patterns to offspring through a DNA methylation-independent mechanism, therefore, we set out to explore other known epigenome regulatory candidates, including histone post-translational modifications.

In somatic cells, PTMs on the N-terminal tails of histones have been associated with specific transcriptional states. For instance, some modifications associate with increased transcription of a genes while others associate with a repressed state (Fuks 2005; Gifford et al., 2013). While there are seemingly countless exceptions to each characterized association, these trends are known collectively referred to as the "histone code" (Jenuwein & Allis, 2001). As previously described, the N-terminal tails of histones H2A, H2B, H3 and H4 are flexible and subject to many distinct chemical modifications, which associate with specific chromatin states and gene expression states (Allfrey et al., 1964). Bulky acetyl marks typically result in a more relaxed chromatin state while methylation can be activating or repressive based on which lysine is modified. Based on this

background knowledge and our data that suggested epimutations are transmitted via DNA methylation-independent mechanisms, we set out to elucidate if epimutations are carried through altered histone post translational modifications in sperm. However, without a specific post translational modification or candidate histone, we employed an unbiased approach. Mass spectrometry represents an optimal approach based on its ability to assess the global histone post-translational modification population in our treatment samples.

Proteomics is the large-scale study of proteins in living organisms (Anderson & Anderson, 1998; Blackstock & Weir, 1999) . Proteins are a class of large biomolecules that are composed of a large chain of amino acid residues (Pauling, Corey, & Branson, 1951). Proteins are encoded by genes in DNA and the eventual end product of translation resulting in a linear chain of residues. These long amino acid residues will fold or bend to form a larger 3D structure that are responsible for a large array of cellular functions (Anfinsen 1972, 1973) . Some examples of the functions proteins carry out are catalyzing enzymatic reactions, responding to stimulus, transporting molecules, transferring signals, and DNA replication. Proteins can be modified after translation, also known as post-translational modifications. These modifications can result in changes to domain function, conformational shifts in shape and change in enzymatic activity (Lee et al., 2006). In regards to histones, post translational modifications to the N-terminus tail result in alterations in chromatin structure, DNA affinity, or recruitment of histone modifiers (Campos & Reinberg, 2009; Jenuwein & Allis, 2001; Zhou, Goren, & Bernstein, 2011). Based on the unique size and shape of proteins, they can be accurately distinguished through a technique known as mass spectrometry (Loo, Udseth, & Smith, 1989; Price

1991). In short, mass spectrometry ionizes proteins and sorts them based on their mass-to-charge ratio (Loo et al., 1989; Price 1991; Tanaka et al., 1988). In other terms, mass spectrometry measures the mass of proteins and enables the identification of specific histone proteins, variants and importantly, the repertoire of posttranslational modifications present.

The laboratory of Dr. Benjamin A. Garcia from the University of Pennsylvania's Perelman School of Medicine has optimized a mass spectrometry methodology that specializes in characterizing histone PTMs (Kori et al., 2017; Sidoli et al., 2017). This technique is highly useful is quantifying the changes in the proteome and posttranslational modifications in response to significant biological events such as our paternal alcohol exposures (Witze, Old, Resing, & Ahn, 2007). The advantage of this method is that it is completely unbiased. Therefore, this method will evaluate the entirety of the retained sperm histone population in order to find differences between our treatment groups. In order to isolate a purified population of histones from mouse sperm samples, optimization of histone acid extraction will need to be performed.

Results

Sperm Isolation from mice.

After concluding a minimum of three matings, males were sacrificed and sperm was collected using a modified swim-up procedure. Briefly, human tubal fluid (HTF) was covered with mineral oil, the dish set in an incubator at 37°C for three hours before males were to be sacrificed. The male reproductive tract was surgically excised and placed into

a petri dish containing warmed fertilization medium. Forceps were used to force the sperm through cuts in the epididymis. Concentrated sperm were placed in 5mL of HTF medium in a polystyrene round bottom tube at a 45° angle in the incubator for 1 hour. After the top 1.5 mls was removed and sperm counted using a hemocytometer for reduced representation bisulfite sequencing (RRBS), the rest of the sperm was layed upon and spun through a Nidacon PureSperm 40/80% mixture, which acts to purify the motile sperm from extraneous cellular debris and seminal plasma through a density gradient. Lastly, the purified sperm pellet was washed in PBS then spun down and flash frozen (Stored at -80°C).

