METABOLIC DYSFUNCTION AS A LEGACY OF PATERNAL PRECONCEPTION ETHANOL ABUSE

An Undergraduate Research Scholars Thesis

by

GABRIELLE I. SUTTON

Submitted to the Undergraduate Research Scholars program at
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by Research Advisor: Dr. Michael Golding

May 2017

Majors: Molecular and Cell Biology
Psychology

TABLE OF CONTENTS

ABSTRACT

Metabolic Dysfunction As A Legacy of Paternal Preconception Ethanol Abuse

Gabrielle I. Sutton
Department of Biology
Texas A&M University

Research Advisor: Dr. Michael Golding
Department of Veterinary Physiology and Pharmacology
Texas A&M University

It is now well-accepted that parental history and exposures encountered prior to conception exert a significant impact on offspring health through epigenetic means, and in some cases, have the potential to induce the development of disease later in life. Preliminary studies in our lab have identified prenatal / postnatal growth restriction and altered epigenetic programming in a mouse model of chronic, preconception male alcohol exposure. These studies challenge the current maternal-centric exposure paradigm and implicate paternal exposure history as an additional mediator of alcohol-induced defects. In clinical studies, fetal growth restriction is associated with the early onset of multiple adult diseases including type II diabetes, non-alcoholic fatty liver disease and a collection of pathologies commonly referred to as metabolic syndrome. How prenatal growth restriction is able to induce this sequela is poorly understood. Recently, disruptions in complex networks of genes regulated through genomic imprinting have emerged as major regulators of metabolism. In this study, we assayed the expression of genes participating in these imprinted regulatory networks within the placenta and fetal liver. Using real-time qPCR, we measured the expression of transcripts encoding the imprinted genes *Cdkn1c, Dcn, Dlk1, Gatm, Gnas, Gh2, Grb10, H19, Igf2, Igf2r, Mest, Ndn, Sgce, Meg3, Peg3,*

Zac1 and *Slc38a4*. From these assays, we observed altered expression of select imprinted genes, including *Grb10* (males) and *Sgce* (females) in the placenta, as well as *Cdkn1c* (males) and *GH2* (females) in the fetal liver. However, all of these candidate genes exhibited decreased expression with no evidence of inappropriate contributions from the normally silent paternal allele. Thus, there is likely another transcriptional mechanism at the root of this growth phenotype.

ACKNOWLEDGEMENTS

I would like to express sincere appreciation to my advisor, Dr. Golding, for his patience, encouragement and guidance. You have a remarkable aptitude for teaching, a quality that I hope to someday develop and share with others.

Thanks to Richard Cheng for his assistance in answering any questions and to Dr. Carlos Pinzón for his assistance in creating my research poster.

CHAPTER I

INTRODUCTION

Until recently, maternal consumption of alcohol has been assumed to be the direct and exclusive origin of fetal alcohol spectrum disorders (FASDs), leading to characteristic growth retardation, facial abnormalities and nervous system defects in offspring. However, recent studies have accumulated a sufficient amount of circumstantial evidence to suggest that preconception paternal consumption of alcohol contributes to low birth weight and cognitive growth deficiencies, complications typically associated with FASDs.[1-2] Thus, paternal consumption of alcohol may contribute to the spectrum of the syndrome and the variance in growth rates and malformations beyond the boundaries traditionally reserved for maternal drinking prevalence.[3] Despite widespread education on the matter within the United States, FASDs remain a perpetual concern with a prevalence of 30.52 to 47.13 incidences per 1,000 generating extensive economic burden.[4] Thus, an understanding of the breadth of factors contributing to this syndrome is imperative in order to manage the ramifications resulting from alcohol-related birth defects.

