THE EPIGENETIC EFFECTS OF PRECONCEPTION PATERNAL ALCOHOL EXPOSURE ON ADULT HEALTH AND DISEASE

A Dissertation

by

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ABSTRACT

Alcohol is a notorious teratogen and a major driver of both mental and physical defects. Recently, alcohol has been discovered to exert intergenerational effects on offspring development. While maternal exposure to alcohol in-utero has been linked to the development of fetal alcohol spectrum disorders, paternal contributions to this disorder remain poorly understood. Emerging evidence suggest the association of paternal environmental exposures and long-term metabolic dysfunction1,2. Using a mouse model, our preliminary studies have identified an association between preconception paternal alcohol use and deficits in both the prenatal and postnatal growth of the offspring. These growth defects are accompanied by altered transcriptomic profiles in the fetal liver at gestation day (GD) 14.5, which persists into the adult stages. In this study, we will examine the mechanism by which paternal alcohol exposure drives the development of prenatal growth retardation and long-term postnatal growth restriction in the offspring, as well as the abnormal metabolic response of the offspring to dietary challenge.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Professor Michael Golding of the Department of Veterinary Physiology and Pharmacology, Professor Robert Burghardt of the Department of Veterinary Integrative Biosciences, Professor Stephen Safe of the Department of Veterinary Physiology and Pharmacology, and Professor Rajesh Miranda of the Department of Neuroscience and Experimental Therapeutics. All other work conducted for the thesis (or) dissertation was completed by the student independently.

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CHAPTER I

INTRODUCTION

Epigenetics

Traditional models depicting the control of gene expression have focused on a "DNA-RNA-Protein" axis that contains two regulatable steps named transcription and translation. However, in this model, the concept that "DNA produces RNA, which produces protein" has not been able to explain instances where gene expression was absent despite the presence of the requisite protein factors necessary to drive the biochemical process of transcription. Thus, it was that researchers began to recognize the importance of non-sequenced based information and the initial rise of epigenetics.

Broadly speaking, epigenetics refers to the study of changes in organism phenotype caused by the modification of gene expression rather than alteration of genetic code itself. In 1999, Dr. Alan Wolffe first proposed that heritable changes to the chromatin fiber, including DNA methylation and chemical modifications of histones, could heritably modify gene expression without a changing the DNA sequence [1-3]. In his pioneering work, Wolffe emphasized that the chromatin template possessed an innate ability to induce transcriptional repression and that this mechanism was a basic component to the transcriptional control of gene expression that suppressed those genes that are 'unnecessary' to a specific cellular phenotype. For instance, his work demonstrated that DNA methylation in promoter regions correlated negatively with gene expression, and that heterochromatin showed a transcriptionally silent condition[1-3]. Since this early work, several distinct epigenetic processes have been described and have been broadly categorized into three core mechanisms that include: DNA methylation, post-translational chromatin modification, and noncoding RNA-mediated gene silencing.

DNA Methylation

DNA methylation refers to the addition of a methyl group to the number 5-carbon position of the DNA base cytosine through a reaction catalyzed by the DNA methyltransferase (DNMT) family of enzymes. Cytosine bases in a cytosine-phosphate-guanine (CpG) dinucleotide arrangement, that exist in clusters and are referred to as CpG islands, are often the target of these methylating enzymes. In the earliest studies, gene silencing was believed to be the biological consequence of DNA methylation and was identified in both plants and animals[4]. In these early models, DNA methylation blocked the binding of transcription factors, which were hypothesized to interact with CpG dinucleotides in the regulatory regions of genes. For instance, binding of Sp1, which is a zinc finger transcription factor that binds to GC-rich motifs found in the regulatory elements of many promoters involving a diverse range of cellular processes, is blocked by DNA methylation[5]. By blocking access of transcription factors, the recruitment and assembly of the polymerase II complex is impeded. In addition, transcription factors are known to influence nucleosome stability and positioning over the site of transcriptional initiation. Today, the role of DNA methylation has been recognized as complex, with the large majority of this epigenetic modification found in both silent transposable elements as well as within the gene bodies of highly transcribed genes[6]. Therefore, DNA methylation has context-specific roles that extend beyond an association with gene silencing.

Chromatin Modification

Chromatin is the protein multimolecular complex bound to genomic DNA that forms a fundamental unit called a nucleosome. The structure of chromatin is now recognized as a "language" that confers epigenetic information through the cell cycle and in select instances, onto the next generation. The concept of the "histone code" was first proposed by Dr. David Allis and

suggested that gene expression is regulated by the combinations of N-terminal post-translational modifications to histone tails, including acetylation, methylation, phosphorylation, ubiquitination, SUMOylating, and ADP-ribosylation[7]. To exert these covalent post-translational modifications (PTMs) to histone proteins, histone-modifying enzymes, chromatin remodeling factors, transcriptional factors, and co-regulators join chromatin complexes to both impart and maintain epigenetic states[8]. Acetylation of histones H3 and H4 by histone acetyltransferase (HATs) associate with increases in gene expression and correlate with an open and accessible chromatin structure. Similar to the processes described in DNA above, histone methylation is performed by methyltransferase enzymes (histone methyltransferases - HMTs), which transfer methyl groups from the donor molecule S-adenosyl-L-methionine onto lysine or arginine residues found in histone proteins. Histone methylation has been associated with both active and silent chromatin states and depends on the specific residue of the histone tail[9]. Several different histone methyltransferases have been identified, many of which have specificity towards a single lysine or arginine residue in the histone tail. As examples, in mammalian cells, SET1, SET7/9, Ash1, ALL-1, MLL, ALR, Trx, and SMYD3 are histone methyltransferases that catalyze methylation of histone H3 at lysine 4 (H3-K4). ESET, G9a, SUV39-h1, SUV39-h2, SETDB1, Dim-5, and Eu-HMTase catalyze methylation of histone H3 at lysine 9 (H3-K9). G9a and polycomb group enzymes such as EZH2 are histone methyltransferases that catalyze methylation of histone H3 at lysine 27 (H3-K27)[10]. Both H3-K9 and H3-K27 methylation enhance heterochromatin formation and are associated with silent chromatin states found in euchromatic regions.

The phosphorylation of histones is highly dynamic, and is targeted predominantly to serine, threonine and tyrosine residues, and adds a significant negative charge to the modified histone proteins, which affects the interaction of nucleosomes with DNA[11]. However, comparatively less

is known regarding the recruitment and function of histone phosphatases on chromatin. For example, mammalian MAPK1 possesses an intrinsic DNA-binding site to tether DNA but the consequence of this interaction on gene function remains poorly described[12].

Histone ubiquitylation is unlike the abovementioned histone modifications since ubiquitin, a 76-amino acid polypeptide, results in the addition of a large covalent side chain on lysine residues and is carried out by E1-activation, E2-conjugating and E3-ligating enzymes[13]. This modification may be removed by isopeptidases called de-ubiquitin enzymes and leads to both gene activity and silencing, depending on the context[14]. Similar to ubiquitylation, SUMOylation involves the covalent attachment of small ubiquitin-like molecules to histone lysine through the series of action of E1, E2, and E3 enzymes. SUMOylation has been detected on all four core histones to function by antagonizing both acetylation and ubiquitylation[15,16]. Consequently, it has been associated with repressive functions, but more evidence is needed to answer the molecular mechanism(s) through which SUMOylating exerts its effect on chromatin. Histones are known to be mono- and poly-ADP ribosylated on glutamate and arginine residues on all four core histones, as well as on the linker histone H1. However, the function of this modification is not clear. Poly-ADP-ribose polymerase (PARP) controls the levels of poly-ADP ribosylated histones and its activity is correlated with a relatively relaxed chromatin structure[17].

Noncoding RNA Silencing

In addition to the well-described phenomenon of DNA methylation and post-translational histone modifications, non-coding RNAs have recently been linked to epigenetic mechanisms of transcriptional regulation. Non-coding RNAs are defined as a group of RNAs that do not encode functional proteins and were originally thought to only regulate gene expression through post-transcriptional mechanisms. Surprisingly, a wide variety of recent studies demonstrate that

miRNAs, endogenous siRNAs, piRNAs, and long non-coding RNAs exert epigenetic control over chromatin structure.

miRNAs

miRNAs are approximately 19-24 nts single stranded RNAs. In the human genome, the development of high-throughput sequencing technologies has identified almost 1,800 putative miRNAs to date[18-20]. The current model of miRNA regulated gene expression is the complementarity between miRNA, target mRNA, and the loading protein Argonaute (AGO) to exert a transcriptome silencing[21].

Among more than 13,000 human annotated genes, histone methyltransferases, methyl CpG binding proteins, and histone deacetylases have been reported as potential targets for miRNAs[22]. Therefore, miRNAs can affect Chromatin status by altering chromatin remodeling enzyme activity. For example, miR-140 targets histone deacetylase 4 (HDAC4) in mouse embryonic cartilage tissue[23,24]. In addition, DNMT3a and DNMT3b have been suggested to be targets of the miR-29 family, which consequently affect DNA methylation[25-27]. In addition to indirect effects, miRNAs have also been reported to modify chromatin structure directly, although the mechanisms through which this occurs remain poorly defined[28].

siRNAs

Endogenous siRNAs are derived from long double-stranded RNA, which is cut by the Dicer into RNA fragments of 19-24 nts. Similar to miRNAs, the AGO protein is necessary to serve as the platform exercising the function of siRNAs to silence gene activity[29,30].

Piwi-interacting RNAs

Piwi-interacting RNAs (piRNA) are approximately 20-31 nts in length and are originally found to bind to Piwi proteins under physiological conditions[31,32]. As for their role in epigenetic

regulatory processes, the Piwi protein-RNA complex silences the homeobox gene cluster by binding to genomic PcG response elements and recruits poly-comb group proteins (PcGs)[33]. In addition, Piwi protein/piRNA complex mediate the methylation of transposons in germ cells (Kuramochi-Miyagawa et al., 2008). However, in contrast to gene silencing, piRNAs have also been found to promote euchromatic histone modifications in Drosophila melanogaster[34]. Therefore, piRNAs exert diverse epigenetic effects.

LncRNAs

Unlike small noncoding RNAs, long noncoding (LncRNAs) are generally larger than 200 nt in length and exert well-characterized epigenetic functions, including the silencing of genes regulated by genomic imprinting, as well as in heterochromatin formation during X chromosome inactivation[35]. The best-characterized noncoding RNA is H19, which is a lncRNA transcribed from the imprinted IGF2-H19 locus. H19 is transcribed, spliced, polyadenylated, and then translocated to cytoplasm. However, the function of H19 remains unclear despite that fact that H19 is the first lncRNA to be associated with genomic imprinting. Recent studies suggest the epigenetic function of H19 RNA may also be related to an enhanced small RNA silencing mechanism by producing a miRNA precursor such as miR-675[36,37]. Another well-studied lncRNA is Xist RNA, which is 17 kb long. Unlike H19 RNA, Xist stays in the nucleus and interacts with the X chromosome to physically coat the surface of the X chromosome[38]. A study reported that a 1.6 kb fragment of Xist, named RepA, recruits the Polycomb repressive complex 2 (PRC2) to induce H3K27 trimethylation and inactivate the X chromosome[39].

Developmental Origins of Health and Disease

The Developmental Origins of Health and Disease (DOHaD) hypothesis was proposed by Barker and colleagues based on a set of studies related to correlations observed between infant

birth weights and adult disease epidemiology[40-43]. Here, under-nutrition at different stages of gestation were linked to a variety of adult disease phenotypes, including cardiovascular disease and type II diabetes[42]. In addition to nutritional deficits during fetal development, other prenatal maternal stresses and disruption of the maternal-placental-fetal axis have now been included in the DOHaD approach. For example, a growing body of studies proposes that maternal psyconeuroendocrine processes, cardiovascular, metabolic, and behavioral deficits all associate with fetal growth restriction, which in turn can be linked to long-term effects on offspring health[44-48].

Epigenetics in DOHaD

To understand how these early life experiences exert a long-term effect on the life course of the offspring, Callinan and Feinberg proposed that epigenetic mechanisms of transcriptional control are programmed to alter offspring phenotype. Specifically, they proposed that "the study of heritable changes other than those in the DNA sequence that encompass two major modifications of DNA or chromatin: DNA methylation, the covalent modification of cytosine, and post-translational modification of histones including methylation, acetylation, phosphorylation and SUMOylation" [49]. were responsible for the phenotypic changes observed in the offspring. Later, Gluckman et al summarize the link between mechanisms for developmental plasticity described in the DOHaD approach and the critical epigenetic processes of DNA methylation and histone modification[50]. Here they specifically cited a role for imprinted genes and genes with metastable epialleles (MEs) that may be modified epigenetically during differentiation and result in alterations to offspring health and growth[51].

Imprinted Genes

Imprinted genes are expressed exclusively from either the paternally or maternally inherited allele, while the opposite allele is silent. This epigenetic mechanism effectively renders the expression of imprinted genes to be functionally haploid and is associated with heritable patterns of DNA methylation. Metastable epialleles (MEs) are non-imprinted genes but have one or both alleles epigenetically regulated and result in varying levels of gene expression[52]. Interestingly, despite potential drawbacks associated with a haploid gene compared with a diploid gene that has a backup copy to be protected from "single-hit" effects of DNA damage, a large number of genes are predicted to be monoallelically expressed, and are proposed to have potential evolutionary benefits as the product of positive Darwinian selection[53]. Recent DOHaD studies suggest that a suboptimal early-life environment can lead to permanent functional changes in certain organs, which associate with an increased risk of developing metabolic syndromes in later life[54-57]. In these studies, imprinted genes and metastable epialleles are suspected to drive the development of these functional changes. However, at this point, only correlations between abnormal expression patterns and disease can be identified.

Fetal Metabolic Syndrome in DOHaD

Metabolic syndromes have been a critical challenge for public health due to the associated medical conditions such as obesity, hypertension, dyslipidemia, non-alcoholic fatty liver disease, hyperglycemia, and insulin resistance[58,59]. The very first key epidemiological study indicating increases of fetal metabolic syndrome-related to alterations in developmental programming were drawn from data gathered from patients that had survived the famine of the Dutch Hunger Winter[60-62]. Supporting these studies, risk factors for metabolic syndromes correlated with a range of pre-, peri-, and post-natal insults that included maternal malnutrition, smoking, maternal obesity, and in

utero exposure to endocrine-disrupting chemicals[63]. In these patient cohorts, an association between low birth weights and different phenotypes of metabolic syndromes, including type-2 diabetes, hypertension, and insulin resistance has been observed. Collectively these data reveal that neonates with low birth weight and who experienced a rapid catch-up growth have an approximate 80% increased risk for development of cardiovascular disease[64]. Although the pathogenic mechanisms remain poorly defined, certain programming mechanisms such as an impaired nitric oxide system, a heightened state of oxidative stress, and chronic inflammation have been proposed as links between the poor fetal and infantile environment and the risk of metabolic syndrome development in later life[65,66].

Maternal Contribution in DOHaD

According to the Pregnancy Risk Assessment Monitoring System (PRAMS), maternal obesity has increased across all categories of age, race, and education. PRAMS data warn that two-thirds of women were not able to gain weight within the recommended range suggested by The Institute of Medicine (IOM)[67]. Racial and ethnic factors are also reported to affect weight gain during pregnancy according to Brawarsky and colleagues. For instance, African American women are more likely to be overweight during pregnancy while white females and Hispanic women were likely to report target gains. Asian women were likely to gain less than PRAMS and IOM recommended weight gain during pregnancy[68]. These increased rates of obesity are now suspected to be a negative modulator of epigenetic programming in the next generation.

Maternal Obesity

Maternal obesity results in a range of negative outcomes for both mothers and their fetuses[69]. Maternal risks during pregnancy include gestational diabetes and preeclampsia, while the fetus may suffer from intrauterine fetal demise and congenital anomalies. Obesity in pregnancy

can also affect health later in life for both mother and child. For women, these risks include increased risks of heart disease and hypertension. In addition, a recent meta-analysis revealed that obese pregnant women have heightened risks of stillbirth that is twice that of women with normal BMI[70,71]. As for the effects of maternal obesity on fetal development, heavier women are less likely to have a pregnancy complicated by a small-for-gestational-age infant or intrauterine growth restriction which appears to disappear as the maternal BMI reaches obesity level (> 30 kg/m^2)[72]. Meta-analysis reveals a dose-dependent relationship between maternal obesity and fetal macrosomia, which is defined as an estimated fetal weight of greater than or equal to 4500 g[71,72]. Maternal obesity is also associated with an increased risk of neural tube defects (NTD) in the offspring. Specifically, Watkins and colleagues proposed that a 1 kg/m2 increase in BMI is correlated with a 7% increased risk of NTD in the offspring[73-75]. The abovementioned evidence suggests that the incidence of maternal obesity continues to increase and has major public health implications. Therefore, not only is the long-term health of the woman impacted by obesity, but the life-long health and well-being of the child are negatively impacted with the offspring displaying increased risks of childhood obesity and diabetes.

Preconceptional Paternal Insults and Transgenerational inheritance

Emerging studies indicate that the father's lifestyle and exposure history are an unappreciated element in determining the long-term health and development of offspring[76]. In rodents, changing the quantity or quality of a male's diet at various developmental time points has been found to induce phenotypic changes in the male's offspring. For example, males exposed to prenatal dietary restriction sire offspring with reduced birth weights and impaired glucose tolerance compared to controls (Jimenez-Chillaron et al., 2009). Similarly, male mice fed a low protein diet sire offspring with altered expression of genes associated metabolism, cholesterol

synthesis and display alternations in insulin sensitivity (A. J. Watkins et al., 2018; A. J. Watkins et al., 2017).

Paternal Lifestyle and It Transgenerational Effects

In addition to paternal nutrition, paternal smoking prior to pregnancy has been reported to be associated with an increased risk of leukemia in the offspring[77], suggesting that smoke-induced genetic or epigenetic changes occur in sperm that are transmitted to offspring. Paternal stress has also been linked to altered epigenetic programming in paternal germ cells and disruption in the hypothalamic-pituitary–adrenal stress axis response of the offspring[78,79]. Finally, paternal exposure to prolonged periods of cold increases brown adipose tissue activity in offspring via epigenetic programming of sperm[80]. Therefore, sperm-inherited changes in epigenetic programming are emerging to be just as relevant as those induced by maternal exposures.

Paternal Obesity and It Transgenerational Effects

Obesity has reached epidemic proportions worldwide and especially in the US. As described above, maternal obesity is known to predispose the offspring to metabolic disorders. Importantly, intergenerational transmission has also been observed in cases of paternal obesity, with select studies describing multigenerational effects on the metabolic and reproductive health of the offspring[81]. Rodent models have suggested that preconception paternal exposure to a high-fat diet (HFD) significantly impairs the metabolic health of subsequent generations[82-85].). In addition, HFD induced epigenetic disturbances in sperm have been linked to metabolic co-morbidities in the subsequent generation[83,86]. These studies suggest that paternal nutrition prior to fertilization may trigger the intergenerational transmission of metabolic and reproductive traits to descendants. Although the underlying mechanisms of paternal programming of the offspring

phenotype remain unknown, a growing body of evidence support the hypothesis that epigenetic processes in sperm may act as potential mediators of these transgenerational effects[87-89].

Sperm Noncoding RNA and Its Role in Paternal Inheritance

It is believed that mammalian sperm carries small noncoding RNAs (sncRNAs) into oocytes during fertilization but it remains unclear whether these sperm-borne sncRNAs contribute to the transgenerational memories induced by paternal insults. Yan and colleagues employed male germline-specific Dicer or Drosha conditional knockout mice to demonstrated a sperm phenotype with a reduced motility and altered miRNA and endo-siRNA expression profiles[90,91]. Importantly, oocytes fertilized by spermatozoa with aberrant small RNA profiles displayed reduced preimplantation development potential[91]. Using a mouse model, Zhou and colleagues reported that sperm tsRNAs play a critical role in transmitting intergenerational metabolic disorders induced by paternal high-fat-diet exposure[92]. Sarker and colleagues recently observed increased expression of sperm tsRNAs in the F1 male offspring born to HFD-exposed dams and demonstrated that these sperm derived tRNAs small RNAs (tsRNAs) partly contribute to the transmission of the phenotypes observed in the offspring, and importantly, are independent of sperm changes in the sperm methylome[93]. Importantly, these small RNAs are transferred to sperm from secreted vesicles called epididymosomes during the transit of sperm through the epididymis[94] and are not imparted during germ cell formation.

Paternal Alcohol Consumption and It Transgenerational Effects

Alcohol is common and prevalent teratogen. In the United States, 6 to 17 children per 1000 live births are diagnosed with fetal alcohol spectrum disorder (FASD)[95]. FASDs are characterized by a spectrum of developmental and behavioral effects and importantly, offspring display life-long growth retardation[95,96]. While maternal exposure to alcohol in-utero is, undoubtedly, a significant

contributing factor to FASDs, several independent studies have emerged indicating a link between chronic alcohol consumption in males and both cognitive and growth defects consistent with those of FASDs[76,97]. Although studies suggest fetuses sired by males exposed to alcohol suffered abnormal organ development and or neurocognitive development[98], the mechanisms by which paternal exposures influence and impact offspring phenotype remain to be identified. Therefore, the ability of preconception male ethanol exposure to impact developmental programming in the male germline and contribute to the lasting growth deficits associated with FASDs remains poorly defined.

Central Hypothesis

While maternal exposure to alcohol in-utero has been linked to the development of fetal alcohol spectrum disorders, paternal contributions to this disorder remain poorly understood. **We hypothesize that fetuses sired by males exposed to alcohol prior to conception will suffer prenatal growth restriction and abnormal metabolic control.** In the following studies, we seek to identify the mechanism by which male preconception alcohol exposure exerts an impact on offspring health. The long-term research goal of this study is to determine the mechanism by which paternal alcohol exposure influences fetal development and alters long-term metabolic programming. Here, we will use a mouse model to examine mechanisms by which paternal alcohol exposure influences offspring health and response to a dietary challenge.

Aim 1: Determine the effects of preconception paternal alcohol consumption on offspring growth. This Aim will evaluate the impact of chronic preconception paternal alcohol exposure on fetal growth and placental function. We will examine the GD 14.5 time point, a period two thirds of the way through gestation. We will conduct transcriptomic analysis of the fetal liver to

determine the impact paternal alcohol use has on the fetal developmental program. The data from this Aim were published in the journal *Epigenetics*.

Aim 2: Determine the effects of preconception paternal alcohol consumption on postnatal growth and metabolic function. In Aim 2, we will determine the effects of paternal preconception paternal alcohol consumption on the long-term growth and metabolic health of the offspring. We will follow postnatal growth from birth to eight-weeks of life and assay metabolic function (glucose tolerance, insulin tolerance) at this time. These data were published in the journal *Epigenetics & Chormatin*.

Aim 3: Determine the influence of paternal alcohol consumption on offspring response to a high-fat-diet challenge. High-fat-diet challenge is known to exert extremely negative impacts on hepatic function and metabolic health. This Aim will determine the impact preconception paternal alcohol consumption has on offspring response to a high-fat dietary challenge. We will examine base metabolic and immune signaling functions of the offspring of alcohol-exposed fathers after undergoing a 12-week high-fat-diet challenge. We will conduct glucose and insulin stress tests and measure components involved in both the insulin and inflammatory signaling pathways, to determine the potential crosstalk between these two systems and their role in mediating these paternally-inherited phenotypes. These data have been submitted to the journal *Molecular Metabolism*.

Aim 4: Determine the role of sperm-inherited noncoding RNAs in the transmission of the alcohol-induced growth phenotypes. Aim 4 will begin to assay the molecular mechanisms by which the epigenetic memory of paternal alcohol exposure transmits through sperm to the offspring. Specifically, we will evaluate the role-sperm-inherited noncoding RNAs have in mediating the observed intergenerational effects. Total RNAs will be isolated from alcohol-

exposed sperm, size selected and small RNAs sequenced. Reads will be mapped to small RNA databases and differences in miRNA, piRNA, tRNA, tRNA-derived small RNAs assayed. This dataset has been published in the journal *Reproductive Toxicology*.

CHAPTER II

DNA METHYLATION-INDEPENDENT GROWTH RESTRICTION AND ALTERED DEVELOPMENTAL PROGRAMMING IN A MOUSE MODEL OF PRECONCEPTION MALE ALCOHOL EXPOSURE*

Rationale

We first sought to determine if preconception male alcohol exposure could impact fetal growth and development using a C57BL/6J mouse model of alcohol exposure. We hypothesize paternal alcohol exposure impairs fetal growth and associates with alterations in the developmental program of the liver. This chapter will evaluate the impact of chronic preconception paternal alcohol exposure on fetal growth and placental function. We will examine the GD 14.5 time point, a period two thirds of the way through gestation. We will conduct transcriptomic analysis of the fetal liver to determine the impact paternal alcohol use has on the fetal developmental program. The data from this chapter were published in the journal *Epigenetics*[99].

Background

It has recently become clear that through epigenetic means, environmental exposures prior to conception exert a significant impact on offspring health and development[100]. 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[83,101-114]. These studies challenge the singular importance of maternal in utero environmental exposures and implicate paternal exposure history as an

* Reprinted with permission from "DNA methylation-independent growth restriction and altered developmental programming in a mouse model of preconception male alcohol exposure." By RC Chang, 2017, ***Epigenetics***, 12(10),841-853, Copyright 2017 by Taylor & Francis.

additional and important mediator of both environmentally-induced disease and dysgenesis[76,98]. However, the capacity of preconception paternal exposures to broadly contribute 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.

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)[95]. This condition is characterized by a spectrum of structural defects, central nervous system disorders and growth deficits that persist well into postnatal life[95,96]. One of the major confounding elements in the study of this disorder is the enormous variation observed in FASD phenotypes and incidence[115]. FASD-associated defects have a wide range of severity, and importantly, while many women who drink alcohol during pregnancy have affected children, a subset of the exposed offspring remain unaffected[116]. The observed variance and inconsistency in FASD phenotypes suggest that multiple factors beyond the incidence of maternal ethanol exposure must play a significant role in the genesis of this disorder.

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[76,117]. 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[118-122]. 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[123-126].

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[124-126]. 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[127,128]. 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[129]. 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[102,111,112]. 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.

Given the recent association between paternal environmental exposures and long-term metabolic dysfunction[98], we sought to examine the capacity of preconception male ethanol exposure to impact developmental programming in the male germline and determine if heritable alterations in the DNA methylation profiles of alcohol-exposed sperm contribute to the prenatal growth deficits associated with FASDs. 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[130]. Our studies associate preconception male ethanol exposure with prenatal growth restriction, decreased placental efficiency, disruptions in the genetic pathways controlling both lipid metabolism and hepatic fibrosis and the abnormal expression of imprinted genes. However, similar to studies examining the inheritance of diet induced metabolic dysfunction[102,111,130], we find evidence to suggest that the transmission of these phenotypes occurs independent of sperm-inherited alterations in DNA methylation.

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[123,124,126]. This technique is known to induce activation of the stress response[131], which has previously been shown to modulate epigenetic programming in sperm[107]. 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[132]. 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. During the first week of the exposure paradigm, males acclimating to the EtOH treatment consumed 9% less fluid during the exposure window than the controls (p=0.03, Fig. 1A). However, after the first week of treatment, no significant differences in fluid consumption were observed. 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[133,134]. Using this model of exposure, experimental males consistently achieved blood EtOH concentrations of 210 mg/dL (Fig.1B). As a reference, this concentration is approximately 2.5 times the legal limit, and reflects a range frequently obtained by binge drinkers and alcoholics[135]. 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[83,86]. Chronic EtOH consumption did not influence paternal body weight, fertility, or the sex distribution of the offspring (Fig.1C-E).

