EFFECTS OF ALCOHOL ON THE REGULATION OF IMPRINTED GENES IN MOUSE STEM CELLS

An Undergraduate Research Scholars Thesis

by

ALYSSA CROCKER

Submitted to Honors and Undergraduate Research
Texas A&M University
In partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

Approved by
Research Advisor: Dr. Michael Golding

May 2013

Major: Animal Science

TABLE OF CONTENTS

ABSTRACT

Effects of Alcohol on the Regulation of Imprinted Genes in Mouse Stem Cells. (May 2013)

Alyssa Crocker
Department of
Animal Science
Texas A&M University

Research Advisor: Dr. Michael Golding
Department of
Veterinary Physiology and Pharmacology

Prenatal alcohol exposure is the leading, preventable cause of birth defects in the United States (5). A structurally important cell layer, the placenta, is responsible for transporting nutrients from the maternal system into the developing fetus. Importantly, this transient organ is profoundly affected by alcohol consumption. Placental development is regulated by a subset of monoallelic expressed genes that are regulated by a specialized transcriptional mechanism called genomic imprinting (4). Expression of specific imprint allele's imperative to proper placental structure and function was further analyzed by exposing trophectoderm cells to alcohol levels at the binge, and 2x binge drinker level (15).

Utilizing trophoblastic stem cells derived from a F1 cross between the C57Black6 and castaneous strains of mice; we have determined the expression of five imprinted genes when exposed to alcohol which have allowed us to measure the impact of prenatal alcohol exposure on the developing fetal epigenome. Through these experiments we have observed intensified and reduced expression of the five genes selected to be subjected to ethanol, and used for our

research. One of the imprinted genes understudy, Peg3 was found to exhibit a significant up-regulation in maternal expression.

ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Michael Golding, and Daria Muller. They allowed Elizabeth Villanueva and I to participate in the study of alcohol effects on imprinted gene regulation in the mouse stem cell. They were both very helpful in teaching, and guiding us through our project, and took time out of their own day to do so. I have learned a lot from the both of them, enjoyed completing my findings, as well as being part of their lab.

NOMENCLATURE

FASD – Fetal Alcohol Spectrum Disorder (5)

Igf2r – *Insulin like Growth Factor 2 Receptor* (7)

Peg3 – *Paternally expressed 3*(7)

H19 – *Fetal Liver mRNA* (7)

Igf2 – *Insulin like Growth Factor 2* (7)

Gtl2 – *Gene Trap Locus 2* (7)

B6 – maternal cDNA sample or expression, (B6) C57BL/6 black 6 strain of mice

Cast – paternal cDNA sample or expression, CAST7 *Mus musculus castaneus* strain of mice

DTT – Dithiothreitol, redox reagent

PCR – polymerase chain reaction

CHAPTER I

INTRODUCTION

During gestation, totipotent stem cells differentiate into the embryo and placenta. The placenta is an invasive structure that serves as the interface between the embryo and the maternal blood supply. Acting as a multifunctional unit, the placenta is responsible for transporting nutrients from the maternal system into the developing fetus. It plays a functional role in removing waste, transporting hormones, and providing a protective, structurally important sac for the developing fetus (2). Placental development is controlled, in part, by a subset of genes that are regulated by a specialized transcriptional mechanism called genomic imprinting. Unlike most of the genes in the genome, which are expressed equally from both parental alleles, imprinted genes are expressed exclusively from a single allele; the other allele is silenced (3).

Epigenetics can be defined as heritable changes in gene expression that occur in the absence of changes to DNA sequence.(6) Epigenetic changes to chromatin structure can be driven by exposure to external environmental factors (5). External influences of teratogens, such as alcohol, on the epigenome could lead to alterations in fetal placental structures during development (4). According to the National Institute on Alcohol Abuse and Alcoholism "prenatal alcohol exposure is the leading preventable cause of birth defects which are labeled under fetal alcohol syndrome disorder in the United States." (5). Fetal alcohol syndrome encompasses a pattern of teratogenic abnormalities that have been proven to lead to "decreased cognitive development, growth malformations, neurodevelopmental disorders, reduced brain

