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

An Undergraduate Research Scholars Thesis

by

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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: Interdisciplinary Studies

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ABSTRACT

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

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Our environment plays a critical role on our growth and development. In recent years epigenetics has captured the interest of many researchers. Epigenetics looks at how environmental factors affect the genetic material during development. One increasingly common disorder is Fetal Alcohol Spectrum Disorder or FASD. FASD is seen in many infants that have been exposed to alcohol, an environmental toxin, during fetal development. This disorder causes distinct mental and physical abnormalities whose origin comes from alternations to the molecular mechanisms controlling development. In this study, we examined how gene regulation is affected when alcohol is introduced into the environment by looking at genomic imprints. Genomic imprinting is the unique programming of genes that exclusively activate the expression of either the maternal or paternal copy of a gene. A change in the specific expression pattern found in these genes signifies an error in epigenetic programming.

By looking at embryonic stem (ES) cells derived from F1 crosses between the C57BL/6 and *Mus musculus castaneus* strains of mice, we have identified the expression of five imprinted genes. The following five genes: Ube3a, Peg3, H19, Igf2r, and Igf2 were examined in ES cells treated

with three different alcohol concentrations to mimic typical consumptions found in today's society. We have found that three out of the five genes show an up regulation in the maternal imprinting pattern at the 95% confidence interval. These results suggest that alcohol does affect the expression of some imprinted genes and aids in the understanding of the observed clinical phenotypes and the role of epigenetics in the etiology of FASD.

ACKNOWLEDGEMENTS

I would like to give a special thanks to Daria Muller for taking the time to train me, help guide me and support me through all the bumps in the road. I also would like to thank Kylee J. Veazey and Mindy Carnahan for the help and support they provided. Lastly, I would like to thank Dr. Michael C. Golding for providing the cells, lab, materials, and the needed guidance to carry out the research.

NOMENCLATURE

C57BL/6	Maternal genotype
Mus musculus castaneus	Paternal genotype
Peg3	Paternally expressed 3, also known as PW1, End4, ASF-1, Gcap4, Zfp102, AL022617, mKIAA0287
Ube3a	Ubiquitin protein ligase E3A, also known as Hpve6a, mKIAA4216, 4732496B02, 5830462N02Rik, A130086L2Rik
Igf2	Insulin-like growth factor 2, also known as Mpr, M6pr, Peg2, Igf-2, Igf-II, AL033362
Igf2r	Insulin-like growth factor 2 receptor, also known as CD222, CI-MPR, Mpr300, AI661837, M6P/IGF2R
H19	H19 fetal liver mRNA, also known as AI747191
FASD	Fetal Alcohol Syndrome Disorder
Bp	Base pair
ES cells	Embryonic Stem Cells
cDNA	Copy DNA

CHAPTER I

INTRODUCTION

Background

Our environment plays a huge role in our development. In recent years epigenetics has become a topic of major interest for many scientists. Epigenetics looks at how our environment and what we expose ourselves to plays a role in human development. Epigenetics works on three levels of a cell: controlling the functions of a gene, modifying RNA splicing into a protein, and controlling the topographic distribution [9].

During mammalian reproduction a subset of genes are regulated through a specialized epigenetic mechanism that restricts patterns of gene expression based on their inheritance from either the mother or father. This "genomic imprint" is passed on by one of the parents to the fetus, causing one allele to be active while other remains silent. [5]. In mammalian development, DNA methyl marks are attached to a parental gene copy and are involved in transcriptional silencing. During early development, various teratogens can alter expression of imprinted genes leading to developmental problems in the embryo [5]. More importantly genes that are regulated by imprinting can have an even larger effect on placentation and embryonic metabolism [7].

Each gene contains a specific combination of base pairs, ATCG, that code for different proteins needed by the cells. Each gene has a specific purpose during development. There are numerous imprinted genes but we choose to look at five particular genes because of the role they play during development. Ube3a is biallelically expressed gene and it is responsible for making E3A ubiquitin protein [10]. This protein places a target on other proteins that need to be broken down

because they are damaged or are no longer needed in the cell [10]. Peg3 is only biallelically expressed in the early embryo and it is responsible for cell proliferation and regulating p53 mediated apotosis [10]. Igf2r is maternally expressed while Igf2 is paternally expressed [10]. These two genes work together whereby Igf2r is the receptor for Igf2 which is insulin like growth factor [10]. Lastly, H19 is a maternally expressed and functions as a tumor suppressor [10]. Errors in the regulation of H19, Igf2r, and Igf2 tend to contribute to Beckwith-Wiedemann disease when their expression is not correctly regulated. Beckwith-Wiedemann is a "growth disorder that causes large body size, large organs, and other symptoms" [10]. Igf2 is also associated with Russell- Silver syndrome, several cancers, and eating and weight disorders [8]. Ube3a when altered causes Angelman syndrome which is a "neuro-genetic disorder that . . . causes developmental delay, lack of speech, seizures, and walking and balance disorders" [10].

In mammalian development, an organism starts from the fertilization of egg and sperm, syngamy, forming a zygote. Through mitosis, the cell replicates until it reaches 32-64 cell mass called a morula. This structure will differentiate into a blastocyst composed of an external and internal cell mass each with different developmental fates. The internal mass of cells will grow to form what is called an embryo; this structure is composed of cells called embryonic stem (ES) cells. Once the embryo reaches its eighth week it is referred to as a fetus and will continue to grow and develop until birth. The external cells will give rise to the placenta, an organ that surrounds the embryo and attaches to the lining of the womb during mammalian development [4]. This organ's purpose is to protect the embryo during development by creating growth hormone (hCS), preventing infection, separating the mother and the baby's blood, providing nutrients, supplying oxygen, and excreting waste [4]. However, the placenta is not able to keep

everything out. If the mother consumes alcohol, it can pass through the placenta and harm the embryo by preventing proper growth and development.

During the development of a baby, it is important for the mother to take care of herself in order to prevent any problems for the baby. However, sometimes the mother is unaware of the pregnancy and could unintentionally introduce alcohol into her body. Some doctors say it is okay to drink small amounts of wine while pregnant [2]. According to the Texas Adoption Resource Exchange, "As many as 12,000 infants are born each year with [Fetal Alcohol Syndrome] FAS" [1]. A child that is born with FAS can display malformations of the face and/or body, as well as poor growth [3]. Along with the physical features, children with FAS can also display cognitive and motor impairment as well as behavioral problems [3].