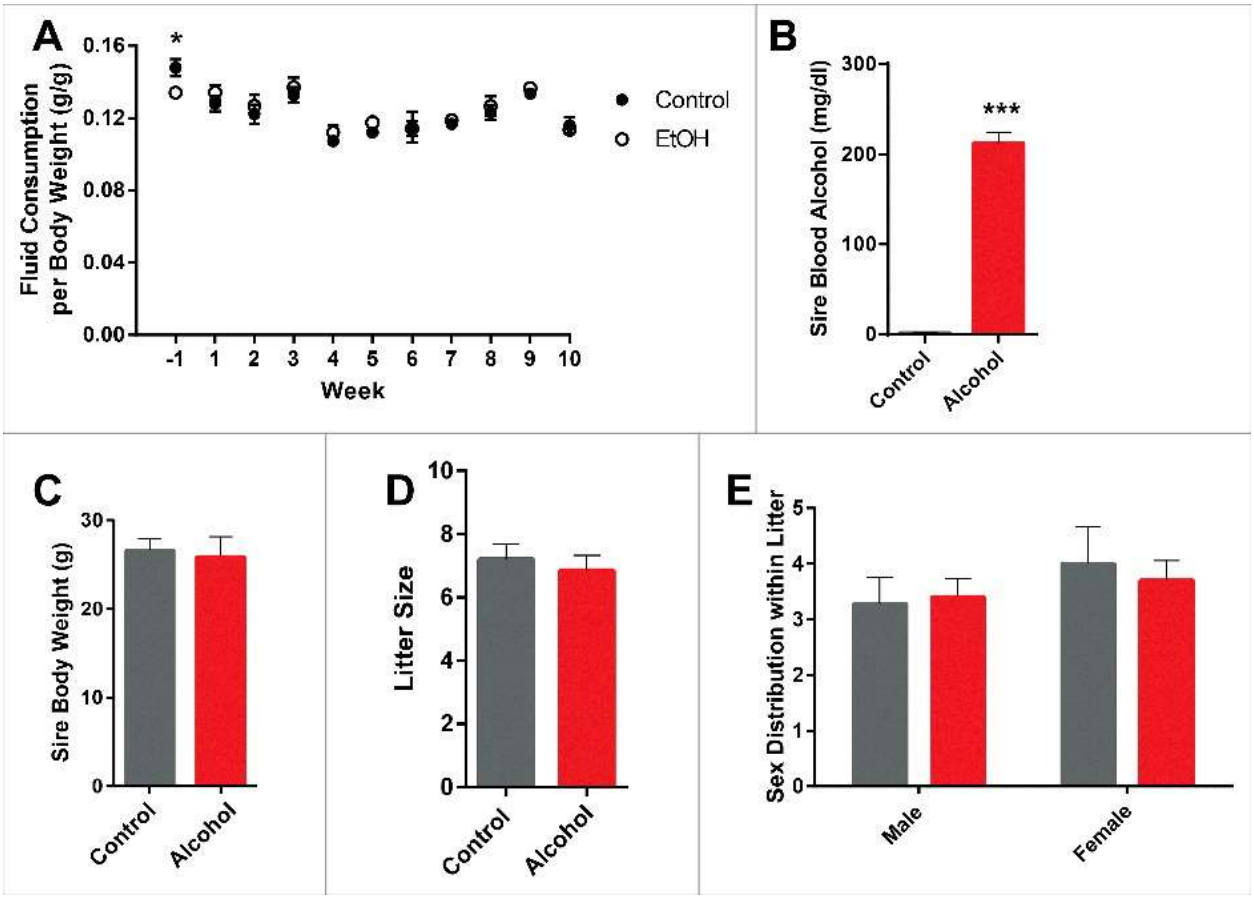


Figure 1 Chronic male alcohol exposure does not impact fertility or sire weight.

(A) Average fluid consumption per gram body weight during the preconception treatment period (n=8 control, 10 alcohol-exposed sires). (B) Average blood alcohol concentrations during the exposure period (n=6 males). (C) Average body weight of sires at the end of the 70-day EtOH exposure period (n=8 control, 10 alcohol). (D) Average litter size and (E) 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.

Male Alcohol Exposure Associates with Fetal Growth Restriction

Once the 70-day milestone was surpassed, males were mated to unexposed dams and fetuses collected at day 14.5 of gestation (GD14.5). During this dissection, the uterus was removed, and the maternal decidua completely separated from the fetal interface. The GD14.5-time point was selected based on our experience reliably excising the fetal component of the placenta away from maternal tissues, thus ensuring that the fetal component of the placenta is minimally contaminated with maternal cells. This approach and mouse model permit accurate assessment of placental patterns of imprinted gene expression[136]. At this developmental time point, chronic paternal EtOH exposure was determined to be significantly associated with a reduction in the weight of the gestational sac (11% in males, 8% in females, $p<0.01$), a reduction in crown rump length (3.6% in males, 3.9% in females, $p<0.05$) and in female offspring only, a 7.4% reduction in fetal weight (Fig.2A-C). In the female offspring of EtOH-exposed males, fetal weight was highly correlated with placental weight, and a 12% decrease in placental efficiency ($p<0.05$) was observed (Fig. 2D-E). 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[137].

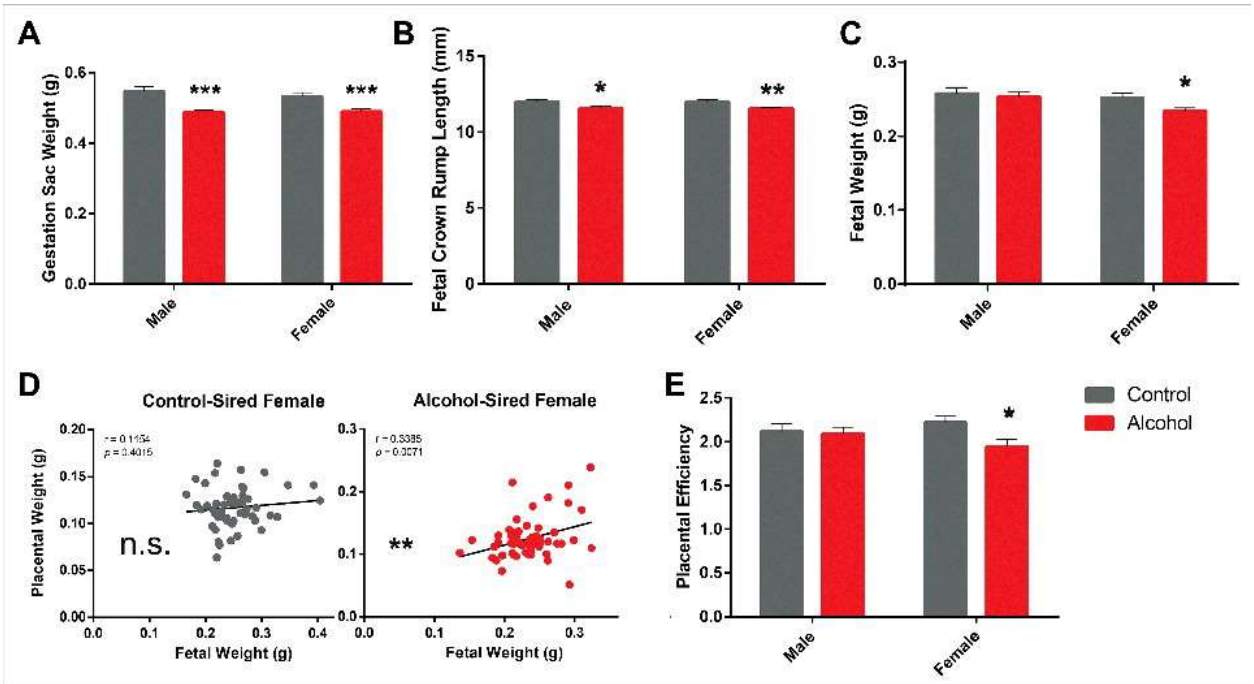
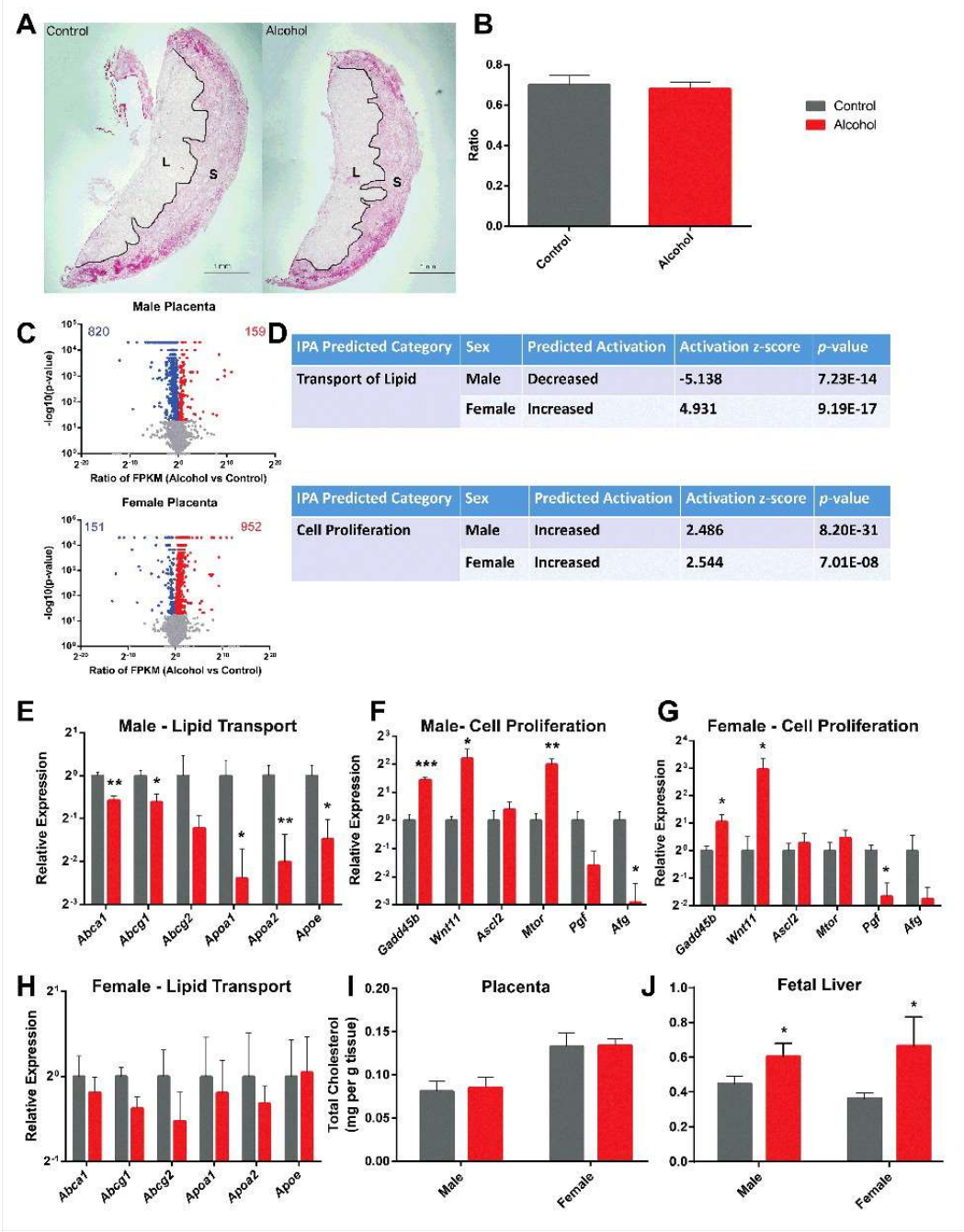


Figure 2 Preconception male alcohol exposure impacts fetal growth.

Preconception male alcohol exposure impacts fetal growth. (A) Average weight of the gestational sac, (B) average fetal crown-rump length and (C) average fetal weights between male and female offspring sired by preconception EtOH-exposed or control males. All measures taken on gestational day 14.5 (control n=46 males, 55 females, alcohol n=56 males, 62 females). (D) Analysis of correlation between fetal weight and placental weight in female offspring sired by EtOH-exposed and control males; (E) placental efficiencies between male and female offspring sired by preconception EtOH-exposed and control sires. Efficiencies obtained by dividing fetal weight by placental weight (control n=46 males, 55 females, alcohol n=56 males, 62 females) Error bars represent SEM, ns = not significant, *P < 0.05, **P < 0.01 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 a one-way ANOVA followed by Sidak post hoc analysis.

Placental alterations in the pathways controlling lipid transport and cellular proliferation

The growth[127,128]. A gross histological assessment of the placenta did not reveal any morphological abnormalities or alterations in the proportional sizes of the labyrinthine and spongiotrophoblast layers between preconception treatments (Fig. 3A-B). Deep-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 (Fig. 3C-H). As one of the predominant pathways to emerge from this analysis was lipid transport, we assayed the concentration of total cholesterol within the placenta and fetal liver. Total cholesterol was significantly increased in the fetal livers of offspring sired by the EtOH-exposed males (Fig. 3I-J). These results significantly correlate preconception male EtOH exposure with disruptions in the genetic pathways regulating lipid homeostasis, which is known to be associated with intrauterine growth restriction in clinical studies[138].



IPA Predicted Category	Sex	Predicted Activation	Activation z-score	*p*-value
Transport of Lipid	Male	Decreased	-5.138	7.23E-14
	Female	Increased	4.931	9.19E-17

IPA Predicted Category	Sex	Predicted Activation	Activation z-score	*p*-value
Cell Proliferation	Male	Increased	2.486	8.20E-31
	Female	Increased	2.544	7.01E-08

Figure 3 Preconception male alcohol exposure alters genes controlling lipid transport and cellular proliferation in the placenta.

(A) Periodic acid–Schiff stained histological sections of gestational day 14.5 placental tissues derived from offspring sired by preconception EtOH-exposed or control males; L =

labyrinthine and S = spongiotrophoblast. (B) Ratio of labyrinthine to spongiotrophoblast surface area compared between preconception treatments (n=5 males, 5 females). (C) Comparison of the placental transcriptome between offspring sired by EtOH-exposed and control males. Volcano plots between male and female offspring (n=4). (D) Top two categories identified using Ingenuity Pathway Analysis. (E) qRT-PCR validation of candidate genes related to lipid transport in male offspring; (F) genes controlling cellular proliferation in male offspring; (G) genes regulating cell proliferation in female offspring and (H) genes related to lipid transport in female offspring. Note the log scale. (I) Total cholesterol levels in the placenta and (J) fetal liver of offspring sired by sired by EtOH-exposed and control males (n=5). For qRT-PCR analyses, measured Ct values were normalized to the geometric mean of Sdha, Hprt, and Mrpl1 and graphed relative to the control treatment. Graphs represent independent replicates (n=6 male, 8 female), with two independent RT reactions and three qRT-PCR measurements for each RT. Error bars represent SEM *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ (comparisons between EtOH and control preconception treatments). Data analyzed using either an unpaired t-test or a one-way ANOVA followed by Sidak post hoc analysis.

Sex-specific impacts on the pathways regulating hepatic fibrosis

Given the observed changes in total cholesterol, we next assayed gene expression in the GD14.5 liver using deep sequencing. These analyses 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 (Fig. 4A-B)[139]. Interestingly, of the 153 genes common between both sexes, 147 (96%) displayed

alterations in transcription that were diametrically opposite between males and females. As examples, transcripts encoding Acta1 and Col18a1 were significantly increased in the livers of male offspring, while the same genes were down-regulated in female offspring (Fig.4C-D). Of the 34 identified genes associated with hepatic fibrosis, 20 (60%) increased transcription in the male offspring, while these same genes were down-regulated in females (Fig. 4E)

To determine if alterations in these genes could be further correlated with the growth restriction phenotype, we separated samples into the smallest and heaviest offspring based on fetal weight and assayed the expression of two pro-fibrotic candidate genes[139]. These experiments identified a strong correlation between altered patterns of transcription and preconception EtOH-dependent reductions in fetal growth (Fig. 4F-I). For example, in the smallest male fetuses, transcripts encoding Col18a1 and TgfB2 displayed preconception EtOH-dependent increases, while the abundance of these transcripts in the largest sized fetuses were not significantly different (Fig. 4F-G). In contrast, the smallest female offspring displayed preconception EtOH-dependent down regulation of both Col18a1 and TgfB2, with no detectable differences in the largest offspring (Fig. 4H-I). Interestingly, transcripts encoding TgfB2 exhibited differences in expression between the smallest and largest control offspring that were distinct between males and females ($p > 0.05$). These observations suggest a sex-specific interaction between fetal growth rate and the transcriptional control of this cytokine.

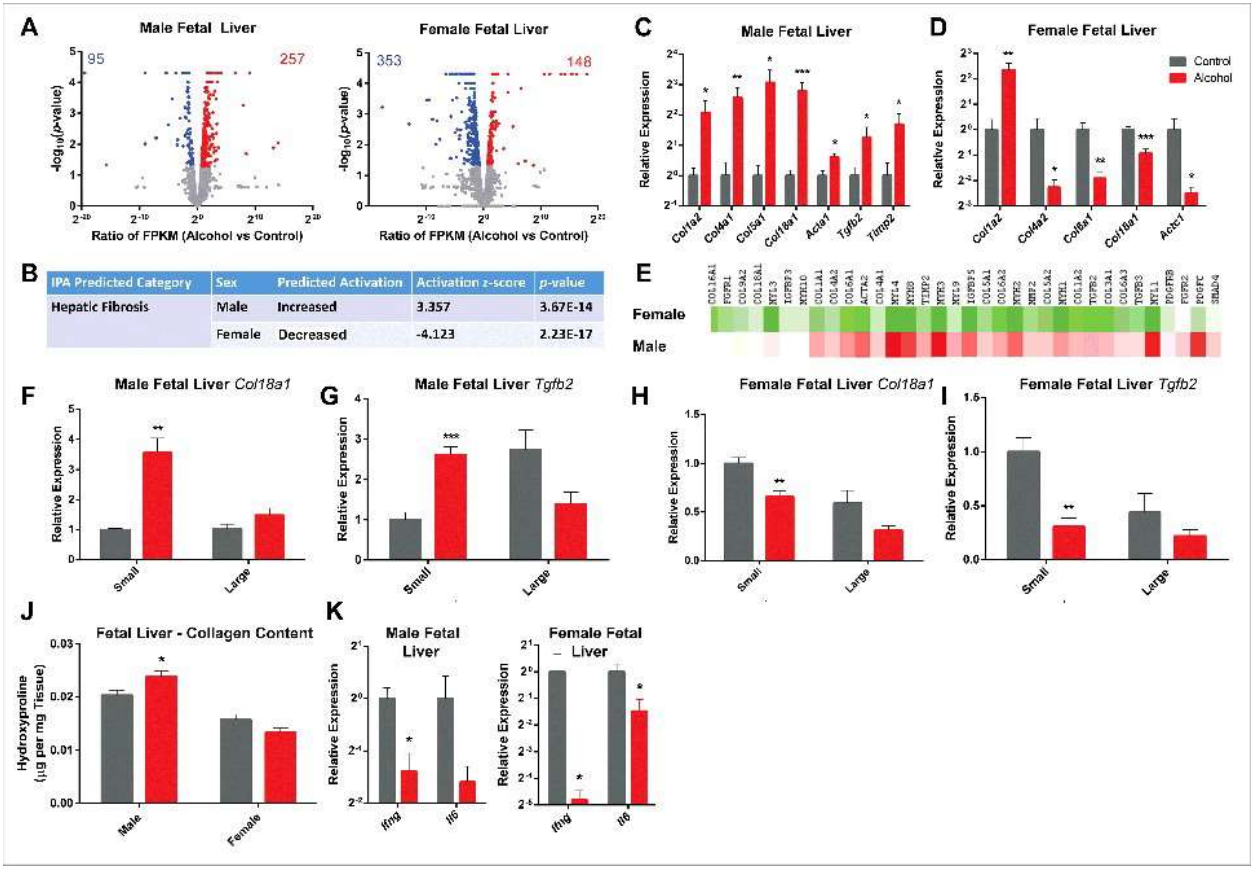


Figure 4 Preconception alcohol exposure activates the genetic pathways controlling hepatic fibrosis.

(A) Comparison of the gestational day 14.5 liver transcriptome between offspring sired by EtOH-exposed and control males. Volcano plots between male and female offspring (n=4). (B) Top catagory identified using Ingenuity Pathway Analysis. (C) qRT-PCR validation of candidate genes in male and (D) female offspring (n=8). (E) Heatmap contrasting the abundance of transcripts encoding genes associated with hepatic fibrosis between male and female offspring. Expression patterns were normalized to the offspring of control animals. (F-I) qRT-PCR analysis of candidate genes between the smallest (bottom 25%), and largest (top 25%) weight offspring sired by EtOH-exposed and control males (n=8). (J) Total levels of cellular hydroxyproline within the gestational day 14.5 livers of offspring sired by EtOH-exposed and control males (n=4) (K)

qRT-PCR analysis of transcripts encoding inflammatory cytokines between the offspring of EtOH-exposed and control males (n=8). For qRT-PCR analyses, measured Ct values were normalized to the geometric mean of transcripts encoding Ywhaz, Hprt, and Mrpl1, and graphed relative to the control treatment. Graphs represent independent replicates, with two independent RT reactions and three qRT-PCR measurements for each RT. Data analyzed using either an unpaired t-test or a one-way ANOVA followed by Sidak post hoc analysis. Error bars represent SEM *P < 0.05, **P < 0.01 and ***P < 0.001 (comparisons between alcohol and control preconception treatments).

Sex-specific induction of hepatic fibrosis and alterations in immune signaling

To determine if alteration of these gene cohorts could be associated with an increased fibrotic response, we next assayed the fetal liver for the abundance of hydroxyproline, which is a commonly employed marker of hepatic fibrosis[139]. Male offspring displayed a 15% increase in hydroxyproline ($p<0.05$), while female livers were identical to the controls (Fig. 4J). Given the link between intrauterine growth restriction, hepatic fibrosis and inflammation in clinical studies[140], we examined the expression of four key pro-inflammatory cytokines using qRT-PCR. These experiments identified significant alterations in transcripts encoding Infg in male and both Infg and Il6 in female offspring (Fig. 4K), while Il1βand Tnf did not display any significant changes in either sex (data not shown).

Alterations in the regulation of imprinted genes

Given the link between imprinted genes, hepatic dysfunction and fetal-placental growth[127,128,140,141], 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 (Fig. 5A-B). In the fetal liver, we observed significant reductions in transcripts encoding Cdkn1c, Dcn and Gnas in male offspring and Meg3 in female offspring (Fig. 5C-D). However, none of the examined imprinted genes displayed significant differences in the contribution from the normally silenced allele (Table 1). These observations associate preconception paternal EtOH exposure with decreased expression of select imprinted genes that is independent of compromised genomic imprinting.

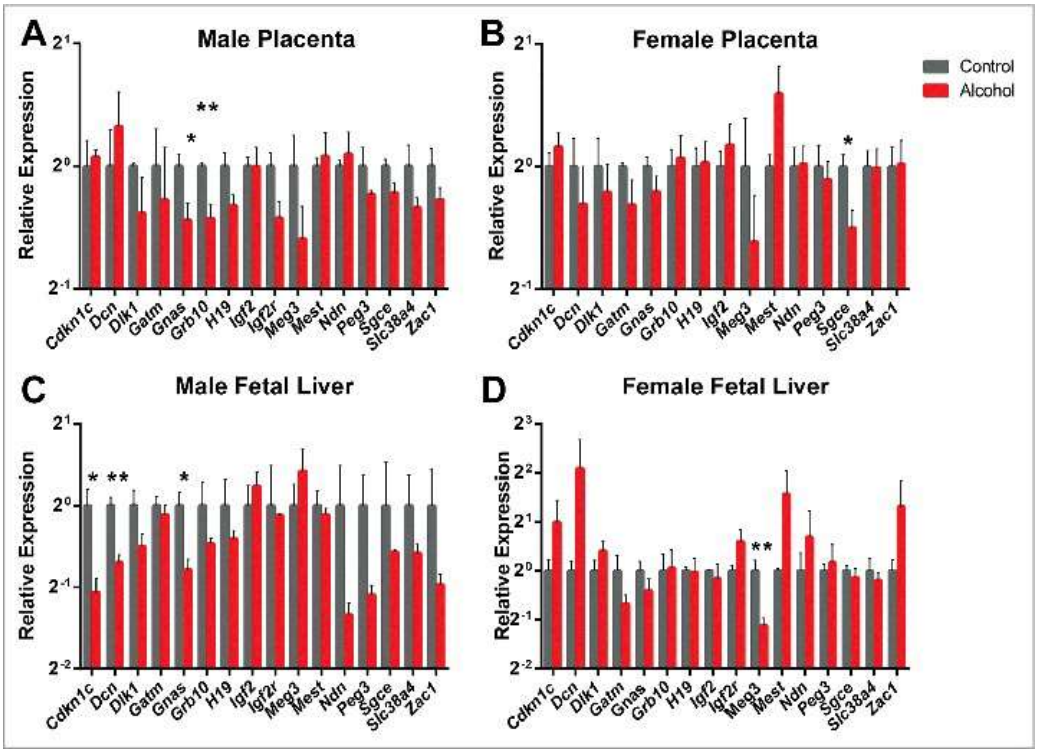


Figure 5 Altered patterns of imprinted gene expression within the offspring of alcohol-exposed males.

(A) qRT-PCR 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 qRT-PCR 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).

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 mRNAs of genes regulated by genomic imprinting.

Preconception EtOH exposure does not influence male fertility or alter levels of the one-carbon metabolite s-adenosylmethionine

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 (Fig. 6A-B). No treatment effects were observed on the weights of the paternal liver or epididymal fat (Fig. 6C-D). Previous studies have suggested that chronic exposure to alcohol disrupts one-carbon metabolism, and in doing so, has the capacity to alter epigenetic programming[142]. In our chronic model of exposure, no significant differences in the abundance of the one-carbon donor s-adenosylmethionine could be identified between the kidney, liver or testis of EtOH-exposed and control males (Fig. 6E).