size, decreased immunity as well as cerebral palsy disorder" (5). These teratogenic abnormalities can result from inefficient hormonal imbalances and growth defects that are thought to be associated with placental growth hormone (hGH) present within the structure of the placenta (3). We believe the structural defects that arise as a consequence of ethanol exposure are a consequence of altered epigenetic control of imprinted gene expression. This altered structural growth has led us to believe that a subset of imprinted genes responsible for placental development will be impaired during in vitro alcohol treatment. "Thus far a generally safe level of alcohol consumption during pregnancy has not been established by research." (1) We will use our research to investigate the effect of ethanol exposure on genomic imprinting of trophectoderm stem cells. These alterations will lead to loss of monoallelic expression within the placenta, which can result in abnormalities associated with FASD such as decreased nutrient absorption, and hormonal transfer during gestation.

CHAPTER II

METHODS

Placental stem cell samples were derived from a cross between a *C57Black6* strain of mice and mice of a *Mus musculus castaneus* background prior to this experiment proposal (Golding et al., 2010). These two strains of mice carry distinct nucleotide polymorphisms within both the promoter regions and messenger RNAs of ten candidate-imprinted genes. By using the identified gene polymorphisms, we can distinguish between maternal and paternal alleles by using a restriction enzyme digest-based assay. Placental stem cells derived from F1 crosses were exposed to two different alcohol concentrations: the binge 120 mg/dl, and two times binge 320 mg/dl limit. (15) We then isolated RNA from these samples and converted it into cDNA for analysis. To isolate RNA, cell membranes were lysed with trizole buffer followed by a series of washes to purify the RNA pellet. Once RNA from the placental stem cells had been extracted it was treated with DNAses to remove any contaminating DNA for 15 minutes at room temperature. Once contaminated DNA was removed we added both dNTP's and random primers then brought the reaction to 70 degrees Celsius for 10 minutes. When complete, we added DTT, and First Standard Buffer to complete the final steps of RNA reverse-transcription resulting in the generation of copy DNA (cDNA). Using the newly transcribed cDNA from the alcohol treated and control samples we utilized polymerase chain reaction (PCR) to target and amplify each mRNA of interest. Each cDNA sample will be mixed with a pre-set master mix, and run in a PCR program specific to each of the five genes provided in the Table 1 below. The PCR program was run overnight to anneal and amplify the target mRNAs. After completion the

samples were loaded into a 1.5% agrose gel and run at 120 volts for 45 minutes. Amplification was then reviewed to see if each sample was present.

Table 1: Chart listing candidate imprinted genes, corresponding restriction enzymes, and resulting band number expression. (9)

Gene	Restriction Enzymes	Expected # Bands (B6)	Expected # Bands (Cast)
H19	Cac8 I RD	**2**	**1**
Igf2	Tas I	**2**	**3**
Peg3	Taa I RD	**1**	**2**
Igf2r	Taq I RD	**1**	**2**
Gtl2	Sfc I RD	**2**	**1**

Once amplification was obtained for all samples we then proceeded to the process of Restriction Enzyme Digest that allowed for digestion of our PCR products into fragments at discrete palindromic sites. DNA restriction endonucleases listed in Table 1 were then added to cleave DNA at specific restriction sites in order to distinguish maternal and paternal expression patterns. Enzyme, buffer, H2O, and DNA were combined in this step. The samples were placed in a hot water bath or a hot plate at appropriate temperatures listed for their corresponding restriction enzymes. Results were then interpreted by viewing gel electrophoresis that was run at 120 volts for 1 hour and 30 minutes. A 1x TAE buffer was combined with agrose powder, heated for 3minutes and 30 seconds, and then mixed with 4ul of Gel Red to make electrophoresis gels. Acrylamide gel electrophoresis was used to view digest of imprinting gene Igf2 in order to separate a smaller range of fragment sizes. A 7.5% gel was made which contained 10.5ml of 30% acrylamide, 27.3ml ultra-pure H2O, 4.2ml 10x TBE, 700ml of 10% APS, and 35ml