Problem

The question that is being examined is, does alcohol affect the mechanisms regulating genomic imprinting? I hypothesize that ES cells exposed to alcohol will display a change in their genomic imprint ultimately leading to a loss of monoallelic expression pattern.

CHAPTER II

METHODS

In this experiment, we will utilize embryonic stem cells derived from F1 crosses between a C57BL/6 (B6) and *Mus musculus castaneus* (CAST) mice. This F1 generation contains several nucleotide polymorphisms that allow for the parental identification of specific imprinted genes. These polymorphisms will help identify which imprinted genes are being expressed in the F1 generation through the use of a restriction enzyme based assay; one allele will be cut while the other remains unaffected. The table below lists the five imprinted genes that will be examined along with their corresponding restriction enzymes used to cut each gene and the number of expected bands.

Table 1: Resulting band sizes following a restriction enzyme digestion of candidate imprinted genes

Gene	Restriction Enzymes	PCR size	B6 band sizes	Cast band size
H19	**CaC8**	**235bp**	**173bp, 62bp**	**235bp**
Igf2	**Tsp 509 IRd**	**202bp**	**178bp, 24bp**	**163bp, 15bp, 24bp**
Peg3	**HpyCH3**	**239bp**	**224bp, 16bp**	**148bp, 76bp, 16bp**
Igf2r	**Taq I**	**388bp**	**388bp**	**210bp, 178bp**
Ube3a	**HpyCH3**	**418bp**	**418bp**	**336bp, 82bp**

Table 1: The table contains all the imprinted genes with the expected band sizes of both the maternal copy, B6, (C57BL/6) and paternal copy, CAST, (*Mus musculus castaneus*) before and after the restriction enzyme digestion [6].

The alcohol concentrations used for this experiment were: 60mg/dl, 120mg/dl, and 320mg/dl. These alcohol concentrations were chosen because they resemble 0.06% blood alcohol level just under the legal limit, 1.5 times the legal limit, and 4 times the legal limit often observed during binge drinking these three levels which are commonly observed in today's society. [11] After the

cells have been exposed to the three alcohol concentrations, the cells were harvested. The RNA was extracted from the cells. Through the use of reverse transcriptase (RT) the RNA was changed to copy DNA (cDNA). The cDNA sample was analyzed using the Polymerase Chain Reaction (PCR). PCR products were run on an agarose gel for analysis. Each of the genes was cut using the specific enzymes listed in Table 1 above. The enzymes cut the DNA at specific polymorphisms sites that will allow us to distinguish between maternal and paternal expression of genes. The digest used to cut the DNA consist of water, buffer, enzyme specific for each gene, and PCR DNA. The digested DNA will then be run on an agarose gel using 1x TAE buffer at a 120V for 1 hour -1 hour and 30 minutes. All of the genes except the Igf2 were run on a 1.5% agarose gel to visualize the digest. A 7.5% acrylamide gel was used to view the Igf2 digest since the bands required a higher degree of resolution.

After the digestion the samples were run on a gel to view the resulting DNA fragments. The gel was viewed on a ProteinSimple imager with AlphaView software. The brightness of each band was analyzed using a densitometry program within the Alphaview software, which calculated the average range of light intensity (i.e pixels) within a given area of the band. The maternal and paternal bands were then compared in a ratio to the control. A 95% confidence interval was then calculated and used to determine if there was a significant difference between the three alcohol concentrations**.** Three trials were carried out for each gene and the average ratios calculated. We then used the 95% confident interval in order to identify which alcohol concentration demonstrated a significant change in expression.

CHAPTER III

RESULTS

The following five genes were examined to see if alcohol affects their expression pattern: Peg3, Ube3a, H19, Igf2r, and Igf2. All five genes were digested using the listed enzymes in Table 1.

Peg3

We first chose to look at the gene Paternally Expressed 3 or Peg3. Figure 2 shows the image that was produced after digesting the Peg3 PCR product with HpyCH3. Following digestion, the maternal sample produced two bands, one band that is 224 bp and another with 16 bp. The paternal band was cut into three bands 148 bp, 76 bp, and 16 bp. When we look at the control we see it cut into four bands 224 bp, 148 bp, 76 bp, and 16 bp. The control and samples contain both the maternal and paternal band patterns categorizing Peg3 as biallelically expressed in ES cells. This expression pattern is seen in all three alcohol samples. This process was repeated 3 times to verify the results.