Optimization of histone acid extraction technique in mouse sperm.

When dealing with isolated histones, which comprise less than 5% of all the DNA associated proteins in sperm, it is challenging to maintain a sufficient concentration of protein. Therefore, samples from the independent males needed to be pooled for analysis. Pelleted sperm sample were washed twice with ice cold 1X PBS with a centrifuge speed of 1,500 RPM for 5 minutes. Sperm pellets were then resuspended in 1 mL of somatic cell lysis buffer and incubated on ice for 15 minutes. Sperm pellet samples were then resuspended in 1 mL of hypotonic lysis buffer. Samples were rotated at 4°C for 30 minutes. Nuclear pellets were resuspended in 0.4N H_2SO_4 and vortexed to get into solution. At this point the sample were rotated overnight at 4°C to allow enough time for all the histones to be extracted. The following morning 100% trichloroacetic acid (TCA) (w/v) was added to bring the final concentration to 20%. Samples were allowed to

precipitate for 2 – 4 hours on ice. Pellets were washed twice with acidified acetone and washed two more times with pure acetone. Pellets were allowed to air dry for 20 minutes and resuspended in 100μL H_2O. Samples were left at room temperature for 2 hours to promote resuspension and stored overnight at 4°C to dissolve into solution. Long-term storage was at -80°C. Extracted histones from our sperm extracts were comparable to results obtained previously (Shahhoseini 2010) (**Figure 23B-C**).