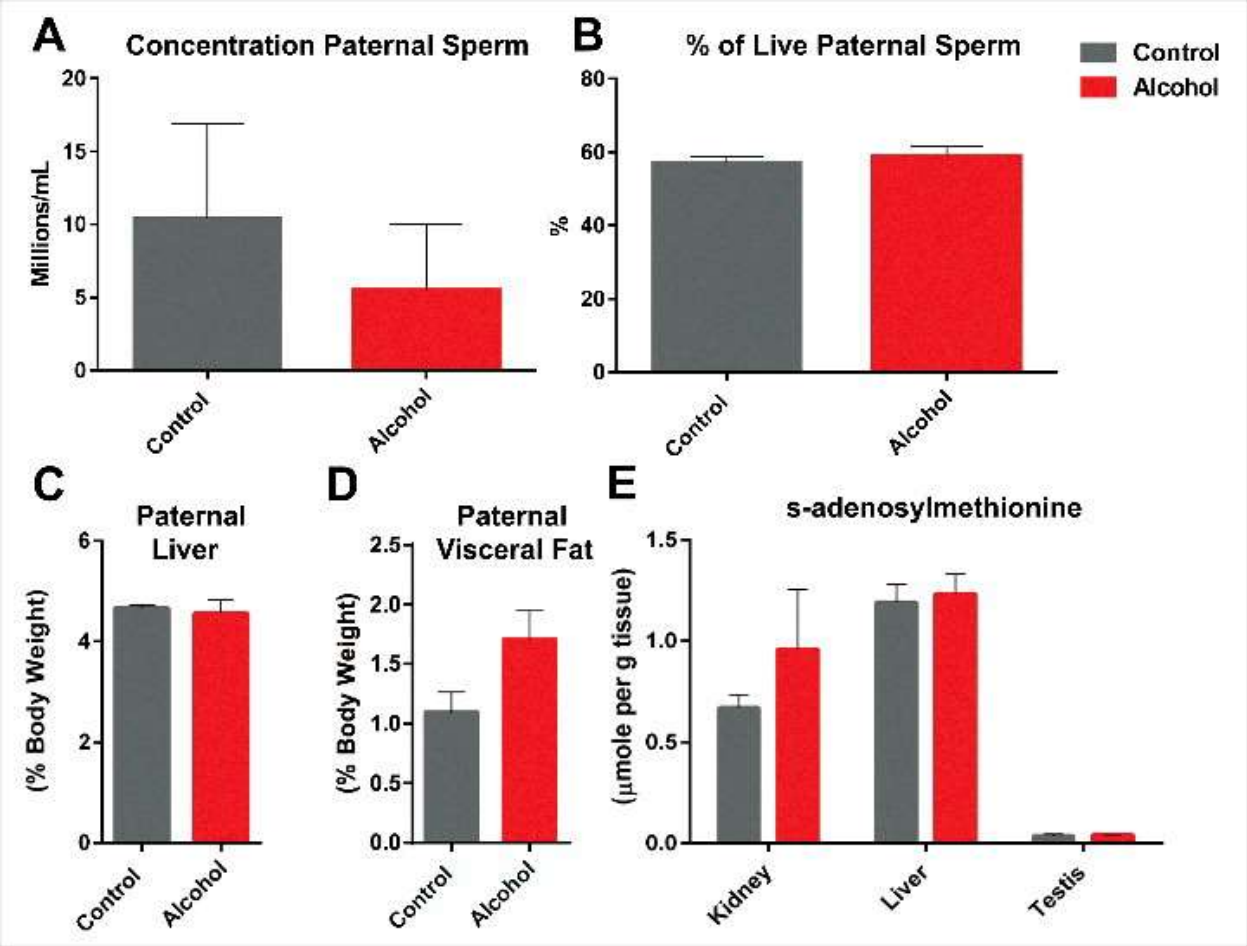


Figure 6 Preconception male alcohol exposure does not impact base fertility or abundance of the one-carbon metabolite s-adenosylmethionine.

(A) Concentration of sperm between EtOH-exposed and control males; (B) percentage of live sperm between EtOH-exposed and control males (n=8). (C) Measures of the percentage of total body weight for the liver and (D) epididymal fat between EtOH-exposed and control males (n = 8). (E) Comparison of total s-adenosylmethionine in the kidney, liver and testis between EtOH-exposed and control males (n=8). Physiological and reproductive parameters were analyzed using either an unpaired t-test or a one-way ANOVA followed by Sidak post hoc analysis.

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 bisulphite 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 (Fig. 7A). 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 (Fig. 7B-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 0.0150, 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. 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 7D). When we randomly selected four of these lower confidence candidate loci to contrast measures between Methyl-seq and PCR-based bisulphite sequencing approaches, only two displayed significant alterations in DNA methylation using both methods (Fig. 7E). Using PCR-based bisulphite sequencing, the two significant regions identified both displayed less than a 25% change. Importantly, no differences in the DNA methylation profiles of any imprinted genes were observed and no correlative changes in the expression of the nearest mappable genes in either the fetal liver or placenta for any candidate loci could be identified.

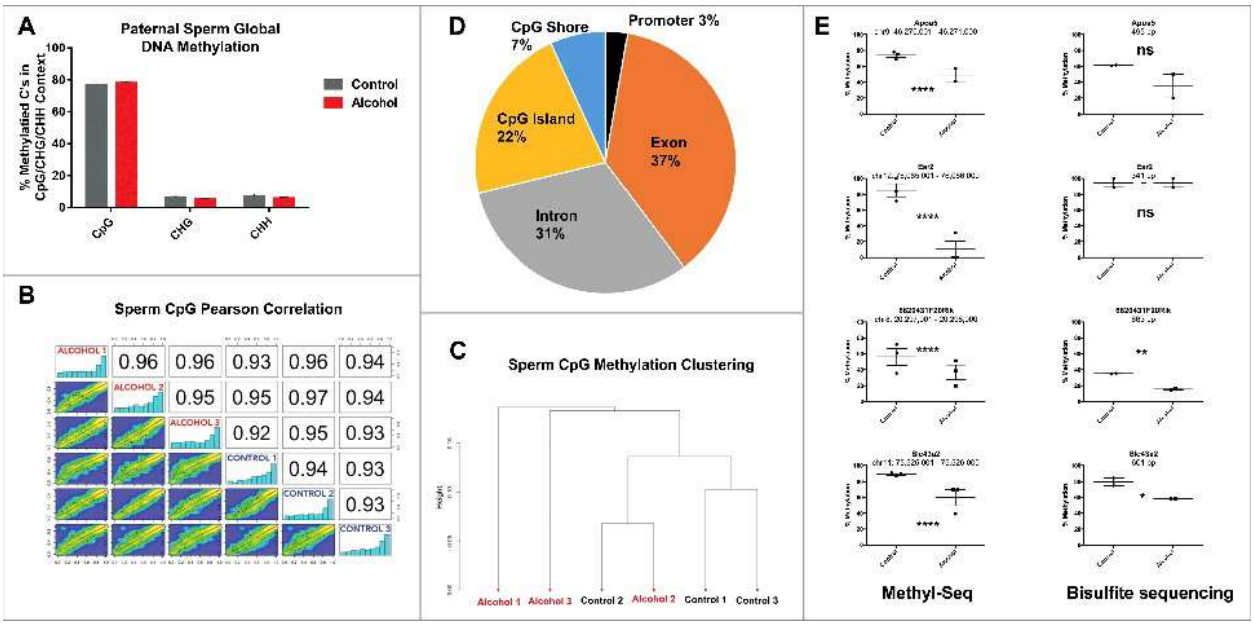


Figure 7 Preconception male alcohol exposure does not impact the DNA methylation profiles of paternal sperm.

(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 Bisulphite sequencing. Bisulphite sequencing data were compared using an unpaired t-test. Error bars represent SEM *P < 0.05, **P < 0.01 and **** P < 0.0001.

Discussion

The objective of this study was to determine the impact preconception male drinking exerts on the incidence of FASD growth deficits, and the relevance the reported alterations in the DNA methylation profiles of EtOH-exposed sperm have on the development of these phenotypes. We find 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, altered cholesterol trafficking and sex-specific indicators of hepatic fibrosis. Interestingly, while hepatic fibrosis has long been one of the clinical manifestations of alcoholic liver disease in adults[143], three isolated case reports have also identified a similar condition in FASD children[144-146]. The identification of increased lipids and markers of hepatic fibrosis in these published case reports, as well as our work, suggest some aspects of this pathology may involve alterations in developmental programming. However, we do not know if the indicators of fibrosis we observed in this model were a primary consequence of altered developmental programming (programming of the sperm to impart a heightened fibrotic state) or a secondary consequence of fetal growth restriction. Indeed, intrauterine growth restriction alters the hepatic control of glucose transport, has been linked to abnormal lipid profiles in the offspring and has also been associated with the development of adolescent non-alcoholic fatty liver disease (NAFLD)[147]. NAFLD shares many common elements with alcoholic liver disease, including both fibrosis and the accumulation of lipids[138,148]. Each of these conditions are also accompanied by alterations in immune signaling, which may account for the sex-specific phenotypes we observe here[149]. We suspect that, similar to other models of developmental programming, a “second hit”

will be required to unmask the full extent of the programmed dysfunction and reveal the breadth of pathologies in our model[147].

Alterations to the developmental program have been proposed to arise through environmentally-induced changes to the epigenome during crucial periods of plasticity[150]. Numerous studies support the notion that gametogenesis is a labile period and that both placental and hepatic function are profoundly impacted by alterations in the developmental program[100,148]. Similar to studies of preconception male alcohol exposure, rodent studies examining maternal periconceptional alcohol exposure also report EtOH-induced changes in fetal growth as well as sex-specific impacts on metabolic programming[151,152]. Importantly, both our model of preconception male alcohol exposure and the periconceptional model of maternal exposure reported by the Moritz group yield fetal growth deficits similar in magnitude to those reported in rodent models of in utero exposure[137]. Collectively, these studies reveal preconception 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[118,124-126]. Similar to these works, we also observe 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[127,128]. 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[141]. 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[124,126,129]. 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[124,129]. 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. For example, a loci that transitions from ~90% methylation down to 80% methylation implies that instead of 1 out of 10 sperm being affected by alcohol, the populations shifts to 2 in 10 being impacted. For changes of less than 5%, the induced change is even less frequent. Yet each of these cited studies demonstrate measurable changes in offspring growth or behavior across the total population[123,124,126]. 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[126]. 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[102,104,111]. Collectively, our results, along with previously published data[123,124,126], 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[91]. Interestingly, these ncRNAs appear to have functional roles during embryonic development and the profile of sperm-inherited RNAs can be modulated by paternal diet[92,153]. Many ncRNAs, including miRNAs, are influenced by alcohol exposure and have roles in the development of FASD phenotypes64. As previous studies have identified a link between paternally inherited miRNAs and stress-induced phenotypes in the offspring[107], 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.

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[95]. 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[154], further investigation is clearly warranted.

Materials and 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[130]. 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[136]. C57BL/6(CAST7) males were employed in a chronic, moderate-dose, voluntary alcohol exposure paradigm referred to as 'Drinking in the Dark'[132]. Here, individually caged, postnatal day 90, adult males were provided limited access to EtOH during a four-hour window immediately after their sleep cycle[18]. 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[155]. 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[155], 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 Zfy and Xist genes conducted.

Measurement of Physiological Parameters

Blood alcohol concentrations were measured using an Ethanol Assay Kit (catalog # ECET100, BioAssay Systems) according to manufacturers protocol. Total cholesterol levels were determined using the Total Cholesterol Assay Kit (catalog # STA- 384, Cell Biolab, Inc), according to the recommended protocol. The levels of hydroxyproline were determined using the Hydroxyproline Assay Kit (catalog # MAK008, Sigma-Aldrich). The concentration of S-adenosylmethionine within the paternal liver, testis and kidney were measured using the Bridge - It® S-Adenosyl Methionine (SAM) Fluorescence Assay Kit according to the recommended protocol (catalog # 1-1-1003B; Mediomics).

RNA Analyses

Total RNA was isolated from E14.5 fetal liver and placenta using the RNeasy Plus Mini Kit, (catalog # 74134, Qiagen) 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 (catalog # RS-122-2001, Illumina) 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[156] and referenced against the Mus musculus genome (UCSC version mm10).

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

Following deep sequencing analysis of 50-bp length 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)[157].

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) 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 CFX38. Primers are listed in Table 2.

Prenatal Sexing			
Primer name	**Fwd/Rev**	**Sequence**	**Reference**
Zfy	Fwd	AAGATAAGCTTACATAATCACATGGA	
	Rev	CCTATGAAATCCTTTGCTGCACATGT	
Xist	Fwd	TTGCGGGATTCGCCTTGAT	
	Rev	TGAGCAGCCCTTAAAGCCAC	
Lipid Transportation			
Primer name	**Fwd/Rev**	**Sequence**	**Reference**
Abca1	Fwd	GGACATGCACAAGGTCCTGA	Tang C et al., 2010
	Rev	CAGAAAATCCTGGAGCTTCAAA	
Abcg1	Fwd	CCTTCCTCAGCATCATGCG	Tang C et al., 2010
	Rev	CCGATCCCAATGTGCGA	
Abcg2	Fwd	GGCTTTCTACCTGCACGAAAACCAGTTGAG	Peng H., 2009
	Rev	ATGGCGTTGAGACCAG	
Apoa1	Fwd	CTTCAGGATGAAAGCTGTGGT	
	Rev	AGATTCAGGTTCAGCTGTTGG	
Apoa2	Fwd	AGAGTAGACGGGAAGGACTG	
	Rev	TCAAAGTATGCCTTGGCCTG	
Apoe	Fwd	CACAAGAACTGACGGCACTG	
	Rev	CCCGTATCTCCTCTGTGCTC	

Table 2 DNA Primer Sequences.

Cell Proliferation Primers

Primer name	Fwd/Rev	Sequence	Reference
Gadd45b	Fwd	GCCCGAGACCTGCACTGCCT	Tian J et al., 2011
	Rev	CCATTGGTTATTGCCTCTGCTCTCTT	
Wnt11	Fwd	CAGTGAAGTGGGGAGACAGG	Hayashi K et al., 2009
	Rev	ACCACTCTGTCCGTAGGG	
Ascl2	Fwd	CTACTCGTCGGAGGAAAG	van der Flier LG et al., 2009
	Rev	ACTAGACAGCATGGGTAAG	
Mtor	Fwd	TTGGAGTGGCTGGGTGCTGA	Siddappa D et al., 2014
	Rev	AAGGGCTGAACTTGCTGGAA	
Pgf	Fwd	CTCTCTGGAACACAGGCAGA	Zhang F et al., 2009
	Rev	CCGTAGCTGTACCACGAAGA	
AfP	Fwd	TCGTATTCCAACAGGAGG	Watt AJ et al., 2007
	Rev	AGGCTTTTGCTTCACCAG	

Imprinted Gene Primers

Primer name	Fwd/Rev	Sequence	Reference
Cdkn1c	Fwd	AACGTCTCAGATGAGTTAGTTTAGAGG	Varrault A et al., 2006
	Rev	AAGCCCAGAGTTCTTCCATCGT	
Gnas	Fwd	AGCGCGAGGCCAACAAAA	Varrault A et al., 2006
	Rev	GTGCGTGGCCCGGTAGA	
Gnas	Fwd	GGGCGTCATCAGGCTGGTTA	Varrault A et al., 2006
	Rev	GCCGACGCGACTGAGTGT	
Gnas	Fwd	CGTCTCTACCGGATCTGATGCT	Varrault A et al., 2006
	Rev	CGGCATCGCTCTGGCTATCT	
Grb10	Fwd	AGGATCATCAAGCAACAAGGTCTC	Varrault A et al., 2006
	Rev	ATTACTCTGGCTGTCACGAAGGA	
H19	Fwd	CTTGTCGTAGAAGCCGTCTGTTC	Varrault A et al., 2006
	Rev	GTAGCACCATTTCTTTCATCTTGAGG	

Table 2 Continued.

Igf2	Fwd	GCACAGAATCCAGACTAGCATTACA	Varrault A et al., 2006
	Rev	CCTCCTTATCAGCTTTAAATATGTCTTTCTT	
Meg3	Fwd	CTTCCTGTGCCATTTGCTGTTG	Agarwal SK et al., 2012
	Rev	TGCAACGTGTTGTGCGTGAAG	
Meg3	Fwd	CGAGGACTTCACGCACAACAC	Agarwal SK et al., 2012
	Rev	CCACGCAGGATTCCAGATGATG	
Peg3	Fwd	TTGGACTGGACAGAGATGATGACA	Varrault A et al., 2006
	Rev	ATTCTGGTATGACTCGGCATCCT	
Sgce	Fwd	GTGATGGAGTCCTGTATGGGTCT	Varrault A et al., 2006
	Rev	GTAGGCAGTTATCTCAATAATAGTTGGTTT	

Fibrosis Signature Primers

Primer name	Fwd/Rev	Sequence	Reference
Acta1	Fwd	GAGGTATCCTGACCCCTGAAGTA	
	Rev	CACACGCAGCTCATTGTAGA	
Acta2	Fwd	GTGGCTATTCCTTCGTGACTAC	
	Rev	GAGCTACATAGCACAGCTTCTC	
Actc1	Fwd	CCTCTCTGGAGAAGAGCTATTGA	
	Rev	AATGAAAGAGGGCTGGAAGAG	
Col1a2	Fwd	CTTCACTCAGACCCAAGGAC	
	Rev	AGTAGTAATCGCTCTTCCACTC	
Col3a1	Fwd	TGGTATGAAAGGACACAGAGG	
	Rev	CAACTTCACCCTTAGCACCA	
Col4a1	Fwd	CTAACGGTTGGTCCTCACTG	
	Rev	CGRGGGCTTCTTGAACATCTC	
Col4a2	Fwd	GGCTTCATCAAAGGAGRCAAGG	
	Rev	CCCAATGRCACCAAAGTCCC	
Col5a1	Fwd	GAGATTGAACAGATGAAGCGAC	
	Rev	ACATAGGAGAGCAGTTTCCCA	
Col6a1	Fwd	CCAGGCGCGGCTAACAACGAC	
	Rev	GGCAATCTCAAAGTTCTGTAGG	
Col6a2	Fwd	GGGACATCGCTAACTCTCCA	
	Rev	CTCACCTTGTAGCACTCTCCA	
Col18a1	Fwd	CTGTGCACTGTCTGGATGAA	
	Rev	GAGCCCAGGTAGTAGAGATGTA	

Table 2 Continued.

		Sequence	Reference
Tgfb2	Fwd	TAAAATCGACATGCCGTCCC	Braga P et al., 2013
	Rev	GAGACATCAAAGCGGACGAT	
Smad4	Fwd	ACTTACCATCATAACAGCACTACC	Nye MD et al., 2014
	Rev	ATCCACATAGCCATCCACAG	
Timp1	Fwd	GCATGGACATTTATTCTCCACTGT	Meng et al., 2010
	Rev	TCTCTAGGAGCCCCGATCTG	
Timp2	Fwd	GGAATGACATCTATGGCAACC	Meng et al., 2010
	Rev	GGCCGTGTAGATAAACTCGAT	

Proimflammtory Gene Primers

Primer name	Fwd/Rev	Sequence	Reference
Il1b	Fwd	TGTAATGAAAGACGGCACACC	Boaru SG et al., 2012
	Rev	TCTTCTTTGGGTATTGCTTGG	
Il6	Fwd	CCAGAAACCGCTATGAAGTTCC	Boaru SG et al., 2012
	Rev	TCACCAGCATCAGTCCCAAG	
Tnf	Fwd	CTCCAGGCGGTGCCTATGT	Boaru SG et al., 2012
	Rev	GAAGAGCGTGGTGGCCC	
Ifng	Fwd	GGATGCATTCATGAGTATTGC	Boaru SG et al., 2012
	Rev	CCTTTTCCGCTTCCTGAGG	

Reference Primers

Primer name	Fwd/Rev	Sequence	Reference
Ywhaz	Fwd	TTGATCCCCAATGCTTCGC	Carnahan et al., 2013
	Rev	CAGCAACCTCGGCCAAGTAA	
Hprt	Fwd	CTGGTGAAAAGGACCTCTCGAA	Carnahan et al., 2013
	Rev	CTGAAGTACTCATTATAGTCAA	
Mprt	Fwd	AACTTCCTCAGCACCAAAATAG	Carnahan et al., 2013
	Rev	GACCACAAACGGACCCAGATT	

Bisulfite PCR Primers

Primer name	Fwd/Rev	Sequence	Reference
Apoa5	Fwd	GGYGGAGTTGATGGAGTAGGTGGGTTTG	
	Rev	TATCATACCRAAAAACCTACAATCTCTAAC	
Esr2	Fwd	AGTGGGTATGGTTAGGTTTTTTTTTTAGTAAGAG	
	Rev	TCTCTAACRAAAACAAATACCCAAAAATCATAAAC	
6820431F20Ri	Fwd	GGCTTTCTACCTGCACGAAAACCAGTTGAG	
	Rev	AATAAATTTCCCRCAAAAAAAACTTTTCCTATAACAC	
Slc43a2	Fwd	AAATTYGGTAGTAGTAATGGTGTTTTGG	
	Rev	TCAACTACCATCTAACTACCAAATAAAAATTAACC	

Table 2 Continued.

Sperm Collection and Analysis

After a minimum of three matings, breeder males were sacrificed and sperm collected using a modified swim-up procedure[158]. 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% CO2, 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 hemacytometer. 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 ug/ml Proteinase K. Incubation was performed for 4 hours at 55°C with frequent vortexing. Samples were combined with 200 uL of 100% ethanol and 200 uL 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 larger 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 (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.

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/). Primers are listed in Table S1 - Primer Sequences.

Statistical Analysis

For all experiments, statistical significance was set at alpha = 0.05. In this study, the effect of two independent variables (sex versus preconception treatment) were assessed using an analysis of variance test (ANOVA), and differences among the means evaluated using Sidak's post-hoc test of contrast. No interactions ($p>0.05$) were observed between fetal weight and sire weight, fetal weight and dam weight, nor litter size and fetal weight.

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. For placental tissues, transcripts encoding succinate dehydrogenase complex, subunit A (Sdha - NM_023281), mitochondrial ribosomal protein L1 (Mrpl1 - NM_053158) and hypoxanthine-phosphoribosyl transferase (Hprt - NM_013556) were measured. For liver, we measured transcripts encoding tyrosine 3-monooxygenase / tryptophan 5-monooxygenase activation protein zeta (Ywhaz - NM_011740), mitochondrial ribosomal protein L1 (Mrpl1 - NM_053158) and

hypoxanthine-phosphoribosyl transferase (Hprt - NM_013556). Each of these reference genes were validated for stability across treatment groups as described previously70. Normalized expression levels were calculated using the ΔΔCt method described previously71. Relative fold change values from each biological replicate 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 comparisons including sex and preconception treatments, an analysis of variance (ANOVA) was utilized and Sidak's analysis applied to comparisons with p-values < 0.05. For single comparisons, an unpaired student's t-test was applied. In all instances, we have marked statistically significant differences with an asterisk. For genes demonstrating significant changes, multiple primer sets targeting the same transcript were employed. Before averaging the results from three primer sets, a mixed two-way ANOVA was applied to ensure no interactions between primer sets. A Sidak post-test was utilized to identify differences between the preconception EtOH and control treatment groups.

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) strains36 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-Squared analysis or, if read counts were less than 5, a Fisher's Exact test.

CHAPTER III

PRECONCEPTION PATERNAL ALCOHOL EXPOSURE EXERTS SEX-SPECIFIC EFFECTS ON OFFSPRING GROWTH AND LONG-TERM METABOLIC PROGRAMMING*

Rationale

Our results presented in chapter II reveal that the offspring sired by alcohol-exposed sires exhibit a molecular signature of hepatic fibrosis and defects in immune signaling[159]. We followed the postnatal growth of the offspring to 8 weeks of life and observed persistant growth retardation in male offspring. We hypothesize that preconception paternal alcohol exposure alters the long-term developmental program of the liver, which impairs offspring growth.

To test this hypothesis, we examined the effects of paternal preconception paternal alcohol consumption on the long-term growth and metabolic health of the offspring. We followed postnatal growth from birth to eight-weeks of life and assayed metabolic function (glucose tolerance, insulin tolerance) at this time. These data were published in the journal *Epigenetics & Chormatin* [159].

Background

In clinical studies, fetal alcohol spectrum disorders (FASDs) associate with three broad developmental defects: distinctive craniofacial malformations, central nervous system defects, and both prenatal and postnatal growth restriction[160,161]. The growth defects are characterized by reductions in height, weight, and body mass index that manifest at birth and continue to persist through young adulthood[162-164]. Indeed, although perceived as a childhood disorder, the growth phenotypes associated with FASDs are lifelong, with long-term growth restriction, as well as

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immune dysfunction, hyperinsulinemia and other endocrine disruptions persisting into adulthood[164-169]. As a consequence of these persistent abnormalities, the life expectancy of patients with fetal alcohol syndrome is 34 years, which is 58% lower than the general population[170]. This dramatic reduction is very likely linked to the capacity of alcohol to significantly alter developmental programming, which promotes the early onset of adult disease[171,172]. However, while much research has focused on the neurological phenotypes of FASDs, the relationship between ethanol exposures and the long-term effects on growth and metabolic programming have received comparatively little attention.

FASD growth phenotypes can be linked to alcohol-induced intrauterine growth restriction mediated by impaired placentation[163,173-176]. Impaired placentation, in turn, associates with poor cognitive development and long-term alterations in metabolic programming within the offspring[177-184]. Thus, the long-term effects of ethanol on growth and adult health may be linked to early developmental insults arising from a compromised fetal-maternal interface. Importantly, emerging research can now link a number of preconception exposures to compromised placentation and long-term alterations in metabolic function within the offspring[185-187]. These data further emphasize the importance parental histories of drug use, social stress and environmental exposures have on child health and may help explain the enormous variation observed in FASD phenotypes and incidence[188].

The impacts parental histories of alcohol use have on child development is poorly explored and represents a significant gap in our understanding of the teratogenic potential of ethanol. This is especially true of preconception male alcohol exposures, which are a largely ignored aspect of patient history and are similarly under-explored in relevant biomedical models[189]. To this point, emerging epidemiological research indicates preconception paternal alcohol exposures have the

ability to significantly influence child health and development[190,191]. For example, clinical data can correlate paternal alcoholism with negative impacts on child behavior[192-197] and cognitive development[198-202]. In addition, clinical associations between paternal drinking and increased rates of congenital abnormalities, as well as decreases in infant birth weight and head circumference have been reported[203-205]. However, in these studies, additional and often uncontrolled factors such as nutrition, poor housing conditions, maternal stress, smoking, and parental alcohol use, all exert independent effects on child growth and development. In this setting, it is virtually impossible to identify a direct link between preconception paternal alcohol use and child development[206,207].

In rodent models, preconception paternal alcohol exposures associate with reductions in birth weight, increased incidences of congenital anomalies and cognitive impairment in the offspring[190,208-217]. These observations join a growing body of work demonstrating the capacity of multiple stressors to alter the sperm-inherited developmental program[218-237] and suggest preconception paternal alcohol exposures may exert a long-term influence on offspring growth and development. In rodent models of maternal exposure, alcohol-induced abnormalities in the germline programming of the hypothalamic-pituitary stress response transmit through multiple generations via the male germline but are not maternally inherited[238]. Thus, alcohol has the ability to exert a lasting impact on the male-inherited developmental program, which, similar to studies employing models of intrauterine ethanol exposure[239-244], may exert long-term impacts on offspring health. However, no studies have yet directly examined the associations between paternal preconception alcohol exposures and long-term growth and metabolic programming within the offspring.

Recent work by our group has linked chronic preconception male alcohol exposure to fetal abnormalities in cholesterol trafficking, sex-specific patterns of growth restriction, disruptions in

the regulation of imprinted genes and alterations in the genetic pathways regulating hepatic fibrosis[217]. The identification of altered cholesterol trafficking and increased markers of hepatic fibrosis within the fetus raise the prospect that these paternally-inherited abnormalities have the capacity to significantly impact the long-term metabolic function of the offspring. This notion is further reinforced by the identification of compromised placental function in the offspring of alcohol-exposed males[217]. On the basis of these considerations, the aim of the present study was to investigate the long-term impact of chronic preconception paternal alcohol exposure on offspring growth and metabolic programming.

Results

Chronic paternal ethanol exposure associates with delayed parturition and intrauterine growth restriction of the offspring

In clinical studies, FASDs are characterized by reductions in height, weight, and body mass index that manifest at birth and persist through young adulthood[162,164]. However, no studies have determined the capacity of chronic preconception male alcohol exposures to contribute to these phenotypes or impact postnatal growth. To address this gap, adult male mice were exposed to ethanol every day for a 70-day period, using a previously described limited access model[245]. Similar to our previous studies[217], no differences in fluid consumption were observed between the ethanol and control preconception treatment groups (Fig. 8A). Ethanol exposures were measured on day 10 and again on day 70 of the preconception treatment course and in the ethanol-exposed males, yielded average plasma alcohol levels of 178 and 245 mg/dL respectively (Fig. 8B). During the course of the 70-day preconception treatments, no differences in weight gain could be detected between ethanol-exposed or control males (Fig. 8C). After 70-days of preconception treatment, exposed males were mated to six- to eight-week-old females. At no point during these experiments

were the females ever exposed to the preconception treatments.