Preliminary studies in our lab have identified prenatal / postnatal growth restriction and altered epigenetic programming in a mouse model of chronic, preconception male alcohol exposure. The growth restriction observed in the offspring of the alcohol-exposed males is significantly associated with placental dysfunction, altered lipid homeostasis, as well as postnatal effects on metabolic function. This phenotype of metabolic irregularity may stem from altered epigenetic mechanisms of transcriptional control, which could be passed onto the offspring through paternal sperm. Specifically, epigenetic mechanisms regulating the transcriptional control of imprinted

genes may be disrupted in this model, given the previously described associations between paternal drinking and altered DNA methylation within sperm.[1-2] Further, alterations in networks of imprinted genes have previously been associated with disruption in the development of the offspring's metabolic health.[5-6] Finally, imprinted genes are exempt from the global epigenetic reprogramming that occurs post fertilization, suggesting they may act as a means for offspring to inherit epigenetic alterations affecting gene transcription.[5]

Genomic imprinting is an epigenetic mechanism, which regulates the transcriptional control of gene expression through the inheritance of parent-specific monoallelic patterns of DNA methylation.[7] The chromosomal regions encoding these genes are modified during gametogenesis by imparting parent-of-origin-specific patterns of DNA methylation at CpG sites within key transcriptional regulatory regions.[7] Importantly, this is done without any effect on the integrity of the genetic sequence.[7] These epigenetic modifications are dynamic in their ability to be erased in early germ line development and reestablished in each generation according to the sex of the conceptus.[7] Imprinted genes have varied functions associated with cellular apoptosis, regulation of the immune system, facilitation of metabolic processes and in the regulation of the cell cycle.[7] Recently, this phenomenon has been linked to the inheritance of a predisposition towards obesity, specifically the Trim28 obese phenotype.[6] Given the link between this study and fetal growth rates, we hypothesized that up-regulation in the transcriptional control of imprinted genes may be at the root of the metabolic phenotype associated with male preconception alcohol exposure. We therefore sought to examine the transcriptional control of imprinted genes within key tissues of the growth-restricted offspring by employing real-time quantitative polymerase chain reactions (qPCR) analysis.

CHAPTER II

METHODS

A. RNA Extraction, cDNA Synthesis and Real-Time qPCR

500 μl of TRIzol reagent was added to 25-50 mg of tissue, homogenized by plastic pestle. 100 μl of chloroform was added to the lysates, which were then vortexed and centrifuged at maximum rpm for 10 minutes at 4°C. The upper aqueous phase was transferred into new 1.5 ml microcentrifuge tubes, and 250 μl of isopropanol was added. After a 10 minute incubation period, the samples were transferred to silica spin columns in new 1.5 ml microcentrifuge tubes to be centrifuged at maximum rpm for 5 minutes at 4°C (Omega Bio-tek). Subsequently, the protocol of the E.Z.N.A. Total RNA Kit was followed (Omega Bio-tek). The sample concentrations were determined through a ThermoFisher NanoDrop 2000 spectrophotometer and standardized to a 1 μg concentration. Reverse transcription was completed on the standardized RNA using the First-Strand cDNA Synthesis Kit (Invitrogen). Three qPCR measurements were completed for two independent RT reactions utilizing SYBR Green Master Mix (Thermo Scientific) in 384-well hard-shell PCR plates (Bio-rad). Primer sequences are listed in Table 1. To analyze gene expression, the replicate cycle threshold (Ct) values for each transcript were compiled and normalized to the geometric mean of the three reference genes: *Mrpl1* (mitochondrial ribosomal protein L1), *Hprt1* (hypoxanthine phosphoribosyltransferase 1) and *Ywhaz* (14-3-3 protein zeta/delta). Normalized expression levels were calculated using the ddCt method.[8] The relative fold change values from each replicate were transferred into the statistical analysis program GraphPad (GraphPad Software, Inc., La Jolla, CA) to complete an unpaired t-

test for single transcript comparisons between control and treatment groups. On all figures, significance ($p < 0.05$) is indicated by an asterisk and error bars represent S.E.M. *P <0.05.

Table 1. Primer sequences for real-time qPCR.