TEMED in order to make acrylamide gels. Acrylamide gels were run overnight at 40 volts in order to separate Igf2 fragments. Upon completion of digest gel results, we were able to determine if samples treated expressed imprinted maternal or paternal bands or lacked banding due to alcohol exposure on placental stem cells. Base pair expression allowed us to quantify the size of gene fragments in order to compare parental expression of samples based on fragment size of bands. Densitometry, a protein simple imager, with alpha view software was then used to compare light intensity (pixels) within a selected area of the band for digest images. Digest image measurements were collected for all five imprint genes and results were compared for three trials of each gene. A maternal versus paternal expression percentage was then calculated for samples at the control, 120mg/dl, and 320mg/dl concentration. We were then able to use calculations to determine the level of significance resulting from data collected of each sample in a 95% confidence interval.

All of these experiments are approved by the Texas A&M institution of biosafety # 2012078.

CHAPTER III

RESULTS

Assaying effects of epigenetic expression on imprinted genes was reviewed at a control concentration, 120mg/dl binge alcohol concentration, and 320mg/dl, two times binge level drinker concentration. The five imprinted genes observed included Peg3, H19, Gtl2, Igf2, and Igf2r at an undifferentiated trophectoderm cell level. All five genes selected to test are necessary for proper placental structure and function for the growing embryo. Genes were subjected to testing three times in order to maximize findings and achieve consistent outcomes.

Following amplification of Peg3 cDNA, samples B6, Cast, control, 120mg/dl concentration, and 320mg/dl, were digested, and then run on a 1.5% agarose gel (Figure. 1A)

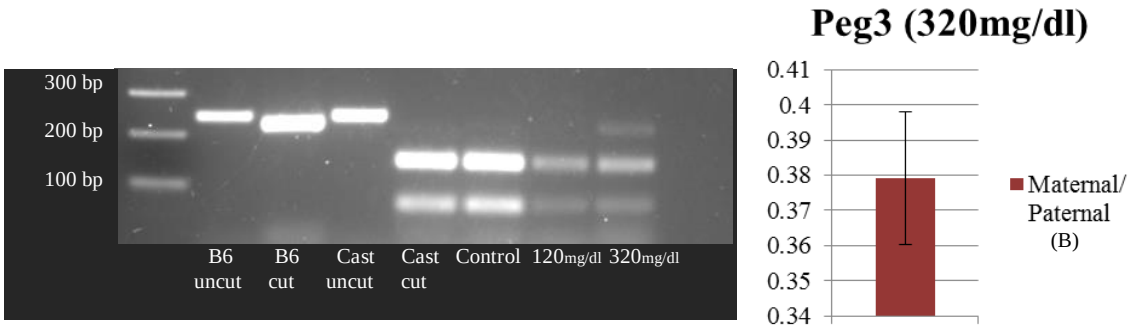

Figure 1A: Digested image and statistical analysis for the gene Peg 3. (A) The sizes of expected fragments following a restriction enzyme digest with Taa I RD for the parental controls B6 and Cast as well as the samples (control, 120mg/dl and 320mg/dl) for the gene Peg3. (B) Statistical analysis of maternal versus paternal expression for Peg 3 at the concentration of 320mg/dl. This data was calculated from results of the densitometry program Alpha View using a 95% confidence interval.

Based on this data we observed that alcohol concentration increased at a level of 320 mg/dl compared to control. Peg 3 imprinting expression in the control TS cells can be seen as paternally imprinted compared to the controls. With exposure to 120 mg/dl, binge level consumption, the Peg3 gene sustains the paternal imprint, but exhibits a decreased expression compared to the control. Surprisingly, TS treated with the concentration of 320 mg/dl acquired an additional maternal band with an equal reduction in the paternal imprint as observed in the 120 mg/dl TS sample. Peg 3 results at the 320mg/dl sample were also compared using using a densitometry program that measured light intensity of imprint banding. This was used to calculate results within a 95% confidence interval. Band intensity of Peg3 for the 320mg/dl concentration was found to be significant for our three tests (Figure 1B). Our data represents conclusive evidence that there must be a predisposition towards bialellic expression at the 320mg/dl concentration level. These results lead us to conclude that high levels of alcohol exposure at the 2x binge level can initiate epigenetic changes of imprinted Peg3 gene expression in mouse placental stem cells.