Litters from each of the preconception control and ethanol-exposed treatment groups were termed and the length of gestation recorded. Unexpectedly, preconception alcohol exposure associated with a 10% increase in the length of gestation ($p < 0.05$), with litters sired by ethanol-exposed males born on day 23 versus day 21 for the control litters (Fig. 8D). No differences in litter size were observed between the preconception treatment groups (Fig. 8E). At one week of age, paternal alcohol exposure associated with a 25% reduction in the body weight of male offspring and a 15% reduction in the body weights of the female offspring (Fig. 8F-G, $p=0.004$, $p=0.017$). Accordingly, the ratio of offspring displaying intrauterine growth restriction[246] was significantly increased in the offspring of ethanol-exposed males (Fig. 8H, $p=0.04$). To determine the long-term impact of the observed growth restriction, fifteen male and fifteen female offspring were randomly selected from across five different litters and their weights tracked for eight weeks. Interestingly, while the female offspring of ethanol-exposed males were able to match the body weights of the controls within the first two weeks of life, male offspring sired by alcohol-exposed fathers continued to display an 11% reduction in weight at five weeks of age ($p=0.005$) and a 6% reduction at eight weeks of age ($p=0.0003$, Fig. 8F-G). To determine if this difference was associated with altered growth parameters, the average growth rates for each group were calculated. Similar to clinical studies of IUGR[247], both the male and female offspring of alcohol-exposed males displayed accelerated postnatal weight gain compared to the controls (Fig. 8I). However, as in clinical reports of FASD children[164], the male offspring of ethanol-exposed fathers remained smaller than the offspring of the controls. For clarity, Additional File 2 contains the data from Figure 1 presented in table form, with the number of replicates, statistical significance, and formula used to derive each measure presented. No significant interactions between litter size and

growth rate were detected using ANOVA.

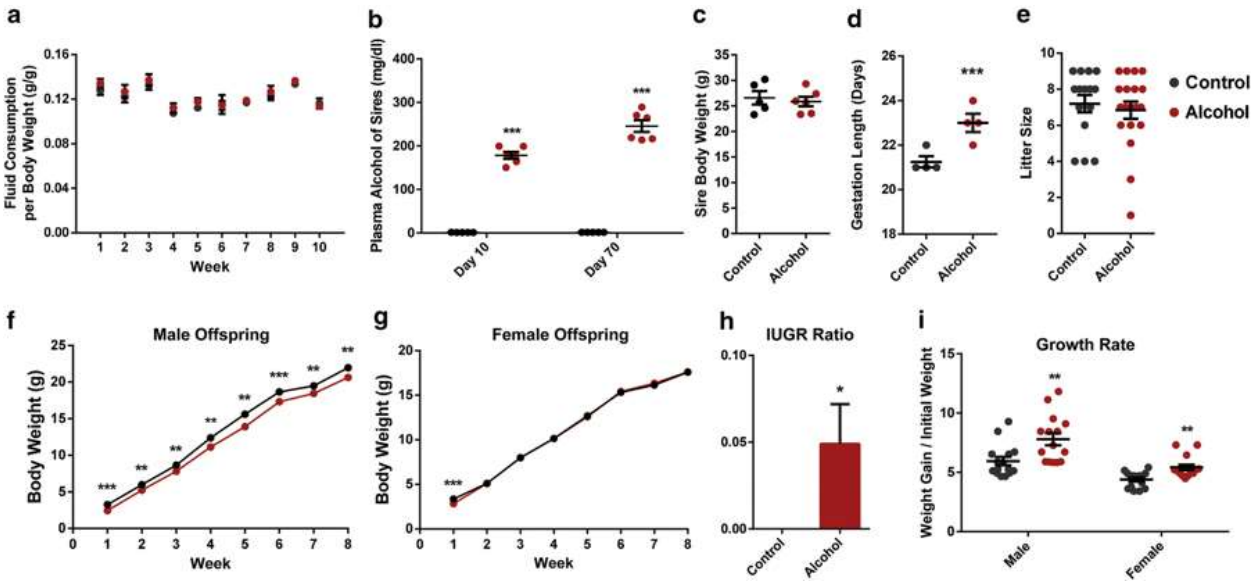

Figure 8 Preconception paternal ethanol exposure associates with intrauterine growth restriction (IUGR) and altered postnatal growth

(A) Average fluid consumption normalized by body weight during the preconception treatment period (n=5 control and 6 experimental males). (B) Plasma alcohol concentrations measured at Day 10 and Day 70 of the 70-day preconception exposure period (n=5 control and 6 alcohol-exposed males). Blood alcohol levels were measured two hours into the four hour exposure period. (C) Average body weight of sires at the end of the 70-day preconception exposure period (n=5 control and 6 experimental males). (D) Average length of gestation for litters sired by alcohol-exposed and control males (n=4). (E) Average litter size between the preconception ethanol and control treatment groups (n= 15 control 19 alcohol). Postnatal weights of (F) male and (G) female offspring sired by ethanol-exposed and control males measured over eight weeks (n= 15 males, 15 females from each treatment group). (H) Average rate of IUGR at one week of age.

The graph depicts the number of IUGR pups/the total number of offspring counted in each group. (I) Rate of bodyweight gain over eight-weeks of postnatal life. Error bars represent SEM *P < 0.05, **P< 0.01 and ***P < 0.001 (comparisons between 10% (w⁄v) ethanol plus 0.066% (w⁄v) Sweet'N Low (alcohol) versus 0.066% (w⁄v) Sweet'N Low alone (control) preconception treatments). Data were analyzed using either an unpaired t-test, arcsine transformed and an unpaired t-test with Welch's correction applied or used in a two-way ANOVA followed by Sidak post hoc analysis.

Chronic paternal ethanol exposure associates with long-term effects on glucose metabolism and insulin signaling

In clinical studies, IUGR children develop impaired insulin responses to glucose and similar observations have been reported in both sheep and rat models of fetal growth restriction[248-251]. To determine if the IUGR observed in the offspring of ethanol-exposed males impacted the long-term regulation of blood sugar homeostasis, glucose and insulin stress tests were conducted. Paternal ethanol consumption associated with a significant decrease in both fasting blood glucose and insulin levels in male offspring, while female offspring displayed an increase in fasting glucose levels only (Fig. 9A-B). At eight weeks of age, these alterations were associated with exaggerated insulin responses in both glucose and insulin tolerance tests within the male offspring, while females displayed a modest impairment in their glucose tolerance test (Fig. 9C-F). To identify the pathophysiological basis to these altered parameters, mice were sacrificed at eight weeks of age, their organs weighed and tissues collected for molecular analysis. The male offspring of ethanol-exposed sires displayed a 13% reduction in visceral fat (p=0.04) and while pancreas weight tended

to be smaller (p=0.1), this did not reach statistical significance (Fig. 9G). No differences in organ weights were noted in the female offspring (Fig. 9H).

To determine if preconception alcohol exposure impacted the insulin signaling pathway, six randomly selected, eight-week-old male and female mice from each preconception treatment group had their livers perfused with insulin to assay *Akt* signaling. *AKT* phosphorylation (Ser473) in the insulin perfused livers was increased by 26% in the male offspring of ethanol-exposed sires (p=0.014), while female offspring were identical to the controls (Fig. 9I-J). Collectively, these observations indicate preconception male alcohol exposure is associated with programmed metabolic dysfunction and are similar to data reported in long-term clinical studies of FASD children, in which disruptions in endocrine regulation of blood glucose levels have been observed[165].

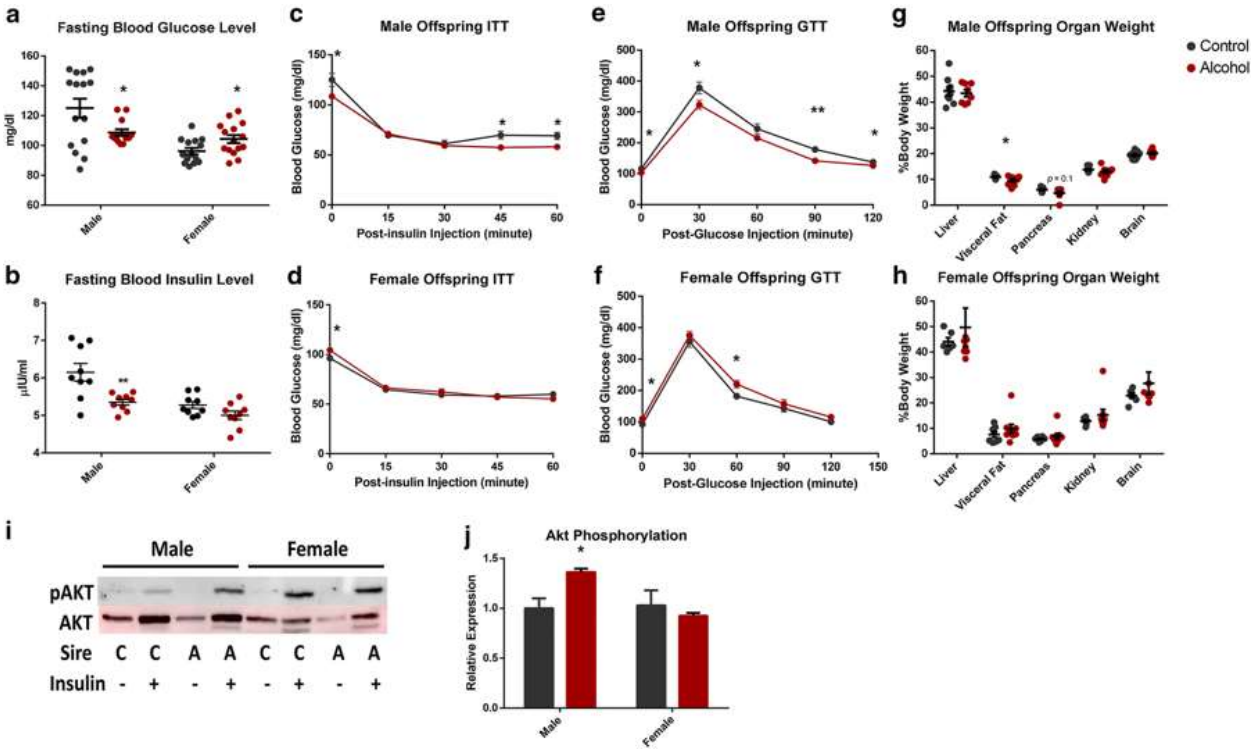

Figure 9 Chronic preconception male ethanol exposure exerts sex-specific effects on offspring metabolic function

(A) Fasting blood glucose levels compared between preconception treatments (n=15 males, 15 females). (B) Fasting insulin levels compared between preconception treatments (n=9 males, 9 females). (C-F) Glucose tolerance (GTT) and insulin tolerance (ITT) tests in the offspring of ethanol-exposed and control males (n= 15 males, 15 females). Organ weights of (G) male and (H) female offspring compared between the two preconception treatment groups (n=9 males, 9 females). (I) Representative immunoblot comparing total and phosphorylated AKT (Ser473) between male and female offspring sired by ethanol-exposed and control males (n=6 males and 6 females). (J) Densitometry analysis of immunoblots comparing total and phosphorylated AKT (n=6 males and 6 females). Data analyzed using either an unpaired t-test or a two-way ANOVA

followed by Sidak post hoc analysis. Error bars represent SEM *P < 0.05 and **P < 0.01 (comparisons between alcohol and control preconception treatments).

Preconception paternal alcohol exposure associates with markers of hepatic fibrosis in the adult offspring

In previous studies, intrauterine growth restriction has been associated with alterations in offspring blood lipid profiles and the development of adolescent non-alcoholic fatty liver disease (NAFLD)[178,252]. In our previous studies examining the fetal development of offspring sired by ethanol-exposed males, we observed alterations in placental cholesterol transport and the emergence of molecular markers associated with hepatic fibrosis[217]. To determine if the legacy of preconception ethanol exposure previously identified in the fetal liver extends into postnatal life, the expression of the genes identified in the transcriptomic analysis of the fetal liver were examined by RT-qPCR[217]. In male offspring, and to a lesser extent females, preconception paternal ethanol exposure continued to associate with persistent alterations in the expression of multiple collagen subtypes and core components of the *TGF-ß* signaling pathway driving hepatic fibrosis[253]; even at eight weeks of age (Fig. 10A-D). To determine if this transcriptional signature associated with increased molecular markers of hepatic fibrosis, we measured total levels of hydroxyproline, a commonly employed biomarker of this condition[254]. Given the trend towards a reduction in weight (Fig. 9G), we also assayed this marker in the pancreas. The male offspring of ethanol-exposed sires displayed a 13% increase in hydroxyproline levels within the liver ($p<0.01$), while levels of hepatic hydroxyproline in the female offspring were identical to the controls (Fig. 10E). No differences in levels of hydroxyproline content could be detected in the pancreas in either males

or females (Fig. 10F). These results indicate that the molecular signature previously identified in the fetal liver[217] persists into postnatal life and suggests the existence of a heightened fibrotic response within the livers of male offspring sired by alcohol-exposed fathers.

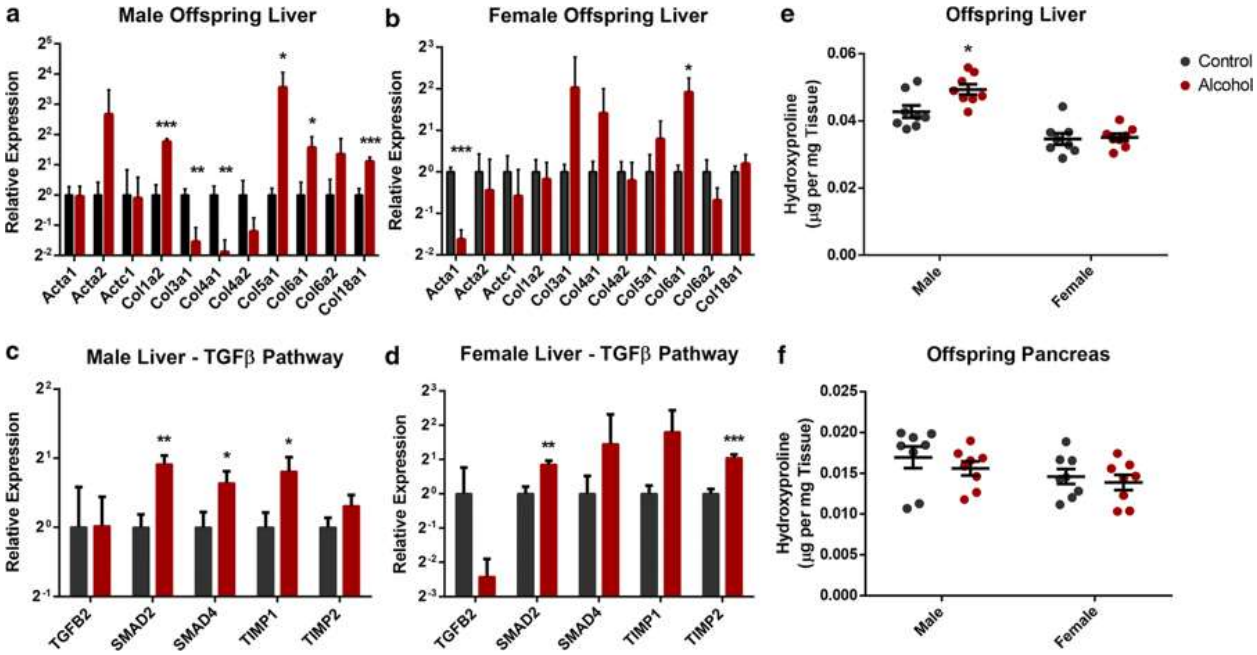

Figure 10 Preconception paternal ethanol exposure imparts a legacy of increased hepatic fibrosis within the male offspring.

RT-qPCR analysis of genes encoding pro-fibrotic structural proteins in the adult liver of (A) male and (B) female offspring (n=8). RT-qPCR analysis of genes within the pro-fibrotic TGF-B signaling pathway in the livers of 8-week old (C) male and (D) female offspring sired by ethanol-exposed and control males (n=8). Total levels of cellular hydroxyproline within the (E) livers and (F) pancreas of 8-week old offspring sired by ethanol-exposed and control males (n=8). For RT-qPCR analyses, measured Ct values were normalized to the geometric mean of transcripts encoding Ywhaz, Hprt, and Mrpl1, and graphed relative to the control treatment. Graphs represent

independent replicates, with two independent RT reactions and three RT-qPCR measurements for each RT. Data analyzed using either an unpaired t-test or a two-way ANOVA followed by Sidak post hoc analysis. Error bars represent SEM *P < 0.05, **P < 0.01 and ***P < 0.001 (comparisons between alcohol and control preconception treatments).

Preconception paternal alcohol exposure associates with disruptions in hepatic gene expression within the adult offspring

Given the persistent changes in gene expression identified between the fetal[217] and adult liver (Figure 10), we next assayed genome-wide patterns of transcription in the adult liver of male offspring using deep sequencing. These analyses identified 548 differentially expressed genes, with 351 down-regulated and 197 up-regulated transcripts identified (q=0.05; Fig. 11A). In these analyses, we observed a high level of variability, where not all of the differentially expressed genes were consistent between the between the offspring of alcohol-exposed sires (Fig. 11B). Using Ingenuity Pathway Analysis, we identified up-regulation of candidate genes participating in the genetic pathways regulating both *LXR/RXR* and *FXR/RXR* activation, as well as down-regulation of genes participating in the cholesterol super-pathway of biosynthesis (Fig. 11C). Although we were able to validate multiple candidate genes participating in *LXR/RXR* and *FXR/RXR* signaling pathways, identified candidate genes participating in pathways regulating cholesterol biosynthesis were not significantly different (Fig. 11D). However, we did observe a 15% increase in the levels of total cholesterol in the livers of the male offspring of alcohol-exposed sires (p=0.04, Fig. 11E). These differences were not linked to specific increases in either high density or low-density lipoproteins, and no differences in their proportional relationship could be detected (Fig. 11F-H).

In contrast to males, female offspring of alcohol-exposed sires displayed a 23% reduction in the proportion of high-density lipoproteins to total cholesterol, as compared to the female offspring of control fathers ($p=0.03$, Fig. 11H). No differences in total cholesterol, high density, and low-density lipoproteins or their proportional relationships were observed within the adult plasma for either sex (Fig. 11I-L).

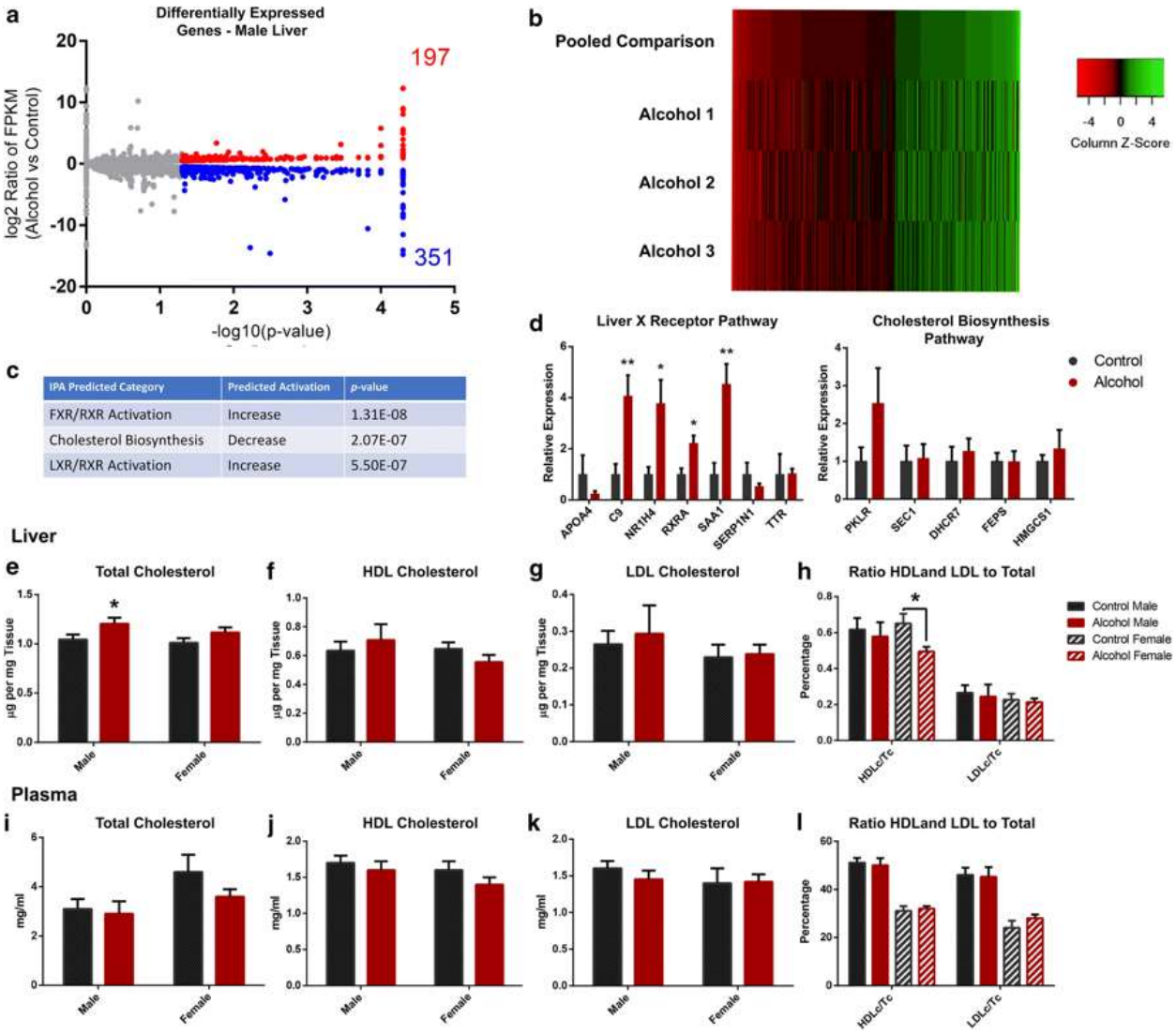

Figure 11 Comparison of the hepatic transcriptome between the adult offspring of ethanol-exposed and control males

(A) Volcano plot displaying differences in gene expression between adult male offspring sired by ethanol-exposed and control fathers (n=3, cutoff q=0.05). (B) Heatmap depicting the differentially expressed genes between the pooled control offspring and each of the three offspring of the alcohol exposed males. (C) Top three categories identified using Ingenuity Pathway Analysis. (D) RT-qPCR validation of candidate genes related to the identified FXR/RXR,

FXR/RXR and cholesterol biosynthesis pathways in the adult male liver (n=8). Levels of hepatic (E) total cholesterol, (F) high density lipoprotein and (G) low density lipoprotein cholesterol esters within the liver of male and female offspring sired by ethanol-exposed and control fathers (n=8). (H) Proportional ratios of HDL cholesterol to total cholesterol and LDL cholesterol to total cholesterol in liver samples derived from the male and female offspring of ethanol-exposed and control males (n=8). Quantification of (I) total cholesterol, (J) high density lipoprotein and (K) low density lipoprotein cholesterol esters within the plasma of male and female offspring sired by ethanol-exposed and control fathers (n=8). (L) Proportional ratios of HDL cholesterol to total cholesterol and LDL cholesterol to total cholesterol in plasma derived from the male and female offspring of ethanol-exposed and control males (n=8). For RT-qPCR analyses, measured Ct values were normalized to the geometric mean of transcripts encoding Ywhaz, Hprt, and Mrpl1, and graphed relative to the control treatment. Graphs represent independent replicates, with two independent RT reactions and three RT-qPCR measurements for each RT. Data were analyzed using either an unpaired t-test, arcsine transformed and an unpaired t-test with Welch's correction applied or used in a two-way ANOVA followed by Sidak post hoc analysis. Error bars represent SEM *$P < 0.05$ and **$P < 0.01$ (comparisons between alcohol and control preconception treatments).

Preconception paternal alcohol exposure associates with long-term alterations in immune signaling within the offspring

Clinical studies of small-for-gestational-age neonates have associated alterations in key inflammatory markers with increased hepatic fibrosis[255,256]. However, the mechanistic basis

underlying these abnormalities has been challenging to decipher. *Liver X receptors* (*LXRs*) are known to modulate numerous aspects of hepatic cholesterol metabolism but have also been found to modulate immune and inflammatory responses in tissue resident macrophages[257]. Specifically, activation of *LXR/RXR* and *FXR/RXR* pathways suppress tissue inflammatory responses via *NFK-B* signaling and block the downstream release of multiple cytokine signaling molecules[258]. We, therefore, assayed the liver, pancreas, and plasma for alterations in *IL1B*, *IL6*, *INFγ* and *TNFα*, which are all established markers of inflammation linked to *LXR* and *NFK*-b signaling[259,260]. In the liver, a ~15% decrease in *IL1B* and a 60% decrease in *IL6* were identified in both male and female offspring, while no alterations in *INFG* and *TNFa* were observed (Fig. 12A-D). In the pancreas, the male offspring of alcohol-exposed sires displayed a ~40% decrease in the levels of *IL1B*, *IL6*, and *TNFα*, while levels of *INFγ* were similar to the controls (Fig. 12E-H). No differences in any of the measured cytokines were observed in the pancreas of female offspring (Fig. 12E-H). In the plasma, only *IL6* could be detected and no significant differences were observed in either males or females (Fig. 12I). These results are similar to previous clinical observations of FASD adolescents, which have also reported immune suppression within this patient group[166-168].

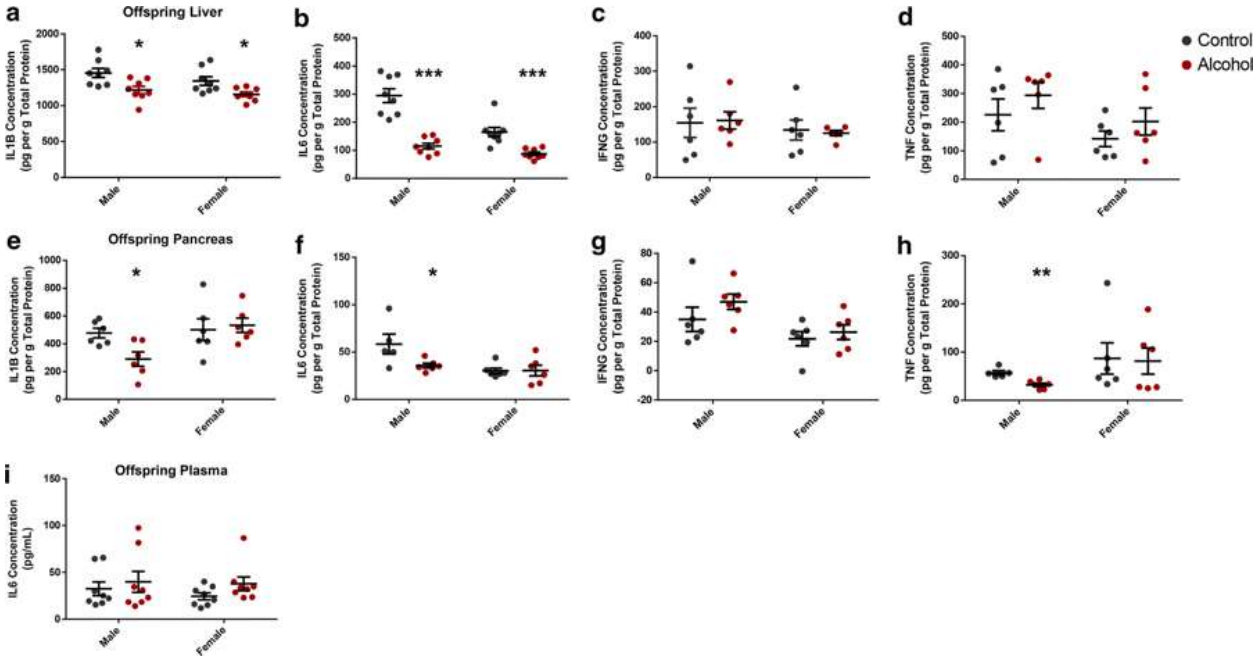

Figure 12 Preconception paternal alcohol exposure associates with long-term alterations in immune signaling within the offspring

Abundance of the inflammatory cytokines IL1B, IL6, INFg and TNFa in the (A-D) liver, (E-H) pancreas and (I) plasma of 8-week old offspring sired by ethanol-exposed and control males (n=8). Data analyzed using a two-way ANOVA followed by Sidak post hoc analysis. Error bars represent SEM *P < 0.05, **P < 0.01 and ***P < 0.001 (comparisons between alcohol and control preconception treatments).