Gene	Forward Sequence	Reverse Sequence
Cdkn1c	AACGTCTGAGATGAGTTAGTTTAGAGG	AAGCCCAGAGTTCTTCCATCGT
Dcn	CATCTTCGAGTGGTGCAGTGTT	GCAGGTCTAGCAAGGTTGTGTC
Dlk1	GACGGGAAATTCTGCGAAATAGAC	GTCCACGCAAGTTCCATTGTTG
Gatm	CCGACCGTCCTTGTCATCAGA	TGGTGTTGGAGGAGTAACTATGGT
Gnas ex1A	GGGCGTCATCAGGCTGGTTA	GCCGACGCGACTGAGTGT
Gnas exon1	AGCGCGAGGCCAACAAAA	GTGCGTGGCCCGGTAGA
Gnas XL	CGTCTCTACCGGATCTGATGCT	CGGCATCGCTCTGGCTATCT
Grb10	AGGATCATCAAGCAACAAGGTCTC	ATTACTCTGGCTGTCACGAAGGA
GH2	CGAGGACTTCACGCACAACAC	CCACGCAGGATTCCAGATGATG
H19	CTTGTCGTAGAAGCCGTCTGTTC	GTAGCACCATTTCTTTCATCTTGAGG
Igf2	CTTGTTGACACGCTTCAGTTTG	GTTGGCACGGCTTGAAGGC
Igf2r	GCACAGAATCCAGACTAGCATTACA	CCTCCTTATCAGCTTTAAATATGTCTTTCTT
Mest	CAACAATGACGGCAACCTGGT	TCTGAATTTCTTCCTTTGATTAATGTACTGTA
Ndn	TGTGCTGTGCTAAACTTGTGAAATAC	TGCTCAGGTCTGCGTTGCT
Peg3	TTGGACTGGACAGAGATGATGACA	ATTCTGGTATGACTCGGCATCCT
Sgce	GTGATGGAGTCCTGTATGGGTCT	GTAGGCAGTTATCTCAATAATAGTTGGTTT
Slc38a4	ACTGTGGCAATACTCTCGCTCTA	ATCCAAATGCTTTCTCGCCCAAT
Zac1	TTTTCTTTGCCTAGCTTAACCTACTACTT	CACAATCCTCTTGGGATACAAACTAA

CHAPTER III

RESULTS

A. Imprinted Gene Expression in the Placenta

In the placentas of the female offspring sired by alcohol-exposed males, *Sgce* (sarcoglycan epsilon) was significantly down-regulated (Fig. 1A), while in the male offspring, *Grb10* (growth factor receptor bound protein 10) was significantly down-regulated (Fig. 1C).