H19 imprinting expression was determined through the same process and replication steps as exhibited in Peg3 imprinted gene amplification. Results proceeding amplification represented maternal expression that was maintained for H19 imprint when subjected to a 120mg/dl, and 320mg/dl alcohol levels. (Figure. 2)

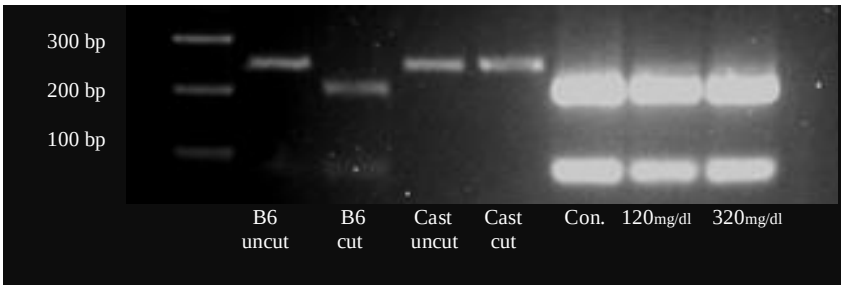

Figure 2: H19 restriction enzyme digest. Descriptive band sizes are listed in the far left column of the gel. The size of expected fragments following a restriction enzyme digest with Cac8 I RD, the parental controls B6 and Cast as well as the samples (control, 120mg/dl and 320mg/dl).

These results lead us to imply that alcohol exposure at a 120 mg/dl, and 320mg/dl levels do not affect imprinting expression of H19.

Gtl2 was a third imprinting gene we chose to examine. During amplification at a PCR, and enzyme digestion level I had difficulty observing a clear paternal banding pattern for my Cast samples. Ultimately, although amplification of cast samples was not accomplished I was able to obtain amplification of Gtl2 cDNA, samples B6, control, 120mg/dl concentration, and 320mg/dl, when digested, and amplified on (1.5%) agrose gel (Figure. 3)

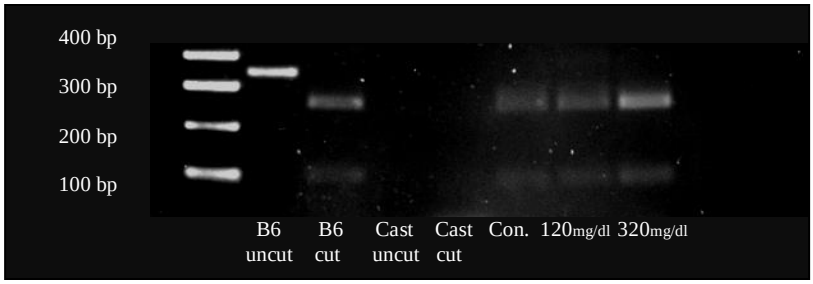

Figure 3: Gtl2 restriction enzyme digest. Descriptive band sizes are listed in the far left column of the gel. The size of expected fragments following a restriction enzyme digest with Sfc I RD, for parental controls B6 and Cast as well as the samples (control, 120mg/dl and 320mg/dl).

Obtained results implicate that maternal expression seems to intensify within trophectoderm cDNA that is exposed to increased alcohol concentrations at a 320mg/dl level. Further testing would be necessary in order to conclude consistency and reliability of results along with amplification of Cast samples.

Subsequent amplification of Igf2 cDNA, samples B6, Cast, control, 120mg/dl concentration, and 320mg/dl, were digested as well, proceeding amplification on a (1.5%) acrylamide gel (Figure. 4A)