Preconception alcohol exposure does not impact the regulation of imprinted genes

This study utilized two strains of mice carrying distinct single nucleotide polymorphisms within both the promoter regions and messenger RNAs of multiple genes. When using F1 hybrid crosses between the B6(CAST7) strain and a C57BL/6J strain, we can distinguish maternal and paternal alleles using C57BL/6(Cast7) and C57BL/6J polymorphisms. Using informatic

approaches, we mined our RNA-sequence data sets to determine if any imprinted genes exhibited abnormal bi-allelic expression (Table 3). We were able to identify exclusively paternal expression for *Peg3*, exclusively maternal expression for *Snrpn* and bi-allelic expression of *Ube3a*, which is only imprinted in the brain. None of the candidate genes examined displayed any detectable abnormalities in imprinted gene expression and no candidate genes were identified as differentially expressed in the RNA-seq dataset.

Gene	SNP location	C57BL/6J	Control 1	Control 2	Control 3	Alcohol 1	Alcohol 2	Alcohol 3
Ascl2	chr7:142,968,971	G	ND	ND	ND	ND	ND	ND
Cdkn1c	chr7:143,460,109	A	A/6	A/6	A/7	A/10	A/3	A/10
Dcn	No SNP found		ND	ND	ND	ND	ND	ND
Dlk1	chr12:109,460,379	A	ND	ND	A/1	ND	ND	ND
Gatm	chr2:122,594,926	C	ND	ND	ND	ND	ND	ND
Gnas	No SNP found		ND	ND	ND	ND	ND	ND
Grb10	chr11:11,954,907	A	A/1	ND	ND	ND	ND	ND
Gtl2	chr12:109,545,837	A	A/1	A/1	ND	ND	ND	ND
H19	chr7:142,577,095	T	ND	ND	T/1	T/1	T/1 C/1	ND
Igf2 (1)	chr7:142,652,037	G	ND	ND	ND	ND	ND	ND
Igf2 (2)	chr7:142,652,936	C	ND	ND	ND	ND	G/2	ND
Igf2r	chr17:12,682,709	C	C/64	C/116	C/171	C/126	C/67	C/87
Mest	chr6:30,747,382	A	A/2	ND	ND	ND	ND	A/1
Ndn	chr7:62,348,457	T	C/2	C/1	C/1	ND	ND	ND
Peg3	chr7:6,706,217	G	T/4	T/12	T/5	T/2	T/8	T/14
Sgce	chr6:4,717,926	T	T/5	T/4	T/1	T/3	ND	T/4
Slc38a4	chr15:96,995,974	C	C/89 A/1	C/78 A/1	C/26	C/117 A/1	C/74 A/1	C/72
Snrpn	chr7:59,983,415	T	T/49	T/76	T/120	T/94	T/48	T/72
Ube3a	chr7:59,228,878	T	T/7 C/5	T/8 C/8	T/3 C/5	T/16 C/10	T/7 C/9	T/6 C/3
Zac1	chr10:13,128,934	G	G/1	G/5	G/3	G/3	ND	G/1
Zim1	chr7:6,675,637	A	ND	ND	ND	ND	ND	ND

Table 3 Abundance of B6(CAST7) and C57BL/6J polymorphisms identified within select imprinted genes

The abundance of B6(CAST7) and C57BL/6J polymorphisms within the RNA-sequencing profiles of the adult liver were compared between the offspring of males exposed to the two preconception treatments.

Discussion

Our group recently reported sex-specific patterns of fetal growth restriction in a mouse model of preconception male ethanol exposure[217]. This growth restriction predominantly impacted the female offspring of ethanol-exposed sires and was accompanied by a 12% decrease in placental efficiency, abnormal placental cholesterol transport and altered markers of hepatic fibrosis within the fetal liver. The aim of the present study was to determine if these paternally-inherited abnormalities cause any long-term impacts on the growth and metabolic health of the offspring. Here, we report that chronic paternal ethanol exposure associated with a prolonged period of fetal gestation and an increased incidence of intrauterine growth restriction, which affected the male offspring to a greater extent than the females. In the male offspring, these growth deficits persisted into adult life and associated with insulin hypersensitivity, increased markers of hepatic fibrosis, and alterations in immune signaling.

How preconception paternal ethanol exposure leads to the sequelae described above is not understood. We suspect that similar to other models of altered developmental programming, ethanol-induced disruptions in the sperm-inherited epigenetic program alter the formation or function of the placenta, which leads to long-term alterations in developmental programming within the offspring[185-187]. In our model, we observed a 7% decrease in the weight of only the female offspring at gestational day 14.5[217], while one week after birth, both the male and female offspring display significant growth restriction (25% in males 15% in females). These observations indicate that the large majority of growth restriction predominantly occurs during the later phases of pregnancy when the mouse fetus experiences a dramatic increase in growth rate[261]. This late-phase growth restriction is similar to the phenotypes reported in studies of placental-specific *Insulin growth factor 2* (*Igf2*) loss of function, as well as prenatal nutrient restriction, which also

predominantly manifest late in pregnancy[262,263]. In these studies, the placenta initially compensates by increasing its overall efficiency through proportional increases in the labyrinthine layer (initially) and up-regulation of System A amino acid transporters (later)[263]. However, these strategies cannot sustain fetal growth through the late phases of pregnancy, when the mouse fetus is normally growing the most rapidly in absolute terms[264]. In our model, we observed decreased placental efficiency at gestational day 14.5, indicating the placenta enters the late growth phase at a functional deficit[217]. We suspect that as pregnancy proceeds, this placental dysfunction progressively gets worse, ultimately causing growth restriction and the delay in partition identified in Figure 1. Further studies examining placental morphology and function between gestational days 16 and 19 are required to define the physiological basis of this growth restriction.

Although very few clinical studies have followed FASD children into adolescence (reviewed here [169]), the few that have, report a number of observations that share many similarities with results presented in this report. For example, a recent study examining a patient cohort from Cape Town South Africa followed the height, weight and head circumference of alcohol-exposed babies through to 13 years of age[164]. In this cohort, alcohol-exposed children had a growth trajectory that was significantly less than the control group at all ages examined. In our model of paternal ethanol exposure, we observed significant growth restriction at one-week of age, which in the male offspring, persisted despite an overall increase in growth rate. Although the Cape Town study did not conduct separate analyses of males and females, other studies indicate that male FASD children have lower postnatal viability compared to females, indicating FASD growth defects may impact boys to a greater extent than girls[265].

We observed insulin hypersensitivity in the male offspring of ethanol-exposed fathers, which correlated with long-term deficits in growth. Our observations indicate this increased response is

due to heightened sensitivity of the hepatic insulin signaling pathway, although the specific molecular mechanisms by which this arises are still under investigation. Interestingly, our data are in direct contrast with both a clinical case report examining the metabolic health of FASD children[165], as well as work conducted using a rat model of prenatal maternal ethanol exposure, in which insulin resistance was observed[266,267]. However, in the rat model, both the male and female F2 progeny of ethanol-exposed offspring displayed hypoglycemia and hyperinsulinemic response patterns[267]. Combined with our work, these observations suggest that ethanol can program an insulin hypersensitive phenotype, which, similar to alcohol-induced disruptions to the hypothalamic-pituitary axis[238], can transmit to the next generation via the germline.

Clinical studies have shown that, even after controlling for low maternal income, maternal smoking, and birthweight, newborns whose mothers drank during pregnancy have a 3-fold increased risk of infection compared to mothers who did not drink[168]. FASD infants exhibit an increased risk of developing upper respiratory tract infections, recurrent otitis media, pneumonia, persistent diaper rash, meningitis, and gastroenteritis[166,167]. These deficits in immune function have been linked to alterations in the number of T helper (*CD4+/CD3+*) and T cytotoxic/suppressor cells (*CDS+/CD3+*)[167], suggesting the processes driving systemic inflammation are blunted in these children. Our studies examining the offspring of ethanol-exposed males identified alterations in hepatic *LXR* signaling, which, through the activity of *NFKb*, is a potent suppressor of multiple cytokine signaling pathways[257,258]. Consistent with these observations, we identified tissue-specific reductions in multiple *NFKb* regulated genes (*IL1B*, *IL6*, and *TNFa*) within the liver and pancreas but not in the plasma. It is unclear if the reductions in immune signaling observed in our model have any relevance to the enhanced predisposition of FASD children to postnatal infection, however, programmed alterations in systemic inflammation appear to be a common theme

emerging in other models of developmental programming[268]. Additional studies are needed to determine how preconception paternal ethanol exposure influences the inflammatory stress response.

Consistent with previous studies[269], placental dysfunction occurring late in gestation predominantly affected the long-term health of the male fetus, while the female offspring were better able to recover. These observations suggest that females are better able to compensate for late-stage insults than males. Interestingly, while hepatic fibrosis is one of the defining symptoms of alcoholic liver disease in adults[270], three isolated case reports have also identified a similar condition in FASD children[271-273]. Our previous studies of the fetal liver identified alterations in the transcription of candidate genes participating in the genetic pathways regulating hepatic fibrosis and stellate cell activation[217]. Although found in both the male and female offspring of ethanol-exposed sires, this signature was diametrically opposite between the sexes; males displayed an up-regulation of pro-fibrotic genes while females suppressed this pathway. In the liver, 72% of genes are expressed in a sexually dimorphic manner and importantly, half of the candidate gene identified in this study follow this pattern[274]. Therefore, sex-specific differences in patterns of hepatic gene expression may explain the contrasting outcomes between the male and female offspring. Importantly, the persistence of this signature in the adult male liver, along with the increased hydroxyproline content, suggests that preconception paternal alcohol exposure may predispose the offspring to hepatic dysfunction and susceptibility to liver disease. If true, this would significantly enhance our understanding of the mechanisms of inheritance at work in the development and progression of alcoholic liver disease. Additional studies are necessary to determine the importance this pro-fibrotic signature has in hepatic disease pathogenesis.

Finally, although we were unable to validate the differential expression of genes participating

in the cholesterol biosynthesis pathway, we did observe a modest increase in total cholesterol levels in the male offspring of alcohol-exposed sires and a proportional reduction in the ratio of high-density lipoproteins to total cholesterol in the female offspring. The failure to identify changes in cholesterol-related transcripts is likely reflective of the variability of this model, which unlike a genetic model, is more prone to produce a subset of offspring that are highly affected while others exhibit a more modest phenotype. Indeed, although select collagen subtypes were present in our RNA-seq datasets, fibrosis did not emerge as an enriched pathway, indicating this phenotype is also highly variable. Future studies will explore the impact the stress of a high-fat diet has on the offspring of alcohol-exposed fathers and will help determine both the penetrance of the metabolic phenotypes and the extent of the sexual dimorphism in this preconception model of exposure.

One potential confound to this study is the use of the 0.066% (w⁄v) Sweet'N Low in both the control and alcohol preconception treatments, which was used to promote the consistent consumption of ethanol[245]. The gut contains glucose receptors that are stimulated by artificial sweeteners causing the release of incretin peptide hormones, which have a significant role in glucose homeostasis, metabolic control and proper ß-cell function[275]. Artificial sweeteners also influence the intestinal microbiota and alter metabolic function indirectly[276]. Therefore, it is possible that some of the effects are the result of metabolic disturbances in the sires. However, we did not observe any differences in weight gain between the preconception treatments and neither group displayed an obese phenotype. In addition, genetic differences unique to the C57BL/6(Cast7) and C57BL/6J cross may influence the observed phenotypes. However, the ability to informatically distinguish the maternal and paternal alleles allowed us to examine imprinted gene expression, which is disrupted in other models of developmental

programming[277]. Future studies will focus on implementing an exposure paradigm that does not involve the use of artificial sweeteners and repeating these observations using both a pure C57BL/6J cross and an outbred line.

Materials and Methods

Animal work

All experiments were conducted under AUP 2017-0308 and approved by the Texas A&M University IACUC. In the outlined experiments, the male mice were of a C57BL/6(Cast7) background, while female mice were C57BL/6J (RRID:IMSR_JAX:000664). The C57BL/6(Cast7) strain of mice were generated in the Bartolomei laboratory and were selected to possess portions of a *Mus* m*usculus castaneus* chromosome 7 and chromosome 12 (where at least 5 imprinting domains and more than 30 imprinted genes reside) bred onto a C57BL/6J background[278]. When using 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 previously[217,279]. The C57BL/6(Cast7) strain of mice is on a C57BL/6J background, which is susceptible to alcohol-induced teratogenesis[280,281].

To investigate the long-term impact of alcohol exposure on the male-inherited developmental program, an established and well-characterized mouse model of chronic alcohol exposure was employed[245]. Here, postnatal day 90, adult males were provided limited access to ethanol during a four-hour window of the night cycle. This rodent model (Drinking in the Dark) promotes the voluntary consumption of ethanol in sufficient quantities to achieve pharmacologically meaningful blood alcohol concentrations, typically in excess of 150mg/dL[217,282]. In a study examining the drinking patterns of 10,424 college freshmen in the United States, 1 in 5 males reported routinely consuming 10+ drinks in a single session, while half

reported drinking beyond the binge level (5+ drinks in a single session)[283,284]. Thus, the blood alcohol levels observed in our model reflect a range frequently obtained by college age males[283-285].

Here, individually caged, postnatal day 90, adult males were fed a standard diet (catalogue# 2019, Teklad Diets, Madison, WI, USA) and maintained on a 12-hour light/dark cycle. Males were provided limited access to ethanol during a four-hour window, beginning one hour after the initiation of the dark cycle[217]. During this four-hour window, experimental males were provided access to a solution of 10% (w/v) ethanol (catalog# E7023; Millipore-Sigma, St. Louis, MO, USA) and 0.066% (w/v) Sweet'N Low (Cumberland Packing Corp, Brooklyn NY, USA), while control males received a solution of 0.066% (w/v) Sweet'N Low alone. After each session, the amount of fluid consumed by each mouse was recorded.

The addition of Sweet'N Low is necessary to encourage male mice to develop consistent drinking habits. Although, prolonged exposure to a 10% Sweet'N Low solution has previously been shown to drive the development of glucose intolerance through functional alterations to the intestinal microbiota, the concentrations employed in our studies were 150-fold lower than those utilized in these previous experiments[276]. In addition, we were careful to ensure that mice in both preconception treatment groups consumed equivalent fluid volumes and therefore, received identical exposures to Sweet'N Low.

Once consistent patterns of drinking were established, 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 spermatids were exposed to alcohol[286,287]. 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 were 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 each male had produced a minimum of three litters.

Pregnant dams were maintained on a Breeder diet (catalogue# 5058; LabDiet, St. Louis, MO, USA), subjected to minimal handling and monitored for delivery twice daily. One week after birth, fifteen male and female offspring from each preconception treatment group were randomly selected from across at least five different litters and monitored for postnatal growth and development. Mice were maintained on a standard diet (catalogue# 2019; Teklad Diets, Madison, WI, USA) and body weight was recorded weekly, for eight weeks. Between seven and eight weeks of age, metabolic function was assayed using glucose and insulin tolerance tests. After eight weeks of age, offspring were terminated and both physiological fluids and tissues were collected.

Insulin and Glucose Tolerance Tests

Beginning at seven weeks of age, mice were fasted overnight for 12 hours and tested for glucose and insulin tolerance, with a minimum of a one-week recovery time between these separate tests. Here, mice received a single intraperitoneal injection of D-glucose (2 g/kg body weight; catalog# SG8270; Millipore-Sigma, St. Louis, MO, USA) or insulin (1 unit/kg body weight; catalog# 89508-914; VWR, Radnor, PA, USA) and blood glucose levels measured using Clarity Plus - Blood Glucose Test Strips (catalog# DTG-GL5PLUS; Clarity Diagnostics, Boca Raton, Florida USA) from 5ul of blood drawn from the tail vein. For glucose tolerance tests, blood glucose levels were measured before the injection of glucose and at 30, 60, 90, and 120 minutes post injection. For insulin tolerance tests, blood glucose levels were measured before the injection of

insulin and at 15, 30, 45, and 60 minutes after insulin injection. Each experimental group contains fifteen male and female animals (n = 15).

Liver Perfusion Assay

One week after the final evaluation of metabolic parameters, six males and six females from each group were randomly selected to evaluate insulin signaling and levels of AKT phosphorylation (Ser473) in the liver. Mice were fasted 12 hours overnight and anesthetized with 2% isoflurane[288], until animals achieved a deep plane of anesthesia, demonstrating a lack of a righting reflex and a ~50% reduction in respiratory rate. At this point, the body cavity of the mouse was opened and the liver perfused with either 37 °C PBS + 0.1% BSA (Control) or 2 units/kg of an insulin solution (10 nM insulin, Sigma, in PBS + 0.1% BSA; catalog# 89508-914; VWR, Radnor, PA, USA) at a flow rate of 100 ml/h. Here, the suprahepatic vessel was clamped and a 27-gauge syringe inserted into the intrahepatic cava. After observing liver perfusion, the hepatic portal vein was cut open. During this procedure, a nose cone containing gauze soaked in isoflurane was kept over the nose of the animal and removed only when the rate of respiration dipped down below 25%. After five minutes, animals were euthanized by cervical dislocation and tissues of interest, including liver, kidney, and pancreas collected and snap frozen in liquid nitrogen. Each experimental group contains six different animals (n = 6).

Western Immunoblot Analysis

Liver tissue samples were collected and homogenized in a Tris lysis buffer including 50 mM Tris, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 1% β-mercaptoethanol, 50 mM NaF, 1 mM Na3VO4; pH 7.5. Samples were separated on 10% sodium dodecyl sulfate–polyacrylamide gels by electrophoresis and transferred to nitrocellulose membranes. The primary antibodies used in this study were as follows: *anti-phosphorylated protein kinase B* (*AKT*) at Serine 473 (catalog

#700392; RRID:AB_2532320; Thermo-Fisher, Waltham, MA, USA) and anti-*AKT* used for loading control (catalog #44609G; RRID:AB_2533692; Thermo-Fisher, Waltham, MA, USA). Blots were visualized by using secondary antibodies conjugated to horseradish peroxidase (catalog# sc-2004; RRID:AB_631746; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and an enhanced chemiluminescence detection system (Pierce, Rockford, IL, USA). Relative *AKT* phosphorylation was derived as a ratio to total *AKT*. Band intensities were quantified by densitometry using ImageJ (RRID:SCR_003070; National Institutes of Health, Bethesda, MD, USA). Each experimental group contains six different animals (n = 6).

Measurement of Physiological Parameters

Male plasma alcohol concentrations were measured using an Ethanol Assay Kit (catalog# ECET100; BioAssay Systems, Hayward, CA, USA) according to manufacturer's protocol. Animals were euthanized by cervical dislocation, blood collected post-mortem and plasma insulin levels determined using the Mouse Insulin ELISA kit (catalogue# EMINS; Thermo-Fisher, Waltham, MA, USA), according to the recommended protocol. Levels of *IL1B*, *IL6*, *INFg* and *TNFa* were determined using commercial ELISA assays (catalog# KMC0061 and KMC0011; Thermo-Fisher, Waltham, MA, USA and catalogue # Ab100689 and Ab100747; Abcam, Cambridge, MA, USA). Total cholesterol levels were determined using the Total Cholesterol Assay Kit (catalog# STA- 384; Cell Biolab, Inc, San Diego, CA USA), according to the recommended protocol. The comparative levels of low density and high density lipoproteins were determined using a Cholesterol Assay Kit (catalog# ab65390; Abcam, Cambridge, MA, USA), according to the recommended protocol. The levels of hydroxyproline, were determined using the Hydroxyproline Assay Kit (catalog# MAK008; Millipore-Sigma, St. Louis, MO, USA), following to the recommended protocol.

RNA analyses

Total RNA was isolated from eight-week old offspring liver 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, USA) and pooled for sequencing on an Illumina HiSeq 2500 at the Whitehead Genome Technology Core (Cambridge, MA, USA). Sequencing data were demultiplexed and aligned using STAR (RRID:SCR_015899) with default parameters[289].

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

Following deep sequencing, Bowtie (RRID:SCR_005476) and Tophat (RRID:SCR_013035) were used to align 50-bp length, paired-end reads into transcripts using the *Mus musculus* (UCSC version mm10) reference genome. To measure the relative abundance of each transcript, the resulting aligned reads were analyzed using the Cufflinks suite (RRID:SCR_014597). 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 transcripts, a volcano plot measuring statistical significance and magnitude of fold-change was generated based on the log2 fold-change (Y-axis) and −log10 p-value from Cuffdiff analysis within the Cufflinks suite (RRID:SCR_014597) (X-axis). Differentially expressed mRNAs were selected on the basis of linear p-value cut off of 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 (RRID:SCR_008653;

Ingenuity System Inc, Redwood City, CA, USA)[290].

Real-time RT-qPCR Analysis of Gene Expression

Total RNA was isolated from 8-week liver 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; Millipore-Sigma, St. Louis, MO, USA) according to the manufacturer's recommendations and seeded into a reverse transcription reaction using the High Capacity cDNA Reverse Transcription Kit (catalog# 4368814; Thermo-Fisher, Waltham, MA, USA), where the reaction mixture was brought to 25°C for 10 minutes, 37°C for 120 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-Fisher, Waltham, MA, USA) according to the recommended protocol. Reactions were performed on a Bio-Rad CFX384. Primer sequences are listed in Additional File 1.

Data Handling and Statistical Analysis

For all experiments, measures were input into the statistical analysis program GraphPad (RRID:SCR_002798; GraphPad Software, Inc., La Jolla, CA, USA) and statistical significance was set at alpha = 0.05. All datasets were first verified for normality using the Brown-Forsythe test. In this study, the effect of two independent variables (sex versus preconception treatment) were assessed using an analysis of variance test (ANOVA), and differences among the means evaluated using Sidak's post-hoc test of contrast. No interactions ($p>0.05$) were observed between fetal weight and sire weight, fetal weight and dam weight, or litter size and fetal weight. Pups were defined as intrauterine growth restricted (IUGR) if their weight at one week of age was less than the average weight of the control group less two standard deviations[246]. The IUGR ratio was calculated by dividing the number of IUGR offspring by the total number of fetuses in each litter.

To determine the significance of IUGR ratio between groups, the IUGR ratio were first transformed by arcsine square root prior to unpaired t-test examination. The offspring were weighed weekly from week 1 through week 8. The average growth rate was calculated using the following equation: [(bodyweight at week 8 − bodyweight at week 1) / bodyweight at week 8].

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. Transcripts encoding *tyrosine 3-monooxygenase / tryptophan 5-monooxygenase activation protein zeta* (*Ywhaz* - NM_011740), *mitochondrial ribosomal protein L1* (*Mrpl1* - NM_053158) and *hypoxanthine-phosphoribosyl transferase* (*Hprt*—NM_013556) were measured as reference genes. Each of these reference genes were validated for stability across treatment groups using methods described previously[291]. Normalized expression levels were calculated using the ddCt method described previously[292]. Relative fold change values from each biological replicate were determined and transferred into the statistical analysis program GraphPad. For comparisons including sex and preconception treatments, an analysis of variance (ANOVA) was utilized and Sidak's analysis applied to comparisons with p-values < 0.05. 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) strains[278] 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 single nucleotide polymorphisms was measured using the Integrative Genome Viewer (RRID:SCR_011793) package and analyzed using either Chi-Squared analysis or, if read counts were less than 5, a Fisher's Exact test.

CHAPTER IV

PRECONCEPTION PATERNAL ALCOHOL EXPOSURE EXERTS SEX-SPECIFIC EFFECTS ON OFFSPRING GROWTH AND LONG-TERM METABOLIC PROGRAMMING*

Rationale

Chapter III examining the adolesent liver identified up-regulation of the liver X receptor pathway in the male offspring of alcohol-exposed males[159]. We, therefore, are interested on how the offspring of alcohol-exposed males will respond to high-fat-diet challenge. We hypothesize that the offspring of alcohol-exposed males will display resistance to high-fat-diet induced-obesity, liver inflammation, and insulin hypersensitivity.

This chapter will determine the impact preconception paternal alcohol consumption has on offspring response to a high-fat dietary challenge. We examined base metabolic and immune signaling functions of the offspring of alcohol-exposed fathers after undergoing a 12-week high-fat-diet challenge. We conducted glucose and insulin stress tests and measure components involved in both the insulin and inflammatory signaling pathways, to determine the potential crosstalk between these two systems and their role in mediating these paternally-inherited phenotypes. These data have been submitted to the journal *Molecular Metabolism*.

Background

Despite clear evidence that parental histories of drug abuse, over or malnutrition, social stress and environmental exposures all influence fetal development, the long-term contributions of preconception history to the development of disease remain a significant gap in our understanding

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* Reprinted with permission from "Programmed increases in LXRα induced by paternal alcohol use enhance offspring metabolic adaptation to high-fat diet induced obesity." By RC Chang, 2019, ***Molecular Metabolism***, 12(30),161-172, Copyright 2019 by ELSEVIER.

of developmental toxicology[100,293]. This is especially true of preconception male exposures, which are a largely ignored aspect of patient history and are dramatically under-explored in relevant biomedical models. Indeed, although maternal exposures to alcohol, cigarettes, and other drugs of abuse have well-established effects on fetal development, the importance of the lifestyle choices of the father have only recently been recognized for their potential to influence offspring health[97]. However, the molecular pathways by which preconception paternal drug exposures exert a long-term influence on offspring growth and development remain poorly defined.

Similar to rodent models examining a wide range of other environmental and metabolic stressors, paternal exposures to alcohol and drugs correlate with alterations to the sperm epigenome, as well as changes in offspring behavior, metabolism and the propensity to develop disease[97,117]. Using a mouse model of voluntary consumption, our lab recently identified an association between chronic paternal alcohol use and long-term effects on the growth and metabolic health of the offspring[99,159,294]. Specifically, the offspring of males continuously exposed to alcohol before conception displayed placental dysfunction and late-term fetal growth restriction[99,294]. Interestingly, the male offspring of alcohol-exposed fathers continued to exhibit growth deficits in postnatal life, which persisted into adolescence[159]. Similar to our studies, Rompala and colleagues have also described alcohol-induced effects on the postnatal growth of male offspring using an inhalation model of alcohol exposure[295]. However, the molecular basis of these growth defects remains unknown.