Figure 1: Peg3 Digest Image

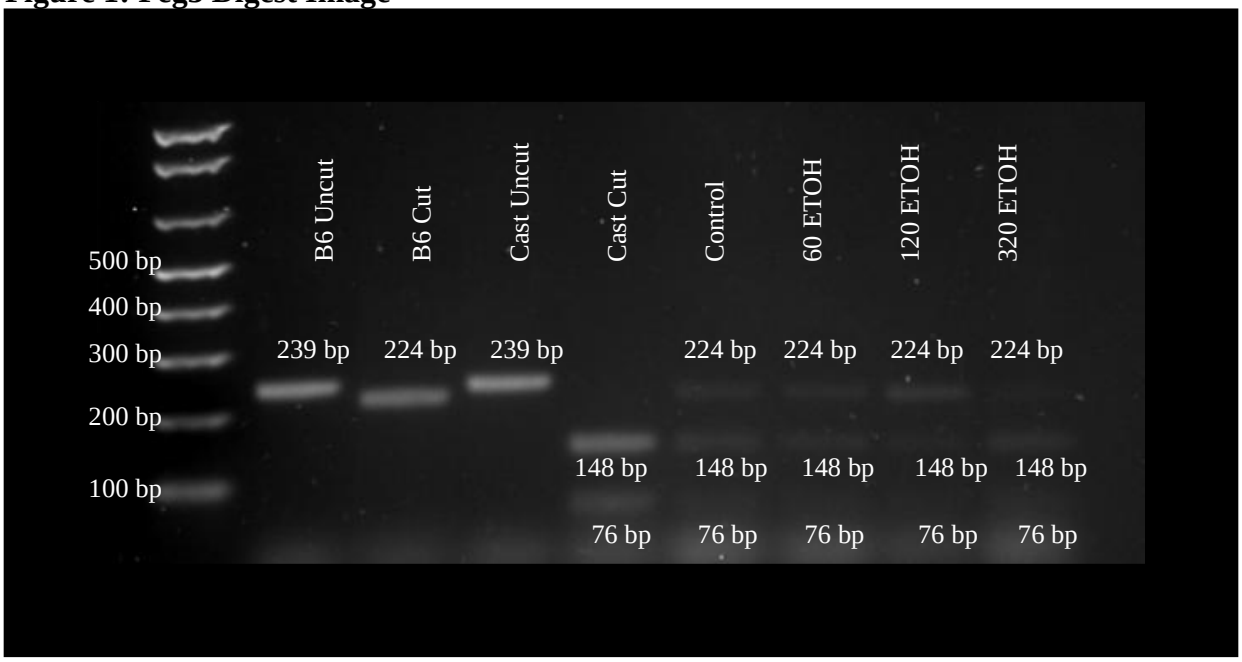

Figure 1. Peg3 digest on a 1.5% agarose gel ran for an hour and 30 minutes. The maternal band was cut into two bands one at 224 bp and 16 bp*. The paternal band was cut into three bands 148 bp, 76 bp, and 16 bp*. The control shows four different bands at 224 bp, 148 bp, 76 bp, and 16 bp*. This shows that Peg3 is biallelic in ES cells. This cut pattern is seen in all three alcohol concentrations. *denotes band sizes too small to be seen on the gel.

The digested gel images were then analyzed using densitometry to measure the brightness of each band that were produced. Figure 2 below shows the average ratio between the maternal and paternal band brightness. The average ratio calculated for the samples are as followed: control 0.6343, 60 mg/dl 0.6541, 120 mg/dl 0.5937, and 320 mg/dl 0.6102. The data shows no significant change in expression for Peg3 at all three alcohol concentrations.

Figure 2: Peg3 Densitometry Results

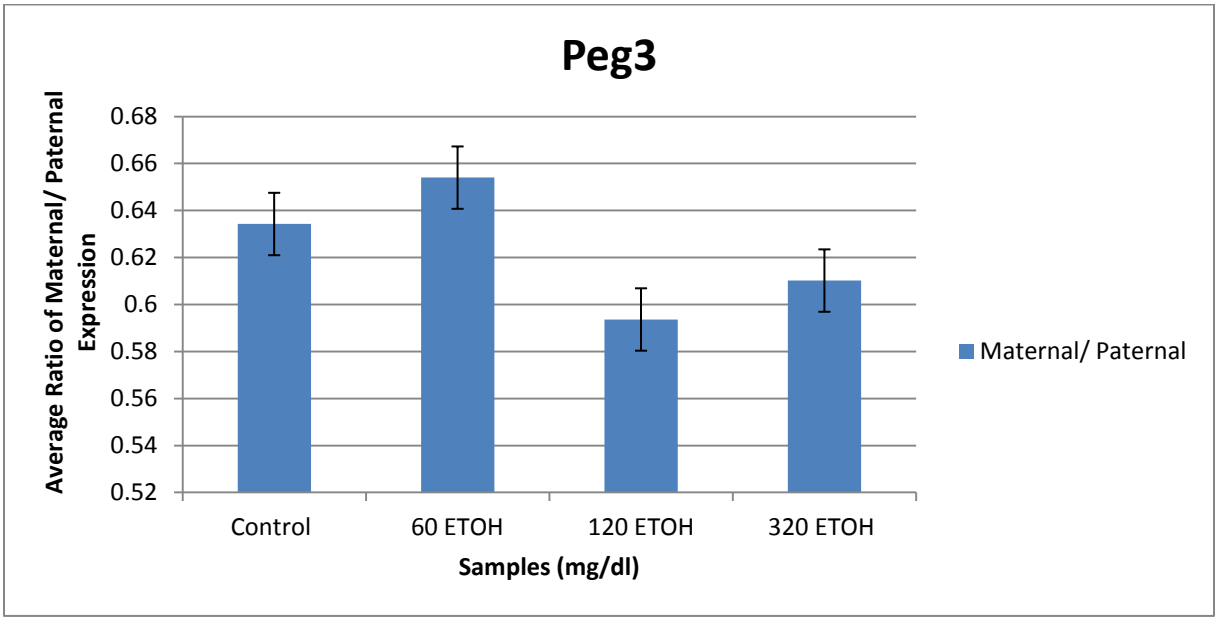

Figure 2: Peg3 densitometry results. The figure above shows the ratio of maternal to paternal expression of imprinted bands for Peg3. Compared to the control, all three alcohol concentrations show no difference in expression ($p > .05$). Sample size was 3 trials (N=3).

H19

The second gene that was looked at was H19 fetal liver mRNA, or H19. Figure 3 shows the image that was produced after digesting H19 with CaC8. When the maternal sample was cut, it produced two bands 173 bp and another at 62 bp. The paternal band doesn't get cut and instead produces one band at 235 bp. When we look at the control we see it cut into three bands 235 bp, 173 bp, and 62 bp. The control and samples contain both the maternal and paternal band patterns categorizing H19 as biallelic expressed in ES cells. This expression pattern is seen in all three alcohol samples. This process was repeated three times to verify the results.

