PRECONCEPTION PATERNAL ALCOHOL EXPOSURE DISRUPTS PLACENTAL GENE EXPRESSION AND ALTERS CHROMATIN BOUNDARY ELEMENTS

A Thesis

by

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ABSTRACT

Fetal alcohol spectrum disorders (FASD) are a spectrum of pathophysiological consequences that include structural, neurological and behavior disorders that arise following prenatal alcohol exposure (PAE). Work by our group and others have begun to associate alcohol-induced alterations in gene expression with heritable alterations in chromatin structure. These observations now include alterations in fetal physiology and gene expression arising from preconception paternal alcohol exposures. In this study, we hypothesized that male preconception alcohol exposure can heritably alter gene expression in placenta and that these changes in transcription are linked to alterations in the positioning of chromatin boundary elements. In this thesis, we begin to examine this assertion by characterizing patterns of gene expression and localizing chromatin boundaries between clusters of dysregulated genes.

Placentas of both male and female offspring from control and alcohol-exposed fathers displayed alterations in the expression of cohorts of genes that are enriched in genetic pathways controlling aspects of molecular transport, protein synthesis, lipid metabolism, small molecule biochemistry, as well as vitamin and mineral metabolism. Importantly, many of the genes displaying alterations in gene expression localized to co-regulated gene clusters, suggesting a higher order of transcriptional control was perturbed. Given the established role of DNA methylation in the control of gene expression within co-regulated clusters, we first assessed the enrichment of the methyl donor S-adenosylmethionine (SAM). These experiments did not identify any significant

alterations in the levels of SAM in the paternal liver, testis, and kidney nor were we able to identify differences in the fetal brain or placenta. Further examination of chromatin structure at co-regulated promoters, enhancers and insulators within three of the most prominent gene clusters revealed that binding of the chromatin boundary protein CTCF was decreased significantly in offspring of males exposed to alcohol prior to conception. This reduced CTCF occupancy was not due to a global decrease in CTCF protein levels in placentas. These observations provided new insights in the study of male preconception alcohol abuse and the heritable impact of preconception exposures on chromatin organization.

DEDICATION

This thesis is dedicated to my beloved maternal grandmother for all these sweet memories you have left with me. Wish you a happy life in the world of Sukhavati.

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NOMENCLATURE

FASD	Fetal Alcohol Spectrum Disorders
PAE	Prenatal Alcohol Exposure
SAM	S-adenosylmethionine
FAS	Fetal Alcohol Syndrome
H3K4	Histone 3 Lysine 4
H3K9	Histone 3 Lysine 9
H3K27	Histone 3 Lysine 27
me1	Monomethylation
me2	Dimethylation
me3	Trimethylation
ac	Acetylation
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
bp	Base Pair
CHIP	Chromatin Immunoprecipitation
seq	Sequencing
E	Enhancer
I	Insulator
CE	Candidate Enhancer
CI	Candidate Insulator
CM	Control Male

EM	Ethanol Male
CF	Control Female
EF	Ethanol Female

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CHAPTER I

INTRODUCTION: THE IMPACT OF PRECONCEPTION PATERNAL ALCOHOL EXPOSURE ON PLACENTAL PATTERNS OF GENE EXPRESSION

1.1 Introduction

Each year, six percent of children worldwide are born with birth defects, which are defined as structural deformities existing at or before birth regardless of cause (Arnold Christianson, 2006). Cumulatively, congenital defects are the leading cause of death among young children and are estimated to cost the health care industry 3.3 billion US$ annually. The top four most prevalent structural birth defects are 1) congenital heart defects, 2) neural tube defects, 3) cleft pallet, and 4) Down syndrome (http://www.childrenshospital.org/).

While genetic studies have identified a genetic basis for some birth defects, the overwhelming majority are classified as non-syndromic, which means that they are not associated with multiorgan defects and cannot be explained by a single gene or chromosomal abnormality. Although their origins remain mostly unclear, recent evidence point to a complex interaction between genetic, epigenetic and environmental factors (Hobbs et al., 2014). Thus, the developmental origins of non-syndromic birth defects cannot be adequately explained by genetics alone, but rather arise as a consequence of complex gene-environment interactions; where common environmental exposures unmask a predisposition for dysgenesis (Hannon, 2010). In order to better

understand the developmental origins of non-syndromic birth defects and dysgenesis, we must gain a better grasp on the role epigenetic processes play in their development.

1.1.1. Fetal Alcohol Syndrome and Birth Defects

Fetal alcohol spectrum disorders (FASD) refer to a spectrum of pathophysiological consequences including structural, neurological and behavior disorders following prenatal alcohol exposures (PAE) (Riley et al., 2011). The adverse effects of FASDs range from mild to very severe and include fetal alcohol syndrome (FAS), which ranks as the most serious manifestation of alcohol-induced teratogenesis. FAS is diagnosed by pre- and post- natal growth deficiency, craniofacial defects, central nervous system damage and confirmed PAE, cumulatively leading to behavioral or cognitive abnormalities (Astley and Clarren, 2000).

Epidemiologic studies have shown that alcohol is the most prevalent teratogen to which humans are exposed, and in the United States, 6–9 infants per 1000 live births are diagnosed with some degree of fetal alcohol spectrum disorder (Fox et al., 2015). However, alcohol wasn't even recognized as teratogen until 1968, when the first group published a report detailing cognitive and morphological changes in a group of 127 children born to alcoholic mothers (Lemoine P, 1968). Five years later, two physicians, (Jones and Smith) from the University of Washington Medical School published two papers in the Lancet, which first coined the term fetal alcohol syndrome (FAS) and described a cohort of children with morphological defects and developmental delays; who again, were born to alcoholic mothers (Jones et al., 1973) (Jones and Smith, 1973).

In these original publications, FAS was defined as a pattern of altered growth and morphogenesis in the offspring of alcoholic mothers. The phenotypes typically include pre- and post- natal growth deficiencies, abnormal facial morphology and brain damage. Building upon these seminal studies, investigators have proven that alcohol has the capacity to behave as a teratogen, which can cause birth defects in the developing embryo or fetus.

In 1988, the Alcoholic Beverage Labeling Act (ABLA) was implemented by the US Surgeon General, which required all alcoholic beverages to be labeled with a warning informing women of the dangers of alcohol related birth defects. Since then, studies have shown a slight decrease in prenatal alcohol consumption. However, 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 (Deaver, 1997).

1.1.2. Fetal Growth Restriction Arising from Alcohol Exposure

One of the diagnostic criteria for FASDs is growth restriction, which is often marked by children being born small for their gestation age (SGA) and having low birth weights at the end of pregnancy. In addition, alcohol can also cause long-term growth restriction, which persists into adult life and is associated with metabolic syndrome, neurocognitive deficits, hypertension, immune dysfunction and increased rates of cancer (Valsamakis et al., 2006) (Moore and Riley, 2015) (Carter et al., 2016). The mechanisms by which alcohol can induce growth restriction are thought to be through impacts on

maternal physiology, altered uteroplacental blood flow and the induction of fetal structural abnormalities. As the maternal interface with the developing embryo/fetus, the placenta functions in nutrient supply, waste elimination, hormone production, immune protection, and gas exchange; and also plays a critical role in mediating prenatal alcohol exposure induced growth restriction (Rossant and Cross, 2001) (Gundogan et al., 2008).

Previous studies have shown that in addition to teratogenic effects on central nervous system, pregnant rats chronically exposed to ethanol gave birth to offspring with growth restriction as measured by reduced fetal weight and length. This intrauterine growth restriction was mainly mediated by impaired placentation through inhibiting aspartyl-(asparaginyl) b-hydroxylase (AAH) expression, which is downstream of the insulin-like growth factor (IGF) pathway and plays an important role in trophoblast cell motility and invasion(Gundogan et al., 2008).

Other studies have also shown that alcohol consumption during pregnancy may alter fatty acid metabolism, endocrine signaling (such as human chorionic gonadotropin (hCG) and IGF2), as well as induce hypoxia, thus causing fetal growth restriction and birth defects (Linask and Han, 2016) (Joya et al., 2015) (Bosco and Diaz, 2012). However, our understanding of the impacts of alcohol on placental development and function remain very poor. Though several studies have characterized the placenta’s role in mediating maternal alcohol exposure induced birth defects, the mechanisms by which alcohol exposure impacts placental function remain unanswered.

1.1.3. Male Environmental Exposures and Alterations in Epigenetic Programming

Recent studies suggest that preconception male exposures to a variety of environmental stressors have the capacity to alter fetal development, and negatively influence the health and fitness of the offspring. To date, pesticide exposures, folic acid deficiencies, high-fat diets, and intense stress have all been significantly associated with alterations in the developmental program of sperm; and are correlated with negative outcomes in the next generation (Bollati and Baccarelli, 2010) (Lambrot et al., 2013) (Donkin et al., 2016) (Gapp et al., 2014). As specific examples, transient exposures to endocrine disruptors in pregnant rats generate offspring with decreased spermatogenic capacity that persists into subsequent generations through the male germ line (Anway et al., 2005). In a Northern California Leukemia Study, paternal smoking was significantly associated with increased childhood leukemia risk (Chang et al., 2006). In male rats, high-fat diet induced obesity has been associated with β-cell dysfunction and impaired insulin secretion and glucose tolerance in their female offspring (Ng et al., 2010). In mice, a paternal low-protein diet results in alterations to lipid metabolism in the next generation (Carone et al., 2010). These observations in rodent models are further supported by epidemiological data in humans, where it was observed that excessive food intake in paternal grandfathers is associated with increased cardiovascular disease, diabetes and mortality in the male grandchildren (Kaati et al., 2002).

The mechanism of how these environmental stressors induce abnormalities remains unanswered, largely because of the misconception that sperm do not transmit information beyond the genetic code (Feng et al., 2010). However, recent studies have

discovered that apart from the genetic code, epigenetic factors including non-coding RNAs, DNA methylation and histone modifications play a significant role in transmitting developmental and metabolic traits to the next generation. For example, paternal chronic stress prior to conception induces significant changes in sperm miRNA content and reduces offspring hypothalamic-pituitary-adrenal (HPA) axis stress responsivity (Rodgers et al., 2015). Pre-diabetic male mice exhibit altered methylation patterns in sperm DNA that overlap with various differentially methylated genes in offspring pancreatic islets, especially across several signaling genes associated with glucose intolerance and insulin resistance in offspring (Wei et al., 2014). In addition, sperm post-translational histone modifications can also be transmitted to offspring. As an example, reduced histone 3 lysine4 (H3K4) methylation in sperm induced by transgenic over-expression of the H3K4 demethylase KDM1A within the testis, impairs health and development of the offspring. This phenomenon persisted for several generations and even in non-transgenic offspring (Siklenka et al., 2015). While alcohol exposure in utero is, undoubtedly, a significant element in the origins of FASD associated birth defects, studies have emerged indicating that 75% of FASD children have biological fathers who were either heavy drinkers or chronic alcoholics (Abel et al., 1983). However, it still remains to be determined if developmental aspects of fetal programming are influenced by paternal ethanol exposure.

1.1.4. PAE and Epigenetics

The term epigenetics refers to heritable traits, which are hypothesized to be transmitted via differential patterns of DNA methylation, histone post-translational modifications and the expression of non-coding RNAs (Bird, 2007). Given the evidence that the epigenome can be altered by environmental exposures and transmitted to future generations, it is not surprising that, as the most prevalent environmental stressors for humans, alcohol has the capability to alter each of the above, both before and after conception. Epigenetic mechanisms are now hypothesized to make a significant contribution to the development of FASDs (Kleiber et al., 2014) (Perkins et al., 2013) (Mason and Zhou, 2015).

1.1.4.1. Impact of PAE on DNA methylation

DNA methylation is probably one of the best-studied epigenetic modifications and mainly occurs symmetrically at palindromic cytosine-guanine (CpG) sites where cytosines are converted to 5-methylcytosine. CpG islands are normally defined as regions longer than 200bp with G+C content above 50% and observed/expected ratio of CpG above 60%. In humans, roughly 70% of genes bear CpG islands in their regulatory regions. DNA methylation within the promoter region is generally associated with transcriptional repression, which is hypothesized to influence the binding of transcription factors and repressors to these regulatory regions. Genome-wide, the majority of CpG islands are unmethylated (Smith and Meissner, 2013). DNA methylation is critical for normal development and plays an essential role in various processes, including X

chromosome inactivation, the control of alternative splicing, genomic imprinting and silencing of transposons (Bestor et al., 2015). Given the fact that CpG island methylation is critical for the regulation of gene expression during development, various studies have examined in both gene-specific and global patterns of DNA methylation in response to PAE.

Alcohol exposure can cause perturbations in DNA methylation and damage to offspring at different stages of development. It has been shown that alcohol exposure during early neurulation leads to growth retardation, abnormal neural tube formation, which correlates with alterations in DNA methylation (Liu et al., 2009). Later, the same group reported that alcohol-induced growth retardation could be correlated with delayed DNA methylation (Zhou et al., 2011). In addition, global DNA methylation can also be altered due to alcohol exposure. Acute alcohol exposure during the gestational days 9-11 in pregnant mice results in a reduced DNA methylome in fetuses and this is mainly mediated by hindered DNA methyltransferase 1(DNMT1) activity (Garro et al., 1991). Besides acting through DNMTs, alcohol can also alter DNA methylation by inhibiting folate absorption and reducing levels of the methyl donor S-adenosylmethionine (SAM), which is a crucial element in the methionine-homocysteine pathway (Halsted et al., 2002). Deficiency in folate is associated with globally decreased DNA methylation and a similar spectrum of developmental abnormalities as FASDs (Kim et al., 1997). Collectively, these results indicate alterations in DNA methylation, both gene specific and globally, resulting from prenatal ethanol exposures, can be correlated with stage specific developmental deficits.

In addition to prenatal exposures, encounters prior to conception have been associated with increased CpG demethylation in two differentially methylated regions (DMRs) in male gametes (Ouko et al., 2009). During preimplantation stage, in utero alcohol exposure leads to reduced methylation of paternal alleles in placenta and embryo as well as placental growth retardation (Haycock and Ramsay, 2009). Further, alcohol exposure during fetal development alters patterns of DNA methylation within the regulatory regions of genes modulating the stress response gene proopiomelanocortin (POMC), which heritably transmits to the next generation (Gangisetty et al., 2014). Taken together, these studies indicate that alterations in DNA methylation induced by exposures both preconception and in utero associate with the adverse developmental effects which manifest during different stages of development.

1.1.4.2. Impact of PAE in post-translational histone modification

In eukaryotic cells, DNA is wrapped around histones, a group of highly alkaline proteins. Core histones H2A, H2B, H3 and H4 exist as dimers and form protein octamers called nucleosomes which are further stabilized by linker histones H1/H5 (Marino-Ramirez et al., 2005). Besides packaging and ordering DNA into cell nuclei, histones play a role in gene regulation. Different post-translational histone modifications have been found to associate with different states of chromatin accessibility. The best-characterized modifications include methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, citrullination and ADP-ribosylation (Tropberger and

Schneider, 2013). Together with DNA methylation, these post-translational histone modifications add another layer of regulation to chromatin accessibility.

Histone modifications are affected by alcohol exposures both in vivo and in vitro. Pregnant rats exposed to alcohol during gestational days 7-21 give rise to offspring with decreased H3K4me3, acetylated H3K9 and increased H3K9me2 within the hypothalamus (Bekdash et al., 2013). Ethanol treatment at gestational day seven in mice leads to augmented G9a activity, a dimethyltransferase, which further increase H3K9me2 and H3K27me2 and promotes apoptotic neurodegeneration in neonatal stages of life (Subbanna et al., 2013). In another study, mice repeatedly exposed to ethanol vapor displayed varied transient histone modifications in different regions of the brain (Finegersh et al., 2015). Numerous studies of alcohol's impact on development have been carried out in vitro using different cell culture models. As examples, cardiac progenitor cells showed dose-dependent elevations in acetylated H3K9 and modulation of genes including GATA4 and Mef2c (Zhong et al., 2010). In previous work from our lab, primary neutrospheres treated with alcohol, displayed alterations in multiple post-translational histone modifications within the regulatory regions of genes responsible for neural precursor cell identity and differentiation (Veazey et al., 2013). Further, when EtOH was removed from cell culture and the cells were allowed to undergo a recovery period, histone signatures distinct from those during the exposure phase emerged (Veazey et al., 2015). This was also observed in our most recent publication using both undifferentiated and fully differentiated cell types (Veazey et al., 2017, accepted). In summary, these findings suggest that post-translational histone modifications are heavily

impacted by alcohol exposure; however, the effect can vary by the timing, dose and tissue type.