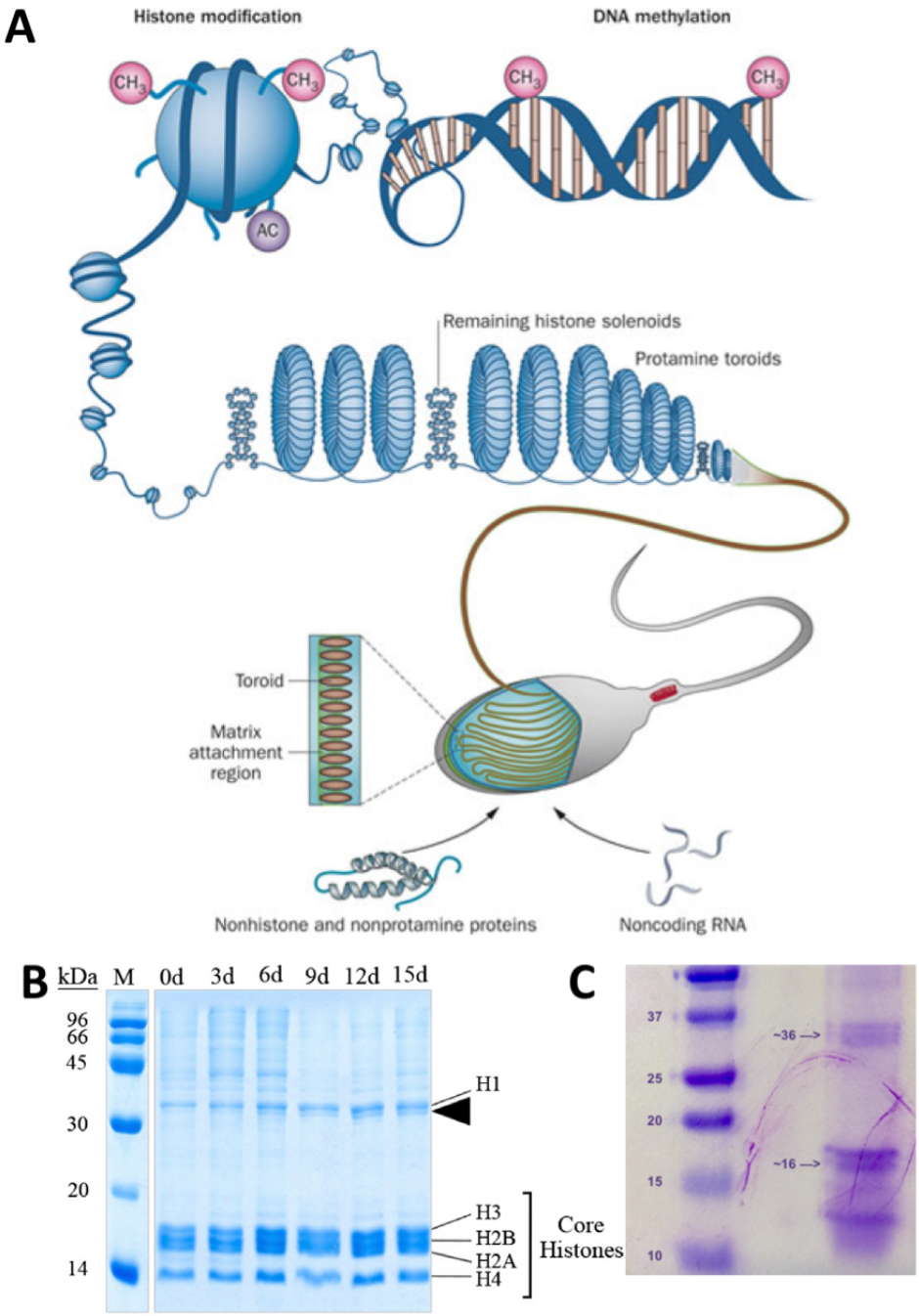

Figure 23. Packaging of DNA in a mature spermatozoa and gel images of successful acid extracted histones from mouse sperm.

A. Overview of sperm epigenetics. Approximately 85% of DNA-binding histones are replaced by protamines during spermiogenesis. Thus, sperm chromatin consists of nucleoprotamines coiled into toroids and attached to matrix attachment regions and nucleohistones coiled into solenoids. Although sperm is known to be a transcriptionally inactive cell, remaining histones are highly acetylated, as well as bearing other modifications such as methylation. As in somatic cells, the DNA in sperm cells is methylated. Sperm also contains non-histone proteins, silent mRNAs (the

function of which is still not known) and noncoding RNAs. Abbreviations: AC, acetyl group; CH3, methyl group. (Schagdarsurengin et al., 2012) **B.** SDS–PAGE pattern of acid-extracted histones of NT2 cells during RA-induced differentiation, at the time points indicated. M, molecular weight marker. The arrow head shows a histone variant with differential expression pattern, identified by mass spectrometry as the H1x variant. (Shahhoseini 2010) **C.** SDS-page gel, stained with comassie blue, from our successfully conducted acid extraction of histones from mouse sperm.

Discussion

There have been very few theories as to how alcohol exposure in adult males can impart an epigenetic signature in sperm capable to transmission to the next generation. Paternal alcohol exposure studies first revealed that alcohol consumption could impact the methylation status of the CTCF binding site in the H19 locus of the offspring providing evidence that DNA methylation could be a means for epimutation propagation. The researchers could only provide evidence of a single altered CpG in the sperm raising questions as to the single CpG's direct contribution to the offspring's observed effects (Knezovich & Ramsay, 2012). Other evidence shows alcohol exposure in the non-pairing region of the Y chromosome that directly regulated POMC expression can acquire aberrant methylation (Chastain & Sarkar, 2017; Sarkar 2016). Due to its physical location on the Y chromosome, it naturally escapes epigenetic reprogramming and therefore transmits its epigenetic signature to the next generation. Providing evidence for an epigenetic transmission to the next generation by inducing alterations in a region that does not experience epigenetic reprogramming at all does not shed any light on the actual nature of the epigenetic mechanisms for transmission. DNA methylation has been shown to be underrepresented (hypomethylated) at retained histone locations providing a potential role

for hypomethylation in maintaining a protamine-free region (Erkek et al., 2013). Aberrant protamine-histone ratios and even protamine 1-protamine 2 protein (P1/P2) ratios have been correlated to infertility and increased DNA fragmentation (Dadoune 1995; Grassetti et al., 2012; Hecht 1990). This provides evidence that incorrect DNA methylation can lead to altered chromatin state more susceptible to DNA damage and potential epimutations (Aoki, Emery, Liu, & Carrell, 2006; Aoki et al., 2005; Torregrosa et al., 2006). While DNA methylation has potential to propagate an epimutation to the next generation, the wave of DNA demethylation that occurs shortly after fertilization is hypothesized to reset these errors raising warranted skepticism to its role in epimutation propagation (Santos et al., 2002).