Figure 3: H19 Digest Image

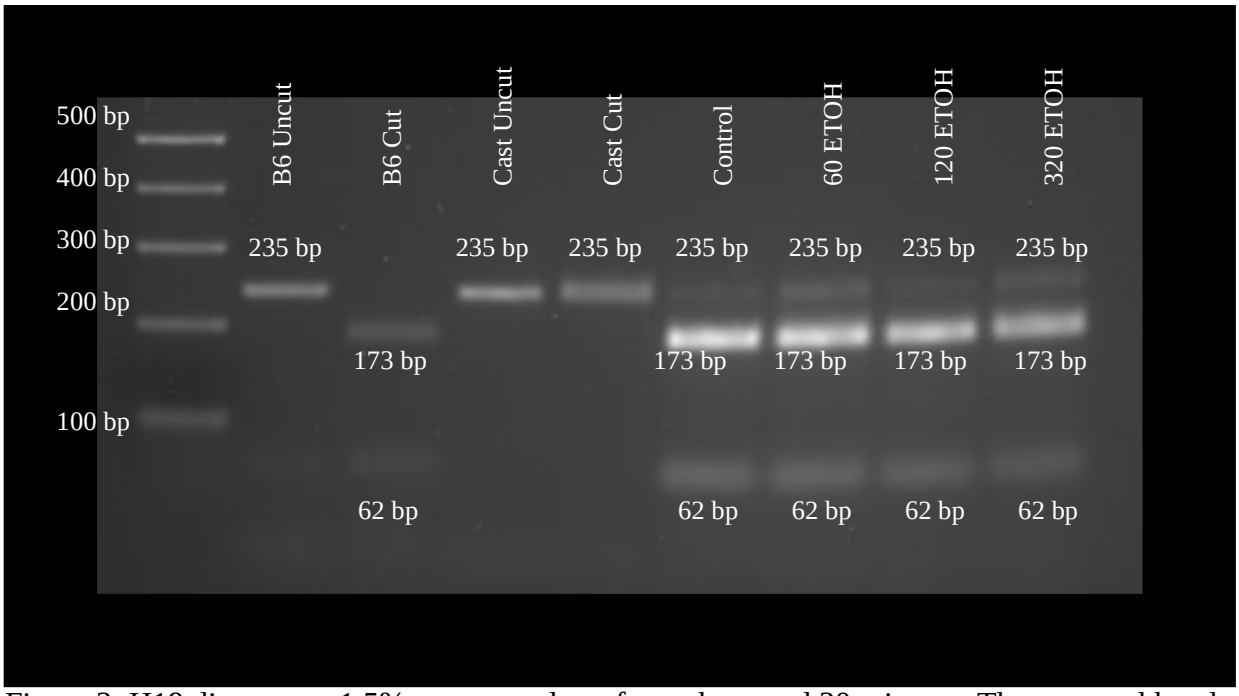

Figure 3: H19 digest on a 1.5% agarose gel ran for an hour and 30 minutes. The maternal band was cut into two bands one at 235 bp and 62 bp. The paternal band only produced a band at 235 bp. The control shows three different bands at 235 bp, 173 bp, and 62 bp. This shows that H19 is biallelic in ES cells. This cut pattern is seen in all three alcohol concentrations.

The digest gel images were then analyzed using densitometry to measure the brightness of each band that was produced. Figure 4 below shows the average ratio between the paternal and maternal band brightness. The average ratio calculated for the samples are as followed: control 0.3012, 60 mg/dl 0.3041, 120 mg/dl 0.2725, and 320 mg/dl 0.2958. The data shows an up regulation in the maternal bands at the 120 mg/dl alcohol concentration ratio compared to the control using a 95% confidence interval. However the 60 mg/dl and 320 mg/dl concentrations show no significant change in expression.

Figure 4: H19 Densitometry Results

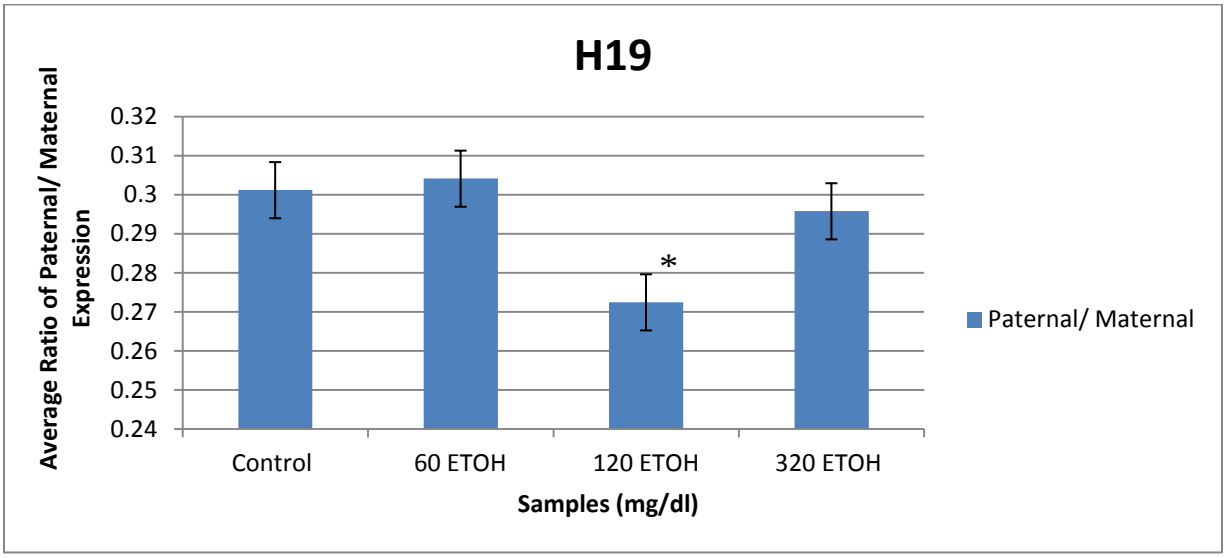

Figure 4: The figure above shows the ratio of paternal to maternal expression of imprinted bands for H19. Compared to the control the 120 mg/dl alcohol concentrations show a significant down regulation in paternal copy and an up regulation in maternal copy. * Denotes a significant difference ($p < 0.05$) in the ratio between the paternal/maternal band expressions. Sample size was three trials (N=3).

Ube3a

The third gene that we chose to look at was ubiquitin protein ligase E3A, or Ube3a. Figure 5 shows the image that was produced after digesting Ube3a with HpyCH3. The maternal band only produced one band at 418 bp. The paternal band produced two bands one at 336 bp and 82 bp. When we look at the control we see it cut into three bands 418 bp, 336 bp, and 82 bp. The control and samples contain both the maternal and paternal band patterns categorizing Ube3a as biallelically expressed in ES cells. This expression pattern is seen in all three alcohol samples. This process was repeated three times to verify the results.