1.1.4.3. Impact of PAE on non-coding RNAs

Non-coding RNAs refer to RNA molecules that are not translated into proteins, which include a large variety of RNAs, such as microRNAs (miRNAs), long non-coding RNAs (lncRNAs), small nuclear RNAs (snoRNAs), and piwi-interacting RNAs (piRNAs) (Birney et al., 2007). Non-coding RNAs are highly abundant and functionally critical; however, the functions of the majority of these noncoding RNAs are poorly understood.

MiRNAs are a group of small non-coding RNA molecules, roughly 22 nucleotides in length, and mainly function in RNA silencing through complementary binding with their target mRNAs, which induces mRNA destabilization or translational inhibition (Bartel, 2004). MiRNAs are critical in regulating the epigenetic machineries and the expression levels of genes participating in multiple cellular pathways. As the most studied non-coding RNA in FASDs, miRNAs are significantly affected by alcohol in various cull culture and animal models. For instance, in mice PAE elevates miR-10a expression and associates with mental retardation in the offspring. Interestingly, in a cell culture model, this impact of alcohol exposure was ameliorated by folate supplementation and with an associated down-regulation of miR-10a and up-regulated target gene Hoxa1 (Wang et al., 2009). In a zebrafish model, ethanol treatment suppressed miR-9 expression and its target genes, which developed a similar cranial

defect as animals with miR-9 loss of function. This alcohol-induced suppression was associated with increased CpG methylation at miR-9-2 locus in murine neural stem cells (mNSCs) (Pappalardo-Carter et al., 2013). Interestingly, acute PAE at gestational day 12 leads to the dysregulation of several miRNAs, which can be reversed by social enrichment (Ignacio et al., 2014). Moreover, in a sheep model, pregnant sheep treated with alcohol during their third trimester give birth to offspring with alterations in the plasma miRNA profiles, which included alterations in miR-9, miR-15b (Balaraman et al., 2014). These studies provide valuable knowledge as to the effects and consequences of alcohol-induced alterations in miRNAs and suggest these changes are both functional and have the potential to serve as biomarkers.

In contrast to miRNAs, the functions and consequences of alcohol-induced changes to other non-coding RNAs remain poorly characterized. In previous work conducted by our lab, linc1354 RNA expression levels were significantly decreased in alcohol treated neurosphere stem cells (Veazey et al., 2013). Similarly, adult mouse brains that are exposed to alcohol exhibit up-regulation of the MBII-52 snoRNA and down-regulation of the MBII-85 snoRNA (Laufer et al., 2013). The biological roles of these non-coding RNAs in various cellular processes remain an unexplored aspect of FASDs and require more investigation.

Given the importance of the described epigenetic phenomena with respect to the phenotypes associated with FASDs, we sought to determine if the growth phenotypes observed in our model of male preconception alcohol exposure could be linked to changes in these epigenetic mechanisms of transcriptional regulation. As alterations in

placental function have previously been correlated with patterns of fetal growth restriction, we elected to focus on this organ system.

1.2. Research Objective and Hypothesis

Objective 1 – determine if preconception alcohol exposure heritably impacts patterns of gene expression within the placenta.

We hypothesize that male preconception alcohol exposure will impact patterns of gene expression within the placenta resulting in the differential expression of cohorts of genes.

Objective 2 – Previous studies of male preconception alcohol exposure have identified alterations in DNA methylaiton within sperm (Bielawski et al., 2002). Here we will determine if the alterations in gene expression can be associated with programmed dysfunction in the metabolism of the methyl donor S-adenosylmethionine (SAM).

We hypothesize that preconception alcohol exposure impacts the metabolism of the methyl donor S-adenosylmethionine (SAM) within the tissues of the paternal liver, paternal testis, paternal kidney, fetal placenta and fetal brain.

1.3. Material and Methods

1.3.1. Animal Studies and Tissue Collection

All experiments were conducted under AUP 2014-0087 and approved by Texas A&M University IACUC. At postnatal day 90, adult C57BL/6(Cast7) males were

provided limited access to EtOH during a four-hour window immediately after their sleep cycle. Males were maintained on a 12-hour light/dark cycle (06:00/18:00 hours) and provided access to either a solution of 10% (w⁄v) EtOH and 0.066% (w⁄v) saccharin (experimental) or 0.066% (w⁄v) saccharin alone (control) for four hours a day. This protocol followed the Drinking in the Dark paradigm established by established by (Brady et al., 2012). Using this limited access model of exposure, experimental males consistently achieved blood EtOH concentrations of 210 mg/dL (Chang et al., unpublished). After 70 days of drinking, which is a period long enough to encompass two complete spermatogenic cycles, exposed males were mated to unexposed C57BL/6J dams and placental and fetal tissues collected at day 14.5 of gestation (GD14.5). After three consecutive successful copulations, breeder males were sacrificed and the paternal liver, testis and kidney collected.

1.3.2. RNA-Sequencing and Data Processing

Total RNA was isolated from isolated E14.5 fetal placenta using the RNeasy Plus Mini Kit, (catalogue # 74134, Qiagen, Germantown MD, USA) according to manufacturer's instructions. RNA-libraries were generated by Illumina RNA-seq preparation kit followed by pooling and high-throughput sequencing on an Illumina HiSeq 2500 (Genomic Core at Whitehead Institute for Biomedical Research, Cambridge, MA). The raw reads obtained from RNA-Sequencing were mapped to mm10 database using tophat (Trapnell et al., 2009) and assembled by cufflinks. Cuffdiff further assigns each gene with its expression in FPKM value and reports statistical significance using its

default settings (Trapnell et al., 2010). Functional analysis of differentially expressed genes is performed by Ingenuity Pathway Analysis (IPA) version 2.0 (Ingenuity Systems, Redwood City, CA).

1.3.3. Sex Determination

Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Cat. No. 69504) and PCR amplification of the Zfy and Xist genes conducted.

1.3.4. RNA Isolation and Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

RNA samples were isolated from placental tissues using Trizol (Cat# 15596026; Invitrogen) and 1ug RNA was treated with DNAase I (Cat# AMPD1; Sigma) according to the recommended protocols. These RNA samples were mixed with 1µL 10mM dNTP (Cat# 18427-013; Invitrogen), 1µL random hexamer oligonucleotides (Cat# 48190011; Invitrogen), 11µL water and incubated in 70℃ for 5min. After cooling down on ice, add SuperScriptII reaction buffer, DTT and SuperScriptII into this mixture according to the SuperScriptII system (Cat# 18064-071; Invitrogen) kit protocol, which was further incubated at 25℃ for 5min, 42℃ for 50min, 45℃ for 20min, 50℃ for 15min and finally 70℃ for 5min. The relative abundance of individual genes was measured by mixing using the Dynamo Flash mastermix, according to manufacturer's protocol, on a Bio-Rad CFX38 PCR system. Primers are listed in Table 1.

Table 1. Primers for RT-qPCR

Primer name	Fwd/Rev	Sequence
p57	Fwd	GATCTGACCTCAGACCCAATTC
	Rev	CTCAGTTCCCAGCTCATCAC
Alb	Fwd	GCAGACTTGCTGCGATAAAC
	Rev	CACTTCCTGGTCCTCAACAA
Gc	Fwd	CACCTACGTGGAACCAACAA
	Rev	CAGAGGCGCTTGTCCATAAT
Afm	Fwd	CATGCTGGATTACAGGGATAGG
	Rev	CAGTCCCTCCATGTCACATAAC
F2	Fwd	CGAACCAGATATGAGCGGAATG
	Rev	GTCACTGAAGGGTACAGGTTTC
C2	Fwd	CGATCCTCTCGGATGGTTAAAG
	Rev	AGGTGAAGTCTTGCTCACAC
C3	Fwd	GTGGTCACTCAGGGATCTAATG
	Rev	TCTGGGAGAGTGTCCTTCTT
APOC3	Fwd	ATGGAACAAGCCTCCAAGAC
	Rev	GCCGGTGAACTTGTCAGTAA
APOA5	Fwd	GACGACCTGTGGGAAGATATTG
	Rev	CTCCACCCTCTGCCTAATAGA
APOE	Fwd	CACAAGAACTGACGGCACTG
	Rev	CCCGTATCTCCTCTGTGCTC
APOM	Fwd	CTCTCTCTCTATGGCCTTCTCT
	Rev	GCTCCCGCAATAAAGTACCA
APOC2	Fwd	TCAGATGCAGGAGGCAAAG
	Rev	TAGTGGCAGGAAGGGACTAT
APOA4	Fwd	GTACCCTCTTCCAGGACAAAC
	Rev	CCAGCTCCTTCTTGATCTCTTC
Trf	Fwd	CCCTCTGTGACCTGTGTATTG
	Rev	GGGTTCTTTCCTTCGGTGTT
Ttr	Fwd	CTCGCTGGACTGGTATTTGT
	Rev	AGGATCCCTCAGAGGTCTTT
Fgb	Fwd	AAGCTGCCGATGATGACTAC
	Rev	GGGTCTCCGTTCTACTTTCTTC
Fgg	Fwd	GAAGGACAGCAGCATCACAT
	Rev	GAGGTCCTGAAAGTCCATTGTC
Fga	Fwd	CCAACGAGAGACTGTGATGATG
	Rev	CTTGCCAGGTCCGGTTAAA

Table 1. Continued

Primer name	Fwd/Rev	Sequence
Fxyd2	Fwd	CATAGTAGAGGCCACAGGAAGA
	Rev	CCTTTGCGGACGGTTTCATA
Fcgrt	Fwd	ACACTCCTGTCTGTCGTCTT
	Rev	CCCGCCTTGTTGATTCTTCA
Afp	Fwd	AGGAGTGCTTCCAGACAAAG
	Rev	GGAGGTTTCGGGATCCAAAT
Cts6	Fwd	GCGCAGTTGGTGATGTTCTA
	Rev	CTGTCCTTCTATGGCACCATTC
4930405D11Rik	Fwd	CGGCAGCACTTGAGATGATT
	Rev	GGCAGATGGCAGAGATTTAAGG
Sepp1	Fwd	ATCTATGACAGATGTGGCCGT
	Rev	CTTACTGCTGCCAAGATGCTC
Rbp4	Fwd	GACAAGGCTCGTTTCTCTGG
	Rev	CTTGAACTTGGCAGGATCTTCAG
Dio3	Fwd	TGGTGGTCGGAGAAGGTGAA
	Rev	AGCGAGTGAAGCAGCAGAGAG
APOA1	Fwd	CTTCAGGATGAAAGCTGTGGT
	Rev	AGATTCAGGTTCAGCTGTTGG
APOA2	Fwd	AGAGTAGACGGGAAGGACTG
	Rev	TCAAAGTATGCCTTGGCCTG
APOB	Fwd	GATCAACTGTAAGGTAGAGCTGGA
	Rev	GCTTGAGTTCGTACCTGGAC
SDHA	Fwd	GCTCCTGCCTCTGTGGTTGA
	Rev	AGCAACACCGATGAGCCTG
Mrpl 1	Fwd	AACTTCCTCAGCACCAAAATAGC
	Rev	GACCACAAACGGACCCAGATT
Hprt	Fwd	CTGGTGAAAAGGACCTCTCGAA
	Rev	CTGAAGTACTCATTATAGTCAAGGGCAT

1.3.5. S-Adenosyl Methionine (SAM) Concentration Measurement

The concentration of S-adenosylmethionine within the paternal liver, testis and kidney, as well as within the fetal placenta and fetal brain of their respective progeny

were measured using the Bridge -It® S-Adenosyl Methionine (SAM) Fluorescence Assay Kit according to the recommended protocol (Cat# 1-1-1003B; Mediomics).

1.3.6. Statistical Analysis

For analysis of gene expression, the replicate cycle threshold (Ct) values for each transcript were compiled and normalized to the geometric mean of the three reference genes: succinate dehydrogenase complex, subunit A (Sdha - NM_023281), mitochondrial ribosomal protein L1 (Mrpl1 - NM_053158) and hypoxanthine-phosphoribosyl transferase (Hprt—NM_013556) (Carnahan et al., 2013). Normalized expression levels were calculated using the ddCt method described previously (Schmittgen et al., 2008). 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, followed by Tukey's HSD analysis applied to comparisons with p-values < 0.05. For single comparisons, a student's t-test was applied. In all instances, we have marked statistically significant differences with an asterisk.

1.4. Results

1.4.1. Preconception Paternal Alcohol Exposure Alters the Placental Gene Expression

Placental RNAs derived from the placentas of both male and female offspring sired by the preconception control and alcohol-exposed males were analyzed by high-throughput RNA sequencing (RNA-Seq) (n=4, 2 male and 2 female). Analysis of these data sets was conducted using the Tophat-Cufflinks pipeline, mapping to the Refseq mm10 genome (Table 2). The normalized RNA expression profiles shared a similar pattern in all samples, as shown in Fig 1A and B.

Table 2. Tophat mapped reads in control and ethanol treated placentas mapped to Refseq mm10 database

Samples	Input reads	Mapped reads	Mapping rate
Control Male 1	67433154	64452595	95.60%
Control Male 2	69782496	66142376	94.80%
Ethanol Male 1	73750124	70900515	96.10%
Ethanol Male 2	76257860	72628587	95.20%
Control Female 1	85001154	81283405	95.60%
Control Female 2	63291562	60104166	95.00%
Ethanol Female 1	58615068	56038879	95.60%
Ethanol Female 2	81447364	78079680	95.90%

Our analysis identified 22036 expressed genes with FPKM value over 0.5. Using the statistical significance provided by a FDR-corrected p-value of $q<0.05$, we identified 287 significantly changed genes between Control Male (CM) and Ethanol Male (EM), with 283 of them displaying decreased expression in EM and only 4 displaying increased expression in EM compared to CM. However, in the female group, among 265 differentially expressed genes, 12 genes were down-regulated and 153 genes up-regulated, when comparing Ethanol Female (EF) to Control Female (CF) (Fig 1C, D, E, F).

To begin the process of identifying the critical genes and pathways underlying the observed placental dysfunction, we analyzed differentially expressed genes from Fig 1C and D using Ingenuity Pathway Analysis (IPA). In both males and females, the top five scored molecular and cellular functions of these 287 differentially expressed genes were molecular transport, protein synthesis, lipid metabolism, small molecule biochemistry, as well as vitamin and mineral metabolism, which all play major roles in placental function during development (Fig 1G and H)

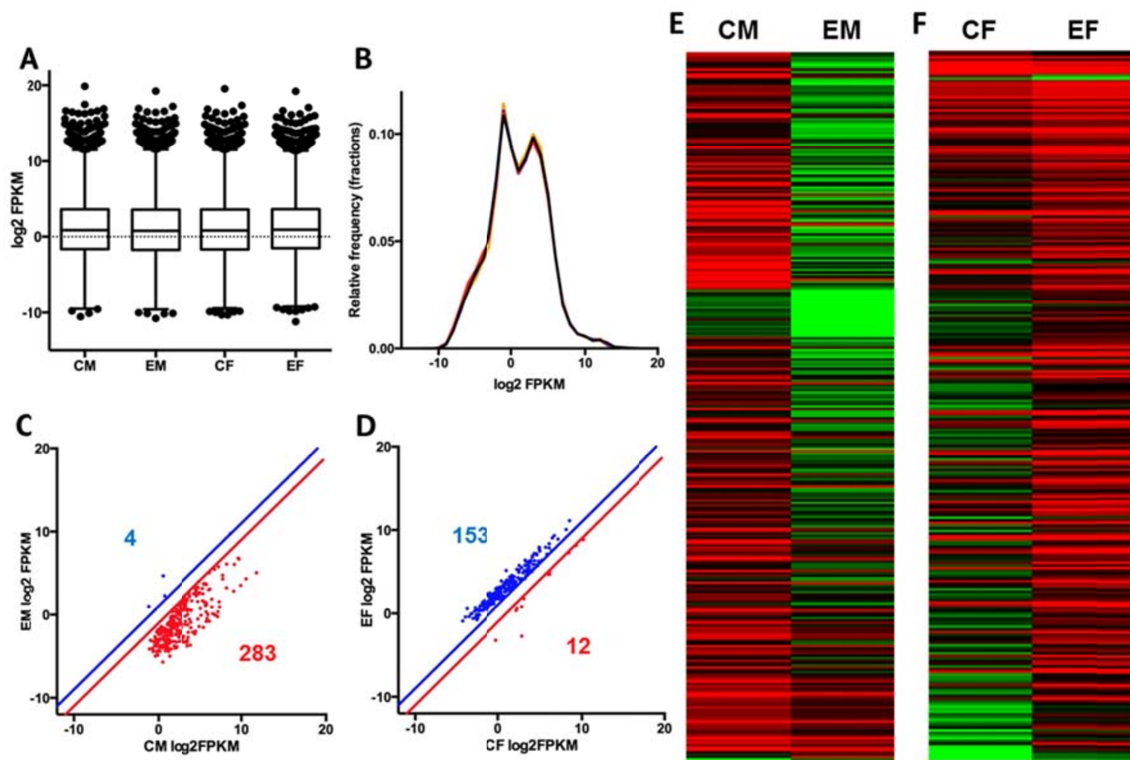


Figure 1. Transcriptome analysis in control and male preconception alcohol-exposed placentas.