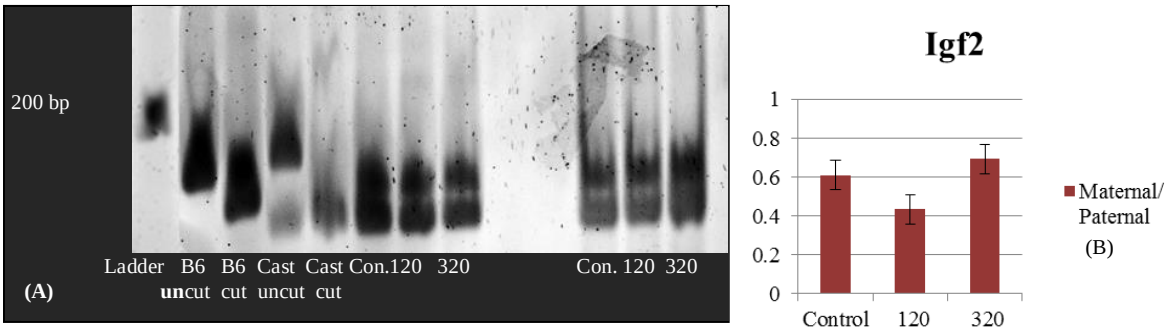

Figure 4: Digested image and statistical analysis for the gene Igf2. Banding following a restriction enzyme digest for the parental controls B6 and Cast as well as the samples (control, 120mg/dl and 320mg/dl). This gel shows results of two Igf2 tests. Parental digests to the left. The second digest following a new PCR amplification of gene Igf2 is shown to the right (control, 120mg/dl, and 320mg/dl). (A) Collected statistical analysis of maternal versus paternal expression for Igf2 control, 120mg/dl, and 320mg/dl. This data was calculated from results of a densitometry program and calculated using a 95% confidence interval to determine level of significance. (B)

Base pair banding of maternal and paternal imprinting have a narrow proximity to each other when run on a gel, therefore we employed acrylamide gel electrophoresis. Acrylamide gel

allows cDNA samples to separate fragments that are in a smaller range to each other in order to view banding more clearly. As observed maternal and paternal banding are small base pair distances from each other represented by all samples. Igf2 banding was compared by using light intensity of a densitometry program that was used to calculate results within a 95% confidence interval. Band intensity of Igf2 for the control, 120mg/dl, and 320mg/dl concentration was not significant within the 95% confidence interval (Figure 4B). This data leads us to conclude variable expression of biallelic Igf2 imprinting gene is expressed through our TS cell samples. Gel amplification after enzyme digest was lastly observed for imprinting gene Igf2r samples B6, Cast, control, 120mg/dl concentration, and 320mg/dl. With our collected data it is perceived that Igf2r is maternally expressed.(Figure. 5)

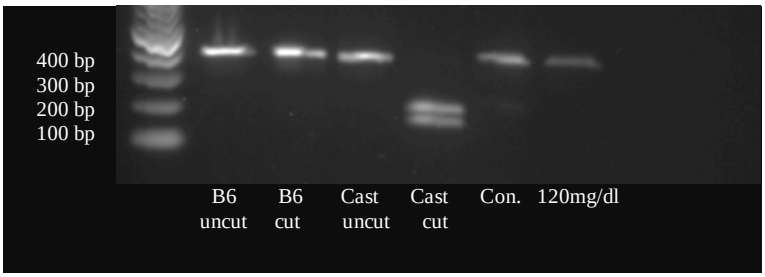

Figure 5: Igf2r restriction enzyme digest. Representative band sizes are listed in the far left column of the gel. The size of expected fragments following a restriction enzyme digest with Taq I RD, the parental controls B6 and Cast as well as the samples (control, and 120mg/dl).

This leads us to assume that concentrations of alcohol at and above a binge level do not affect the epigenetic mechanism of Igf2r imprinting gene.

CHAPTER IV
CONCLUSION

Discussion

The five imprinting genes Peg3, H19, Igf2, Igf2r, and Gtl2 play an important role in the development of placental structure and embryogenesis. Epigenetic expressions of these imprinting genes can be compromised when exposed to teratogens such as alcohol. As can be seen from our data of Peg3, *Paternally expressed 3 gene*, there is a prominent change in imprinting pattern in our 320mg/dl sample. Compared to the control and 120mg/dl TS cell samples, which are paternally expressed, the 320mg/dl sample gained a maternal band initiating biallelic Peg3 expression (Figure 2A). We observed this imprinting pattern consistently over all three Peg3 trials. We confirmed these observations using a densitometry program to further support that the gain in maternal expression is in fact significant (Figure 2B). Our collected data provides evidence that alcohol has an effect on imprinted gene pattern due to significant up-regulated expression in TS cells at a 320mg/dl concentration. This observed biallelic expression at a 320mg/dl concentration in Peg 3 could explain why there is a depression in normal "tumor suppression activity found in glioma and ovarian cells." (7) Proliferation of glioma cells play a role in tumor development of the brain and central nervous system which can lead to serious effects in development (14). Similarly, exposure to alcohol during development has been linked to abnormalities of the central nervous system listed in the fetal alcohol spectrum disorder (5). H19, *Fetal Liver mRNA* gene did not show a significant change in expression when exposed to alcohol, like Igf2, or *Insulin like Growth Factor 2*. Although expression changes were not