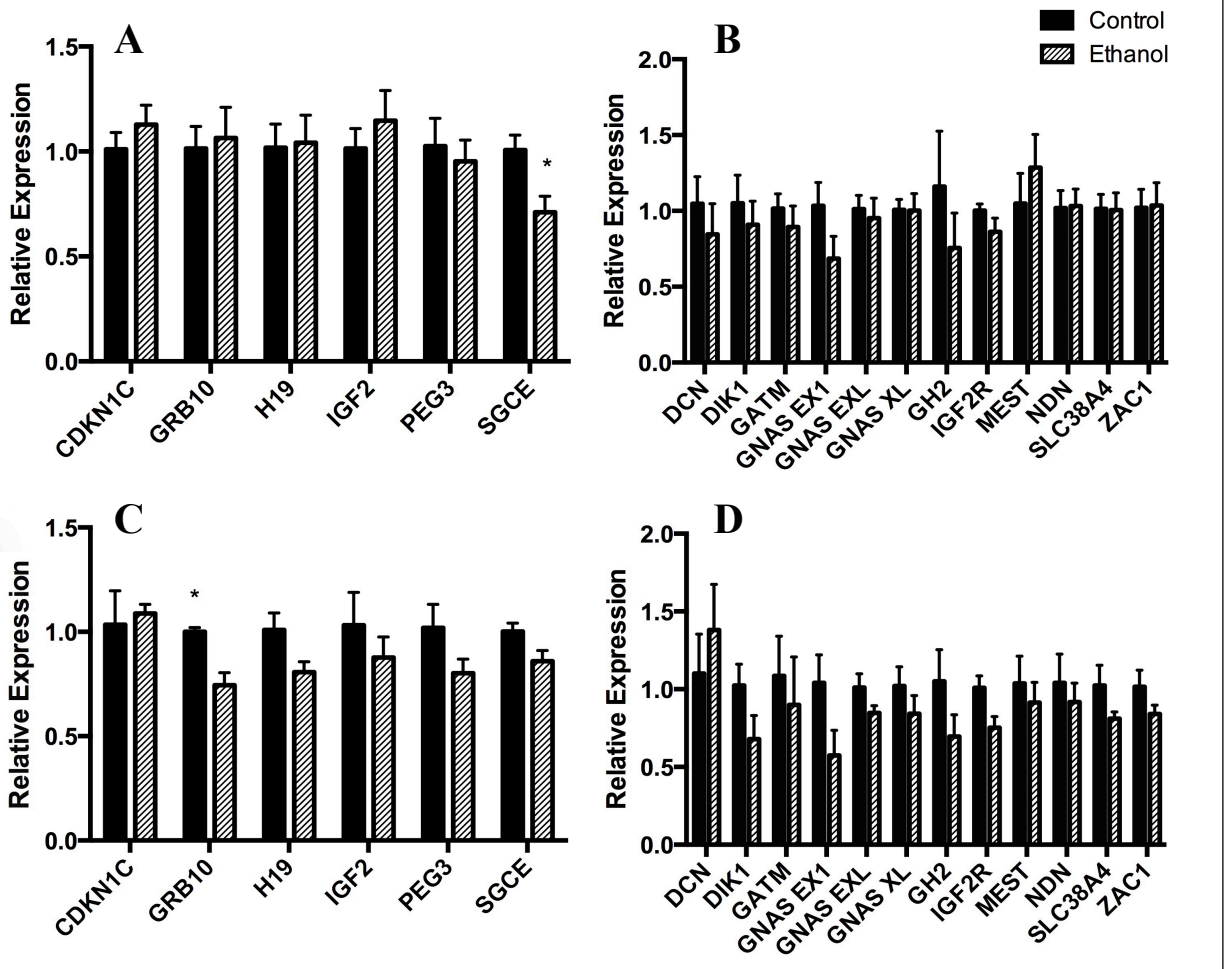

Figure 1. Relative RNA transcript expression in placenta tissue following paternal ethanol abuse. (A-B) Imprinted gene expression in the female placenta. (C-D) Imprinted gene expression in the male placenta.

B. Imprinted Gene Expression in the Fetal Liver

In female offspring of alcohol-exposed males, the fetal liver exhibited down-regulation of *GH2* (growth hormone 2) (Fig. 2B), while in the male fetal liver, *Cdkn1c* (cyclin dependent kinase inhibitor 1C) was significantly down-regulated (Fig. 2C).