Figure 5: Ube3a Digest Image

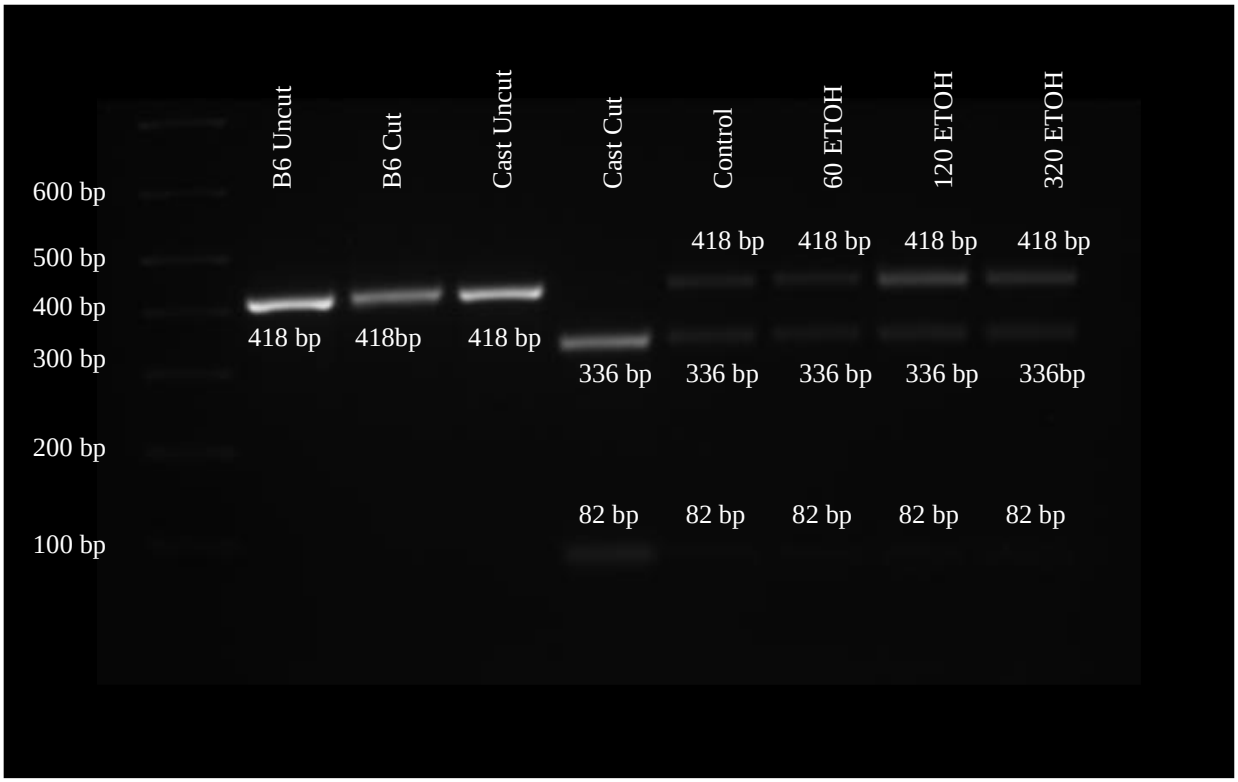

Figure 5: Ube3a digest on a 1.5% agarose gel ran for an hour and 30 minutes. The maternal band only produced one band at 418 bp. The paternal band was cut into two bands at 336bp and 82bp. The control shows three different bands at 418 bp, 336 bp, and 82 bp. The digest shows that Ube3a is biallelic in ES cells. This cut pattern is seen in all three alcohol concentrations.

The digest gel images were then analyzed using densitometry to measure the brightness of each band that was produced. Figure 6 below shows the average ratio between the maternal and paternal band brightness. The average ratio calculated for the samples are as followed: control 0.7999, 60 mg/dl 0.8822, 120 mg/dl 1.1897, and 320 mg/dl 0.9420. The data shows no significant change in the ratio expression for all three alcohol concentrations.

Figure 6: Ube3a Densitometry Results

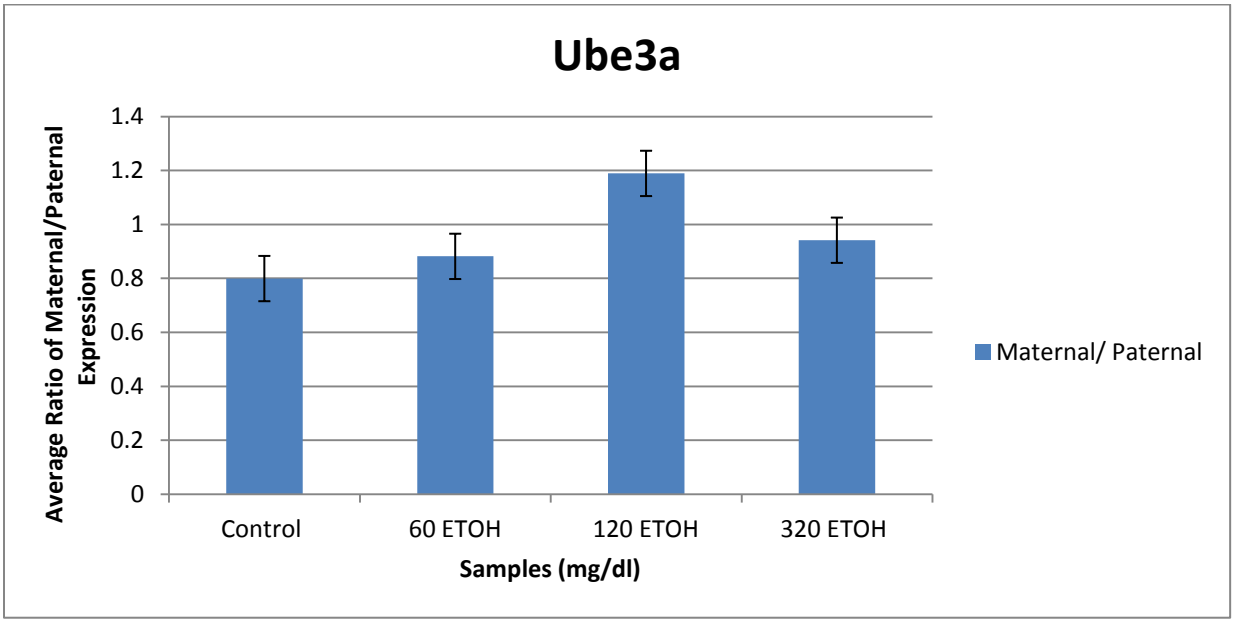

Figure 6: Ube3a densitometry results. The figure above shows the ratio of maternal to paternal expression of imprinted bands for Ube3a. Compared to the control for all three alcohol concentrations no difference in expression was observed ($p > .05$). Sample size was three trials (N=3).