A and B. Distribution of RNA profile in control male (CM), ethanol-treated male (EM), control female (CF), ethanol-treated female (EF) placentas. A. Box and whisker plot represents RNA abundance (log2 FPKM) of all expressed genes. B. Frequency distribution of all expressed genes. C, D. Significantly differentially expressed genes in CM, EM, CF and EF based on cuffdiff FDR-corrected p-value ($q<0.05$); E and F. Heatmap of significantly changed genes from C and D; G, H. Ingenuity Pathway Analysis of significantly differentially expressed genes: top 5 scored molecular and cellular functions in G. EM/CM and H. EF/CF.

G

Molecular and Cellular Functions	P-value	Score
Molecular Transport	3.42E-04 - 2.93E-18	44
Protein Synthesis	3.20E-04 - 1.14E-14	42
Lipid Metabolism	3.42E-04 - 7.23E-14	35
Small Molecule Biochemistry	3.42E-04 - 7.23E-14	33
Vitamin and Mineral Metabolism	2.82E-04 - 7.58E-13	31

H

Molecular and Cellular Functions	P-value	Score
Molecular Transport	3.42E-04 - 2.93E-18	44
Lipid Metabolism	3.20E-04 - 1.14E-14	42
Small Molecule Biochemistry	3.42E-04 - 7.23E-14	35
Vitamin and Mineral Metabolism	3.42E-04 - 7.23E-14	33
Protein Synthesis	2.82E-04 - 7.58E-13	31

Figure 1. Continued

To validate these in silico observations, we selected 29 differentially expressed genes and validated them using qRT-PCR (Fig 2). As is shown in Fig 2A, various physiologically important genes are down-regulated upon paternal preconception alcohol exposure, consistent with our RNA-seq results. For example, in male offspring, transferrin (Trf) an iron-binding blood glycoprotein, which is critical in erythropoiesis, is significantly reduced in alcohol-exposed group. In addition, the Fc fragment of IgG receptor and transporter (Fcgrt) is down-regulated in alcohol group. Transthyretin (Ttr) is a transport protein that carries the thyroid hormone thyroxine (T4) and retinol-binding protein, which can be synthesized, secreted and internalized by trophblasts, plays a role

in transferring thyroid hormone from mother to fetus. Both display decreased expression in the offspring of alcohol-exposed males. In addition, other plasma proteins, such as Selenoprotein P (Sepp1), Retinol binding protein 4 (Rbp4), Complement component 2(C2) and Complement component (C3) all displayed reduced expression in the preconception alcohol group.

In addition to the emergence of several functional groups, several gene families displayed uniform reductions in expression within the male offspring of alcohol-exposed males (Fig 2B, C, D). As examples, the expression level of APOA1/C3/A4 from the APO gene cluster, which is located on mouse chromosome 9 but at a further distance from APOA5, showed a significant decrease while the transcription level of APOA5 remained unchanged (Fig 2B). In addition, other apolipoproteins, including APOA2, APOB, APOC2, APOE, APOM, were also significantly reduced in the placentas of male offspring sired by alcohol-exposed fathers. In addition to the apolipoproteins, we also observed reduced transcription of the fibrinogen cluster (Fig 2C) and the Albumin family (Fig 2D). Here, the four transport proteins Alb, Afp, Afm and Gc, which are highly abundant in placentas of control offspring, decreased dramatically in the offspring sired by alcohol-exposed males. While the RNA-seq results identified within the male offspring displayed consistent profiles, the female offspring displayed large amounts of variation and did exhibit the same degree of consistency that the males did (Fig 2E, F, G, H).

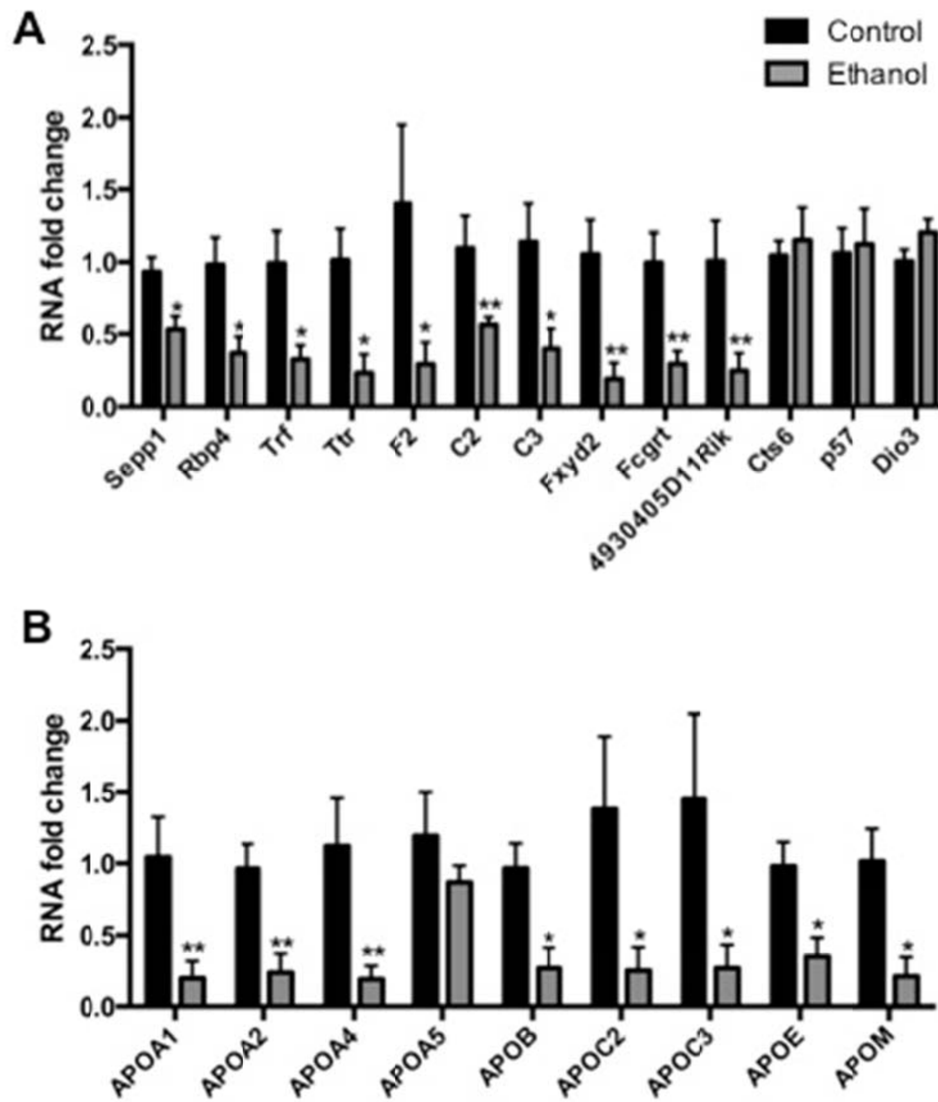


Figure 2. Validation of significantly changed genes by RT-qPCR.
Differences were evaluated using an unpaired student's t-test. Statistical significance is determined by p-value less than or equals to 0.05. A, B, C, D: male offspring placenta, n = 6; E, F, G, H: female offspring placenta, n = 8.

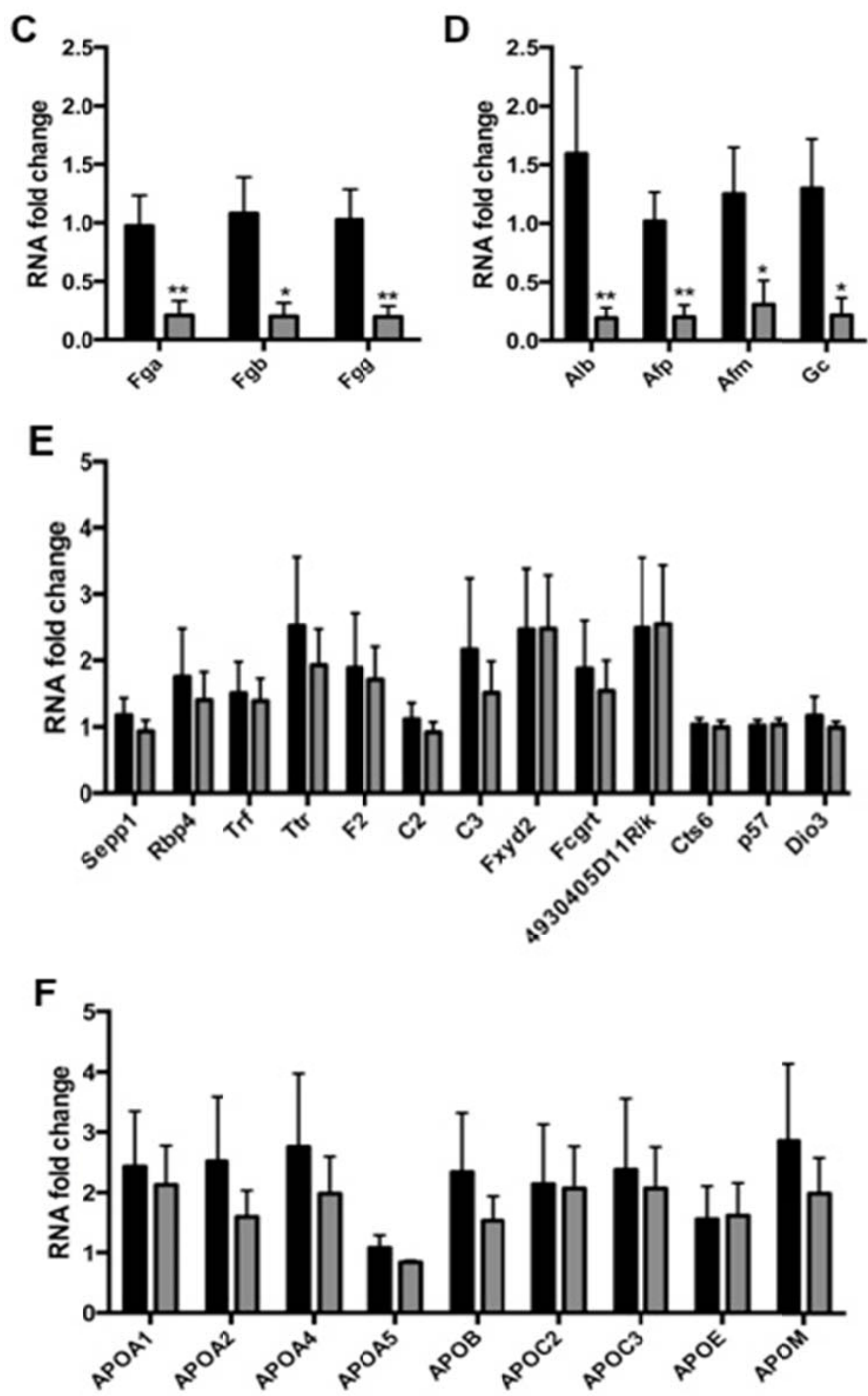


Figure 2. Continued

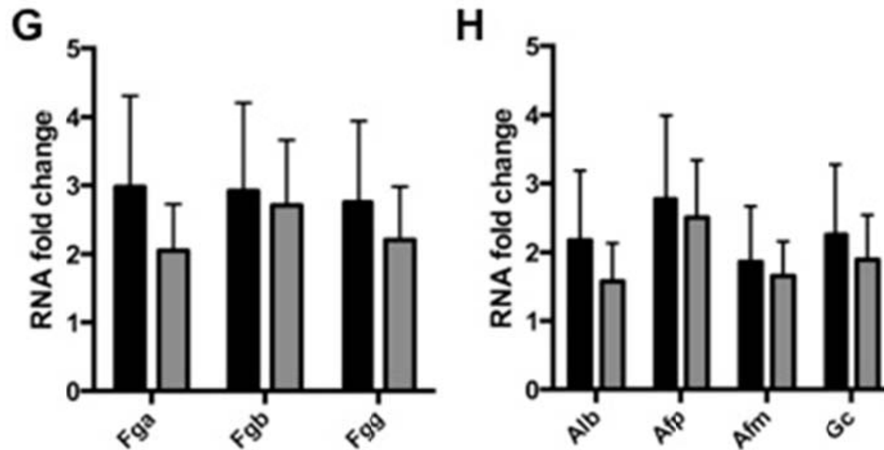


Figure 2. Continued

1.4.2. SAM Levels Were Not Altered by Male Preconception Alcohol Exposure in Both Adult and Fetal Tissues

From our RNA-seq analysis and validation, we observed altered gene expression in placentas from alcohol-exposed fathers, especially in the male group. Previous studies has shown that male alcohol exposure can change the patterns of DNA methylation in sperm. Here we sought to determine if paternal preconception alcohol exposure could impact the levels of the methyl-donor SAM. To this end, we assessed SAM levels in the paternal liver, kidney and testis as well as placental and fetal brain tissues from their offspring (Fig 3). None of the tissues we examined displayed significant alterations in the abundance of SAM between control and alcohol groups, which indicates that alcohol consumption does not have direct impact on SAM levels within the tissues of the adult males nor the placenta and fetal brain of their offspring. These observations suggest that

the altered patterns in gene expression in placenta are not the consequence of alterations in DNA methylation imparted to deficiencies in SAM bioavailability.

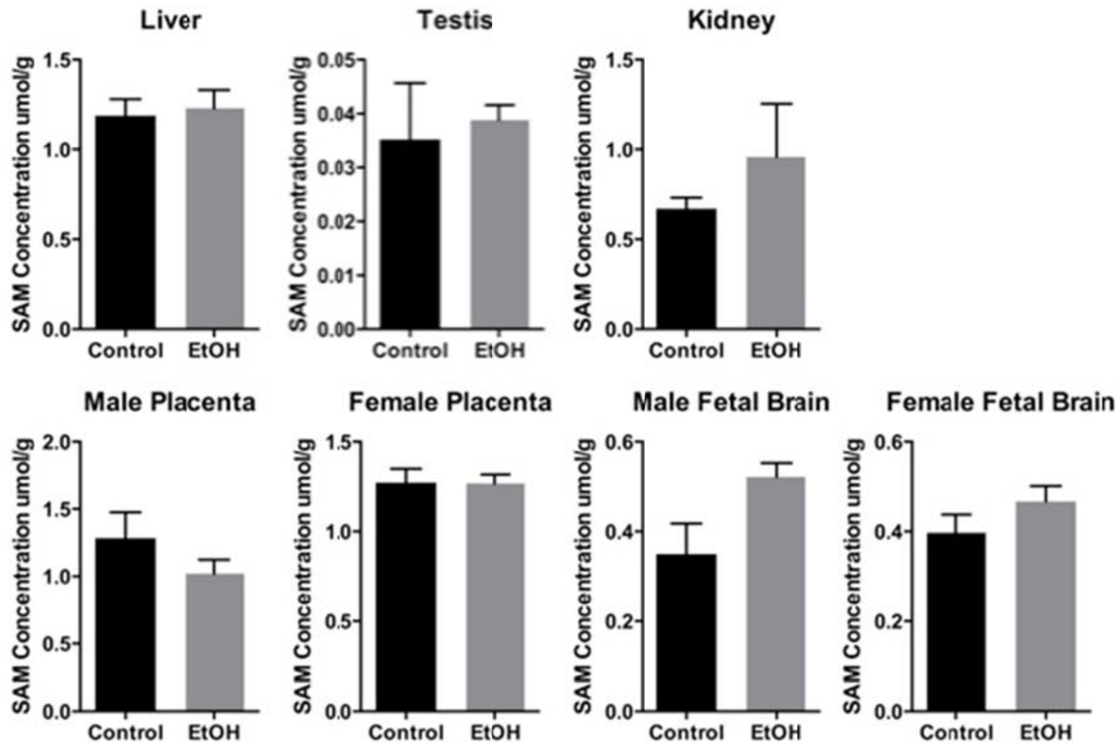


Figure 3. SAM concentrations of adult male liver, testis, kidney and placenta, fetal brain in both control and alcohol groups.
SAM concentration are measured in control and alcohol exposed male liver (n = 3), testis (n = 3), kidney (n = 3) and placenta (n = 10), fetal brain (n = 10) from their offspring. Statistical significance was determined by unpaired Student's t-test as p-value less than or equal to 0.05.