Research has shown that targeted manipulation of post-translational modifications on retained sperm histones cause detrimental effects such as varying levels of infertility (Jenkins & Carrell, 2011). These histones are not randomly distributed and focused in areas surrounding imprinted gene domains, non-coding RNA clusters and genes crucial in early embryonic development and genome activation. Utilizing a powerful and unbiased technique, mass spectrometry, we will be able to pull back the curtain and view global differences that will shed light on alcohols effects on these post-translational modifications (Anderson et al., 2016). This information will not answer the question, how, but will give researchers a more focused target in which to pursue the mechanisms involved in establishing these modifications (or failure to establish these modifications).

Materials and Methods

Sperm Collection and Analysis

After a minimum of three matings, breeder males were sacrificed and sperm collected using a modified swim-up procedure (Zalenskaya et al., 2000). Briefly, human tubal fluid (HTF) medium (catalog # GMHT-100, Life Global Group) supplemented with 4mg/ml of BSA fraction V (catalog # A5611, Sigma Aldrich,) and 1µl/ml of Gentamicin (catalog # 15750-060, Invitrogen) was covered with mineral oil, the dish set in an incubator at 37°C in a 5% CO_2, 5% O_2, 90% N_2 atmosphere for three hours before males were to be sacrificed. The male reproductive tract was surgically excised and placed into a petri dish containing warmed fertilization medium. Forceps were used to force the sperm through cuts in the epididymis. Concentrated sperm were placed in 5mL of HTF medium in a polystyrene round bottom tube (catalog # 353058, BD Falcon) at a 45° angle in the incubator for 1 hour. The top 1.5 mls were removed and sperm counted using a hemocytometer. This portion of the sperm was used for reduced representation bisulfite sequencing (RRBS) as used in previous chapters. The remainder of the sperm sample to be used for acid extraction of histones was layered upon and spun through a Nidacon PureSperm 40/80% mixture (Nidacon PSK-020), which acts to purify the motile sperm from extraneous cellular debris and seminal plasma through a density gradient. For all experiments, sperm samples were judged to be >99% pure, as assessed by microscopy.

Acid Extraction of Histones

Pelleted sperm sample were washed twice with ice cold 1X PBS with a centrifuge speed of 1,500 RPM for 5 minutes. Sperm pellets are then resuspended in 1 mL of somatic

cell lysis buffer (.1% SDS, .5% Triton X-100) and incubated on ice for 15 minutes. Sperm pellet samples are resuspended in 1 mL of hypotonic lysis buffer consisting of 10mM Tris-HCl (pH 8), 1mM KCl, 1.5mM $MgCl_2$, 1mM DTT, and 1X protease inhibitor. Samples were rotated at 4°C for 30 minutes. Nuclear pellets were resuspended in 0.4N H_2SO_4 and vortexed to get into solution. Rotated overnight at 4°C. 100% TCA (w/v) was added to bring the final concentration to 20%. Samples were allowed to precipitate for 2 – 4 hours on ice. Pellets were washed twice with acified acetone (0.1% HCl in acetone) and washed two more times with acetone with a centrifuge speed of 10,000 RPM for 10 minutes at 4°C. Pellets were allowed to air dry for 20 minutes and resuspended in 100µL H_2O. Samples were left at room temperature for 2 hours and stored overnight at 4°C to dissolve into solution. Long-term storage was at -80°C.

Quantification and Purity Assessment

Quantification of histones was achieved using the Pierce BCA Protein Assay Kit (ThermoFisher 23225) following the standard directions and quantified on a NanoDrop 2000 (ThermoFisher ND-2000). For visualization of bands, samples were run on a 15% SDS-Page running gel with a 4% stacking gel for loading. Gel was washed three times with pure H_2O for 5 minutes each and stained with Imperial Protein Stain for 2 hours on a rocker (ThermoFisher 24615). De-staining was achieved by H_2O wash overnight.