observed in our data for H19 and Igf2, retesting would be important to confirm the results. Phenotypic abnormalities of these genes are linked to Beckwith-Wiedemann Syndrome, Wilms tumor, and Silver-Russell Syndrome. Beckwith-Wiedemann Syndrome results in "overgrowth" of fetus during development (8). Wilms tumor is said to lead to attention deficit-hyperactivity disorder, ADHD, and also thought to be associated with low brain development (8). Infants born with Silver-Russell Syndrome are also observed to have "delayed learning," reduced birth rates, as well as prominent facial abnormalities (8). The irregular expression of H19 and Igf2 could also lead to the similar resulting symptoms seen in Fetal Alcohol Disorder, some of which are delayed growth, facial abnormalities, flat nasal bridge, small eyes, reduced brain size, and characteristics of the learning disorder ADHD (5). Unfortunately, we were not able to successfully amplify the cast sample for gene Gtl2, *Gene Trap Locus 2*. There are several methods which we can use to retest the samples for Gtl2 and eliminate variation from sample to sample. By changing the PCR programming we can run the samples in a temperature gradient which will anneal and amplify the target mRNAs more accurately thus allowing us to recover a valuable Gtl2 cast sample. Gtl2 is a tumor suppressor expressed in many tissues. Expression is lost in multiple cancer cell lines of various tissue origins. For Igf2r, *Insulin like Growth Factor 2 Receptor*, our data showed variability which would make this gene necessary for retesting. Igf2r plays a phenotypic role in lysosome trafficking, T cell co-activation, and also serves as a receptor for Igf2 (7). T cell co-activation of lymphocytes or white blood cells, help aid in immunity by fighting off cancer and virus infected cells (8). Low immunity is a symptom of Fetal Alcohol Disorder which could be an effect of irregular expression in Igf2r gene. Phenotypes expressed by infants diagnosed with Fetal Alcohol Disorder express similar characteristics as seen by mutations previously noted for Peg 3, H19, Igf2, Gtl2, and Igf2r. To draw further conclusions

and eliminate variation, methods of improvement in testing are necessary to continue our research. As in the case of Gtl2 improving the PCR protocol will help amplify our gene of interest more accurately. PCR of samples can also be run through a purification column in order to eliminate buffer and salts and isolate pure cDNA to be digested. Purification will improve restriction enzyme digest results by cleaning up the PCR to ensure fragments at discrete palindromic sites are cut. Completing multiple testing of samples, will increase consistency and improve our level of significance at a 95% confidence interval. By improving pipetting, utilizing different alcohol concentrations and cell types, such as neural or embryo stem cells, as well as confirming the accuracy of densitometry data, can help support our results in the near future.

Final Conclusion

The five selected imprinting genes each play important roles throughout gestation that aid in growth, tumor suppression, and other mechanisms needed for healthy development. Similar to Peg 3, H19 and Gtl2 genes are linked to tumor suppressor activity. Beckwith-Wiedemann Syndrome, Wilms tumor, and Silver-Russell syndrome disorders have been linked to mutated imprinting genes H19 and Igf2 (7). Igf2 gene also has a significant role in the epigenome due to its involvement in aiding "fetal development and amplifying glucose mediated insulin secretion (8)." A receptor for Igf2 is Igf2r imprinting gene which is involved in the activation of "growth factor, and lysosome enzymes intracellular trafficking (8)." Specifications in imprinting expression of these genes are the key to the genetic engine that initiates synchronized growth of the placenta and embryo to prevent developmental abnormalities. Abnormalities arise when imprinting expression encounters epigenetic changes driven by external environmental factors