In our model, the long-term growth restriction identified in the male offspring associated with heightened insulin sensitivity, increased markers of hepatic fibrosis, suppressed cytokine profiles within the liver and up-regulation of genes in the liver x/retinoid x/farnesoid x receptor pathways[159]. Previously, male-inherited alterations in the expression of liver x receptor alpha

(LXRα) were described in a mouse model of intrauterine growth restriction (IUGR) induced by late-term maternal malnutrition[296]. Here, multigenerational, paternally programed repression of hepatic LXRα expression induced glucose intolerance and the misregulation of lipogenic genes in liver[296]. However, in contrast to the effects propagated through IUGR males, the male offspring of alcohol-exposed fathers displayed an up-regulation of LXR stimulated pathways[159].

Liver X receptors regulate numerous aspects of cholesterol, fatty acid, and glucose homeostasis, and have well-defined roles in controlling immune function and neurodevelopment[297]. Therefore, this nuclear receptor family may represent a mechanistic hub mediating male-inherited alterations in metabolic phenotypes and could be linked to the wide variations and sex-specific outcomes observed across multiple models of altered developmental programming. In previous studies, treatment with LXR agonists suppressed activation of glycogenic pathways in the liver and protected mice from diet-induced obesity[298-300]. Based on these observations, we hypothesized that the male offspring of alcohol-exposed sires would exhibit programmed resistance to diet-induced obesity and improved metabolic adaptation to a high-fat diet. Therefore, we set out to test this assertion and define the molecular mechanisms by which paternally-programmed increases in LXR activation influence the regulation of glucose homeostasis in the offspring of males chronically exposed to alcohol.

Results

Preconception Paternal Alcohol Exposure Protects Offspring from Diet-Induced Obesity and Improves Metabolic Adaptation

Previously, in the offspring of alcohol-exposed males, we identified increased stimulation of genetic pathways regulated by Liver X receptors[159]. Similar to multigenerational studies examining the offspring of IUGR males[296], we could not identify any differential expression of

known Lxr transcriptional regulators, including PPARα, PPARϒ, and HNF4A. To determine if programmed increases in LXR activity could improve metabolic adaptation and protect the male offspring of alcohol-exposed sires from diet-induced obesity, we returned to our model of chronic paternal alcohol consumption and exposed post-natal day 90 adult males to either the preconception control or alcohol treatments. We did not observe any differences in weight gain or fluid consumption between the two preconception treatment groups. Plasma alcohol levels averaged 127mg/dL, and, similar to previous studies, matings between alcohol-exposed sires and naive females produced growth-restricted offspring, as measured at gestational day 16.5 (Figure 13A-B). This growth restriction was accompanied by a significant reduction in placental efficiency (Figure 13C).

To determine the postnatal response of alcohol-exposed offspring to a high-fat dietary challenge, we randomly assigned male littermates sired by alcohol-exposed and control fathers between standard chow (CD) and high-fat diet (HFD) treatment groups. We did not observe any differences in food consumption between the alcohol or control preconception treatment groups for either dietary treatment. After 3 weeks of exposure to a HFD, male offspring from both preconception treatment groups were significantly heavier than animals maintained on the CD (Figure 1D, p-value = 0.0013). However, after 8-weeks HFD treatment, male offspring sired by alcohol-exposed fathers displayed significant reductions in weekly weight gain as compared to the male offspring of control fathers (Figure 13D). In the male offspring of alcohol-exposed sires maintained on a HFD, we observed a ~10% reduction in fasting blood glucose levels, while fasting insulin concentrations were identical between the two preconception treatment groups (Figure 13E-F). In the HFD treatment group, the observed reductions in blood glucose concentrations and weekly weight gain observed in the offspring of alcohol-exposed sires associated with improved

performance in both glucose and insulin tolerance tests, as compared to offspring sired by control males (Figure 13G-I). Collectively, these observations indicate that the male offspring of alcohol-exposed sires exhibit modest protection from high-fat diet-induced obesity and improved glucose homeostasis under conditions associated with obesity-induced insulin resistance.

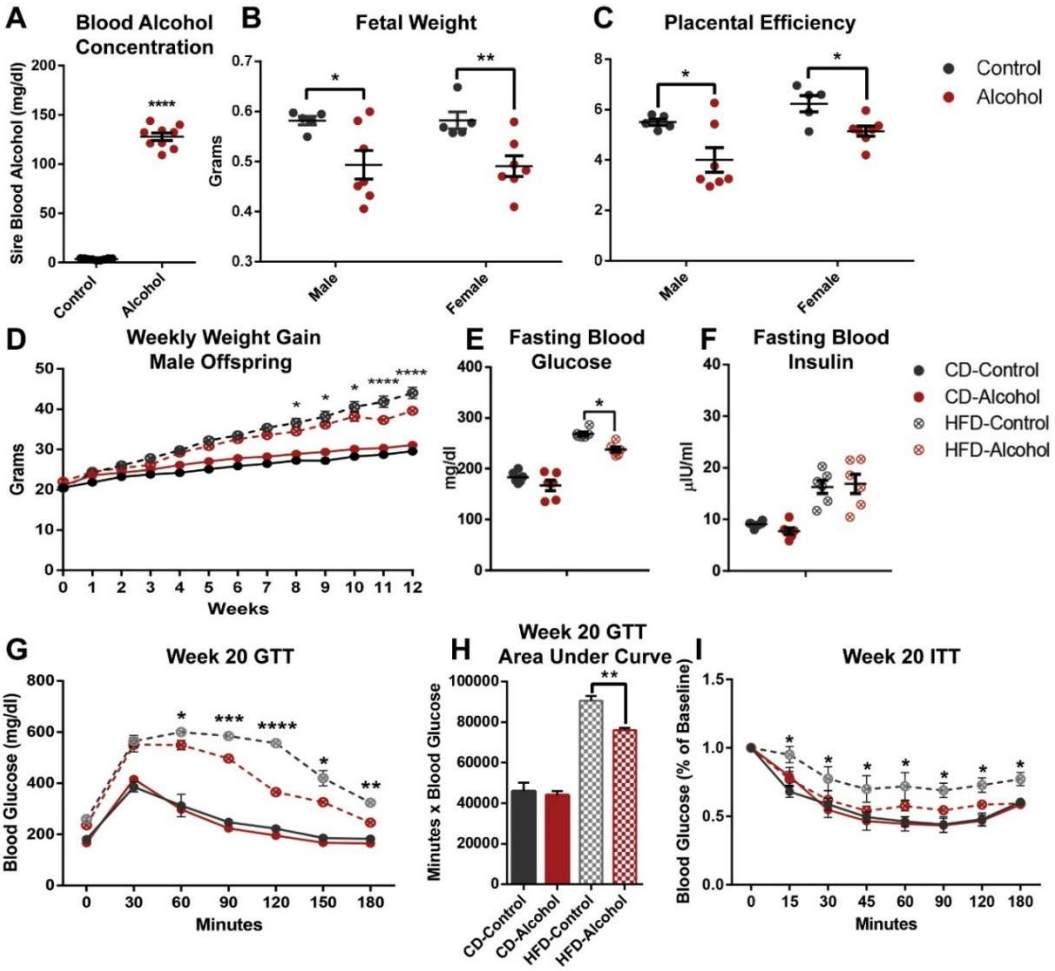

Figure 13 The male offspring of alcohol-exposed fathers display enhanced metabolic adaptation and resistance to high-fat diet-induced obesity

(A) Average blood alcohol concentrations between control and alcohol-exposed sires. (B) Comparisons of average litter weights, separated by sex, between the offspring of control and

alcohol-exposed males. (C) Placental efficiency (gram of fetus produced per gram of placenta) compared between litters sired by control and ethanol-exposed sires. (D) Weekly weight gain, (E) fasting blood glucose and (F) fasting insulin levels compared between the male offspring of control and alcohol-exposed fathers. Comparisons made within the chow diet (CD) and high-fat diet (HFD) postnatal treatment groups. (G) Glucose tolerance test (H) area under the curve analysis and (I) insulin tolerance tests comparing glucose homeostasis between the male offspring of control and alcohol-exposed fathers within the dietary treatment groups. Error bars represent SEM *P < 0.05, **P< 0.01, ***P < 0.001 and ****P<0.0001.

Liver-specific alterations in LXRα programming associate with suppression of proinflammatory NFkB target genes

After 12-weeks of treatment (20 weeks postnatal life), mice were terminated and tissues collected. No differences in organ weights were noted between preconception treatment groups. Using RT-qPCR, we examined the expression of liver x receptors alpha and beta, as well as the farnesoid x and retinoid x alpha receptors in the liver (gene names Nr1h3, Nr1h2, Nr1h4, and Nr2b1 respectively). The expression of Lxrα and Rxrα were significantly increased in the male offspring of alcohol-exposed fathers, regardless of dietary treatment (Figure 14A). Although we did observe increased Fxr transcript levels, it was only in mice maintained on the standard chow diet and not under the HFD treatment. We did not observe any differences in Lxrβ transcripts in any treatment group. Increased expression of Lxrα in the male offspring of alcohol-exposed sires was confirmed using western blotting (Figure 14B). In contrast to the liver, we did not observe

significant changes in any of these candidate receptors in other metabolically relevant tissues, including the pancreas and visceral fat (Figure 14A).

In the liver, LXRα is known to suppress glycogenic pathways through direct binding of the promoters of genes encoding phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase catalytic subunit (G6PC) (Laffitte et al., 2003; Herzog et al., 2007). Suppression of these genes could explain some of the improvements in glucose homeostasis observed in the male offspring of alcohol-exposed fathers maintained on a high-fat diet. However, we could not detect any differences in the expression of either of these genes in the liver, pancreas or visceral fat, under any experimental conditions (Figure 14C).

In addition to regulating cholesterol and glucose homeostasis, LXRα also exerts anti-inflammatory effects by repressing the production of a core set of nuclear factor k B (NFkB)-induced inflammatory cytokines (Joseph et al., 2003; Zelcer and Tontonoz, 2006; Im and Osborne, 2011). Through interactions with the Toll-like receptor (TLR) and the inhibitor of nuclear factor k B Kinase β (Ikkβ) complex, NFkB inflammatory cytokines are powerful suppressors of insulin signaling (Arkan et al., 2005; Cai et al., 2005). The alleviation of this inhibitory influence by increased LXRα activity could be responsible for the improved glucose homeostasis observed in our model. Therefore, we compared the abundance of a core group of metabolically relevant inflammatory cytokines between the male offspring of alcohol-exposed and control fathers, across both dietary treatment groups (Figure 14D). In the livers of the male offspring of alcohol-exposed sires maintained on a HFD, we observed significant reductions in the levels of TNFα, IL1β, and IL6, as compared to the offspring of control males. These reductions were specific to the liver and did not appear in either the pancreas or visceral fat. We did not observe any differences in cytokine abundance in animals maintained on the standard chow diet in any tissue.

When SUMOylated, agonist-bound LXRα exerts trans-repressive activity by forming a complex with the N-CoR (nuclear receptor corepressor) and/or SMRT (silencing mediator for retinoid and thyroid hormone receptors) corepressor complexes on AP-1 and NFκB target genes (Ghisletti et al., 2009). Using chromatin immunoprecipitation, we assayed the enrichment of LXRα at known p50 binding sites within the promoter regions of TNFα, IL1β, and IL6, as well as in the previously identified LXRα binding site in the promoter region of the Pepck gene (Herzog et al., 2007; Falvo et al., 2010). In these experiments, enrichment of LXRα at an untranscribed region of chromosome 6 (Untr6) served as a non-specific control (Vakili et al., 2013). In both dietary treatment groups, the male offspring of alcohol-exposed fathers exhibited a 10 to 20% increase in the enrichment of LXRα on the TNFα, IL1β, and IL6 gene promoters (Figure 14E). Enrichment of LXRα was modestly higher in males maintained on the chow diet as compared to those in the HFD treatment group. Unlike the proinflammatory NFκB target genes examined, the promoter region of Pepck did not exhibit any LXRα enrichment and remained equivalent to the non-specific control. Collectively, these observations indicate that the offspring of alcohol-exposed sires exhibit liver-specific programmed enhancement of LXRα but not LXRβ, which associates with increased binding at NFκB target genes and reductions in the production of inflammatory cytokines.

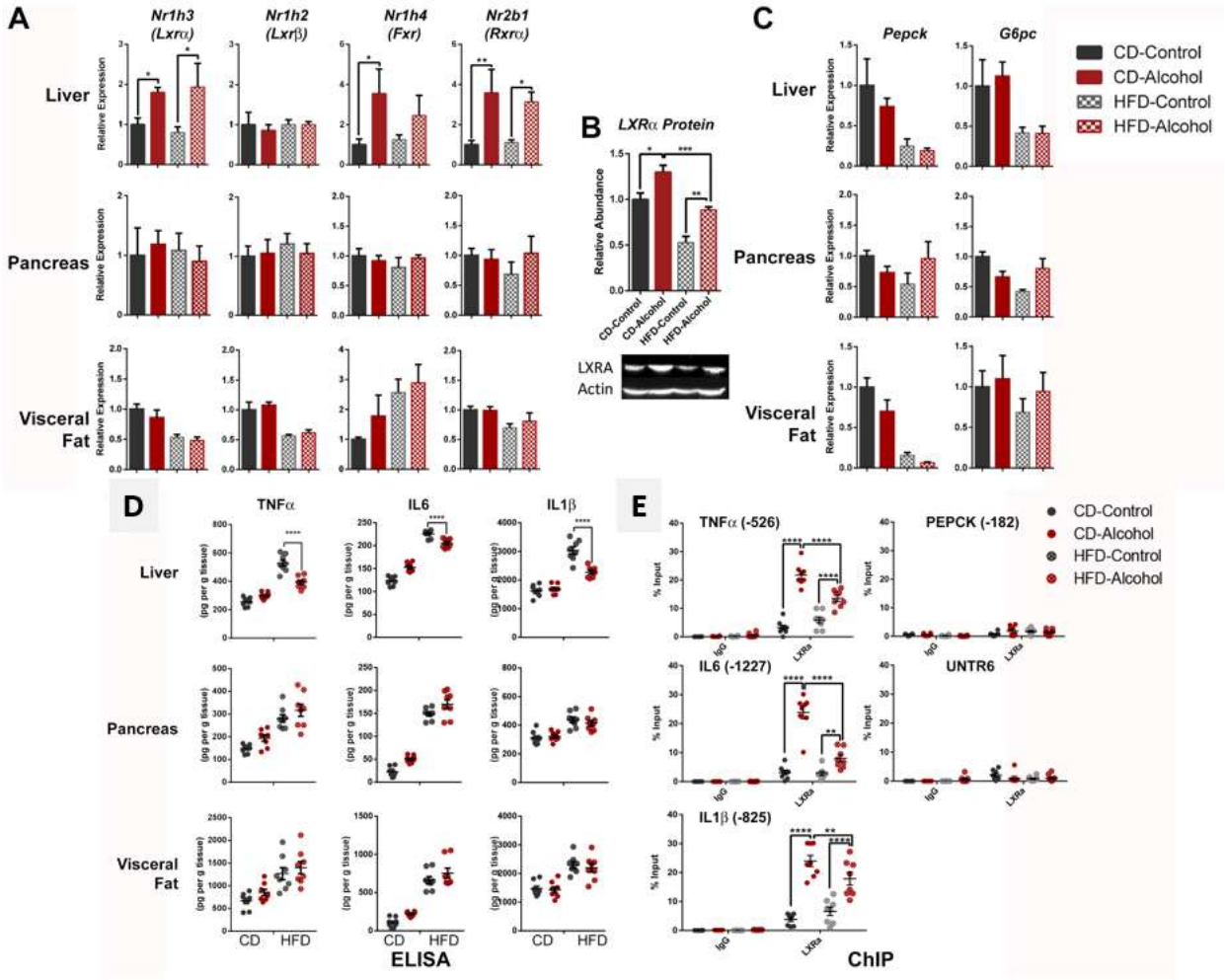

Figure 14 Liver-specific programed up-regulation of Liver X Receptor alpha suppresses NFκB inflammatory cytokine production in the male offspring of alcohol-exposed fathers

(A) RT-qPCR analysis of nuclear receptor expression in the liver, pancreas and visceral fat. (B) Western blot analysis of hepatic LXRα expression. (C) RT-qPCR analysis of LXRα-regulated glycogenic genes in the liver, pancreas and visceral fat. (D) ELISA analysis contrasting the abundance of inflammatory cytokines in the liver, pancreas and visceral fat. (E) Chromatin immunoprecipitation analysis of LXRα enrichment within the regulatory regions of the indicated genes within the liver. Comparisons made between the male offspring of control and alcohol-

exposed sires within the chow diet (CD) and high-fat diet (HFD) treatment groups. Error bars represent SEM *P < 0.05, **P< 0.01, ***P < 0.001 and ****P<0.0001.

Enhanced insulin signaling in the offspring of alcohol-exposed sires

Mice carrying a hepatocyte-specific genetic deletion of toll-like receptor 4 (TLR4) exhibit enhanced insulin sensitivity, similar in magnitude to that observed in our model (Jia et al., 2014). To explore the impact LXRα-mediated reductions in cytokine production have on insulin signaling, we began by assaying the phosphorylation status of Ikkβ. Ikkβ is directly downstream of TLR4 and becomes phosphorylated upon cytokine stimulation. Using western blot analysis, we identified a ~20% reduction in phosphorylated (Tyr 199) Ikkβ in the male offspring of alcohol-exposed sires maintained on the HFD but not in males maintained on the chow diet (Figure 15A). The inhibitory effects of Ikkβ on insulin signaling are primarily mediated by blocking the association of insulin receptor substrate 1 (IRS1) with the p85 subunit of phosphatidylinositol 3-kinase (Arkan et al., 2005; Cai et al., 2005). Therefore, we quantified levels of phosphorylated IRS1 in insulin perfused livers of the offspring of alcohol-exposed and control males under both dietary treatments. We observed a ~50% increase in phosphorylated (Ser 307) IRS1 in the insulin perfused litters of the male offspring of alcohol-exposed sires maintained on a HFD, while male offspring maintained on the chow diet did not exhibit any identifiable changes (Figure 15B). Consistent with increased levels of phosphorylated IRS1, we observed a corresponding ~45% increase in phosphorylated (Ser 473) Akt in the male offspring of alcohol-exposed sires maintained on a HFD (Figure 15C). These observations indicate that, in the male offspring of alcohol-exposed

sires, LXRα mediated decreases in cytokine production alleviate Ikkβ-mediated inhibition of insulin signaling, which enhances metabolic adaptation to a high-fat diet.

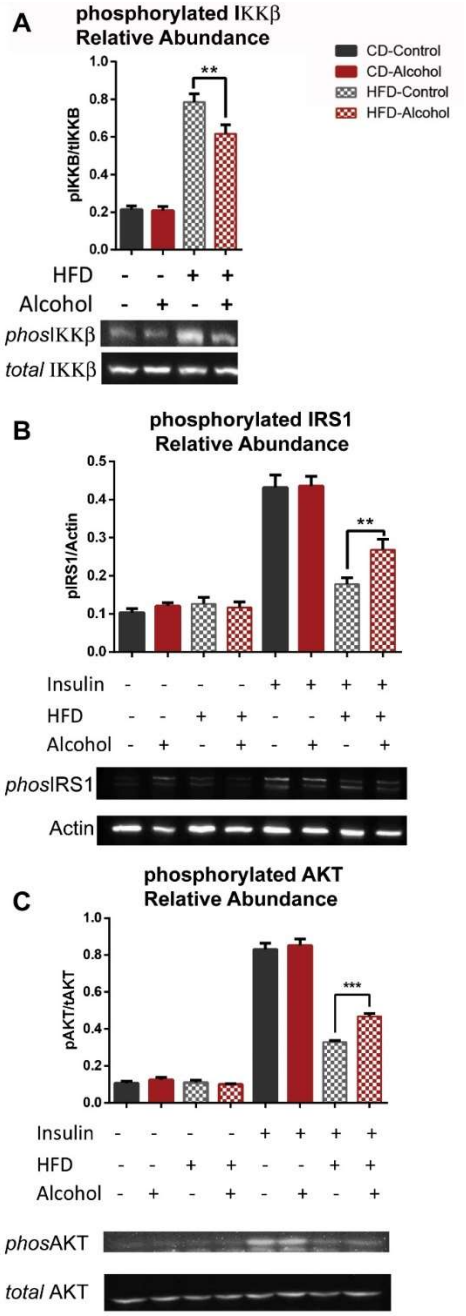

Figure 15 Reduced Ikkβ inhibition and enhanced hepatic insulin signaling in the male offspring of alcohol-exposed fathers

Relative abundance of phosphorylated (A) Ikkβ, (B) IRS1 and (C) AKT were compared between the livers of the male offspring of control and alcohol-exposed sires, within the two

dietary treatments. (CD=Chow Diet, HFD=High-Fat Diet, n =) Error bars represent SEM, **P< 0.01 and ***P < 0.001.

LXRα-induced alterations in lipid metabolism and increased hepatic fibrosis in the offspring of alcohol-exposed sires

Given that paternally-inherited increases in LXRα activity were able to moderate the effects of a high-fat diet, we next examined other metabolic outcomes associated with the use of LXR agonists. In previous studies, treatment with LXR agonists decreased levels of hepatic cholesterol by engaging the genetic pathways driving cholesterol efflux (Naik et al., 2006). Similar to mice treated with the non-steroidal LXRα-specific agonist T0901317 (Grefhorst et al., 2002; Xie et al., 2012), we observed a ~15% decrease in total hepatic cholesterol levels in the male offspring of alcohol-exposed sires maintained on a HFD (Figure 16A). No differences in the proportion of either LDLs or HDLs were observed (Figure S3A-C). In contrast, we did not observe any differences in total cholesterol levels in male offspring maintained on the standard chow diet. Although synthetic LXR agonists inhibit atherosclerosis, their efficacy as therapeutic agents is complicated by the fact that they cause hepatic steatosis and hypertriglyceridemia (Grefhorst et al., 2002). Hepatic steatosis is postulated to be the consequence of increased fatty acid synthesis caused by the induction of lipogenic enzymes, including fatty acid synthase (FASN) and sterol regulatory element binding transcription factor 1 (SREBF1). Consistent with studies examining LXR agonists (Grefhorst et al., 2002), we observed a ~15 to 20% induction of Fasn in the male offspring of alcohol-exposed sires across both dietary treatments. Although levels of Srebf1 appeared to follow a similar trend, transcript levels were only significantly increased in animals

maintained on the chow diet and not under the HFD treatment ($p<0.05$, Figure 16B). However, in previous studies, agonist-induced activation of LXR signaling led to Srebf1-mediated suppression of Apolipoprotein A5 (Jakel et al., 2004). Similar to this report, we observed a significant decrease in Apoa5 transcript levels in the offspring of alcohol-exposed sires across both dietary treatments (Figure 4B). Finally, consistent with the increased expression of lipogenic enzymes, we observed a ~10% increase in hepatic triglyceride levels in the male offspring of alcohol-exposed sires maintained on a HFD, indicating the emergence of steatosis in the offspring of alcohol-exposed sires (Figure 16C).

Hepatic steatosis is considered part of the spectrum of nonalcoholic fatty liver diseases and has been linked to the emergence of hepatic fibrosis (Bataller and Brenner, 2005). During fetal development, the male offspring of alcohol-exposed sires displayed transcriptional up-regulation of TGF-β-driven pathways and increased expression of multiple collagen subtypes (Chang et al., 2017). In both the male and female offspring of alcohol-exposed sires, this signature persisted into adolescence and associated with increased markers of fibrosis at 8-weeks postnatal life (Chang et al., 2019). To determine the impact treatment with a HFD had on the expression of TGF-β-driven profibrotic genes, we assayed the expression of the previously identified candidate genes using RT-qPCR. In the male offspring of alcohol-exposed sires maintained on an HFD, we observed an increased abundance of transcripts encoding Tgf-β, Timp1, and Smad2, as well as the collagen subtypes Col4a1, Col5a2, and Col6a2 (Figure 16D-E). We correlated this transcriptional up-regulation with a 50% increase in levels of liver hydroxyproline (Figure 16F), an established marker of hepatic fibrosis (Ding et al., 2013). Importantly, this signature of fibrosis was present in the offspring of alcohol-exposed sires regardless of dietary treatment but became exacerbated under the HFD treatment.

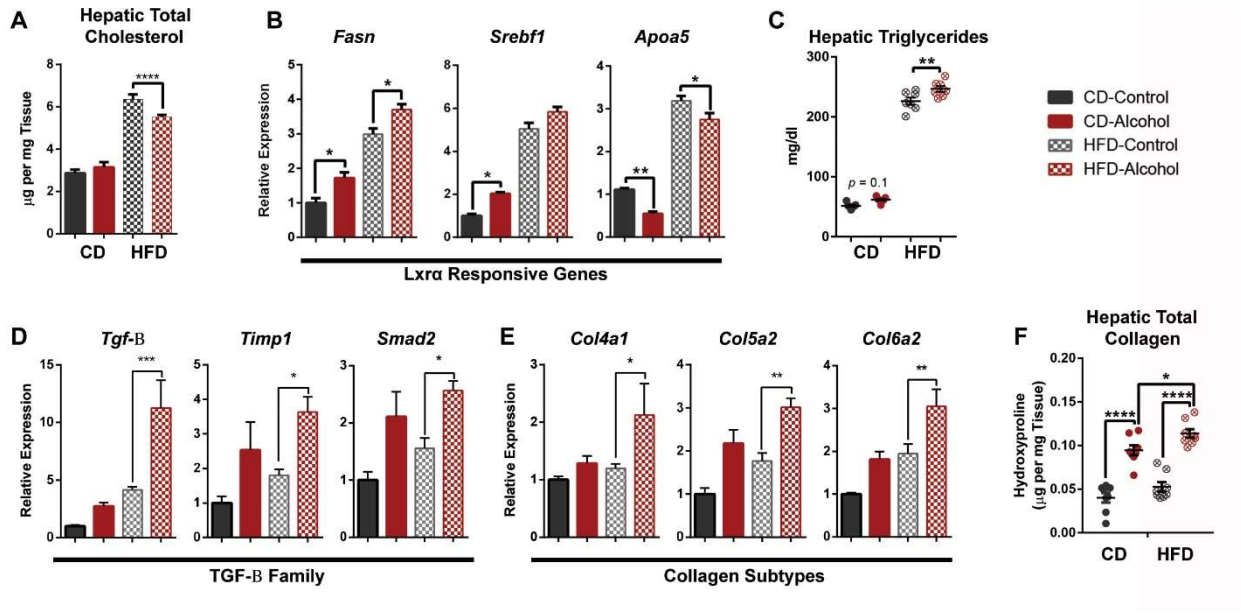

Figure 16 Programed increases in LXRα expression induce hypertriglyceridemia, which correlates with increased hepatic fibrosis in the male offspring of alcohol-exposed fathers

Analyses contrasting hepatic tissues from the male offspring of control and alcohol-exposed sires, across or within the two dietary treatments. (A) Hepatic cholesterol levels, (B) RT-qPCR analysis of LRXα-regulated lipogenic genes, (C) hepatic triglyceride levels, RT-qPCR analysis of genes encoding pro-fibrotic (D) signaling and (E) structural proteins, and (F) total levels of cellular hydroxyproline. Error bars represent SEM *P < 0.05, **P < 0.01 and ***P < 0.001.