Quantitative Mass Spectrometry

Purified sperm histone samples were flash frozen in liquid nitrogen and shipped overnight on dry ice to Dr. Benjamin A. Garcia's laboratory at the Perelmen School of

Medicine at the University of Pennsylvania for quantitative mass spectrometry assessment.

CHAPTER VII

DISCUSSION AND CONCLUSIONS*

Upon exposure to an environmental stress, an organism's germ cell chromatin structure can be modulated and these changes have the capacity to persist on into later developmental stages. The objective of this research was to determine how common environmental exposures modulate chromatin structure in an effort to better understand the nature of epigenetic change. Furthermore, this body of work sought to elucidate whether environmentally induced alterations in chromatin persist, and are heritable within the offspring of exposed individuals. These subjects are all relevant to better understanding the developmental origins of disease. Our overarching hypothesis was that environmental exposures could induce epimutations in an organism, that these changes have the capacity to persist, and that they would correlate with diseases or disorders in the offspring of the exposed individuals.

To gain a better understanding of the potential impact of oxygen exposures during *in vitro* embryo culture on the regulation of chromatin structure, we evaluated the capacity of differing oxygen concentrations to affect the transcriptional control of chromatin modifying genes and the regulation of imprinted gene expression. Our data indicate that

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exposure to atmospheric oxygen negatively impacts embryonic development and is broadly associated with a reduction in the level of transcripts encoding the major factors driving embryonic patterning. Taken in conjunction with our *in vitro* cell culture model, our data reveal that oxygen has the power to influence the transcriptional control of crucial gene networks involved in the establishment and maintenance of chromatin structure and the control of imprinted genes. Further, these transcriptional changes correlate with altered regulation of gene networks controlling foundational aspects of stem cell pluripotency and lineage identity. However, our results only correlate transcriptional changes with either reduced developmental potential (bovine embryos) or alterations in cellular phenotype (ES cells). Further studies examining the protein levels of select candidates is warranted. Collectively, these data demonstrate that early developmental exposures modify gene expression patterns, in particular, genes responsible for larger epigenetic regulation. Further studies examining the impacts to chromatin states are necessary to determine the full extent of the observed transcriptional changes. While much is left to be unraveled, this data indicates that even transient exposures to atmospheric oxygen may have the potential to dysregulate the transcriptional control of common epigenetic modifiers. The extent to which our findings are relevant to current ART industry practices that commonly expose sperm/oocytes and preimplantation embryos to atmospheric levels of oxygen remain to be determined.

While environmental exposures during both the preimplantation period and in utero development have been heavily studied, the preconception environment has recently been revealed to also be a significant modifier of dysgenesis and the development of

environmentally-induced disease. For example, to date, Fetal Alcohol Spectrum Disorders (FASDs) have been exclusively associated with maternal exposures, yet emerging evidence suggests male-inherited alterations in the developmental program of sperm may be relevant to the growth-restriction phenotypes of this condition. It has already been shown that the level of obesity or diet in the father can affect the sperm epigenome (Craig, Jenkins, Carrell, & Hotaling, 2017), but little research has gone into the effects of paternal alcohol consumption on the sperm epigenome.

Using our mouse model, we found that preconception paternal EtOH exposure associates with fetal growth restriction, as measured on day 14.5 of gestation, as well as proportional increases in the placental weight of female offspring (reduced placental efficiencies). These phenotypes are accompanied by alterations in imprinted gene expression, and other indicators of compromised epigenetic programming. Importantly, our observations are similar to work examining the periconceptional period in females, where maternal exposure to alcohol is also able to yield fetal growth deficits similar in magnitude to those reported in rodent models of *in utero* exposure (Gundogan et al., 2008). Collectively, these studies reveal preconception and periconception alcohol exposure to be a significant, yet under recognized contributor to FASD biology and an important factor in the broad variation of FASD growth and neurocognitive defects.