1.5. Discussion

The objective of this first study was to determine if preconception alcohol exposure heritably impacts patterns of gene expression within the placenta. We hypothesized that male preconception alcohol exposure would impact patterns of gene expression within the placenta resulting in the differential expression of cohorts of

genes. In this study, we examined the placental transcriptome of offspring from both control and alcohol-exposed fathers, and observed that the expression of numerous physiologically critical genes is disrupted.

In the male offspring, among the 25 tested significantly changed genes, ~20 of the candidate genes are plasma proteins, which function as carriers transporting substances from the mother to fetus. These metabolically crucial molecules include cations, fatty acids, hormones, bilirubin, and vitamin D. As examples, Trf plays a key role in iron transport between fetal and maternal systems (Morris Buus and Boockfor, 2004). Therefore, it is possible that decreased Trf in male offspring sired by alcohol-exposed fathers may contribute to the dysfunction of placenta and the growth restriction phenotype. In addition, the Fc fragment of IgG receptor and transporter (Fcgrt) which transfers IgG from mother to fetus through placenta (Ishikawa et al., 2015). Previous studies have shown that children with FAS have impaired immunity, which might be due to incomplete IgG transport through placenta (Johnson et al., 1981). Whether the offspring of alcohol-exposed males show similar alterations in immune function remains to be determined. Transthyretin (Ttr) is a transport protein that carries the thyroid hormone thyroxine (T4) and RBP, which can be synthesized, secreted and internalized by trophoblasts are essential in the transfer thyroid hormone from mother to fetus. (Landers KA, Li H, placenta, 2013) Fetal neurological development requires proper levels of thyroid hormone, and reduced Ttr could have an impact on the neurological development of these offspring. In addition to these candidates, the plasma proteins Selenoprotein P (Sepp1), Rbp4, Complement component 2(C2) and Complement

component (C3) also function as carriers for other biomolecules between the mother and fetus. Deficiencies in the function of the albumin family could lead to placental dysfunction through multiple different pathways.

While expression analysis of male placenta revealed consistent patterns between RNA-seq results and qPCR validation, the placenta from female offspring displayed a surprising amount of variation between individual samples within the sample group. This lead to a failure to identify differentially expressed candidate genes from RNA-seq data, with 23 out of 29 genes displaying patterns of expression inconsistent with our RNA-seq data. To determine the basis for the differences between male and female groups, we examined two possibilities: 1) that fetal weight strongly influenced expression and by segregating the specimens by weight we could identify clear trends or 2) that patterns of gene expression could be correlated with placenta weight. We postulated that separating the data sets by fetal or placental weight could both explain and compensate for the large variation observed between samples isolated from female offspring. We therefore selected 4 samples from each of the lightweight animals (smallest 20%), four samples from the medium population (40%-60%) and four samples from the heavy population (>80%). As shown in Fig 4A, male offspring placenta consistently showed decreased expression of 5 tested genes in medium group, but lost its significance in light and heavy groups. However, none of the three groups in the female offspring placenta showed significantly differential expression (Fig 4B). Similarly, R square analysis could not identify a correlation between gene expression and placental

weight (Fig 5). These experiments emphasize the importance of examining each gender independently.

One important observation to emerge from these studies is that this dysregulation tended to occur in gene clusters. As examples, APO, fibrinogen and albumin clusters showed simultaneously reduced expression levels. Since genes in the same cluster are proposed to have evolved from the same gene through duplication and divergence, regulatory elements within the gene clusters are often identical and serve to co-regulate their expression (Yu et al., 2003). This piqued our interest as it suggests that the regulatory mechanisms of these entire gene clusters were impacted, rather than individually. This focused our subsequent studies on the DNA cis regulatory elements within these clusters, including interactions between promoters, enhancers and insulators.

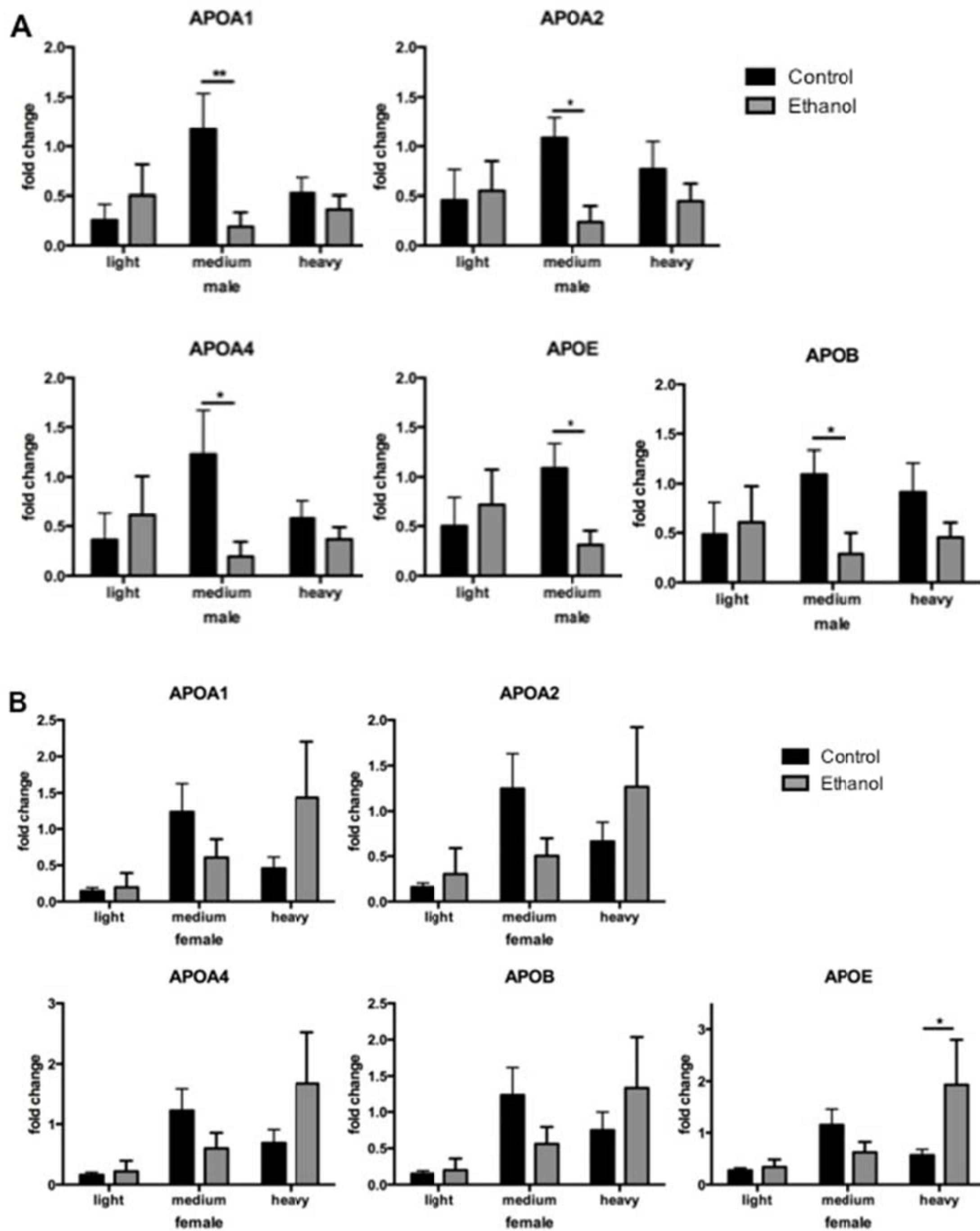


Figure 4. Candidate gene expression in placentas selected by fetus weight.
Samples are selected from light population (smallest 20%), medium population (40%-60%) and heavy population (>80%). Significance is calculated by Student's t-test, n=4. Statistical significance is determined by p-value less than or equals to 0.05. A. Male offspring placenta; B. female offspring placenta.

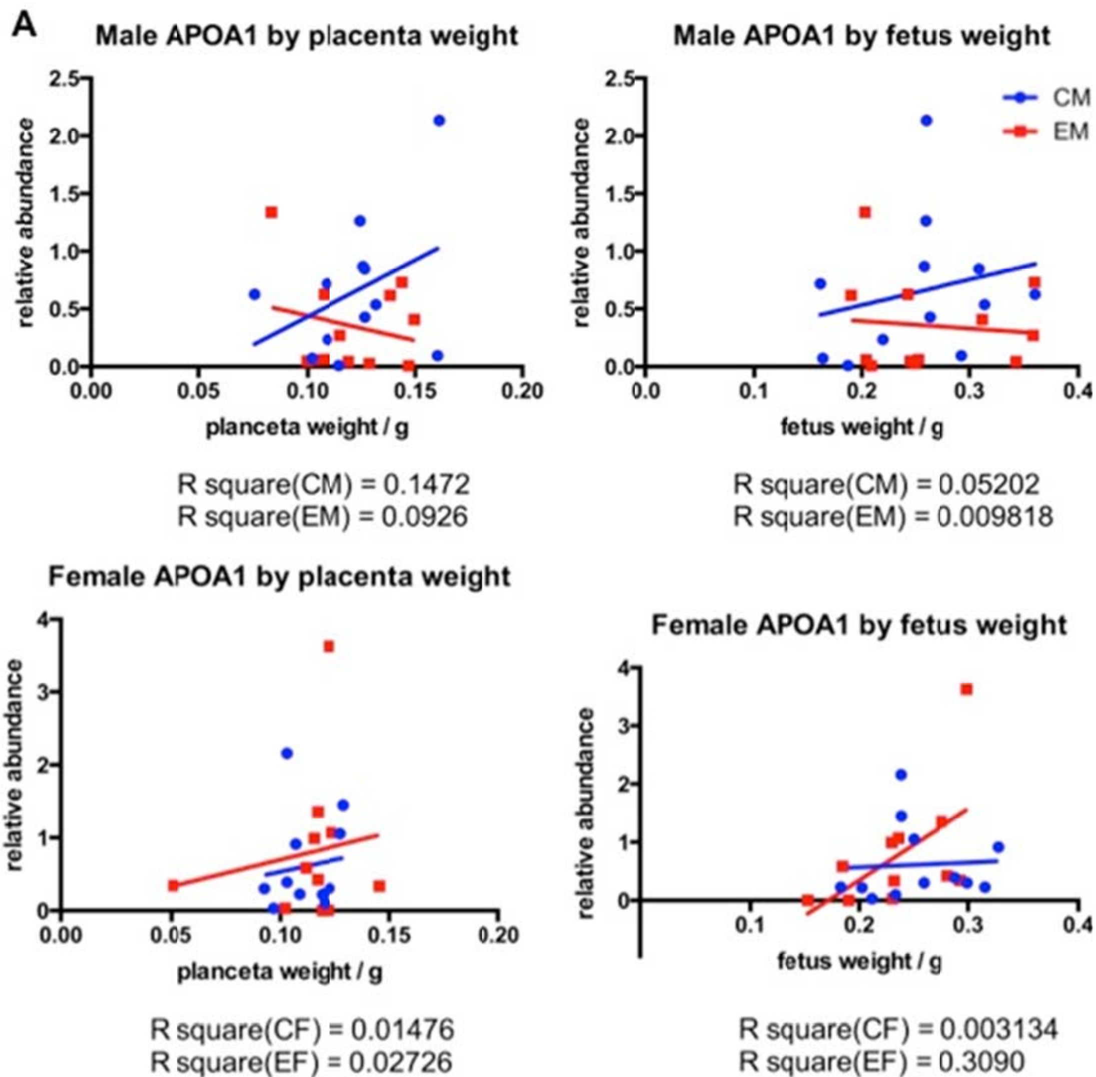


Figure 5. Correlation of relative candidate gene expression level to placenta or fetus weight.

Correlations of relative expression level for APOA1 or APOA2 to placental or fetus weight are calculated by linear regression and represented by R square value, n=12. Blue indicates control group and red indicates alcohol group.

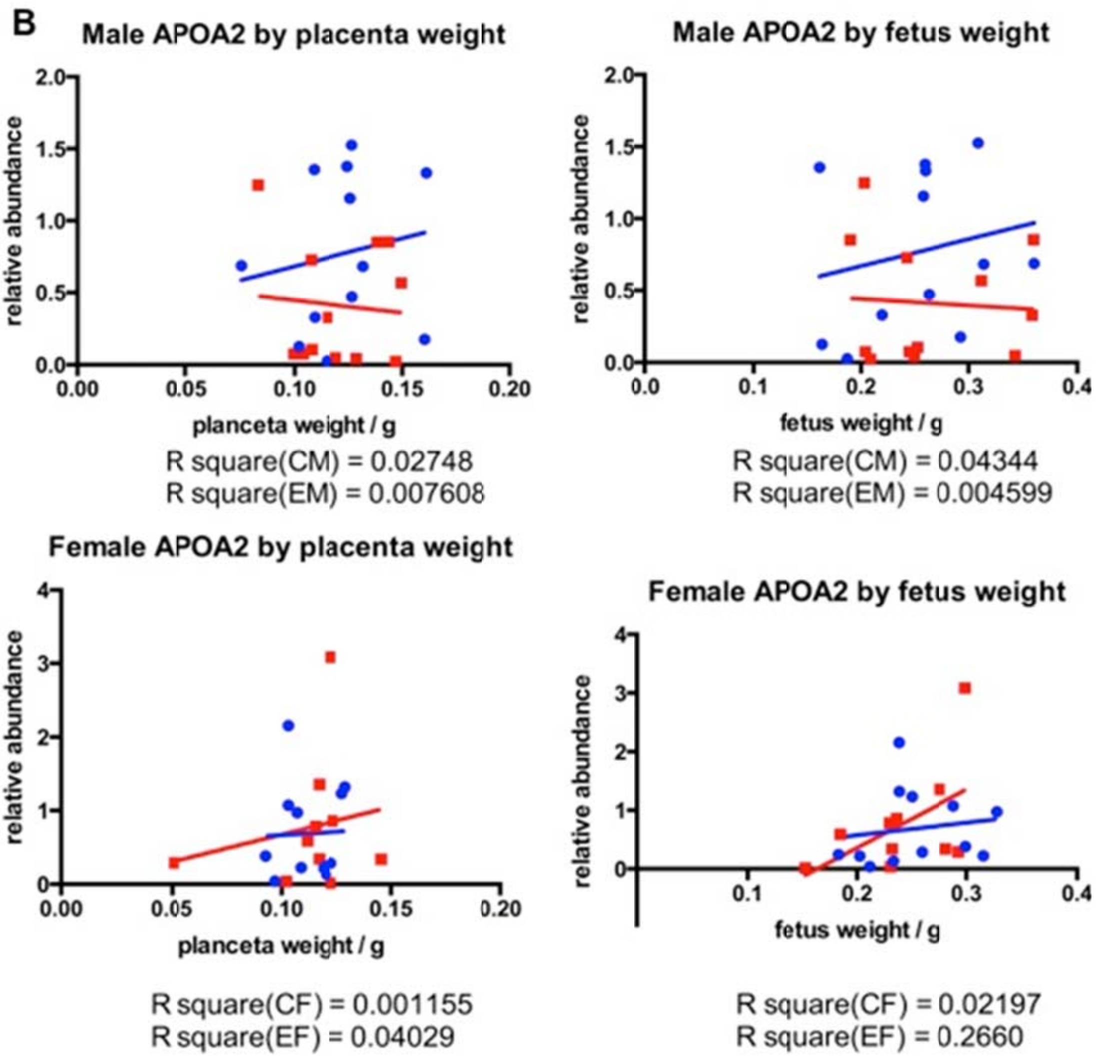


Figure 5. Continued

CHAPTER II

MALE PRECONCEPTION ALCOHOL EXPOSURE ALTERS CHROMATIN BOUNDARY ELEMENTS IN PLACENTA

2.1. Introduction

In the past decade, the importance of chromatin organization has been recognized as crucial in the regulation of the transcriptional programs driving development. Remodeling of chromatin has been extensively studied in development, disease, evolution and in the reprogramming of induced pluripotent stem cells (iPSCs) (Ho and Crabtree, 2010) (Hang et al., 2010) (Vietri Rudan et al., 2015) (Onder et al., 2012). Co-regulated patterns of gene transcription are coordinated by multi-gene complexes formed through chromosomal interactions. These effects are mainly mediated by cis regulatory elements including enhancers, insulators and promoters, with assistance of regulatory proteins including transcription factors.