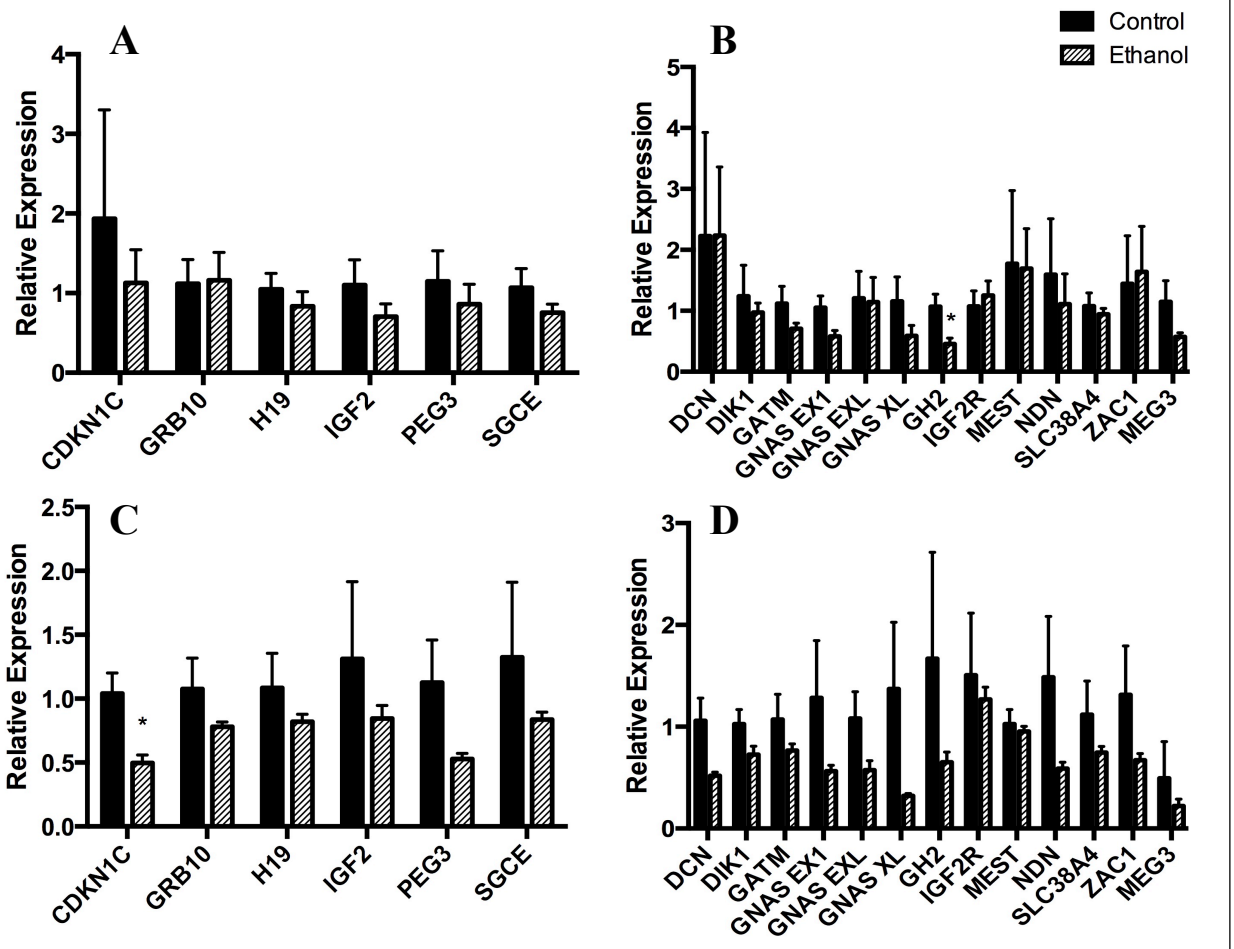

Figure 2. Relative RNA transcript expression in fetal liver tissue following paternal ethanol abuse. (A-B) Imprinted gene expression in the female fetal liver. (C-D) Imprinted gene expression in the male fetal liver.

CHAPTER IV

CONCLUSION

Recently, a trend has emerged in associating the metabolic profile of offspring to that of a parent through the epigenetic landscape.[5-6,9-10] It has been well-established that alcohol exposure within the intrauterine environment can act as a trigger in initiating epigenetic alterations, particularly in disrupting patterns of DNA methylation linked to the transcriptional control of imprinted genes.[11] In this study, we observed a down-regulation in the expression of *Grb10* (males) and *Sgce* (females) in the placenta, as well as *Cdkn1c* (males) and *GH2* (females) in the fetal liver. Given the importance of previously identified interactions between networks of imprinted genes in the control of both fetal metabolism and postnatal growth, we had hypothesized that alterations in the transcriptional control of imprinted genes may be at the root of the metabolic phenotype associated with male preconception alcohol exposure. However, we observed decreased expression of candidate imprinted genes. These disruptions in transcriptional control are likely not the mechanistic basis underlying the metabolic syndrome associated with fetal growth restriction from paternal preconception ethanol abuse.