One common element to emerge from epigenetic focused studies of both preconception and gestational models of EtOH exposure has been disruptions in the transcriptional control of imprinted genes (Finegersh & Homanics, 2014; Knezovich & Ramsay, 2012; Liang et al., 2014; Ungerer, Knezovich, & Ramsay, 2013). Similar to these

works, we also observed altered expression of imprinted genes in our preconception male model of exposure, including Gnas, Grb10 (males) and Sgce (females) in the placenta, as well as Cdkn1c, Dcn, Gnas (males), and Meg3 in the fetal liver. Cdkn1c, Dcn, Gnas, and Grb10 are all expressed predominantly from the maternally-inherited allele and act to regulate both fetal and placental growth (Coan et al., 2005; Piedrahita 2011). Meg3 is a maternally expressed non-coding RNA and in the liver, its over-expression has been associated with the inhibition of stellate cell activation and liver fibrosis (He et al., 2014). However, in our model, all of these candidate genes exhibited decreased expression with no evidence of inappropriate contributions from the normally silent paternal allele. Moreover, using methyl-seq with an average of 90 million reads per sample, we could find no evidence to suggest that these, or any other transcriptional changes, were due to alterations in the sperm-inherited DNA methylation profiles. However, we cannot rule out the possibility that a higher dose of alcohol could influence the profile of sperm-inherited DNA methylation.

Although previous reports examining alcohol-exposed sperm have identified alterations in DNA methylation within imprinted loci, these changes tend to be very modest (Knezovich & Ramsay, 2012; Liang et al., 2014; Ouko et al., 2009). For example, Knezovich et al. reported EtOH-dependent decreases of 1.75% for CpG # 7 within the H19 regulatory region and a 2.2% decrease for CpG # 10 within the Snrpn locus. Similarly, Liang and colleagues identified a 3--6% EtOH-dependent decrease within the H19 regulatory region, while the Peg3 regulatory region displayed a 7% increase. These reported changes tend to be very minor and often only involve single cytosines, while the

levels of DNA methylation for the overall loci remain unchanged (Knezovich & Ramsay, 2012; Ouko et al., 2009). Each sperm carries a single haploid genome and a change of 10% or less suggests that an incredibly small proportion of the total population was impacted. Yet, each of these cited studies demonstrate measurable changes in offspring growth or behavior across the total population (Bielawski et al., 2002; Knezovich & Ramsay, 2012; Liang et al., 2014). Given that the profiles of EtOH-exposed sperm reported in both these studies, as well as our genome-wide analysis, exhibited less than a 20% overall change, it is very challenging to reconcile how the observed differences in sperm DNA methylation, especially at single CpG resolution, could influence the resulting phenotypes. Further, while changes in the somatic DNA methylation profile of the resulting offspring have been reported, they again tend to be very modest (less than 10%) and cannot be correlated with alterations in the transcriptional control of affected genes (2014). Previous studies examining the transmission of metabolic dysfunction through sperm have provided evidence to both support and refute the involvement of sperm-inherited changes in DNA methylation in the transmission of paternally-inherited phenotypes (Carone et al., 2010; Radford et al., 2012; Shea et al., 2015) . Collectively, our results, along with previously published data, (Bielawski et al., 2002; Knezovich & Ramsay, 2012; Liang et al., 2014) strongly suggest a DNA methylation independent mechanism in the transmission of epigenetic errors induced by preconception paternal alcohol exposure.

Recently, it has been revealed that mature sperm carry populations of non-coding RNAs (ncRNAs) that include microRNAs (miRNAs), transfer RNA fragments and

endogenous short interfering RNAs (Yuan et al., 2016). Interestingly, these ncRNAs appear to have functional roles during embryonic development and the profile of sperm-inherited RNAs can be modulated by paternal diet (Chen et al., 2016; Sharma et al., 2016). Many ncRNAs, including miRNAs, are influenced by alcohol exposure and have roles in the development of FASD phenotypes (Miranda et al., 2010). As previous studies have identified a link between paternally inherited miRNAs and stress-induced phenotypes in the offspring (Gapp et al., 2014), an important next step will be to determine if alcohol exposure can impact the ncRNA population of sperm, and if these RNAs represent the transmissible epigenetic factor driving development of the observed growth restriction phenotypes.