Igf2r

The fourth gene that was looked at was insulin-like growth factor 2 receptor, or Igf2r. Figure 7 shows the image that was produced after digesting Igf2r with Taq I. The maternal band only produced one band at 388 bp. The paternal band produced two bands one at 210 bp and 178 bp. When we look at the control we observed that it cut it into three bands 388 bp, 210 bp, and 178 bp. The control and samples contain both the maternal and paternal band patterns categorizing Igf2r as biallelically expressed in ES cells. This expression pattern is seen in all three alcohol samples. This process was repeated three times to verify the results.

Figure 7: Igf2r Digest Image

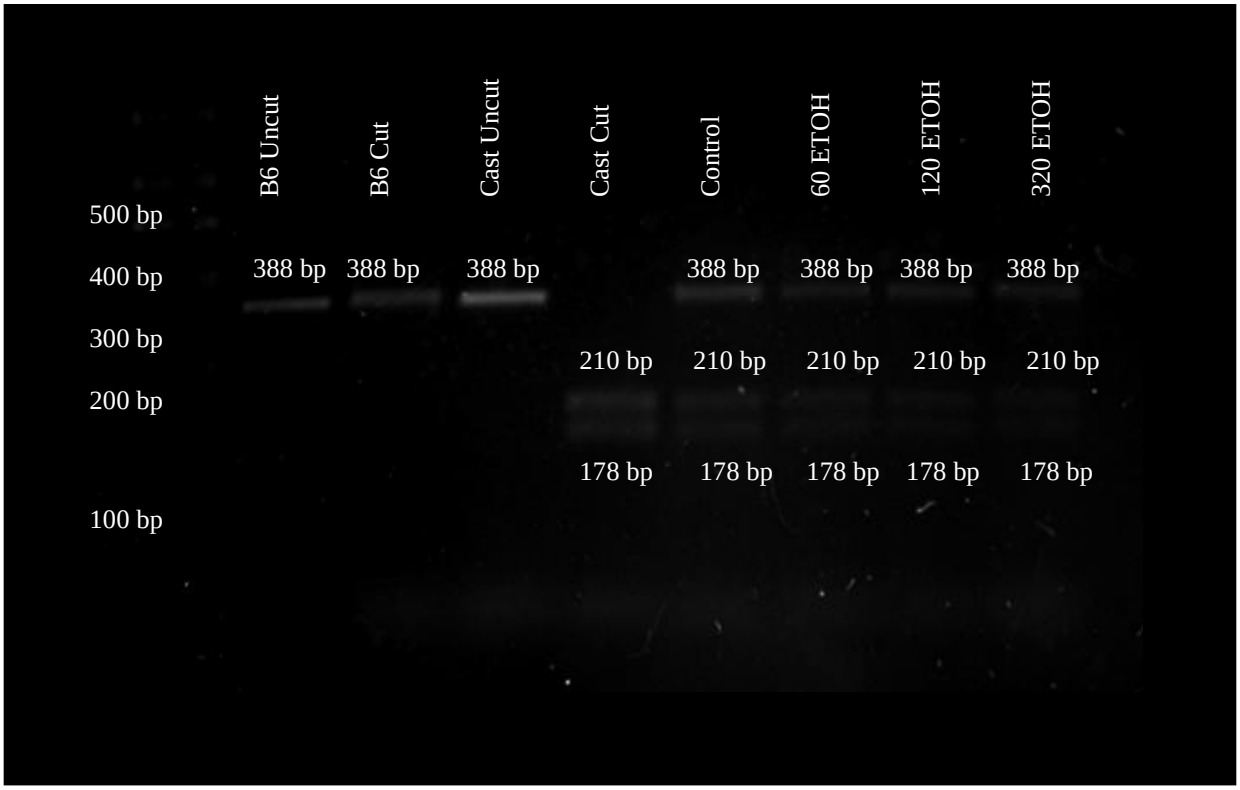

Figure 7: Igf2r digest on a 1.5% agarose gel ran for an hour and 30 minutes. The maternal band only produced one band at 388 bp. The paternal band was cut into two bands at 210 bp and 178 bp. The control shows three different bands at 388 bp, 210 bp, and 178 bp. This shows that Igf2r is biallelic in ES cells. This cut pattern is seen in all three alcohol concentrations.

The digested gel images were then analyzed using densitometry to measure the brightness of each band that was produced. Figure 8 below shows the average ratio between the maternal and paternal band brightness. The average ratio calculated for the samples are as followed: control 0.5202, 60 mg/dl 0.6181, 120 mg/dl 0.5338, and 320 mg/dl 0.5521. The data shows an up regulation in the maternal copy for the samples treated with the concentration of 60 mg/dl compared to the control using a 95% confidence interval. However the 120 mg/dl and 320 mg/dl concentrations show no significant change in expression.