Of the four imprinted genes with modifications in expression, only *Sgce* is paternally expressed; however, as *Sgce* is a tissue-independent methylated gene with a minor association to fetal growth and development, the findings of decreased RNA transcript levels within the female placenta do not provide evidence for a contribution to an irregular metabolic phenotype.[12-13] Preliminary studies in our lab revealed a reduction in fetal weight for females and reduction in

weight of the gestational sac and crown rump length in both sexes. If *Sgce* was a significant contributing factor, an up-regulation in place of down would have occurred. Furthermore, additional work in examining the methylation profile of sperm conducted in a separate study presented no concrete indication that these epigenetic changes were the result of paternal alcohol abuse. These factors indicate that imprinting is not the direct mechanism by which this phenotype is acquired; however, there are other components to consider. Specifically, a recent study revealed an obese "on/off" phenotype to be an effect of a Trim28 haploinsufficiency, caused by a down-regulation in paternally expressed imprinted genes.[6] Similar to our study, there was no evidence of altered DNA methylation within the loci controlling individual imprinted gene loci. However, this study did identify alterations within multiple imprinted gene enhancers and the expression patterns of guide recruitment factors. [6] Therefore, future studies in our model need to delve into histone variation and chromatin looping profiles which may amend the conclusion of this study with this particular imprinted gene dysregulation acting as a secondary effect to some unknown epigenetic mechanism at which the metabolic syndrome originates.

REFERENCES

1. Day J, Savani S, Krempley BD, Nguyen M, Kitlinska JB. Influence of paternal preconception exposures on their offspring: through epigenetics to phenotype. *Am J Stem Cells*. 2016;5(1):11–18.

2. Abel E. Paternal contribution to fetal alcohol syndrome. *Addict Biol*. 2004;9(2):127-133.

3. Sittig LJ, Redei EE. Paternal genetic contribution influences fetal vulnerability to maternal alcohol consumption in a model of fetal alcohol spectrum disorder. *Plos One*. 2010;5(4):e10058.

4. Roozen S, Peters GJ, Kok G, Townend D, Nijhuis J, Curfs L. Worldwide prevalence of fetal alcohol spectrum disorders: a systematic literature review including meta-analysis. *Alcohol Clin Exp Res*. 2016;40(1):18-32.

5. Soubry A, Guo L, Huang Z, Hoyo C, Romanus S, Price T, Murphy SK. Obesity-related DNA methylation at imprinted genes in human sperm: results from the TIEGER study. *Clin Epigenetics*. 2016;8(1).

6. Dalgaard K, Landgraf K, Heyne S, Lempradl A, Longinotto J, Gossens K, … Pospisilik JA. Trim28 haploinsufficiency triggers bi-stable epigenetic obesity. *Cell*. 2016;164(3), 353–364.

7. Barlow DP, Bartolomei MS. Genomic imprinting in mammals. *Cold Spring Harb Perspect Biol*. 2014;6(2).

8. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. *Nat Protoc*. 2008;3(6):1101-1108.

9. Fullston T, Palmer NO, Owens JA, Mitchell M, Bakos HW, Lane M. Diet-induced paternal obesity in the absence of diabetes diminishes the reproductive health of two subsequent generations of mice. *Hum Reprod.* 2012;27(5):1391-1400.

10. Soubry A. Epigenetic inheritance and evolution: A paternal perspective on dietary influences. *Prog Biophys Mol Biol*. 2015;118(1-2):79-85.

11. Ungerer M, Knezovich J, Ramsay M. In utero alcohol exposure, epigenetic changes, and their consequences. *Alcohol Res.* 2013;35(1):37-46.

12. Yuen RK, Jiang R, Peñaherrera MS, McFadden DE, Robinson WP. Genome-wide mapping of imprinted differentially methylated regions by DNA methylation profiling of human placentas from triploidies. *Epigenetics Chromatin*. 2011;4(1):10-25.

13. McCullough LE, Miller EE, Mendez MA, Murtha AP, Murphy SK, Hoyo C. Maternal B vitamins: effects on offspring weight and DNA methylation at genomically imprinted domains. *Clin Epigenetics*. 2016;8(8).