Conclusions

Despite the implementation of numerous community outreach and educational programs, alcohol-related birth defects remain a significant public health concern, and a tremendous economic burden (Roozen et al., 2016). The identification of one of the defining FASD-associated defects in an established mouse model of preconception male exposure strongly suggest the lifestyle choices of the father are extremely relevant to the genesis of this debilitating disorder. Given that 70% of men drink and 40% drink heavily (Naimi et al., 2003), further investigation is clearly warranted.

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APPENDIX

Gene	*SNP Location*	*Increased Contribution of Silenced Allele?*
Ascl2	chr7:142,968,971	No
Cdkn1c	chr7:143,460,109	No
Dcn	No SNP found	-
Dlk1	chr12:109,460,379	No
Gatm	chr2:122,594,926	No
Gnas	No SNP found	-
Grb10	chr11:11,954,907	No
Gtl2	chr12:109,545,837	No
H19	chr7:142,577,095	No
Igf2 (1)	chr7:142,652,037	No
Igf2 (2)	chr7:142,652,936	No
Igf2r	chr17:12,682,709	No
Mest	chr6:30,747,382	No
Ndn	chr7:62,348,457	No
Peg3 (1)	chr7:6,706,207	No
Peg3 (2)	chr7:6,706,217	No
Sgce	chr6:4,717,926	No
Slc38a4	chr15:96,995,974	No
Snrpn	chr7:59,983,415	No
Ube3a	chr7:59,228,878	No
Zac1	chr10:13,128,934	No
Zim1	chr7:6,675,637	No

Table 1: Analysis of single nucleotide polymorphisms within the encoded mRNA sequence of genes regulated by genomic imprinting.
For RNA sequence-based comparisons of allelic patterns of imprinted gene expression, the proportion of identified single nucleotide polymorphisms were analyzed using either Chi-Square or, if read counts were less than 5, a Fisher's Exact test.

IMPRINTED GENE	DIFFERENTIAL METHYLATION DETECTED? (**LIVER**)	DIFFERENTIAL METHYLATION DETECTED? (**PLACENTA**)
ASCL2	**No**	**No**
CDKN1C	**No**	**No**
DCN	**No**	**No**
DLK1	**No**	**No**
GATM	**No**	**No**
GNAS	**No**	**No**
GRB10	**No**	**No**
GTL2	**No**	**No**
H19	**No**	**No**
IGF2	**No**	**No**
IGF2R	**No**	**No**
MEST	**No**	**No**
NDN	**No**	**No**
PEG3	**No**	**No**
SGCE	**No**	**No**
SLC48A4	**No**	**No**
SNRPN	**No**	**No**
UBE3A	**No**	**No**
ZAC1	**No**	**No**
ZIM1	**No**	**No**

Table 2: Detection of differentially methylated CpG residues in either the base and/or tile (1000bp) levels of select imprinted gene.
Detection of differentially methylated loci (Individual base and/or tile) for Methyl-Seq data sets at determined through the MethylKit package (R). Differentially methylated cytosine cutoff (Methylation difference ≥ 25%, and q-value less than 0.01). Differentially methylated regions (1Kb non-overlapping tiling window) cutoff (Methylation difference ≥ 25%, and q-value less than 0.01).

Sperm - Differential Methylation Annotation: CpG - Tiles (1000bp)
Genes with nearest TSS