Figure 8: Igf2r Densitometry Results

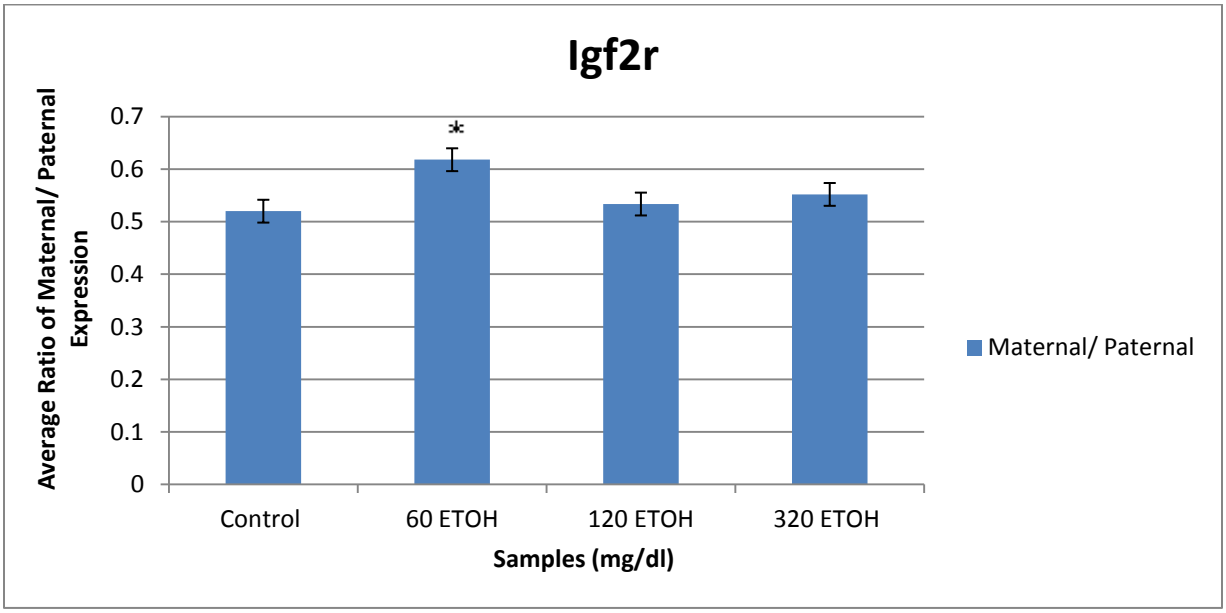

Figure 8: The figure above shows the ratio of maternal to paternal expression of imprinted bands for Igf2r. Compared to the control the 60 mg/dl alcohol concentration shows an up regulation in the maternal copy and a down regulation in the paternal. * Denotes a significant difference ($p < 0.05$) in the ratio between the maternal/paternal band expressions. Sample size was three trials (N=3).

Igf2

Lastly we looked at insulin-like growth factor 2, or Igf2. Figure 9 shows the image that was produced after digesting Igf2 with Tsp 509 IRd. The B6 maternal sample only produced one band at 178 bp. The castaneus paternal sample produced one band at 163 bp. The control was cut into two bands 178 bp, and 163 bp. The control and ethanol treated samples contain both the maternal and paternal band patterns categorizing Igf2 as biallelically expressed in ES cells. This expression pattern is seen in all three alcohol samples. This process was repeated three times to verify the results.

Figure 9: Igf2 Digest Image

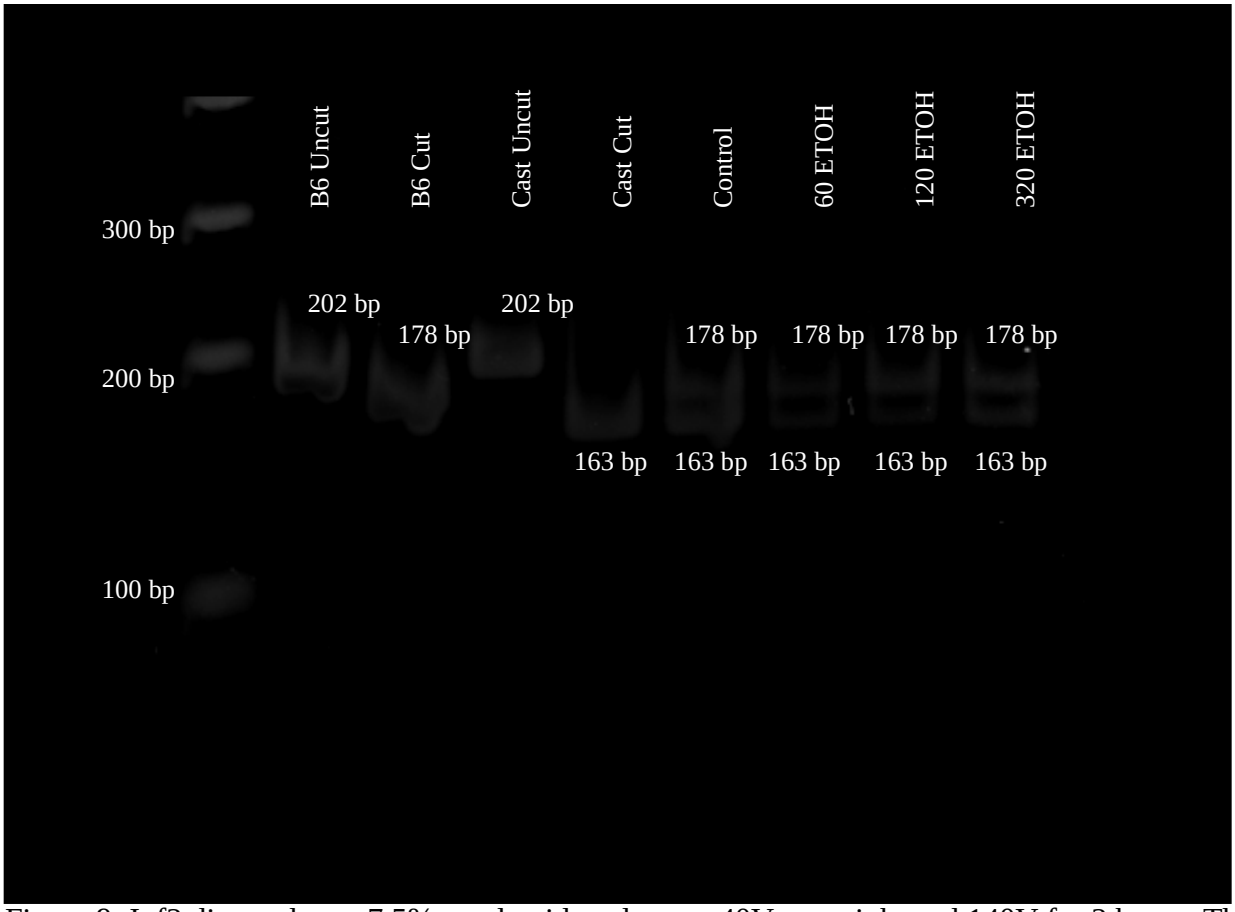

Figure 9: Igf2 digested on a 7.5% acrylamide gel ran at 40V overnight and 140V for 2 hours. The maternal band produced two bands at 178 bp and 24 bp*. The paternal band was cut into three bands at 163 bp, 24 bp*, and 15 bp*. The control shows four different bands at 178 bp, 163 bp, 24 bp*, and 15 bp*. This shows that Igf2 is biallelic in ES cells. This cut pattern is seen in all three alcohol concentrations. *Denotes a band too small to be seen on the gel image.