2.1.1. A Brief Introduction to Enhancer Biology

Enhancers are short DNA elements that can enhance transcription of regulated genes and locate as far as several kilobases or even megabases away from the target gene body. The first enhancer was isolated from the simian virus 40 (SV40), which could be placed at various locations and orientation with respect to an unrelated gene and promote its expression (Banerji et al., 1981). Soon after, the first mammalian enhancer was identified in the immunoglobulin M (IgM) heavy chain locus, which is located between

the joining and μ constant regions and enhances the transcription of IgM heavy chain. Unlike the SV40 enhancer, this IgM heavy chain regulatory element functions only in tissue-specific contexts and functions only in lymphocyte-derived cells (Banerji et al., 1983). A critical component that determines enhancer activity is the type (or types) of histone modification(s) associated with that region. Histone 3 Lysine 4 monomethylation (H3K4me1) is now understood to be associated with poised enhancers that may or may not be actively associated with transcription of the genes that they regulate. It has been shown by several groups that other histone modifying enzymes interact with H3K4me1-marked regions to activate or repress such poised enhancers. For example, the localization of the histone acetyltransferase p300 and the enrichment of Histone 3 Lysine 27 Acetylation (H3K27 ac) can predict with a very high degree of certainty, whether an enhancer is active. Specifically, high H3K4 me1 but low or absent H3K27 ac is indicative of a silent enhancer while high H3K4me1 and high H3K27ac demarcate active enhancers (Heinz et al., 2015) (Creyghton et al., 2010).

Enhancers can potentially activate gene expression across large genomic regions, which despite the linear distance still impart tight control over genes to keep the non-target genes silenced (West and Fraser, 2005). This is achieved by another important DNA regulatory element called an insulator, which functions as a genetic boundary either by blocking enhancer-promoter interactions when placed in between and/or serving as barrier region to block repressive chromatin region from affecting the flanking regions (West et al., 2002). The complex regulatory relationship between enhancers, promoters and insulators is best exemplified by the locus control region

(LCR) of the human beta-globin gene. First associated with five DNAse hypersensitive sites (HS1-5), the human β-globin cluster contains five stage-specific genes in the order of 5'-epsilon-Ggamma-Agamma-delta-beta-3', which are tightly regulated by the LCR located 6-22 kb upstream of the epsilon-globin and drives transcription in a tissue-specific manner. These genes are co-regulated, with periods of expression emerging during different developmental stages. For example, the epsilon-globin gene is expressed in embryonic cell types, gamma-globin during fetal stages and the beta-globin gene in adult stages. In addition to driving high-level expression of beta-globin genes, this LCR is different from traditional enhancers in two core areas: first, position of LCR is critical for normal epsilon-globin expression during the yolk-sac stage of embryogenesis; second, the orientation of LCR cannot be inverted (Grosveld et al., 1987). Among the five DNAse hypersensitive sites of LCR, the 5’ most HS5 functions in a totally different way from the remaining hypersensitive regions and serves to block enhancer activity from spreading inappropriately (Tanimoto et al., 1999) (Chan et al., 2008). Importantly, the 5’HS4 in the chicken beta-globin locus is also reported as an insulator, which requires the binding of the CCCTC-binding factor (CTCF) (Hebbes et al., 1994), a critical transcription factor required for chromatin insulation function (Zuin et al., 2014).

Our studies in Chapter 1 identified wide-spread alterations in gene expression within the APO, fibrinogen and albumin clusters. The identification of altered expression of these co-regulated genes suggests that preconception alcohol exposure may influence the epigenetic control of their expression through a mechanism involving enhancer-promoter or chromatin boundary-based mechanisms.

2.1.2. Current Studies of Regulatory Elements in APOA1/C3/A4/A5 Cluster

The human APO gene cluster is ~44kb in size, contains four genes APOA1/C3/A4/A5 and is located on human chromosome 11. The APOA1/C3/A4 extends ~17 kb and APOA5 is approximately 30kb upstream. APOA1/A4/A5 are transcribed in the same direction whereas APOC3 is transcribed in the opposite direction (Guardiola et al., 2014). Regulatory elements of the human Apo cluster partition the region into different subsects and coordinate the differential expression of APOA1/C3/A4/A5 in different tissues and cell lines. Differential enrichment of key post-translational histone modifications are associated with the tissue specific expression of the Apo genes. For example, the APOC3 enhancer is located 590-900 nucleotides upstream of the APOC3 promoter and differential histone modification associate with altered expression of APOA1/C3/A4 in liver and intestine (Li et al., 2008) (Kan et al., 2000). One of the better studied models is in Hela cells where three insulators were identified as target binding sites for the chromatin boundary element CTCF, as well as Rad21 enriched-sites, which partition the Apo cluster into two separate loops: one with the APOC3 enhancer and APOC3/A4/A5 promoters in the same loop; and another with the ApoA1 promoter in another loop. Depletion of CTCF or Rad21 disrupts this looping structure, which leads to significant alterations in the expression of these genes as well as disrupted localization of hepatocyte nuclear factor-4alpha (HNF-4a) and RNA polymerase II at the Apo promoters (Fig 6) (Mishiro et al., 2009).

As the major component of physiological lipoproteins, the APOA1/C3/A4/A5 cluster is tightly associated with control of plasma lipids and linked with

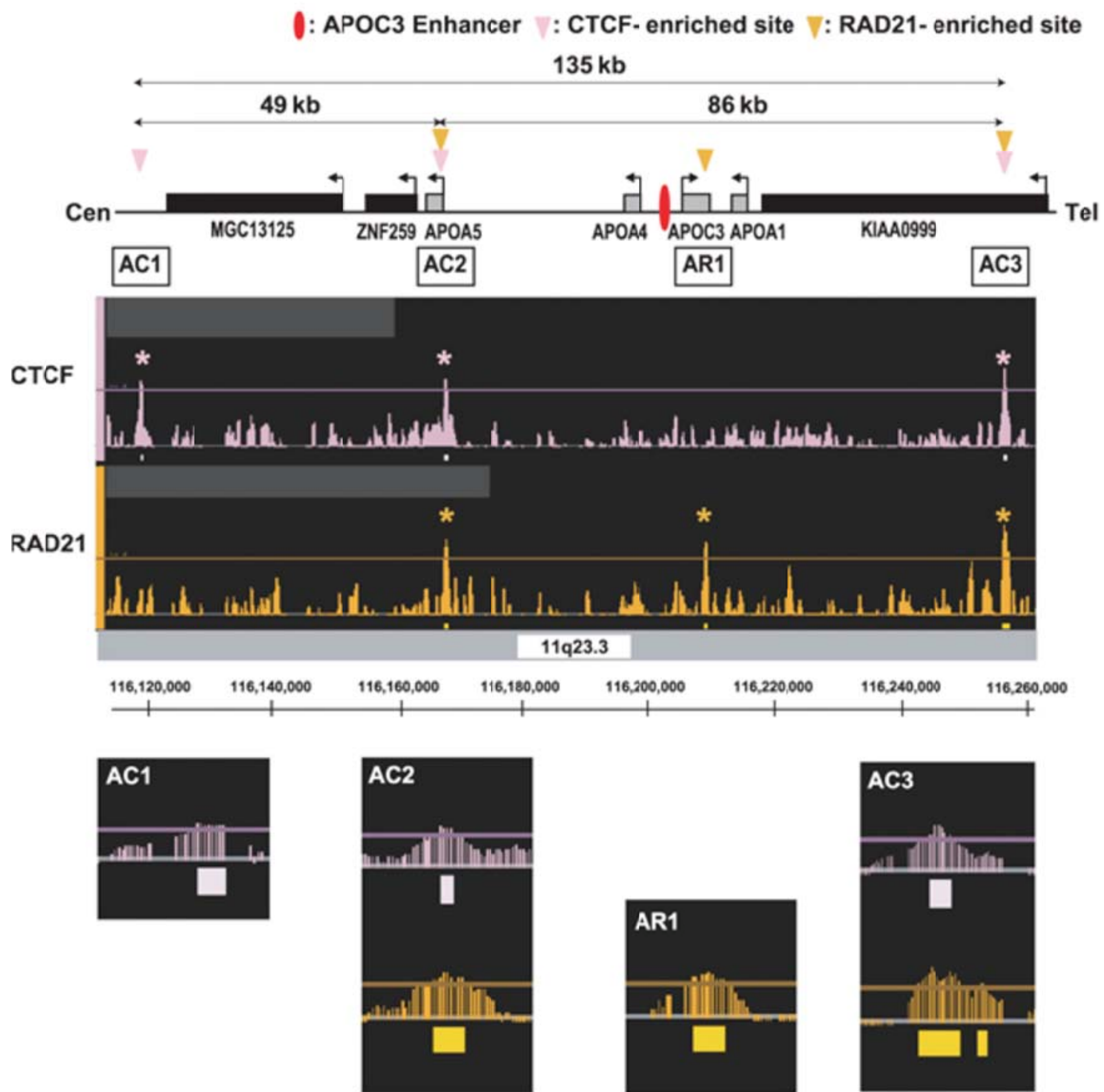


Figure 6. CTCF and Cohesin enriched reigion on human APO cluster in Hela cells. The CTCF enriched sites (pink) are named AC1, AC2 and AC3 and the Rad21 enriched sites (orange) are named AR1, which indicate insulators on Apo cluster. An APOC3 enhancer is indicated in red (Mishiro et al., 2009).

development of cardiovascular disease (Lai et al., 2005). Several studies have suggested that alcohol consumption leads to alterations in Apo expression and is positively correlated with cardiovascular disease rate. However, no studies have examined alterations with respect to preconception paternal alcohol exposures, the effects on placental lipid transport and the impacts on fetal development.

2.1.3. Current Studies of Regulatory Elements in Fibrinogen Gene Cluster

Fibrinogen is a hexamer, with two sets of 3 polypeptide chains α, β and γ, which are encoded by 3 genes: fibrinogen alpha (Fga), fibrinogen beta (Fgb) and fibrinogen gamma (Fgg) respectively. Plasma fibrinogen levels are associated with various physiological and pathological states. An increase of fibrinogen concentration above normal range results in increased cardiovascular disease risk (Danesh et al., 2005). Pregnancy and inflammation are also associated with elevated fibrinogen levels (Page, 1993). It is reported that fibrinogen plays a critical role in stabilizing placental-maternal attachment during embryo implantation, and that depletion leads to retarded embryo-placental development and abortion (Iwaki et al., 2002). In addition, high plasma fibrinogen is strongly associated with increased placental weight to birth weight and reduced growth in early life (Barker et al., 1992). Fibrinogen levels are disrupted by environmental factors, including obesity, smoking and alcohol (Kaptoge et al., 2007). However, the mechanisms by which these exposures influence fibrinogen expression are not understood.

Despite the clinical significance, the study of the regulatory mechanisms of the fibrinogen cluster has thus far been very limited. The human fibrinogen cluster is located on chromosome 4. Under basal conditions, two transcription factors, including CCAAT-box/enhancer-binding protein (C/EBP) and hepatocyte nuclear factor-1 (HNF-1), are recruited to this locus and drive the transcription of Fga and Fgb. During acute inflammation, elevated fibrinogen levels are augmented by interleukin-6 (IL-6), which targets the IL-6 responsive elements within the promoter regions of these genes (Fuller and Zhang, 2001). Interestingly, while several transcription factors are suspected to control the fibrinogen genes, only one regulatory sequence has been reported in this gene cluster; namely, a conserved region between human and zebrafish, termed CNC12, located between Fga and Fgb, which can activate transcription as a liver-specific enhancer (Fort et al., 2011). Previous studies have reported that in fibrinogen expressing cells, low methylation at this site can be correlated with high expression levels. Importantly, this locus overlaps with strong CTCF peaks identified using ChIP sequencing (Vorjohann et al., 2013).

2.1.4. Current Studies of Regulatory Elements in Albumin Gene Cluster

The albumin family includes serum albumin, alpha-fetoprotein, vitamin D-binding protein (or named group-specific component) and afamin, which are encoded by gene Alb, Afp, Gc and Afm respectively. All four genes are expressed primarily in the liver and quite similar to the human β-globin gene cluster, the mouse albumin gene family is also expressed during different stages of development. Afp, Alb and Gc

expression are highly abundant in fetal liver and while Afp transcription is rapidly silenced after birth, Alb and Gc expression remain high in the adult liver (Krumlauf et al., 1985) (Song et al., 1998) (Gualdi et al., 1996). Hepatic expression of Afm initiates during the perinatal period and continues to be expressed in adults (Belanger et al., 1994).

In mice, Alb, Afp and Afm are transcribed in the same direction, with Alb approximately 14 kb upstream of Afp and Afm ~10 kb downstream of Afp; while Gc is distally located at around 1Mb upstream of Alb and transcribed in the opposite direction (Fig 7). The Alb enhancer can drive high-level albumin expression in adult liver but has relatively weak activity in fetal liver (Pinkert et al., 1987). In addition, all the three Afp enhancers are able to enhance transgene expression in yolk sac, fetal liver and remain active in adult liver (Hammer et al., 1987) (Camper and Tilghman, 1989). In another study, the Afp enhancers were linked to the control of both Afp and Alb transcription during early liver development and Afp reactivation in liver regeneration, which is mediated through RNA polymerase II recruitment at Afp and Alb promoters (Jin et al., 2009). Besides the four enhancers mentioned above, there are two hypersensitive sites (HSs) located in the Gc intron, which might function as enhancers for Gc, as deletion of this intronic locus control region repressed Gc expression (Hiroki et al., 2007).

Although the albumin family is extensively studied in development, current knowledge of its transcription modulation through regulatory elements is still very restricted to the enhancers identified above. Further these studies have largely been

focused in the liver, yolk sac and intestine, while the regulation in the placenta remains quite mysterious.

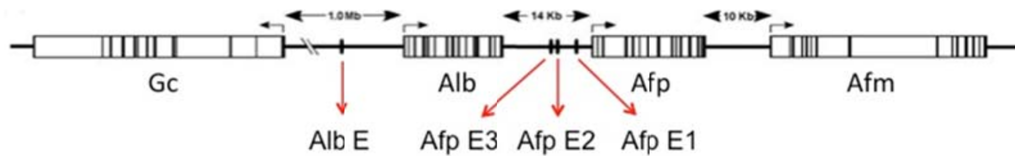


Figure 7. Distribution of mouse albumin gene cluster and enhancers.
Gc, Alb, Afp and Afm genes are represented as boxes. Alb enhancer (Alb E) and three Afp enhancers (Afp E1, E2, E3) are pointed using red arrows (Jin et al., 2009).

2.1.5. Dysregulated Gene Clusters and Disease

In the human alpha-globin gene cluster, four upstream DNA elements identified through DNase I mapping studies (hypersensitive sites (HSs) or multispecies conserved sequences (MCS-R1-4) have been identified as the major regulatory elements for this gene cluster. Genetic mutations within this cluster have been associated with the pathology known as alpha-thalassemia. Here, impaired alpha globin chain production leads to excess beta chains (Galanello and Cao, 2011). Previous studies have reported that in some patients, deletion of these conserved DNase HSs, especially HS-40, results in reduced expression of alpha-globin in alpha-thalassemia patients (Coelho et al., 2010). While in the case of beta- thalassemia, impaired LCR can lead to decreased expression of beta-globin genes (Levings and Bungert, 2002).

Recently it was found that chromosomes are spatially segregated into sub-domains ranging from thousands to millions bp in length, which are collectively referred to as topologically associating domains (TADs). Within one TAD, frequent interactions occur between *cis* regulatory elements, whereas distinct TADs are separated by

boundaries and interactions between boundaries are rare. These TAD boundaries are known to be enriched in CTCF and cohesion binding (Pombo and Dillon, 2015). This suggests that if an enhancer is separated from its promoter by a TAD boundary, they are less like to interact. Disruption of TAD boundaries can impact the expression of genes nearby and cause diseases. For example, disruption of the TAD boundary, which spans through the WNT6/IHH/EPHA4/PAX3 locus, can cause human limb malformations through abnormal interaction between a limb enhancer and their target genes (Lupianez et al., 2015b).