such as alcohol thus causing a change in chromatin structure. Although all imprinting genes do not exhibit an epigenetic change when exposed to alcohol, it is important to study the expression of these genes in order to draw further conclusions. A leading preventable cause of birth defects, prenatal alcohol exposure, is linked to a pattern of teratogenic abnormalities labeled under an umbrella of fetal alcohol syndrome disorders.(5) Our data enhances the knowledge of epigenetic effects governing imprinted gene expression, and aids in a greater understanding of the epigenome. Due to some variability in our results, such as in the case of Igf2r, further testing will be required in order to pinpoint the specific levels of alcohol that would induce an effect on epigenetic expression. Attaining a greater understanding of how alcohol consumption affects imprinting genes during gestation is vital to educating the public and reducing the risk of acquiring Fetal Alcohol Disorder. Additional research of alcohol effects on imprinted genes is imperative to finding consistent evidence that will epitomize a safe level of alcohol consumption during pregnancy.

REFERENCES

(1) Comer, Ronald. Abnormal Psychology. 8th ed. New York, NY: Worth Publishers, 2010. 553. Print.

(2) Diaz, E., and C. Bosco. "Placental hypoxia and foetal development versus alcohol exposure in pregnancy." PubMed. March 2012.47(2):109-17 (2012): n. page. Web. 7 September 2012.

(3) Evangelista, Marco, Ornella Parolini, and Maddalena Soncini. "Placenta-Derived stem cells: new hope for cell therapy?." Cytotechnology. September 28, 2008 (2008): 58(1): 33-42. Web. 7 September 2012

(4) Evers, J., and E. Nelissen. "Epigenetics and the placenta." First published online. October 19 2010. n.page. Web. 13 Sep. 2012.

(5) "Fetal Alcohol Exposure." National Institute on Alcohol Abuse and Alcoholism(NIAAA). U.S.
Department of Health and Human Services, March 2012. Web. 8 Sep 2012

(6) Genetic Science Learning Center. "Epigenetics." Learn.Genetics. Web. 15 March 2013 <http://learn.genetics.utah.edu/content/epigenetics/>

(7) "Gene Cards." Weizmann Institute of Science. n. page. Web. 15 Mar. 2013. < www.genecards.org>.

(8) "Genetics Home Reference ." . U.S. National Library of Medicine, n.d. Web. 15 Mar 2013. <http://ghr.nlm.nih.gov/>.

(9) Golding, Michael. “Generation of Trophoblast Stem Cells”. 925: 49-59. (2012) Pubmed.Web.20 January 2013.

(10) Handwerger, S., and M. Freemark. "The roles of placental growth hormone and placental lactogen in the regulation of human fetal growth and development.." Pub Med. April 13, 2000.(4):343-56 (2000): n. web. 12 Sep. 2012.

(11) Kellam, Nyholt. "Growth hormone and gene expression of in vitro-matured rhesus macaque oocytes." PubMed. 77(4):353-62.2010 April (2010): Web. 8 September 2012.

(12) Messerschmidt, Daniel M. "Should I stay or should I go: Protection and maintenance of DNA methylation at imprinted gene." 7, Issue 9.September 2012 (2012): 969-975. Web. 8 Sep. 2012.

(13) Smirnova, L., A. Sittka, and A. Luch. "On the role of low-dose effects and epigenetics in toxicology." PubMed. 2012.101:499-550 (2012):. Web. 7 September 2012.

(14) Susumu , Otsuka, Maegawa Shinji , Takamura Ayumi , Kamitani Hideki , Watanabe Takashi , Oshimura Mitsuo , and Nanba Eiji . "Aberrant promoter methylation and expression of the imprinted PEG3 gene in glioma." PubMed. 85(4).April 2009 157–165. Web. 15 Mar. 2013. <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3524298/>.

(15) White, Aaron M., Courtney L. Kraus, and Harry Scott Swartzwelder. “Many College Freshmen Drink at Levels Far Beyond the Binge Threshold.” Department of Psychiatry, Duke University Medical Center. 27 May, 2005. Web. 14 September 2012.