Filter

5X coverage minimum *qvalue 0.01 cutoff*
pvalue 0.05 cutoff *meth.diff > 25%*

ACCESSION	GENE ID	ACCESSION	GENE ID	ACCESSION	GENE ID	ACCESSION	GENE ID
NR_015573	1700012D14Rik	NM_001115087	Fancf	NM_172563	Hlf	NM_001080771	Prdm13
NR_045435	2010106C02Rik	NM_008011	Fgfr4	NM_177265	Ifitm10	NM_001110146	Prg4
NR_040655	4930556G01Rik	NR_045156	G630093K05Rik	NM_183144	Inpp5a	NM_008976	Ptpn14
NR_046208	4933436H12Rik	NR_104449	Galnt9	NM_176922	Itga11	NM_001252455	Ptprs
NR_030708	6820431F20Rik	NM_008107	Gdf1	NM_008417	Kcna2	NM_173184	Rln3
NM_008992	Abcd4	NM_182696	Ggn	NM_001291222	Kif7	NM_027187	Rnaseh2a
NM_175086	Agtr1b	NR_040280	Gm12295	NM_021366	Klf13	NM_178280	Sall3
NM_001080933	Ankfn1	NM_177843	Gm14461	NM_001039653	Lhx3	NM_175155	Sash1
NM_080434	Apoa5	NM_001033774	Gm1661	NM_011698	Lin7b	NM_001243072	Sema3a
NM_001033436	Atxn7l1	NR_030701	Gm20554	NM_027400	Lman1	NM_029100	Sepn1
NM_009635	Avil	NM_001270553	Gm21119	NM_010723	Lmo4	NM_001164355	Ska1
NM_001164186	Barhl1	NR_131058	Gm27162	NM_013589	Ltbp2	NM_177388	Slc41a2
NR_040670	BC039966	NR_110488	Gm38509	NM_008520	Ltbp3	NM_173388	Slc43a2
NR_040459	C230079O03Rik	NR_045285	Gm5083	NM_016693	Map3k6	NM_001161431	Slitrk2
NM_178418	Ccdc144b	NR_033461	Gm9999	NM_001083341	Mboat2	NM_001311070	Smad2
NM_026222	Ccdc39	NM_026240	Gramd3	NM_001290489	Med27	NM_025664	Snx9
NM_146239	Cdk17	NM_026630	Gtsf1l	NM_027212	Med30	NM_146064	Soat2
NM_153599	Cdk8	NR_132443	Hist1h2al	NM_023403	Mesdc2	NM_029367	Spaca3
NM_028083	Chaf1b	NM_172563	Hlf	NM_040642	Mipep	NM_001166064	Syde2
NM_178078	Cnot1	NM_177265	Ifitm10	NR_037292	Mir3105	NM_001252342	Syt1
NM_007760	Crat	NM_183144	Inpp5a	NR_030150	Mir466	NM_172443	Tbc1d16
NM_001310635	Crispld2	NM_176922	Itga11	NR_105833	Mir6406	NM_011552	Tcof1
NM_027344	Ctsll3	NM_008417	Kcna2	NR_106069	Mir7210	NM_011902	Tekt2
NM_026648	Dnaaf1	NM_001291222	Kif7	NM_001111016	Nav2	NM_009354	Tert
NM_172708	Dok7	NM_021366	Klf13	NM_010897	Nf1	NM_001304439	Tmem132e
NM_001267626	Dpf3	NM_001039653	Lhx3	NM_027116	Nkpd1	NM_177260	Tmem154
NM_001177625	Ect2	NM_011698	Lin7b	NM_008746	Ntrk3	NM_009396	Tnfaip2
NM_027446	Eef2kmt	NM_027400	Lman1	NM_177028	Oacyl	NM_009408	Top1
NM_023479	Elac2	NM_010723	Lmo4	NM_001164497	Papd5	NM_009464	Ucp3
NM_001163522	Emcn	NM_013589	Ltbp2	NM_020606	Parva	NM_030215	Wrnip1
NR_104386	Esr2	NM_008520	Ltbp3	NM_024229	Pcyt2	NM_001025594	Zbtb43
NM_011934	Esrrb	NM_016693	Map3k6	NM_001003948	Pid1	NM_145492	Zfp521
NM_001143802	Fam196a	NM_026630	Gtsf1l	NM_009090	Polr2c	NM_144516	Zmynd11
NM_001033980	Fam92b	NR_132443	Hist1h2al	NM_001164082	Polr3d		

Table 3. Differential methylation annotation in sperm of CpG. Analysis compares 1000bp tile sections at determined through RStudio package, MethylKit. Annotated genes determined by the proximity of their transcriptional start site (TSS) to the 1000bp tile section. 5X Coverage minimum, *P*-value cutoff (0.05), q-value cutoff (0.01), methylation difference > 25%.