Super-enhancers are clusters of enhancers with extremely strong transcriptional coactivator binding, which locate close in genomic proximity and drive high levels of transcription. In embryonic stem cells, super enhancers are highly enriched in and transcription factors including Oct4, Sox2, Nanog, Esrrb, Klf4 and densely occupied by Mediator which collectively serve to drive genes controlling pluripotency. In differentiated cells, super enhancers recruit tissue-specific transcription factors and regulate the expression of genes controlling cell identity (Whyte et al., 2013). In addition to control the cell identity, super enhancers are found to be disease related by altering the expression of genes defining cell identity, which can often lead to pathologies of differentiation, like cancer. For example, cancer-specific super enhancers at oncogenes are major drivers of carcinogenesis (Hnisz et al., 2013).

In general, genes can be controlled in clusters by distal regulatory elements, no matter if they are originated from the same ancestral gene and locate in the same gene

cluster, or as non-homologous genes located in a broader region of the genome. Disruption of these intricate regulatory networks can lead to various disease conditions.

2.2. Research Objectives and Hypothesis

Objective 3 – given the importance of chromatin structure in developmental programming and organizing the chromatin landscape, we will examine the impact preconception alcohol exposure has on chromatin boundaries and enhancer function.

We hypothesize that preconception alcohol exposure heritably impacts chromatin organization within the placenta, altering chromatin boundary element placements, which heritably influence patterns of gene expression.

2.3. Materials and Methods

2.3.1. Chromatin Immunoprecipitation (CHIP) Analysis

Placentas were dispersed into single cells using 100 µm cell strainer. Chromatin Immunoprecipitations (ChIP) were performed as previously described (Veazey et al., 2017, accepted) (Golding et al., 2010). Eluted DNA was further purified by Qiaquick PCR Cleanup kit (Cat# 28106; QIAGEN). Antibodies used include: anti-H3K4me1 (Cat# 39297; Active Motif); anti-H3K27ac (Cat# 39133; Active Motif); anti-CTCF (Cat# 07-729, Millipore); anti-Rad 21 (Cat# ab992; Abcam). All antibodies were used at 1 ug/reaction, including IgG (Cat# SC-2027; Santa Cruz). Primers designed to assay the enrichment of the indicated proteins were used in real-time PCRs performed on Bio-Rad

CFX38 PCR system using Dynamo Flash supermix (Cat# F-415XL; Thermo Scientific). Primer sequences are listed in Table 3.

Table 3. Primers for CHIP-qPCR.

Primer name	Fwd/Rev	Sequence
Fga promoter	Fwd	CTCTGCTCCACAGAAGTGATAG
	Rev	TGAGCAAGAGTTTCTGGGATAC
Fgb promoter	Fwd	AGACAGGGCTCTTTACAGAATG
	Rev	GTTCACTTGTTGGCTGAACTG
Fgg promoter	Fwd	GGTGGTGTACTGGAAGTAGTTG
	Rev	AGGTTTAAGCTCCTCCCTTTG
CNC12	Fwd	GGGTGACTGTGATTACAACAGA
	Rev	TGTAGGTGTGCAACCTTGAG
Fgg CI1	Fwd	TCGTGAGGCTCAGCAGAAA
	Rev	CATTCCTCCTCCCAAGTTCATC
Fgg CE1	Fwd	CATGGCATCGCAGGTATAGT
	Rev	CTGACTGCAGAAAGAGGAGAG
Fgg CE2	Fwd	GGAACCTGTCTTCCAGTCATC
	Rev	CCTCCTTTCATGTCTAAGGTCTG
Fgb CE1	Fwd	AGTGATGCTTGGGTACTTGG
	Rev	CCAGATTCCCACCACTTTCA
Fgb CE2 region1	Fwd	GTGAACTGACCCTCATGGATATG
	Rev	ACCTCTGATCCTCAGCTCTATC
Fgb CE2 region2	Fwd	ACTGGTTCTACAGTGGTCCT
	Rev	GTGGATCTGATTCTCCGCTTAC
Fgb CE3	Fwd	AGATGCTTAACCCAAGTTCCC
	Rev	CAGATGGTGACGACCAAGAAG
Fgb CE4	Fwd	CTCCTGAGTGCTGGGATTAAA
	Rev	CACATGGGAGAGAAAGACACA
Alb E1	Fwd	CACTGCCTGGCTACAATCTAT
	Rev	TGCTCACAGTCTGTATGTTCTC
Afp promoter	Fwd	CCACTCTGAAGTGGTCTTTGTC
	Rev	CCTGTTTAAGGGATGCCTGTT
Afm promoter	Fwd	GGAGGATTATTCTTACCCTGTGG
	Rev	GTCCTGGTGCAAATTTCTAGGT
Gc promoter	Fwd	TCCCAGACTCTCCTCCATATTC
	Rev	CCAGAAGCAAGGGACAATCA
Afp E1	Fwd	CATGTGGCAGATAACGGAGA
	Rev	GAACGGTACACAGGGACATAG

Table 3. Continued

Primer name	Fwd/Rev	Sequence
Afp E2 region1	Fwd	GTCACATTGTACCTGGGAAGAT
	Rev	CTCAAAGCGCAGTCCTAAGT
Afp E2 region2	Fwd	GCTGTCTCTGATTCTGCTCTATC
	Rev	TTGGCTTATCGCTTCCTATCTC
Afp E2 region3	Fwd	AGGTGGCTCACAGCATACTA
	Rev	CTGACAACGTCTAACGCTCTTT
Afp E3	Fwd	CACTCCACTGACATTGCTGTAG
	Rev	GAGCTGCCTGTCGAACTTAAA
APOA1 promoter	Fwd	ACAGAGCTGATCCTTGAACTC
	Rev	AGCTCTTCTTCCCTGGTCTA
APOC3 promoter	Fwd	TGTCTCACCGACCTCATCTA
	Rev	GAGGAGTTGAGAAATCCCTCTG
APOA4 promoter	Fwd	TGGTACCATCTCTGTAGCTGA
	Rev	GTGCCAGTCTGAGAGAACAA
APOA5 promoter	Fwd	CTGGCCAGTCATTCAGTAAGT
	Rev	CCTCGAGCCTTTACTAAGTGTG
APOC3 E	Fwd	CCCTGCTCCTATCTCAGGATTA
	Rev	GTGAGAGAAGGACAAAGGTCAC
APOA1 CE1	Fwd	GGGAGAGGCGCATATAAGAAAG
	Rev	GCCCATCACTGGTTCCAAA
APOA1 CE2	Fwd	GTGCTGAGACAGAAGTTGTGA
	Rev	CTCCAAAGTGACTCCTGGATTG
APOA1 CE3	Fwd	AGTGAAGGAGCCCACAATTC
	Rev	TCCCGAGGTGTATGTCTTCT
APOA1 CE4	Fwd	CAGAGAGACCAGTGAGAAGATG
	Rev	GTTACCCAACAGCCAGATGA
APOA1 CI1	Fwd	GCATGGGATTGTCAGAGTGAT
	Rev	ATGGGTGCTGGAAGAGAGA
APOC3 CE1	Fwd	AGAAATGGAAGCCGCTACAC
	Rev	TAAACAGGCGGAAAGGAAGG
APOC3 CI1	Fwd	ATCAGAACCTCAGTGCCTTC
	Rev	GCTGCATTGTAGCTGACCTTA
APOA4 CE1	Fwd	ACAGCTACCAATCAGCCTTAC
	Rev	AAGAGCAGACCACCTTATGC
APOA4 CE2	Fwd	ACATGCAAGGACATCAGACC
	Rev	CCCACCATGTGACTTGAGAA
APOA4 CI1	Fwd	ACAGAAGGATCACGGAAGGA
	Rev	GCCATCAAGTATCTGGTGAAGG

Table 3. Continued

Primer name	Fwd/Rev	Sequence
APOA4 CI2	Fwd	GTTTCTGCTGCTCGGTATCT
	Rev	CCGTCCTGGCTTCAATACAA
APOA4 CE3	Fwd	GTGGGACAACGGGAAAGTAA
	Rev	CCTCTGATGTCCAGTTCAGTTC
APOA4 CE4	Fwd	GTTTCCACTGACCCTGACAA
	Rev	GGATATGAAGAAGCAGCAGGAG
APOA5 CI1	Fwd	GCAAACGAGGGAGCTAGATT
	Rev	GATGACTTCCTCAGACAACAGG
APOA5 CI2	Fwd	GCTATGGTAAGTCCCACCATTC
	Rev	GCCGATGTAACATGATCCTACC
APOA5 CI3	Fwd	GACCACTGTCTGCCTTGTTT
	Rev	TTATGCAGCCTATGGCACTG
Alb promoter	Fwd	CCAAGGCCCACACTGAAATG
	Rev	CCCATGCACAAGACTTTGTC
Gapdh	Fwd	TCCTATCCTGGGAACCATCACC
	Rev	TCTTTGGACCCGCCTCATTT

2.3.2. Western Blot

Placentas were homogenized in pH 7.5 Tris lysis buffer including 50 mM Tris, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 1% β-mercaptoethanol, 50 mM NaF and 1 mM Na3VO4. Samples were separated on 8% sodium dodecyl sulfate–polyacrylamide gels by electrophoresis and then transferred to nitrocellulose membranes. Primary antibodies used in this study were anti-CTCF (Cat# 07-729, Millipore); anti-beta actin (Cat# ab8227, Abcam). Blots were visualized by secondary antibodies conjugated to horseradish peroxidase (Life Technologies,Waltham, MA) and enhanced chemiluminescence detection system (Pierce, Rockford, IL). Relative protein expression for CTCF was reported as a ratio to b-actin. Band intensities were quantified using

ImageJ (National Institutes of Health, Bethesda, MD). Each experimental group contains eight different animals (n = 8).

2.3.3. Statistical Analysis

For all experiments, statistical significance was determined by p-value less than or equals to 0.05.

For quantitative analysis of candidate gene regulatory region enrichment, ChIP samples were first normalized to 1% input, using the formula previously described (Mukhopadhyay et al., 2008). To independently examine alterations in each post-translational modification, the means from each independent sample were normalized to the control. The results of 3 independent experiments were then tabulated, cumulative means calculated and standard error of the mean derived. Values from each biological replicate were transferred into the statistical analysis program GraphPad (GraphPad Software, Inc., La Jolla, CA), verified for normality using the Brown-Forsythe test, and a student's unpaired t-test applied to assess differences.

2.4. Results

2.4.1. Male Preconception Alcohol Exposure alters Chromatin Boundary Elements and Enhancer Function in the Offspring

We have observed altered gene expression in placental tissues derived from the offspring of alcohol-exposed sires. We next sought to examine the impact of

preconception alcohol exposure alcohol on placental chromatin structure, especially at critical regulatory regions such as promoters, enhancers and insulators. Here we selected the APO, albumin and fibrinogen gene clusters, as these regions all displayed seemingly co-incident patterns of dysregulation.

All enhancers (E) characterized from these loci to date are listed in Table3. Candidate enhancers (CE) were selected based on their enrichment of H3K27 ac and H3K4 me1 from SRA CHIP-seq profiles; Candidate insulators (CI) were selected by enrichment of CTCF and Rad 21 also from SRA CHIP-seq profiles (Shen et al., 2012). As shown in Fig4, CTCF, Rad21, H3K27 ac and H3K4 me1 enrichments were examined at identified regulatory regions in the APO, fibrinogen and albumin gene clusters. Around 50% of our tested regulatory elements showed a significant decrease in CTCF binding in the offspring of alcohol-exposed males, a trend that was not observed for Rad21 enrichment. In addition, only Fgb CE2 region 2 showed a slightly decrease for H3K27 ac enrichment in alcohol group. Reduced enrichment of H3K4 me1 was observed in alcohol group only at Fgb region 2, Alb promoter and Afp E2 region3.

In the APOA1/C3/A4/A5 cluster, APOA1 CE2 and CI1, which flank the ~3kb region upstream of APOA1, and APOA1 CE3 and are located in the intronic region of the gene body, showed a reduced enrichment in CTCF binding in the preconception alcohol group, but no significant changes in other enrichments. APOC3 CE1 (between APOA1 and APOC3), APOC3 CI (intronic region of APOC3 gene body), and APOC3 E (reported in human), which is located between APOC3 and APOA4, all showed a reduced enrichment of CTCF in alcohol group. A similar pattern is also observed in

APOA4 CE1 (between APOC3 and APOA4), APOA4 CE2 (intronic region of APOA4), APOA4 CE3 (between APOA4 and APOA5), APOA4 CI2 (between APOA4 and APOA5), APOA5 CI1 (intronic region of APOA5) and APOA5 CI2 (between downstream genes Zpr1 and Bud13) (Fig 8 A and B). A similar pattern of decreased CTCF binding was also observed in the fibrinogen cluster, specifically in regions including Fgb promoter, Fgg promoter, Fgb CE2 region 1 (upstream of Fgb), Fgb CE2 region (upstream of Fgb), Fgb CE3 (between Fga and Fgb), Fgb CE4 (between Fga and Fgb) and Fgg CI1 (upstream of Fgg). Fgb CE2, a 3-kb region highly enriched in H3K4 ac and H3K4 me1, which is located upstream of Fgb and dissected into two regions here, showed a significant decrease in H3K4 me1 and H3K27 ac in the offspring of alcohol-exposed sires. Notably, the fibrinogen enhancer CNC12, previously reported in human liver, showed no significant change among any of the enrichments between control and alcohol groups. (Fig 8 C and D) The albumin family also displayed reduced CTCF binding at Alb, Afm promoters, and Afp E2. Interestingly, while CTCF binding of Afp E1 and E3 remains unchanged, all the three regions of the Afp E2 showed significantly reduced CTCF binding in alcohol group. (Fig 8 E and F)

A

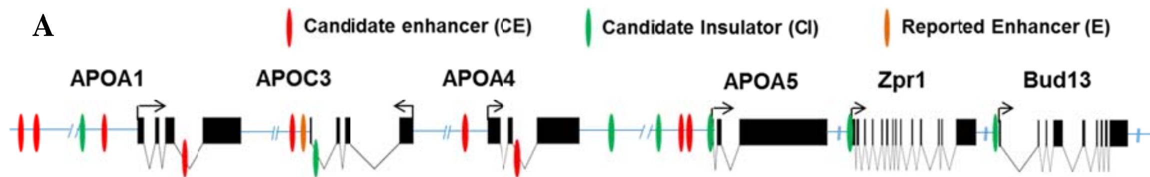


Figure 8. Relative enrichment of CTCF, Rad21 and histone modification H3K27 ac, H3K4 me1 at specific regulatory sites in control and paternal alcohol-exposed placenta is measure by CHIP-qPCR.
A. APO family; C. fibrinogen family; E. albumin family. Statistics significance is determined by unpaired t-test with p-value less than or equals to 0.05, n=5. B, D and F. Proximate location of regulatory sites on each cluster: B. APO family; D. fibrinogen family; F. albumin family.