Discussion

Liver X receptors function as dynamic regulators of cholesterol metabolism and lipid biosynthesis but also sensitize the insulin signaling pathway and suppress the innate immune system (Maqdasy et al., 2016). Despite these far-reaching effects, the contribution of this nuclear receptor family to the metabolic phenotypes observed in models of altered developmental

programming remain poorly described. Consistent with studies examining the effects of Liver X Receptor agonists (Grefhorst et al., 2002; Laffitte et al., 2003), the male offspring of alcohol-exposed sires displayed enhanced metabolic adaptation and slowed weight gain when challenged with a high-fat diet, as well as several of the undesirable side effects of LXR stimulation, including increased hepatic cholesterol efflux and hypertriglyceridemia. However, the molecular mechanisms by which the memory of alcohol exposures persist into the next generation and influence LXRα activity remain unknown.

Multiple studies have suggested that changes in sperm DNA methylation persist through early development and exert a lasting influence on offspring physiology, while others indicate a causative role for sperm-inherited noncoding RNAs (Donkin and Barrès, 2018). However, despite the fact that multiple models of compromised paternal programming consistently exert long-term effects on offspring metabolism, the majority of which appear to converge on the regulation of glucose and lipid homeostasis, no consistent cohort of differentially methylated loci or population of small RNA has been identified that can explain the observed changes in metabolic function. Although Martinez et al., identified reductions in DNA methylation within the Lxrα 5' UTR that were consistent between sperm and the resulting offspring (Martínez et al., 2014), we were unable to identify any changes in DNA methylation within the sperm of alcohol-exposed males (Chang et al., 2017). However, the 2% change identified by Martinez et al., was below our threshold of detection. Further, although we and others have described alcohol-induced changes in sperm-inherited noncoding RNAs (Rompala et al., 2018; Bedi et al., 2019), the identified candidates have either been inconsistent with other metabolically focused studies or associated with deficits in glucose homeostasis and increased susceptibility to the effects of a high-fat diet. Interestingly, none of the nuclear receptors examined in this study were differentially expressed in the fetal liver

(Figure S4), indicating that the up-regulation of LXRα activity in the adult offspring of alcohol-exposed sires is very likely a downstream outcome of altered paternal programming rather than the direct epigenetic memory. The identification of compromised placentation in this and other models of paternal programming suggest the strong possibility that the sex- and tissue-specific effects observed are all secondary symptoms of a compromised maternal-fetal interface (Cheong et al., 2016).

The life expectancy of patients with fetal alcohol syndrome is 34 years, which is 58% lower than the general population (Thanh and Jonsson, 2016). Although clinical studies can correlate maternal alcohol exposures during gestation with dose- and sex-dependent effects on the metabolic health of the offspring (Fuglestad et al., 2014; Werts et al., 2014), no studies have ever examined the long-term consequences of paternal alcohol use on offspring growth and metabolic health. Researchers in the field must now extend their perspective beyond maternal models of exposure and more carefully consider the importance of paternal lifestyle to the developmental origins of alcohol-induced disease.

Materials and Methods

Animal work

All experiments were conducted under AUP 2017-0308 and approved by the Texas A&M University IACUC. This study utilized C57BL/6J mice (RRID:IMSR_JAX:000664), which were obtained from and housed in the Texas A&M Institute for Genomic Medicine, and maintained on a 12-hour light/dark cycle.

Preconception paternal alcohol exposures

We maintained individually caged postnatal day 90 adult males on a standard diet (catalog# 2019, Teklad Diets, Madison, WI, USA) and exposed them to either a control or alcohol preconception treatment using a previously described limited access model of voluntary exposure (Chang et al., 2017; Chang et al., 2019; Bedi et al., 2019). In this model, males are provided limited access to the preconception treatments during a four-hour window, beginning one hour after the initiation of the dark cycle. Specifically, we provided experimental males access to a solution of 10% (w⁄v) ethanol (catalog# E7023; Millipore-Sigma, St. Louis, MO, USA) and 0.066% (w⁄v) Sweet'N Low (Cumberland Packing Corp, Brooklyn NY, USA), while control males received a solution of 0.066% (w⁄v) Sweet'N Low alone. At the conclusion of each four-hour period, we recorded the amount of fluid consumed by each male mouse and weighed the males on a weekly basis.

After a 70-day preconception treatment period, corresponding to the approximate length of two spermatogenic cycles, we placed two naturally cycling females into a new cage along with each exposed male. During the matings, we did not give the males access to the alcohol or control preconception treatments. After six hours, we confirmed matings by the presence of a vaginal plug and returned both the male and female mice to their original cages. We rested males for 72-hours, where the preconception exposure was resumed, and then used them again in a subsequent mating. We repeated this procedure until we obtained the requisite number of pregnancies.

Postnatal dietary treatments

We subjected dams to minimal handling, maintained them on a Breeder diet (catalog# 5058; LabDiet, St. Louis, MO, USA), and monitored them for delivery twice daily. After weaning, we randomly separated male littermates sired by control and alcohol-exposed males into two

treatment groups, with three males, each derived from different sire x dam pairings, co-housed in a single cage. We maintained the male offspring on a standard diet (catalog# 2019, Teklad Diets, Madison, WI, USA) until 8-weeks of age. After 8 weeks, one group continued to receive the standard chow diet (CD - catalog# 2019, Teklad Diets, Madison, WI, USA), while we fed the other a high-fat diet, with 60% kcal derived from fat (HFD - catalog#D12492; Research Diet Inc., New Brunswick, NJ, USA). To monitor the progress of diet-induced obesity, the body weight gain and food intake of each group of males was measured weekly. At 20 weeks of age, mice were fasted overnight, terminated and both physiological fluids and tissues collected.

Insulin and Glucose Tolerance Tests

At 6 and 12 weeks of age, we fasted the male offspring of alcohol-exposed and control sires overnight and assayed differences in glucose and insulin tolerance, with a minimum of one-week recovery time between these separate tests. Here, we injected mice with a single intraperitoneal injection of either D-glucose (2 g/kg body weight; catalog# SG8270; Millipore-Sigma, St. Louis, MO, USA) or insulin (1 unit/kg body weight; catalog# 89508-914; VWR, Radnor, PA, USA) and measured blood glucose levels using Clarity Plus - Blood Glucose Test Strips (catalog# DTG-GL5PLUS; Clarity Diagnostics, Boca Raton, Florida USA) from 5ul of blood drawn from the tail vein. For glucose tolerance tests, we measured blood glucose levels at 0, 30, 60, 90, 120, 150 and 180 minutes post-injection. For insulin tolerance tests, we measured blood glucose levels at 0, 15, 30, 45, 60, 120 and 180 minutes after insulin injection. Each experimental group contains six males (n = 6).

Liver Perfusion Assay

At twenty weeks of age, we randomly selected six males from each treatment group to evaluate insulin signaling by quantifying levels of IRS1 (Ser307) and AKT (Ser473) phosphorylation in the liver. We fasted mice for 12 hours and then anesthetized them with 2% isoflurane (Overmyer et al., 2015), until the animals achieved a deep plane of anesthesia, demonstrating a lack of a righting reflex and a ~50% reduction in respiratory rate. At this point, we opened the body cavity of the mouse and perfused the liver with either 37 °C PBS + 0.1% BSA (Control) or 2 units/kg of an insulin solution (10 nM insulin, Sigma, in PBS + 0.1% BSA; catalog# 89508-914; VWR, Radnor, PA, USA) at a flow rate of 100 ml/h. During this procedure, we clamped the suprahepatic vessel and inserted a 27-gauge syringe into the intrahepatic cava. After observing liver perfusion, we cut the hepatic portal vein open. Throughout the procedure, we kept a nose cone containing gauze soaked in isoflurane over the nose of the animal and only removed it when the rate of respiration dipped down below 25%. After five minutes, we euthanized the animals by cervical dislocation and collected tissues of interest, including liver, kidney, and pancreas, which were snap-frozen in liquid nitrogen. Each experimental group contains six different animals (n = 6).

Western Immunoblot Analysis

Liver tissue samples were collected and homogenized in a Tris lysis buffer including 50 mM Tris, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 1% β-mercaptoethanol, 50 mM NaF, 1 mM Na3VO4; at pH 7.5. 20µg of protein was separated on 10% sodium dodecyl sulfate–polyacrylamide gels and transferred to nitrocellulose membranes. The primary antibodies used in this study were as follows: anti-phosphorylated protein kinase B (AKT) at Serine 473 (catalog #700392; RRID:AB_2532320; Thermo-Fisher, Waltham, MA, USA), anti-AKT (catalog

#44609G; RRID:AB_2533692; Thermo-Fisher, Waltham, MA, USA) anti-phosphorylated Inhibitor Of Nuclear Factor Kappa B Kinase Subunit Beta (IKK beta) at Tyrosine 199 (catalog #ab59195; RRID:AB_943810; Abcam, Cambridge, MA, USA), anti-IKK beta (catalog #ab124957; RRID:AB_10975710; Abcam, Cambridge, MA, USA), anti-phosphorylated Insulin receptor substrate 1 (IRS1) at Serine 307 (catalog #ab5599; Abcam, Cambridge, MA, USA; RRID:AB_304975), anti-LXRα (catalog# ab41902; RRID:AB_304975; Abcam, Cambridge, MA, USA) and anti-beta actin (catalog #ab49900; RRID:AB_867494; Abcam, Cambridge, MA, USA). Blots were visualized by using secondary antibodies conjugated to horseradish peroxidase (catalog# sc-2004; RRID:AB_631746; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and an enhanced chemiluminescence detection system (Pierce, Rockford, IL, USA). Relative AKT phosphorylation was derived as a ratio to total AKT, relative IKK beta phosphorylation was derived as a ratio to total IKK beta, and relative IRS1 phosphorylation was derived as a ratio to beta actin. Band intensities were quantified by densitometry using ImageJ (RRID:SCR_003070; National Institutes of Health, Bethesda, MD, USA). Each experimental group contains protein extracts derived from six to eight different animals (n = 6-8).

Measurement of Physiological Parameters

We measured male plasma alcohol concentrations using an Ethanol Assay Kit (catalog# ECET100; BioAssay Systems, Hayward, CA, USA) according to manufacturer's protocol. We determined plasma insulin levels post-mortem using the Mouse Insulin ELISA kit (catalog# EMINS; Thermo-Fisher, Waltham, MA, USA), according to the recommended protocol. We measured levels of IL1B, IL6, and TNFa using commercial ELISA assays (catalog# KMC0061 and KMC0011; Thermo-Fisher, Waltham, MA, USA and catalog # Ab100689 and Ab100747; Abcam, Cambridge, MA, USA). To determine total cholesterol levels, we used the Total

Cholesterol Assay Kit (catalog# STA- 384; Cell Biolab, Inc, San Diego, CA USA), according to the recommended protocol. To compare the levels of low-density and high-density lipoproteins, we used a cholorfmetirc Cholesterol Assay Kit (catalog# ab65390; Abcam, Cambridge, MA, USA), according to the recommended protocol. We used the Triglyceride Assay Kit (catalog# ab65336; Abcam, Cambridge, MA, USA) to contrast the abundance of hepatic triglycerides, according to the suggested protocol. To compare the levels of hydroxyproline, we used the Hydroxyproline Assay Kit (catalog# MAK008; Millipore-Sigma, St. Louis, MO, USA), and followed to the recommended protocol.

RNA isolation and RT-qPCR analysis of gene expression

Total RNA was isolated from twenty-week old liver, pancreas and visceral fat using an 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; Millipore-Sigma, St. Louis, MO, USA) according to the manufacturer's recommendations and seeded into a reverse transcription reaction using the High Capacity cDNA Reverse Transcription Kit (catalog# 4368814; Thermo-Fisher, Waltham, MA, USA), where the reaction mixture was brought to 25°C for 10 minutes, 37°C for 120 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-Fisher, Waltham, MA, USA) according to the recommended protocol. Reactions were performed on a Bio-Rad CFX384. Procedures for normalization and data handling are described below, primer sequences are listed in Table S2.

Chromatin Immunoprecipitation (ChIP) Analysis

We performed chromatin Immunoprecipitations (ChIP) using a previously described protocol (Veazey et al., 2015; Veazey et al., 2017), with the following modifications. Briefly,

50mg of liver tissue was thawed on ice in cold PBS and dispersed into single cell suspensions using a 100µm cell strainer (catalog# 352360; Corning Life Sciences, Corning, NY, USA). Cells were washed twice with PBS containing protease inhibitor cocktail (catalog# 78437; Thermo-Fisher, Waltham, MA, USA) and re-suspended in cell culture medium (DMEM F-12 catalog# 11320-033; Thermo-Fisher, Waltham, MA, USA) containing 0.1 volume of 10x crosslinking solution (10.8% Formaldehyde, 1M NaCl, 10mM EDTA, 500 mM HEPES pH 7) for fifteen minutes. After quenching with 125 mM glycine for five minutes, cells were washed three times in ice-cold PBS containing protease inhibitor cocktail and lysed in ChIP lysis buffer (150 mM NaCl, 25 mM Tris·Cl, pH 7.5, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate). Cellular extracts were sonicated using a Bioruptor sonication system (Diagenode, Denville, NJ, USA) and DNA fragment lengths visualized using agarose gel electrophoresis. Immunoprecipitations were performed with antibodies recognizing anti-LXRα (catalog# ab41902; RRID:AB_776094; Abcam, Cambridge, MA, USA) overnight, at 4ºC, in dilution buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris, pH 8.1, 500mM NaCl), under constant rotation. Antibodies recognizing rabbit IgG (catalog# SC-2027; RRID:AB_737197; Santa Cruz, Santa Cruz, CA, USA) served as the nonspecific control. All antibodies were used at a concentration of 1 µg/ChIP reaction. Chromatin was precipitated using protein A/G sephadex beads (catalog# 17513801 and 17061801; GE Healthcare, Marlborough, MA, USA), washed in ice-cold Low Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris, pH 8.1, 150mM NaCl), High Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris, pH 8.1, 500mM NaCl), LiCl Wash Buffer (0.25M LiCl, 1% IGEPAL, 1% Deoxycholate Na, 1mM EDTA, 10mM Tris, pH 8.1) and eluted in 70ºC Elution Buffer (10% SDS, 1M NaHCO3). After reversing crosslinks and proteinase K digestion, DNA was isolated using a Qiaquick PCR Cleanup kit

(catalog# 28106; Qiagen, Germantown, MD,USA). For analysis of candidate loci, real-time PCR was performed using the Dynamo Flash supermix (catalog# F-415XL; Thermo-Fisher, Waltham, MA, USA) according to the recommended protocol. Reactions were performed on a Bio-Rad CFX384 Touch PCR system. Data was analyzed using the formula previously described (Mukhopadhyay et al., 2008).

Data handling, experimental replicates and statistical analysis

For all experiments, measures were input into the statistical analysis program GraphPad (RRID:SCR_002798; GraphPad Software, Inc., La Jolla, CA, USA) and statistical significance set at alpha = 0.05. All datasets were first verified for normality using the Brown-Forsythe test. In this study, the effect of two independent variables (preconception treatment v.s. dietary treatment) were assessed using a two-way analysis of variance test (ANOVA), and differences among the means evaluated using Sidak's post-hoc test of contrast. For comparisons between two treatment groups, an unpaired t-test was employed. In all instances, we have marked statistically significant differences with an asterisk.

For the analysis of blood alcohol levels, 9 control and 9 alcohol-exposed males were compared using an unpaired t-test. For comparisons of fetal weight, we first separately averaged the weights of the male and female fetuses in each litter and then compared each group using a two-way ANOVA (n = 5 control and 7 alcohol). Placental efficiency was calculated by dividing the average fetal weight per litter by the average placental weight per litter. Male and female efficiencies were calculated separately. For the analysis of weekly weight gain for the male offspring, comparisons were made using a two-way ANOVA (chow diet-control n = 12 males from 5 separate litters, chow diet-alcohol n = 21 males from 7 separate litters, high-fat diet-control n = 12 males from 5 separate litters, and high-fat diet-alcohol n = 21 males from 7 separate litters.

For the analysis of fasting blood glucose levels, fasting blood insulin levels and both the glucose and insulin tolerance tests, differences were compared using a two-way ANOVA (n=6). For analyses using Western blotting of LXRa, ELISA, and ChIP, as well as investigations of hepatic cholesterol levels, hepatic triglyceride levels and hepatic hydroxyproline content, values were compared using a two-way ANOVA (n=8). For RT-qPCR 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. In the liver, transcripts encoding tyrosine 3-monooxygenase / tryptophan 5-monooxygenase activation protein zeta (Ywhaz - NM_011740), mitochondrial ribosomal protein L1 (Mrpl1 - NM_053158) and hypoxanthine-phosphoribosyl transferase (Hprt—NM_013556) were measured as reference genes. In the pancreas and visceral fat, transcripts encoding H2A Histone Family Member Z (H2afz - NM_001316995), mitochondrial ribosomal protein L1 (Mrpl1 - NM_053158) and hypoxanthine-phosphoribosyl transferase (Hprt—NM_013556) served as reference genes. Each of these reference genes were validated for stability across treatment groups using methods described previously (Carnahan et al., 2013). Normalized expression levels were calculated using the ddCt method described previously (Schmittgen and Livak, 2008). Relative fold change values from each biological replicate were determined using Microsoft Excel, values transferred into the statistical analysis program GraphPad and differences assessed using a two-way ANOVA (n=8).

CHAPTER V

ALTERATIONS IN SPERM-INHERITED NONCODING RNAS ASSOCIATE WITH LATE-TERM FETAL GROWTH RESTRICTION INDUCED BY PRECONCEPTION PATERNAL ALCOHOL USE*

Rationale

Studies show sperm contain small RNAs in various proportions and that environmental exposures have the capacity to alter their enrichment[94] suggesting this epigenetic signature may respond to paternal treatments and potentially transmit to offspring. We hypothesize alcohol consumption alters the profile of small noncoding RNAs transmitted through sperm. This chapter will begin to assay the molecular mechanisms by which the epigenetic memory of paternal alcohol exposure transmits through sperm to the offspring. Specifically, we will evaluate the role-sperm-inherited noncoding RNAs have in mediating the observed intergenerational effects. Total RNAs will be isolated from alcohol-exposed sperm, size selected and small RNAs sequenced. Reads will be mapped to small RNA databases and differences in miRNA, piRNA, tRNA, tRNA-derived small RNAs assayed. This dataset has been published in the journal *Reproductive Toxicology*[294].

Background

Developmental plasticity refers to the dynamic ability of one genotype to produce multiple phenotypes in response to different environmental stimuli[301]. This phenomena enables the best chances of reproductive success but can also associate with the development of functional deficits and disease. Indeed, there are multiple instances where the predisposition of offspring to develop

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diseases later in life can be traced to a fetal compensation to an early life stressor[302]. For example, small-for-gestational-age babies have an increased risk of developing metabolic diseases, such as type two diabetes, and both cardiovascular morbidity and mortality as adults[50,303]. These observations helped found the Developmental Origins of Health and Disease hypothesis, and established the current recognition of the importance intrauterine development has in lifelong health.

Less well defined are the abilities of parental life history to influence organism phenotype. Although not as well characterized as intrauterine encounters, parental exposures prior to conception also exert a significant impact on offspring health and development[100]. Thus, in addition to uterine programming, the processes of germline programming that occur during gametogenesis also have the ability to impact offspring phenotype and influence the developmental plasticity of the next generation. Recently, preconception male exposures to a range of environmental factors have been linked to alterations in the developmental program of sperm and correlated with increased rates of structural and metabolic defects in the next generation[80,83,92,99,101,103-114,128,153,295]. These studies challenge the singular importance of maternal in utero exposures and implicate paternal exposure history as an additional and important mediator of both developmental defects and environmentally-induced disease.

The molecular mechanisms by which preconception stressors heritably influence cellular phenotypes are still very poorly understood. Molecular processes that allow the stable propagation of either chromatin-states or epigenetic information from one generation of cells to the next are hypothesized to play a role in transmitting the cellular memories of past exposures through gametogenesis to the offspring[98]. Specifically, mature sperm carries epigenetic information in patterns of DNA methylation, the region-specific retention of histones and DNA binding factors

(like CTCF), as well as populations of small noncoding RNAs (ncRNAs). However, studies examining paternally-inherited abnormalities in growth and metabolic function have provided evidence to both support and refute the involvement of sperm-inherited changes in DNA methylation in the transmission of these phenotypes[80,99,102-104,110,111]. Similarly, although select regions of the sperm genome retain histones, it is unclear if this epigenetic information persists through the remodeling of the paternal genome during syngamy, or have the ability to transmit through the cell cycle[304,305]. The strongest candidate to date has been the transmission of paternally inherited ncRNAs. In these studies, the injection of either total sperm RNAs or a subset of sperm RNAs (for example, microRNAs or tRNA-derived small RNAs) into normal zygotes can recapitulate some paternal phenotypes induced by mental or metabolic stressors[92]. However, the mechanisms through which the effects of sperm-inherited ncRNAs persist into later life remain poorly defined[306].

In the United States, 70% of men drink and 40% drink heavily, with 8.3% reporting the routine consumption of more than two drinks per day[135,154]. Despite the nearly ubiquitous and constant exposure during reproductive ages, we currently have a very poor understanding of the effects chronic preconception alcohol use has on male reproductive physiology and the sperm-inherited developmental program. Using a mouse model of voluntary consumption, our lab recently described an association between chronic preconception paternal alcohol use and deficits in both placental function and offspring growth[99,159]. Importantly, these phenotypes did not associate with any measurable alterations in sperm-inherited patterns of DNA methylation[99]. Therefore, the question of how the memory of chronic alcohol use transmits to the offspring remains unresolved.

Mechanistic studies in rodents have revealed that alcohol impairs the endocrine-reproductive axis, indicating male alcohol use may impact foundational aspects of reproductive function[307,308]. In addition, multiple studies in humans and rodents have indicated that chronic alcohol use negatively impacts the integrity of the sperm nucleus[309-314]. Finally, using an inhalation model of exposure, Rompala and colleagues recently described alcohol-induced alterations in the profile of sperm derived ncRNAs[97]. However, whether facets of male reproductive function, sperm nuclear structure or the profile of sperm-inherited ncRNAs are altered in our model of voluntary alcohol consumption and associate with the development of the observed growth defects remains to be resolved.

Results

Daily ethanol exposures induce pharmacologically meaningful blood alcohol concentrations but do not impact paternal body weight.

To define the long-term impact alcohol exposure has on both reproductive function and the male-inherited developmental program, we returned to our established mouse model of chronic alcohol exposure[99,159]. Here, postnatal day 90 adult males were provided limited access to either the ethanol or control preconception treatments during a four-hour window, that began one hour into the night cycle. Once consistent patterns of alcohol consumption were established, males were maintained on this protocol for a period of 70 days, which corresponds to the length of two complete spermatogenic cycles and ensures that sperm formed prior to alcohol treatment are not able to confound the resulting phenotypes. For each male, the amount of fluid consumed per day was recorded. No differences in fluid consumption between the two preconception treatment groups were observed (Fig. 17A).

The rodent model we employed (Drinking in the Dark) promotes the daily, voluntary

consumption of ethanol in sufficient quantities to achieve pharmacologically meaningful blood alcohol concentrations, typically around 125 mg/dL or 1.5x the legal limit (Fig. 17B). In the United States, 16% of men report engaging in high-risk drinking, which is defined as exceeding five or more standard drinks on any day within a given week and importantly, 8.3% of men routinely consume more than two drinks per day[315]. Therefore, the blood alcohol levels observed in our model of preconception male alcohol exposure are physiologically relevant. Further, chronic, daily alcohol use among males is both prevalent and a significant health concern. During the treatment course, no differences in body weight or changes in the rate of weight gain were observed between the preconception treatment groups (Fig 17C-D).

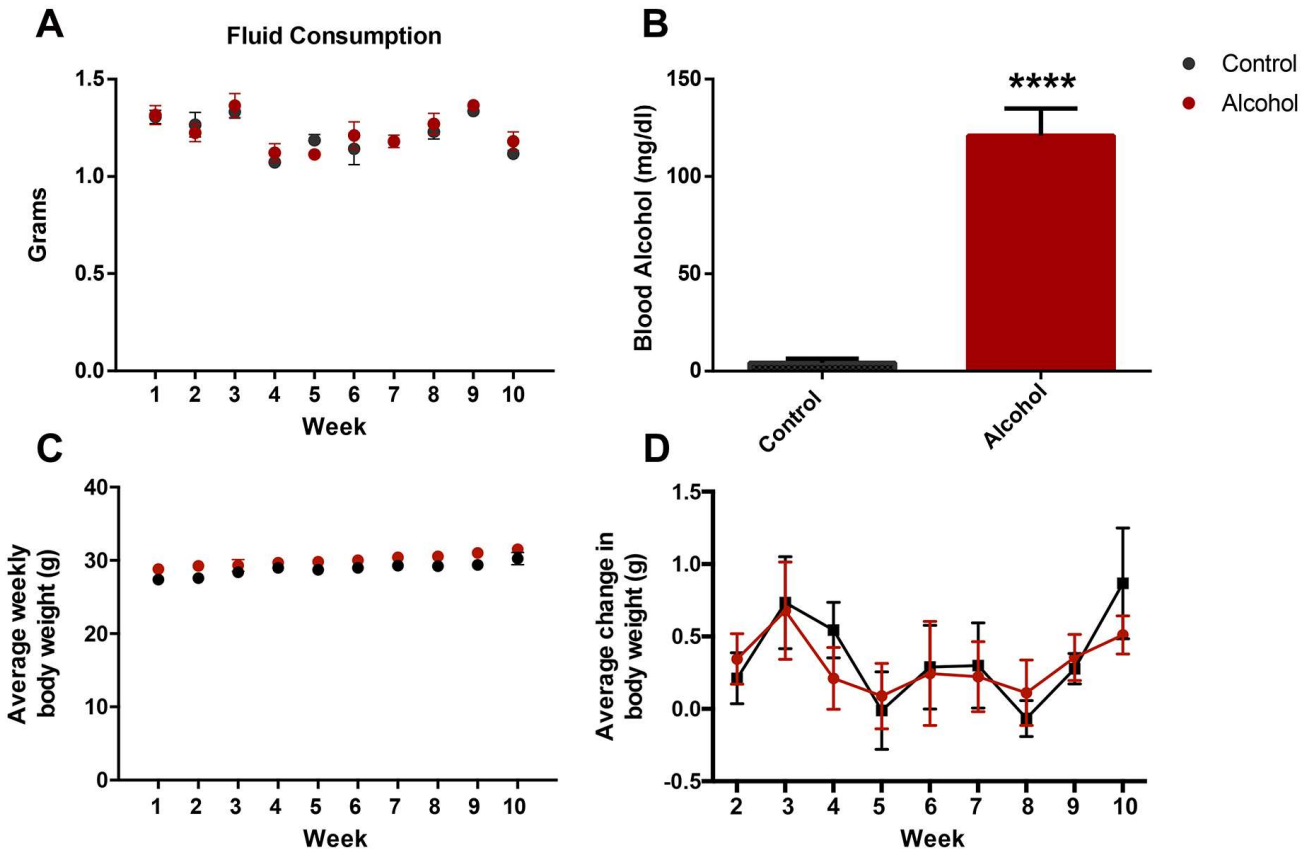

Figure 17 Chronic alcohol exposure using a limited access model induces physiologically relevant plasma alcohol levels but does not alter paternal weight

A) Average daily fluid consumption compared between the preconception treatment groups (n=11). B) Average plasma alcohol levels between preconception treatment groups, as measured one month after the beginning of treatment (n=9). C) Average weight of males in each treatment group over the experimental course (n=11). Average weekly weight gain of males in each preconception treatment group (n=11). Data were analyzed using an unpaired t-test, error bars represent the standard error of the mean (**** $p < 0.0001$).