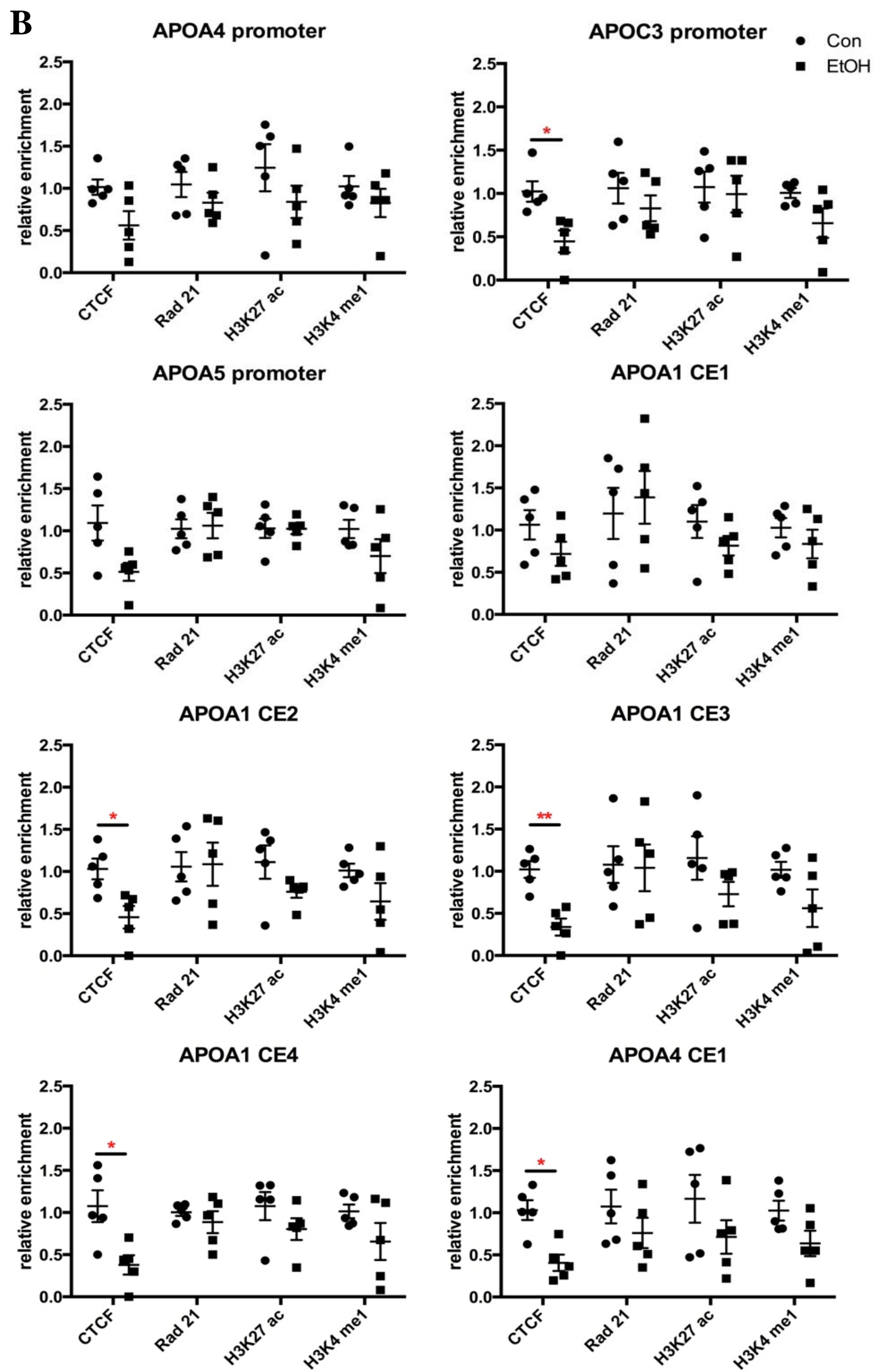


Figure 8. Continued

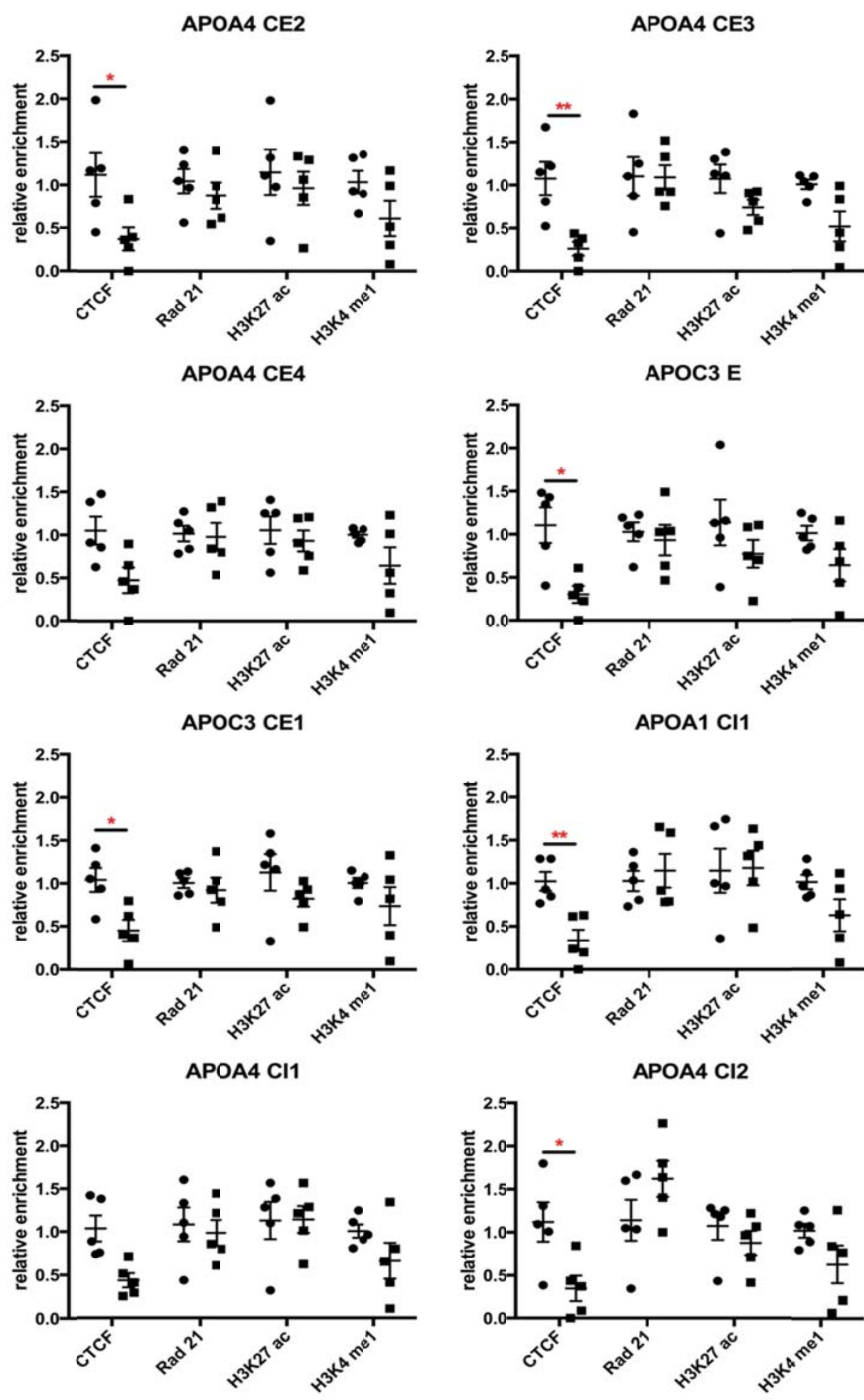
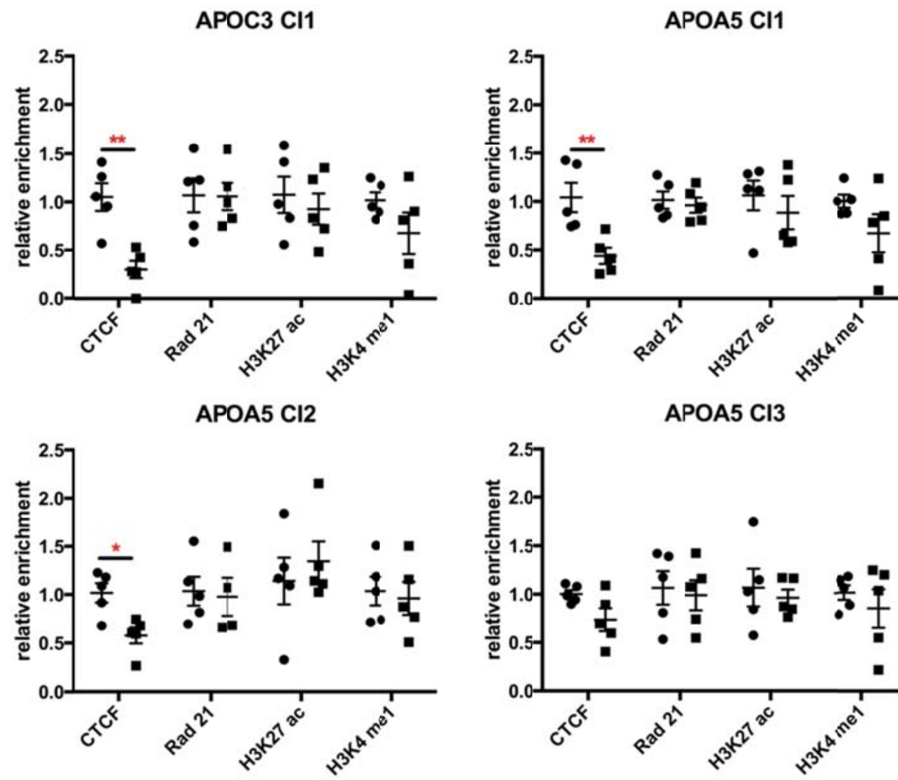


Figure 8. Continued



C

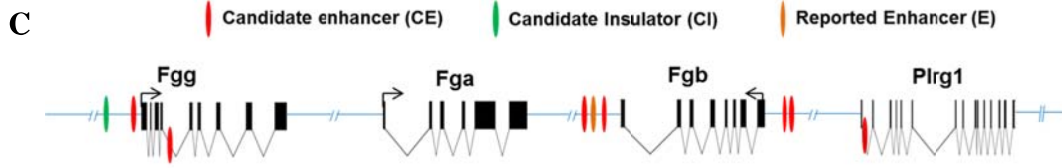


Figure 8. Continued

D

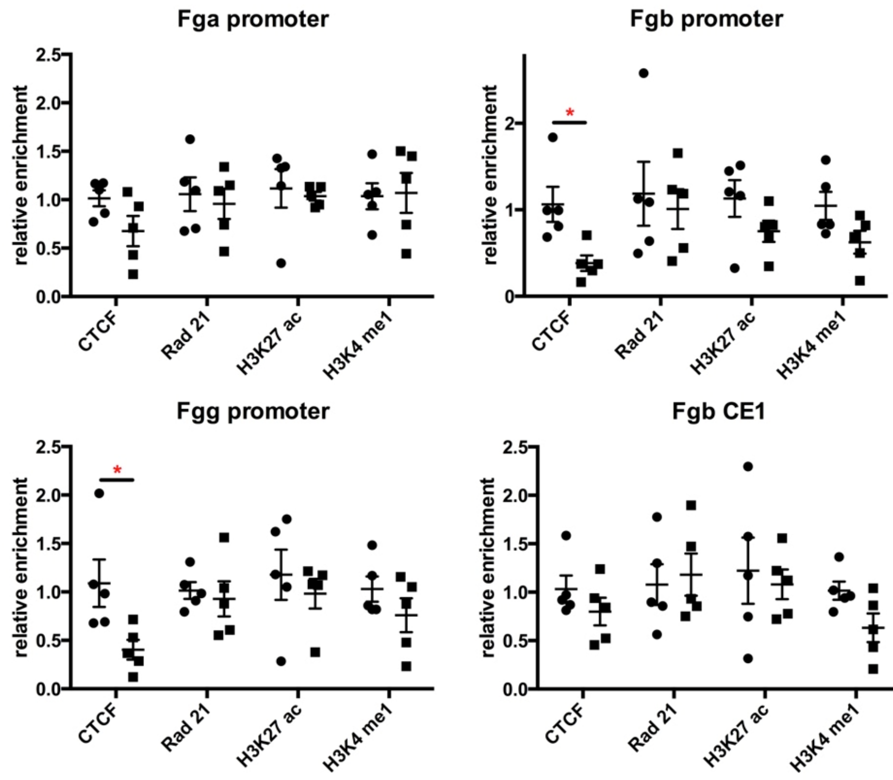


Figure 8. Continued

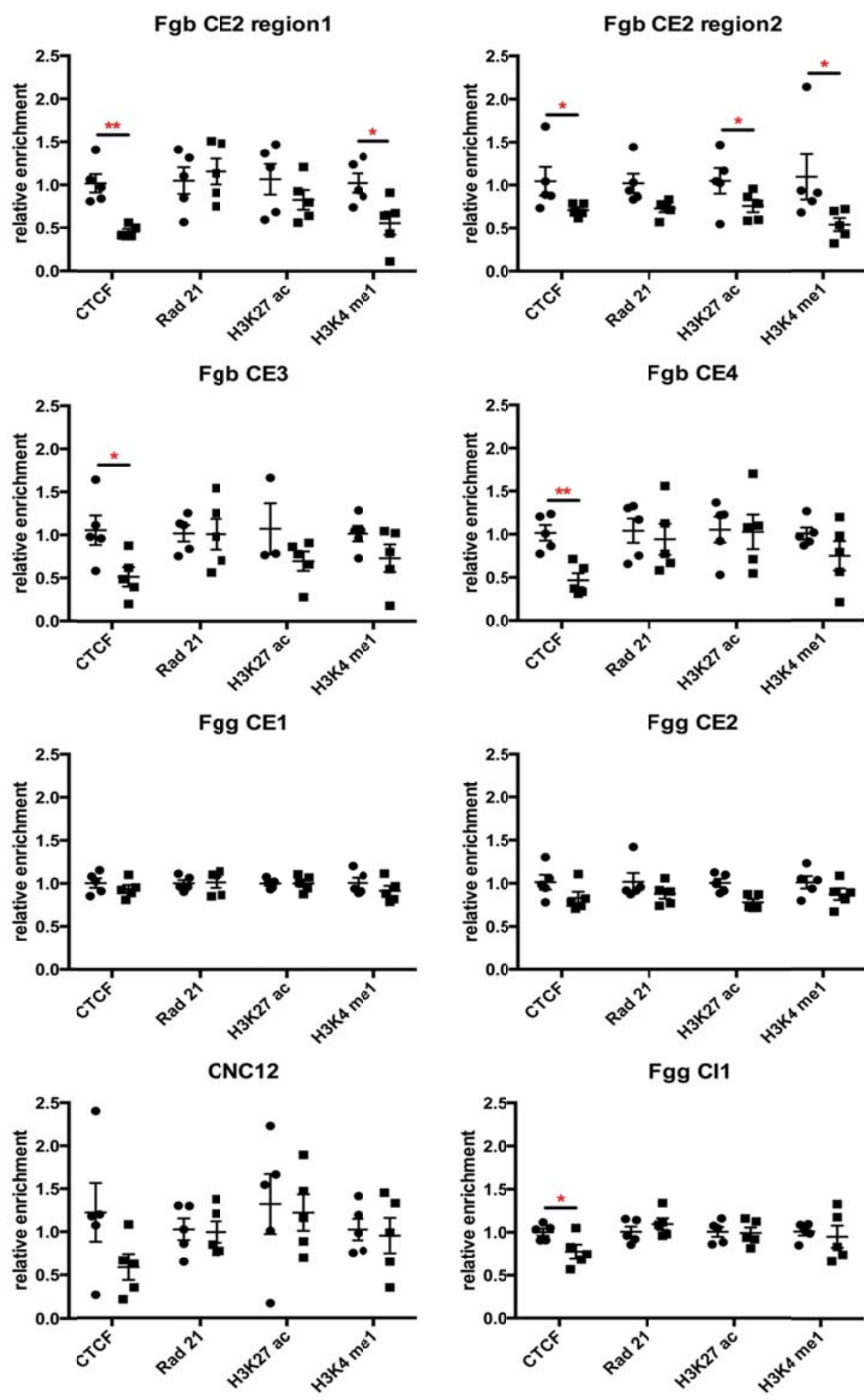
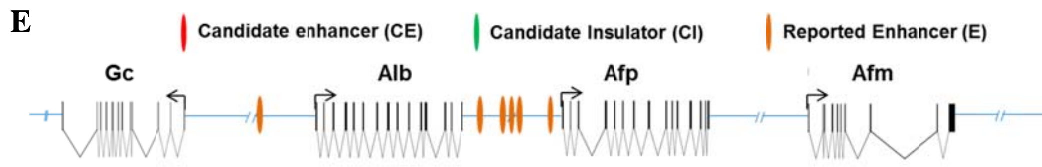


Figure 8. Continued

E



F

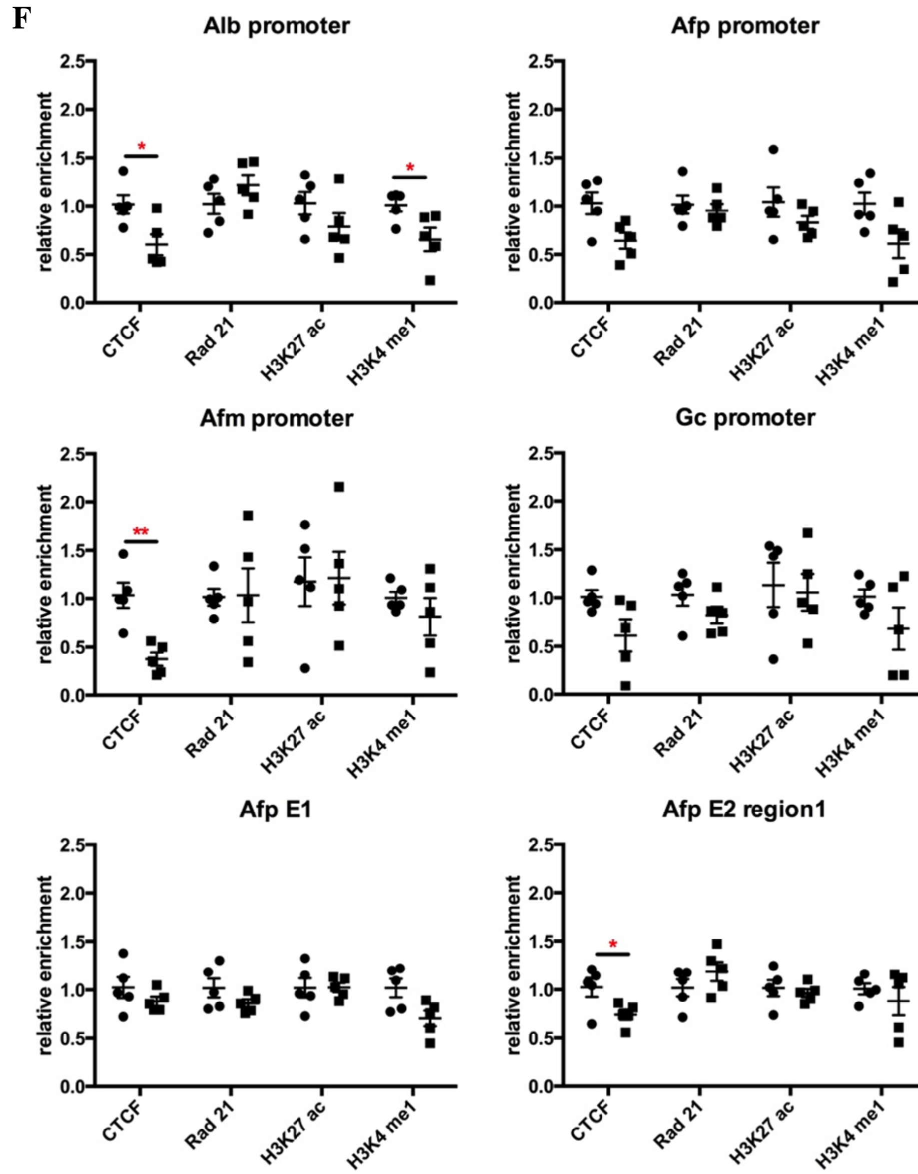


Figure 8. Continued

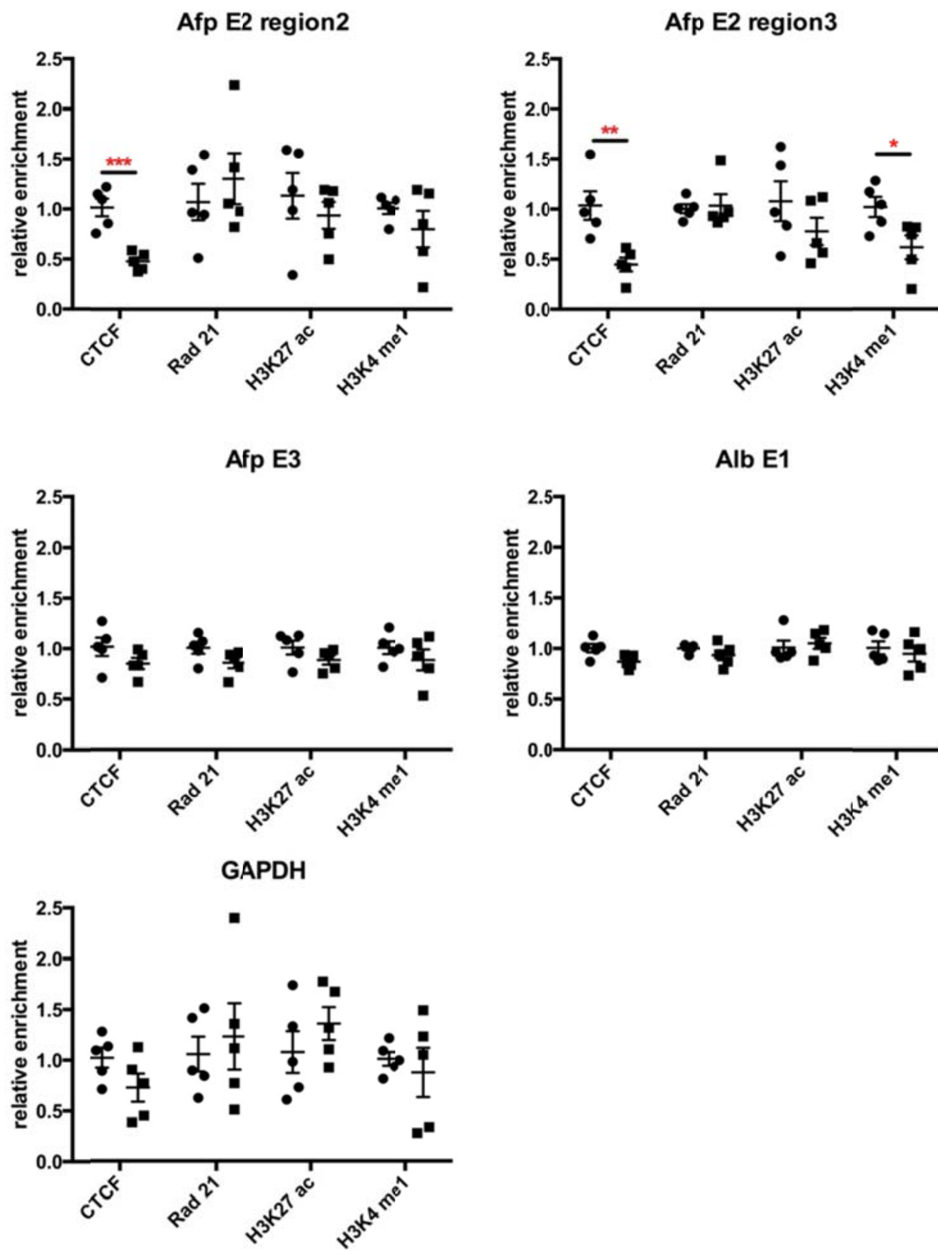


Figure 8. Continued

2.4.2. No Significant Global Alteration of CTCF Protein Level

The broad reduction of CTCF binding at these regulatory sites lead us to speculate that perhaps this protein was broadly decreased within the offspring of alcohol-exposed males. Surprisingly, CTCF protein abundance varies greatly between different individuals, even within the same treatment group (Fig 9A). No significant differences in global CTCF abundance could be observed (Fig 9B).

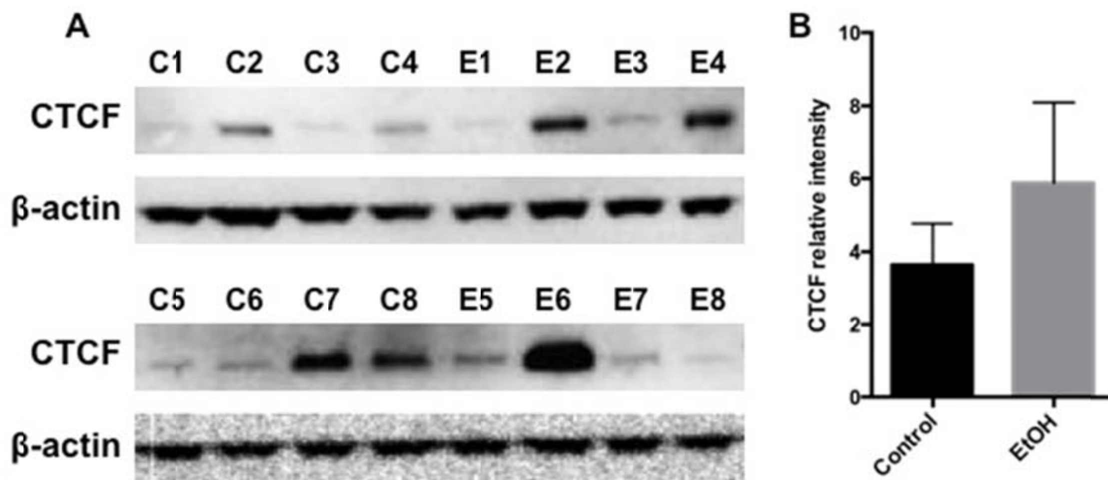


Figure 9. Western blotting analysis of CTCF expression in control and paternal alcohol-exposed placentas.
Quantification of western blot results: band intensities were normalized to β-actin using Image J. Statistics significance was determined by unpaired Student's t-test, p-value = 0.5534, n = 8.

2.5. Discussion

The objective of our second study was to determine if placental chromatin structure is heritably disrupted by male preconception alcohol exposures. We

hypothesized that preconception alcohol exposure heritably impacts chromatin organization within the placenta, which influences patterns of gene expression. In this study, we examined the chromatin structure within the placentas of offspring from both control and alcohol-exposed fathers. Key markers of chromatin structure associated with enhancer function and CTCF binding at various promoters, enhancers and insulators are significantly decreased in the male offspring of alcohol-exposed sires.

CTCF is a major and key regulator in chromosome organization, including chromatin looping and TAD establishment. It is still unclear if the effects of CTCF positioning influence gene transcription, since CTCF binding has been associated with both the promotion and repression of gene expression (Phillips and Corces, 2009). CTCF was first identified as a binding factor at the regulatory region proximate to the chicken c-myc promoter where it functions as a transcriptional repressor (Lobanenkov et al., 1990). However, in a subsequent study where CTCF binding was identified within to the amyloid beta-protein precursor promoter, it was associated with the promotion of transcription (Vostrov and Quitschke, 1997). Therefore, CTCF can activate or repress gene transcription depending on the context. CTCF binding is also enriched in insulators, where it serves to repress transcription by blocking enhancer-promoter interactions (Bell et al., 1999). The identification of its enhancer blocking properties was the first major clue to its involvement in the partitioning of the genome into active and silent compartments. As such, recent work has confirmed to importance of CTCF as a crucial regulator of TAD boundaries, where disruption can lead to abnormal patterns of gene expression associated with disease and dysgenesis (Lupianez et al., 2015a). In our

study, we showed a reduced CTCF binding profile at regulatory elements including known promoters, enhancers and insulators. This altered binding profile was associated with decreased gene expression, which is consistent with the loss of appropriate inter-TAD transcriptional interactions. In related studies, siRNA-mediated suppression of CTCF and Rad21 leads to more compact chromatin state. In these studies, the authors hypothesized that the compact chromatin was due to a decreased number of short-range loops while long-range interactions remained unperturbed (Tark-Dame et al., 2014). This hypothesis was supported by subsequent studies, which proposed that CTCF is largely required for short-range interactions within TADs (Handoko et al., 2011). These observations might explain our observations of reduced CTCF and the association of repressed gene expression. Further studies will be required to assay these loci for signatures of compact chromatin structure.

Our analysis of CTCF protein levels did not show a reduced level in the offspring of alcohol-exposed males. It is likely that CTCF protein levels are not changed in the placentas from alcohol exposed fathers, but that specific binding of CTCF is impacted. It has been shown that CTCF preferentially binds to hypomethylated sites and alterations in DNA methylation could disrupt chromatin looping through impaired CTCF/cohesion binding (Kang et al., 2015). Global analysis reveals that majority of CTCF sites are cell-specific, and 41% of this variable CTCF binding is associated with differential methylation (Wang et al., 2012). It is also possible that paternal alcohol exposure can lead to DNA mutation at CTCF binding sites, which weakens its ability to recruit CTCF and its related transcription factors. A previous study examining mutations at CTCF

sites in tumor cells revealed a predominate point mutation in A-T base pairs at these sites, which is caused by defects of DNA polymerase E exonuclease domain (Katainen et al., 2015). Thus, additional possibilities remain that mutations at DNA sequences, or possibly the binding of other transcription factors could interfere with CTCF binding at these sites.

In summary, our study provides new evidence that male preconception alcohol exposure can impact chromatin structure. In this second study, we hypothesized that preconception alcohol exposure heritably impacts chromatin organization within the placenta, altering chromatin boundary element placements, which heritably influence patterns of gene expression. Further, this altered chromatin structure appears to mainly occur at intra-TAD chromatin boundaries and is associated with reduced CTCF binding. However, the mechanism by which paternal preconception alcohol exposure affects CTCF binding and mechanistically, how this reduced CTCF binding affects gene transcription remains to be fully defined.

CHAPTER III

OVERALL DISCUSSION AND CONCLUSION

In this study, we examined the impact of male alcohol exposure on the transcriptional control of gene expression within the placenta.

First, we sought to determine if male preconception alcohol exposure can heritably impact patterns of gene expression with the placenta. The transcriptome of placental tissues isolated from both male and female offspring sired by both control and alcohol exposed fathers were characterized. According to our bioinformatic analysis, numerous physiologically important genes were significantly different between the control and alcohol preconception treatment groups, in both male and female offspring. Subsequent pathway analysis using IPA revealed that these differentially expressed genes are mainly enriched in molecular functions such as molecular transport, protein synthesis, lipid metabolism, small molecule biochemistry, as well as vitamin and mineral metabolism. These observations indicate that preconception alcohol exposure leads to placental insufficiency through impaired nutrition and supply. These differentially expressed genes were further validated in vivo. Interestingly, while the placentas from male offspring consistently showed similar pattern disruptions as those from the RNA-seq data sets, around 80% of the candidate genes identified in female offspring did not follow the pattern identified in the analysis of the transcriptome. This inconsistency was not due to variable fetal weight or placenta weight.

Interestingly, around 98% of the significantly altered genes in male placenta showed reduced expression in alcohol groups. This high level of reduction in gene expression lead us to speculate that there was a perhaps a global reduction in transcription. Previous studies have identified alterations in DNA methylation within sperm after male alcohol exposure (Bielawski et al., 2002). Our next objective was to determine if the alterations in gene expression could be associated with programmed dysfunction in the metabolism of the methyl donor S-adenosylmethionine (SAM). Our studies indicate that SAM levels were not altered in paternal liver, testis, kidney, and no alterations could be identified in the placenta or fetal brain. This refuted our hypothesis that male preconception alcohol exposure could alter global methylation by interfering with SAM metabolism.

One of the main findings to emerge from our work is that many of the dysregulated genes localized to clusters and exhibited simultaneous reductions in expression in preconception alcohol group. Previous studies have shown that gene clusters from the same ancestor sharing common regulatory elements, genes regulated by super enhancers and genes co-localized within TADs can all be impacted simultaneously though the disruption of common regulatory elements such as enhancers, insulators and promoters (Coelho et al., 2010) (Lupianez et al., 2015b) (Hnisz et al., 2013). Therefore, to investigate how these regulatory elements are impacted by male preconception alcohol exposure, we examined CTCF and RAD 21 occupancy, H3K4 me1 and H3K27 ac enrichment at hypothesized regulatory sites within three of the most prominently impacted gene clusters. Very few of these regulatory elements identified in

previous publications had been functionally validated, and most were selected from published CHIP-seq files based on their enrichment of insulator (CTCF and Rad21) and enhancer (H3K4 me1 and H3K27 ac) makers in cultured placental stem cells. Our results identified a significant reduction of CTCF occupancy in almost 50% regulatory elements we tested in the preconception alcohol group. However, Rad21, H3K4 me1 and H3K27 ac at most regulatory sites showed no difference between these two groups. In fact, other histone modifications including H3K9 dimethylation and H3K4 trimethylation were not changed in many of these elements we tested (data not shown). This is supported by our most recent publication that gene expression does not necessary correspond with the profile of histone modifications (Veazey et al., 2017, accepted). In this case, we observed reduced gene expression while the major histone modifications associated with the control of gene expression remain unchanged. CTCF is a major transcription factor affecting chromatin structure. In this work, reduction of CTCF binding in these regulatory elements lead us to question whether the CTCF abundance is reduced from the protein level, since this reduction is not element-type-specific nor gene-specific. However, our results demonstrate CTCF abundance varied largely between different individuals and did not suggest CTCF protein levels alone could explain these changes.

Our observations that CTCF binding is reduced at regulatory sites in the male placentas from alcohol-exposed fathers provides the first evidence those chromatin boundaries could be impacted by paternal drinking. This is supported by a previous study that CTCF is critical transcription factor for sperm genome organization (Carone et al., 2014). However, further studies are needed to make any firm conclusions as to the

role that CTCF plays in these altered patterns of gene expression. Future studies will need to employ CHIP-seq on placentas sired from control and alcohol exposed fathers to determine if CTCF occupancy is reduced at certain sites or globally. Further, these studies would facilitate the de novo identification of significantly changed regulatory elements. Subsequent studies using chromosomal conformation capture (3C) based techniques would then be needed to provide more insight regarding the actual disruption of chromatin looping patterns that occur due to the modified enhancer histone landscape.

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