Chronic paternal alcohol exposure induces late-term fetal growth restriction and reductions in placental efficiency within the offspring.

In our previous studies examining the offspring of alcohol-exposed males, we identified fetal growth restriction in only the female offspring at gestational day 14.5[99], while at birth, both male and female offspring exhibited significant growth restriction[159]. Therefore, we examined fetal growth at day 16.5 of gestation, which corresponds to the period when the mouse fetus experiences a dramatic increase in growth rate[316]. After 70 days of exposure, males undergoing the described preconception treatments were mated to unexposed females, and at gestational day 16.5, dams were sacrificed and offspring evaluated for growth. No differences in litter size were observed between the preconception treatment groups (Fig. 18A). At this developmental stage, the male and female offspring of alcohol-exposed sires displayed a ~15% reduction in fetal weight ($p < 0.01$), a ~10% reduction in crown-rump length ($p < 0.01$) and a respective 9% and 14% reduction in the weight of the gestational sac ($p < 0.05$) (Fig. 18B-D). These reductions in fetal growth were accompanied by an 18% increase in the placental weight of the male offspring of alcohol-exposed sires, while placental weights of the female offspring were identical to the controls (Fig. 18D). Collectively, a respective 28% and 17% reduction in placental efficiency (grams of fetus produced per gram of placenta[317]) was observed for the male and female offspring of the alcohol-exposed sires (Fig. 18E). These observations indicate that the growth restriction associated with paternal alcohol use predominantly manifest during the later phases of pregnancy and correlate with reductions in placental efficiency.

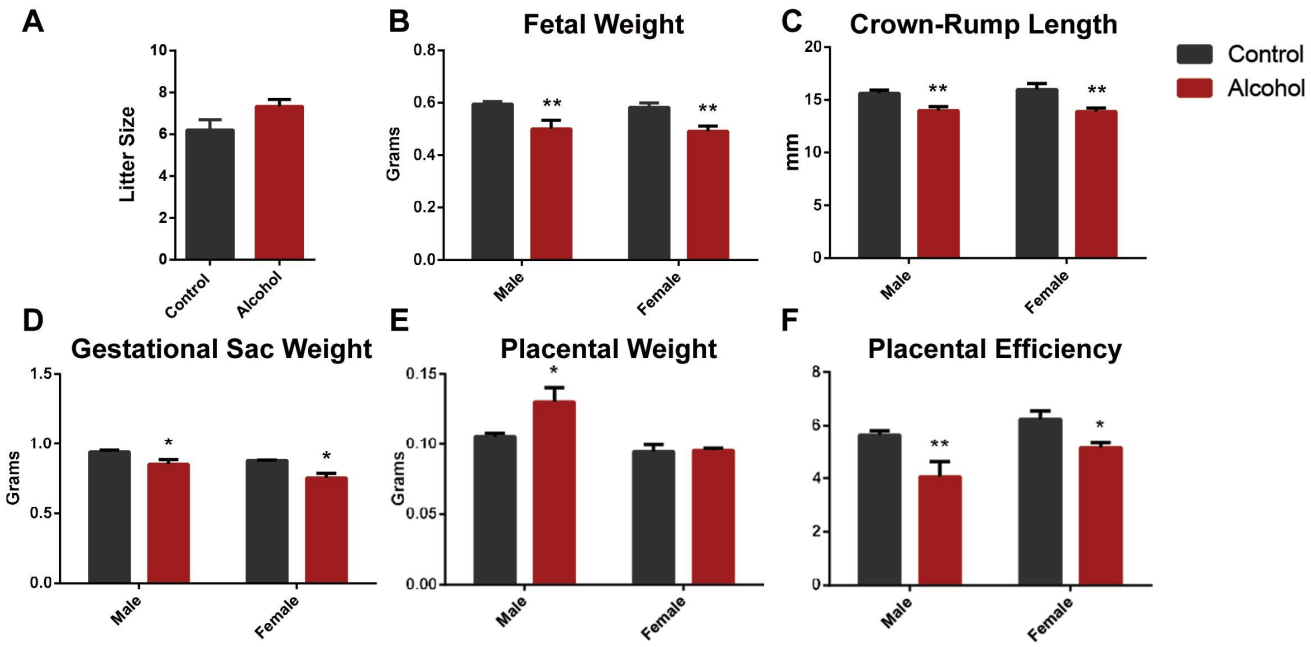

Figure 18 Chronic preconception paternal alcohol exposure induces fetal growth restriction and decreased placental efficiency in the offspring at gestation day 16.5

A) Comparison of litter size between matings sired by control and ethanol-exposed males (n=5 control 6 alcohol). Comparisons of B) fetal weight, C) crown-rump length, D) gestational sac weight and E) placental weights between male and female offspring sired by control and ethanol-exposed males (n=10 male and 12 female offspring). F) Placental efficiencies (gram of fetus produced per gram of placenta) compared between the male and female offspring of ethanol-exposed sires (n=10 male and 12 female offspring). Data were analyzed using either an unpaired t-test or a two-way ANOVA followed by Sidak's post hoc analysis. Error bars represent the standard error of the mean (* $p<0.05$, ** $p < 0.01$).

Chronic paternal alcohol alters the profile of sperm-inherited non-coding RNAs

Using an inhalation model of exposure, Rompala and colleagues recently described alterations in sperm derived ncRNAs induced by a 5-week exposure to alcohol[318]. These studies achieved similar blood alcohol levels (125-175mg/dL) to those observed in our model. However, whether these separate models of exposure and different durations induce similar or distinct impacts on the profile of sperm-inherited ncRNAs is unknown. To examine this further, mature sperm were collected from the cauda epididymides and vas deferens of the control and ethanol-exposed males used to sire the offspring analyzed. The purity of sperm was judged to be greater than 99% as evaluated by microscopy. Similar to previous reports[319], isolated RNAs predominantly ranged from 20 to 40 nucleotides in length (Figure 19A). Small RNAs from control and ethanol-exposed males were subjected to deep sequencing analysis, with an average of 25 million mappable reads obtained per sample (n=4). Similar to previous studies describing the small RNA profiles of mouse sperm[153,318,319], we found that the majority of small RNA reads mapped to transfer RNA-derived small RNAs (~60% tRFs) and Piwi-interacting RNAs (~30% piRNAs) (Fig. 19B). The remaining small RNAs predominantly mapped to transfer RNAs (~5% tRNAs) and microRNAs (~5% miRNAs) (Fig 19B). A recent study by Sharma et al., described proportional changes in the ratio of tRFs and piRNAs as sperm undergo maturation in the epididymis[94]. Here, a progressive increase in tRFs and a loss of piRNAs were observed as sperm mature. Although sperm derived from alcohol-exposed males tended to have proportionally fewer mappable tRFs and a greater abundance of piRNAs, individually, these trends did not reach statistical significance ($p=0.0552$ and $p=0.1086$) (Fig. 19C-D). However, a ratio comparing tRFs:piRNAs revealed a significant shift ($p<0.05$) between the two preconception treatment groups (Fig. 19E). Further, while populations of tRNAs were similar between treatments, we observed a significant ($p=0.03$),

~30% increase in the abundance of miRNAs in sperm derived from alcohol-exposed males (Fig 19F-G). These observations reveal that chronic alcohol consumption shifts the profile of sperm-inherited non-coding RNAs, with miRNAs exhibiting the greatest change.

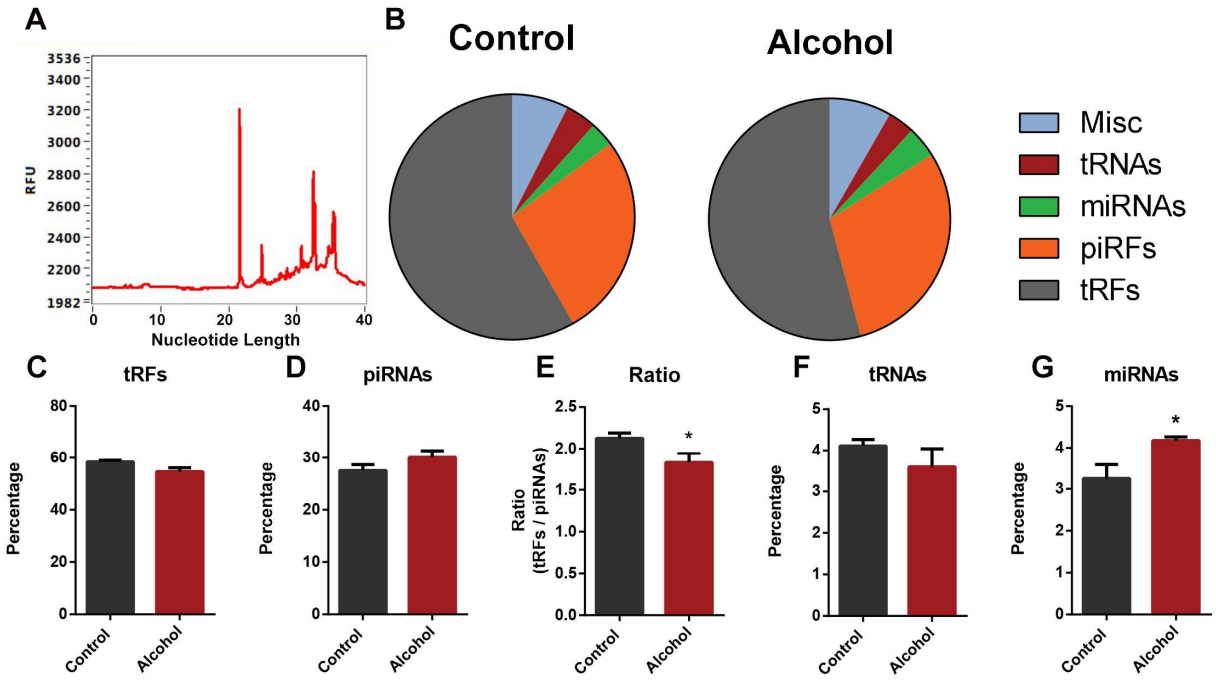

Figure 19 Alcohol-induced alterations to the profile of sperm-inherited non-coding RNAs

A) Representative graph depicting the size distribution of RNAs isolated from sperm. B) The proportional abundance of transfer RNA-derived small RNAs (~60% tRFs) Piwi-interacting RNAs (~30% piRNAs), transfer RNAs (~5% tRNAs) and microRNAs (~5% miRNAs) between sperm derived from the two preconception treatment groups (n=4). Individual comparison of the percentage of C) tRFs and D) piRNAs mapped between preconception treatment groups. E) The ratio of tRFs:piRNAs in sperm derived from control and alcohol-exposed males. Individual comparison of the percentage of F) tRNAs and G) miRNAs mapped between preconception treatment groups. For comparison of percentages mapped, data were arcsine transformed and an unpaired t-test with Welch's correction applied. All other comparisons were conducted using an unpaired t-test. Error bars represent the standard error of the mean (* $p<0.05$).

Alterations in the abundance of miR21, miR30, and miR142 in alcohol-exposed sperm

Using the Bowtie2 and miRDEEP2 pipelines, we compared the abundance of individual candidate small non-coding RNAs between control and alcohol-exposed sperm. No differentially enriched tRFs, piRNAs or tRNAs could be identified between treatment groups (Fig. 20A-C). In contrast, three differentially enriched miRNAs could be identified between treatments (miR21, miR30, and miR142) (Fig. 20D). Of these, miR21 and miR142 were abundantly enriched in both treatment groups (miR21 500 (C) and 900 (A) fpkm, miR142 600 (C) and 300 (A) fpkm). However, differences in the abundance of these two candidates offset each other, and therefore, do not explain the 30% increase in miRNA enrichment observed in alcohol-exposed sperm (Fig. 19F). In contrast to these candidates, the remaining miRNAs identified displayed large variations both across and within treatment groups (Fig. 20E). Collectively, these observations indicate that the 30% increase in miRNA abundance represents a general increase and is not linked to any specific candidate. Recently, alterations in the profile of sperm-inherited miRNAs induced by chronic stress have been directly linked to increased circulating levels of corticosterone[320]. We, therefore, assayed the levels of this hormone in our model. No differences in corticosterone could be identified between preconception treatment groups (Fig. 20F). Therefore, the 30% increase in miRNA enrichment and differences in miR21, miR30 and miR142 cannot be linked to alcohol-induced changes in the profile of circulating corticosterone.

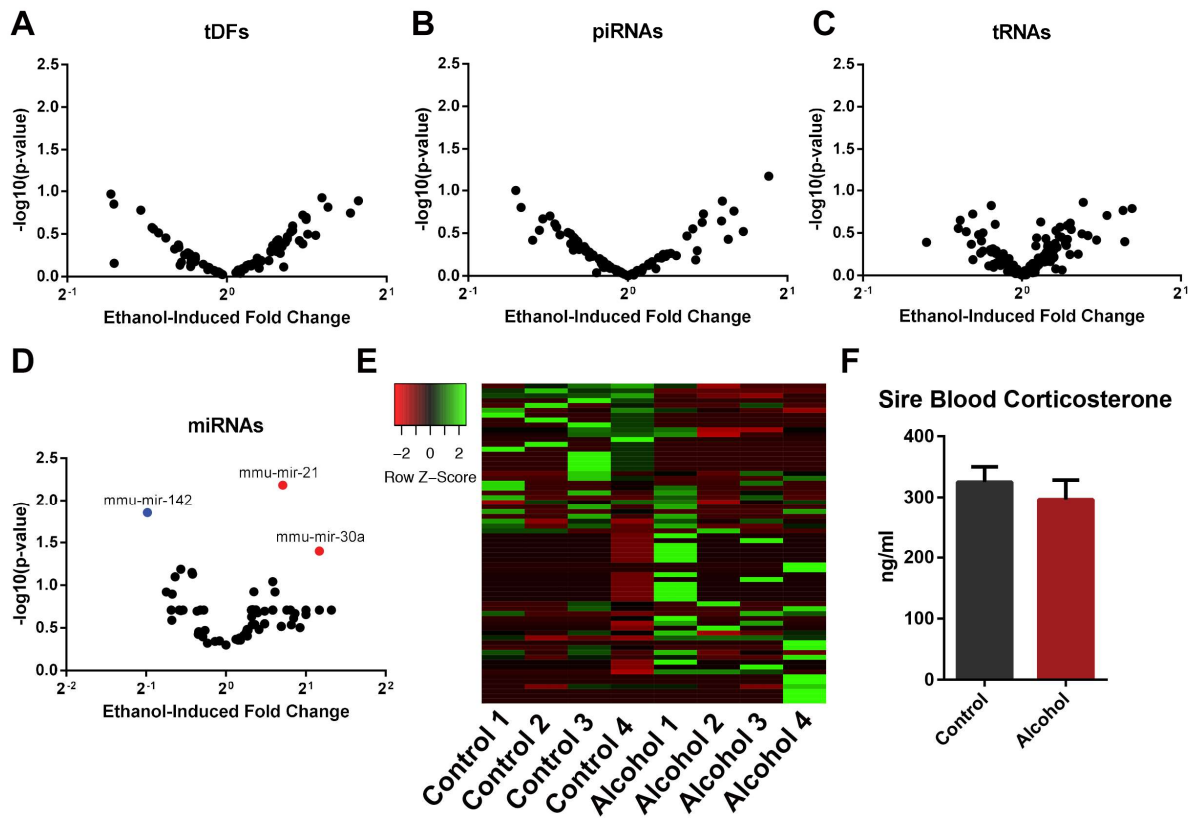

Figure 20 Alcohol-induced changes in the abundance of sperm-inherited miR21, miR30, and miR142

Volcano plots comparing the differential enrichment of candidate A) tRFs, B) piRNAs, C) tRNAs and D) miRNAs of sperm derived from the two preconception treatment groups. E) Heatmap comparing the variation of sperm derived miRNAs between treatment groups. F) Comparison of circulating levels of corticosterone between preconception treatment groups. An unpaired t-test was applied to compare the levels of corticosterone. Error bars represent the standard error of the mean, miRNAs identified with either blue or red dots were differentially enriched ($p<0.05$).

Discussion

Using a mouse model of voluntary alcohol consumption, our group recently described an association between chronic preconception paternal alcohol use and deficits in both placental function and fetal growth within the offspring[99]. Subsequent studies revealed that these alcohol-induced growth phenotypes were accompanied by a prolonged period of fetal gestation and sex-specific patterns of postnatal growth restriction[159]. These deficits in growth are similar to phenotypes described in long-term clinical studies of children with fetal alcohol spectrum disorders[96,169] and join a growing body of literature indicating preconception paternal alcohol use is a significant, yet under-recognized contributor to alcohol-induced growth defects (reviewed here[76,97,117]). However, the question of how the memory of chronic alcohol use transmits from father to offspring remains unresolved.

The literature examining the impacts of chronic alcohol use on male reproductive physiology is varied and highly inconsistent[321]. To this point, of three published studies using similar rodent models of exposure, two identified systemic decreases in testosterone concentrations[322,323], while the third was unable to identify any reproducible changes[324]. Similarly, the human literature is equally varied, with studies describing both alcohol-associated decreases and increases in testosterone levels[325-327]. However, combined with the negative correlations observed between alcohol use and successful outcomes in human in vitro fertilization[328,329], the prevailing feeling is that this teratogen exerts a negative effect on male reproductive function[321].

In this study, we returned to our voluntary model of alcohol consumption to determine the impact chronic ethanol use has on male reproductive physiology and the association of sperm-inherited noncoding RNAs with the transmission of alcohol-induced growth defects. Here, we first confirmed that chronic preconception paternal alcohol exposure induced fetal growth restriction

in the offspring and extended our previous findings by demonstrating that these deficits in growth primarily manifest during the later phase of pregnancy (Fig. 2). We then assayed large-scale measures of male reproductive health, including testicular, epididymal and seminal vesicle weights, as well as testicular morphology and testosterone levels. However, we were unable to identify changes in any of these criteria. In addition, no differences in total sperm counts, sperm DNA fragmentation or sperm nuclear packaging were observed between the preconception treatment groups. Combined with the observed similarities in litter size between preconception treatments, we conclude that the dose and duration of alcohol exposure employed in our model do not impact macro-measures of male reproductive physiology.

As no observable changes in either sperm DNA methylation[99] or macro-measures of nuclear structure (Fig. 4) could be observed, we examined alcohol-induced alterations in the profile of sperm-inherited noncoding RNAs. Using an inhalation model of exposure, Rompala and colleagues recently identified alcohol-induced changes in the profile of tRFs and miRNAs, as well as select mitochondrial small mRNAs in sperm[318]. In contrast to these observations, we were only able to identify differences in select miRNAs, none of which were consistent with this published study. Although both models result in sex-specific patterns of postnatal growth restriction[295], these two separate models of exposure may induce distinct epigenetic changes. However, we did observe some similarities between our dataset and previous works examining the impact of paternal stress on offspring phenotype. Specifically, miR-30 and miR-21 were both up-regulated in the present study as well as in that of three previous reports examining stress-induced changes in sperm noncoding RNAs[107,320]. Although the levels of plasma corticosterone observed in our mice were higher than those reported by Rodgers et al.[330], we presume this difference is due to the techniques used to measure corticosterone levels and note that our results are consistent with those of two

other publications employing ELISA-based measurements[69,320]. Regardless, we could not identify any changes in the levels of plasma corticosterone between treatment groups, indicating alcohol may alter sperm-inherited noncoding RNAs through distinct mechanisms. Importantly, the three differentially enriched candidate miRNAs identified in this study are all known to be modulated by alcohol exposure[331,332].

Recent studies indicate that dynamic changes in the levels of tRFs, piRNAs, and miRNAs are a core feature of sperm maturation[94]. Specifically, progressive increases in tRFs are observed during epididymal transit, while conversely, piRNAs become reduced. These changes are hypothesized to be integral to sperm maturation and the reproductive success of the conceptus[299]. In this study, we observed a shift in the ratio of tRFs and piRNAs indicating that the sperm of alcohol-exposed males has proportionally fewer tRFs and more piRNAs. This may indicate that the complement of non-coding RNAs in alcohol-exposed sperm are less mature than the controls. Of note, miR21, which is comparatively rare in testicular sperm populations, is nearly absent in the in the epididymal soma but is highly abundant in epididymosomes of the caput region of the epididymis[333,334]. This suggests that the alcohol-induced shifts in noncoding RNAs may primarily be mediated by effects on epididymal trafficking. However, further studies are needed to confirm this hypothesis.

At this point, we do not know if the modest changes in noncoding RNA abundance identified in this study are directly linked to the alcohol-induced growth restriction phenotypes observed in the offspring. The comparative contribution of sperm-inherited RNAs to the vast repertoire found in the early conceptus is negligible[335] and further, no mechanisms have been identified by which this small contribution could stably alter the gene expression profile of the early embryo and the long-term health of the offspring. In light of this, it is challenging to see how

modest changes (50% decrease in miR142, 1.5 fold increases in miR21 and miR30) in the identified candidates could induce fetal growth restriction with the consistency observed in our model, as well as mediate the long-term impacts on offspring metabolic health. One possibility is that the paternally-inherited RNAs are post-transcriptionally modified to confer dramatically enhanced stability, which may potentiate their impacts beyond preimplantation development and influence the processes of lineage specification[92]. It may also be that unrelated alterations in sperm histone retention and chromatin looping mediate the observed effects or a combinatorial interaction between multiple epigenetic mediators. Finally, we have not ruled out the possibility that these phenotypes may be due to alcohol-induced changes in the seminal plasma. In previous studies, ablating the seminal vesicle gland induced placental hypertrophy in late gestation and sex-specific effects on the long-term growth and metabolic health of the offspring[336]. Although the effects on offspring growth were opposite to those observed in our model, the impact on placental growth is compelling. Future studies using IVF will be necessary to determine the impact of male seminal plasma in mediating the effects of paternal alcohol use on the offspring.

Materials and Methods

Animal work

All experiments were conducted under AUP 2017-0308 and approved by the Texas A&M University IACUC. Individually caged C57BL/6J (RRID:IMSR_JAX:000664) postnatal day 90 adult males were obtained and housed in the Texas A&M Institute for Genomic Medicine, fed a standard diet (catalog# 2019, Teklad Diets, Madison, WI, USA) and maintained on a 12-hour light/dark cycle. In this study, we employed a voluntary model of alcohol exposure known as Drinking in the Dark. This model of exposure avoids the stress associated with forced feeding and mitigates the known impact of stress on the sperm-inherited epigenetic program[107]. Using methods

described previously[99], males were provided limited access to ethanol during a four-hour window, beginning one hour after the initiation of the dark cycle. During this four-hour window, experimental males were provided access to a solution of 10% (w/v) ethanol (catalog# E7023, Millipore-Sigma, St. Louis, MO, USA) and 0.066% (w/v) Sweet'N Low (Cumberland Packing Corp, Brooklyn NY, USA). Control males received a solution of 0.066% (w/v) Sweet'N Low alone. After each session, the amount of fluid consumed by each mouse was recorded.

Once consistent patterns of drinking were established, males were maintained on this protocol for a period of 70 days. Subsequently, two naturally cycling females were placed in 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 were 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 each male had produced a minimum of two pregnancies. Subsequently, males were euthanized by CO2 asphyxiation and cervical dislocation, blood collected post-mortem and the male reproductive tract excised.

Pregnant dams were maintained on a Breeder diet (catalog# 5058, LabDiet, St. Louis, MO, USA), subjected to minimal handling and euthanized by CO2 asphyxiation and cervical dislocation on gestational day 16.5. The female reproductive tract was excised, the gestational sac removed and fetal tissues weighed.

Sperm RNA Isolation

Approximately 10 million mature sperm per sample were lysed in 1mL TRIzol Reagent (catalog# 15596018, Thermo-Fisher, Waltham, MA, USA) plus 10 µl β-ME (catalog# M3148, Millipore-Sigma, St. Louis, MO, USA) using homogenizing pestles (catalog# 64788-20, Electron

Microscopy Sciences, Hatfield, PA, USA). Subsequently, 200µl of 1-Bromo-3-chloropropane (catalog# B9673, Millipore-Sigma, St. Louis, MO, USA) was added to separate the aqueous phase, which was collected and precipitated using an equal volume of isopropanol. After two ice-cold, 70% ethanol washes, RNA samples were reconstituted in RNase free water and stored at -80ºC.

Sperm RNA Sequencing

Illumina single-end cDNA libraries were synthesized from size-selected RNAs (<50 bases) derived from 100 ng of sperm total RNA using the TruSeq Stranded mRNA kit. Four biological replicates per treatment group were multiplexed and sequenced on a single HiSeq2000 lane (Illumina) within the sequencing core of the Whitehead Institute. Using Bowtie2 (RRID:SCR_016368) and Tophat (RRID:SCR_013035), small RNA reads were aligned to the Mus musculus (UCSC version mm10) reference genome. Small RNA reads with a single allowable mismatch were selected for further analysis. Small RNA annotation was performed by separately aligning reads to the microRNA database(http://www.mirbase.org/), tRNA database(http://gtrnadb.ucsc.edu/), and piRNA database(http://pirnabank.ibab.ac.in/index.shtml). Differentially expressed microRNAs were quantified using the miRDEEP2 ver2.0.0.7 pipeline. The referenced tRNA database holds sequences for both tRNA anticodons and tRNA fragments (tRFs), which are selected as reads partially matching the tRNA anticodon but are less than 34 nucleotides in length. To quantify differentially expressed tRFs, piRNAs, and tRNAs, total counts of mapped reads were calculated using the featureCounts pipeline and then normalized to the total mapped reads of each class of small RNA species, as described previously. Generated volcano plots contrast the differential enrichment of ncRNAs by raw p-value and not by FDR selection.

Data Handling and Statistical Analysis

For all experiments, measures were input into the statistical analysis program GraphPad (RRID:SCR_002798; GraphPad Software, Inc., La Jolla, CA, USA) and statistical significance was set at alpha = 0.05. For all datasets, normality was first verified using the Brown-Forsythe test. In this study, the effect of treatment was assessed using either an unpaired Student's t-test or two-way analysis of variance test (ANOVA), with differences among the means evaluated using Sidak's posthoc test of contrast. In all instances, we have marked statistically significant differences with an asterisk. For the comparisons of testicular, epididymal and seminal vesicle weights expressed as a percentage of total paternal body weight, as well as the percentage of TUNEL and CMA3 positive sperm, data were arcsine transformed and an unpaired t-test with Welch's correction applied. To calculate the tRFs:piRNA ratio, the percentage of mapped tRFs per sample was divided by the percentage of mapped piRNAs and differences compared between treatments using an unpaired student's t-test.

CHAPTER VI

CONCLUSION

The presented results reveal significant growth restriction in the offspring of alcohol-exposed males. The inappropriate activation of the genetic pathways regulating hepatic fibrosis identified in the offspring of alcohol-exposed males would be expected to impede hepatic functions and negatively impact long-term growth. In this study, we also determine if the signature hepatic fibrosis identified during the fetal stages persists into postnatal life and in doing so, understand: 1) the development of liver malfunction arising from fetal stress and the persistence of this condition into adolescence and adulthood and 2) explore this intergenerational effect on offspring metabolic health by determining the capacity of the offspring to respond to high-fat-diet induced liver stress. Finally, by contrasting 1) the molecular signatures regulating the crosstalk between liver inflammation and liver insulin insensitivity in alcohol-sired obese offspring and 2) sperm small RNA profile altered by alcohol exposure, we can pinpoint the effect of paternal alcohol exposure in a molecular level to explore novel therapeutic strategies.

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