The digested gel images were then analyzed using densitometry to measure the brightness of each band. Figure 10 below shows the average ratio between the brightness of the maternal and paternal band. The average ratio calculated for the samples are as followed: control 1.0302, 60 mg/dl 1.0688, 120 mg/dl 1.1200, and 320 mg/dl 1.0356. The data shows an up regulation in the maternal copy for the 120 mg/dl alcohol concentration ratio compared to the control using a 95%

confidence interval. However the 60mg/dl and 320 mg/dl concentrations show no significant change in expression.

Figure 10: Igf2 Densitometry Results

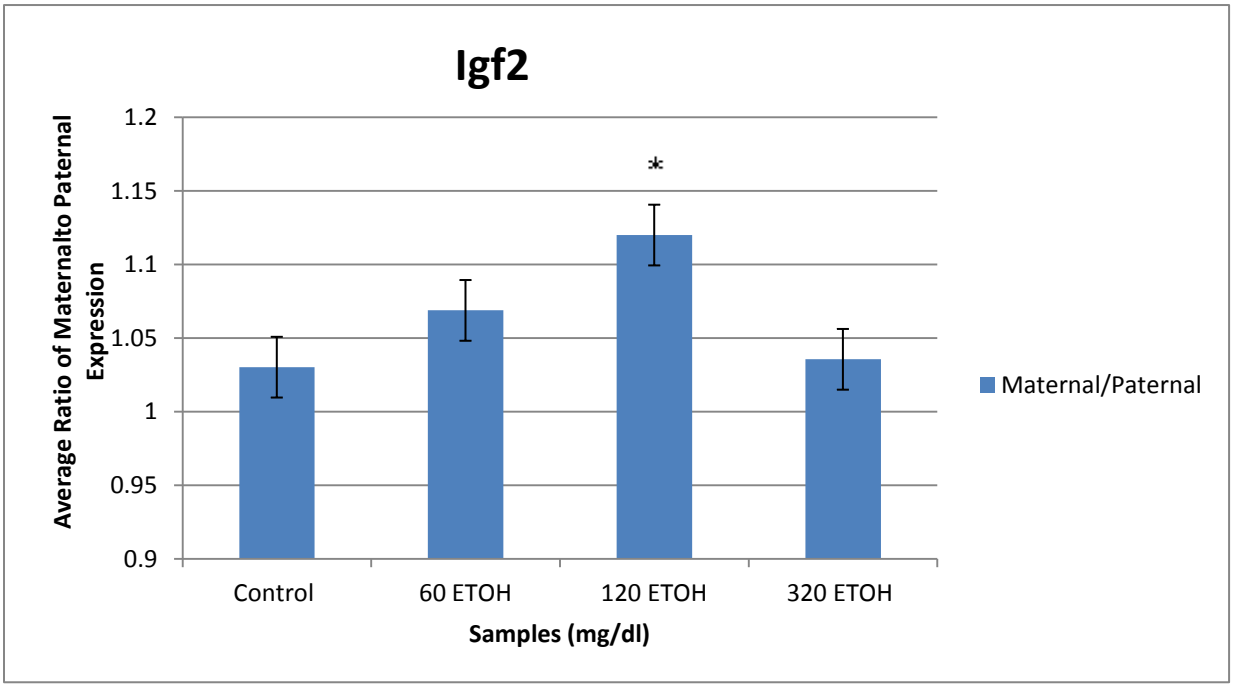

Figure 10: The figure above shows the ratio of maternal to paternal expression of imprinted bands for Igf2. Compared to the control, the 120 mg/dl alcohol concentration shows an up regulation maternal copy and a down regulation in the paternal copy. * Denotes a significant difference ($p < 0.05$) in the ratio between the maternal/paternal band expressions. Sample size was three trials (N=3).

CHAPTER IV

CONCLUSION

Discussion

Imprinting genes Peg3, Ube3a, Igf2r, Igf2, and H19 are some of the key regulatory players in a developing embryo. In this experiment we observed how these genes are affected under various alcohol concentrations. The ES cell samples were exposed to three different alcohol concentrations and digested to view the specific imprinting patterns.

After analyzing the imprinting patterns of genes Peg3 and Ube3a we noticed that while there were changes in the expression patterns for each trial, the changes were not significant enough ($p > 0.05$) to conclude that alcohol is in fact having an effect on the expression of these imprinted genes. However while Peg3 and Ube3a didn't prove to show a significant change at the 60 mg/dl, 120 mg/dl and the 320 mg/dl ETOH concentrations, we did find that H19, Igf2, and Igf2r showed a significant change.

When analyzing the imprinting pattern of the gene H19 we found an up regulation of the maternal imprint in the 120 mg/dl concentration. Igf2 also shows a similar result in that it produces an up regulation in the maternal imprint for the 120mg/dl concentration. Alternatively, while Igf2r also shows an up regulation in the maternal copy, we found a significant change in the 60 mg/dl concentration instead of 120 mg/dl.

Also, we noticed that all two of the three genes, H19 and igf2 are all found on the same chromosome; chromosome 7 in mouse cells. Not only are these genes found on the same chromosome but they all contribute to similar syndromes when mutations occur. Both H19 and Igf2 can lead to Beckewith-Wiedemann syndrome. H19 is can also cause Wilma's tumor and Igf2 can also lead to Russell- Silver syndrome. The symptoms seen in these three syndromes are closely linked to the symptoms displayed in children with FASD.

This signifies that teratogens such as alcohol can have an effect on the expression of the imprinted genes. While the mechanism behind alcohol's effects on imprinting is yet to be determined the results from this experiment holds plenty of promise for future studies in this field. The evidence further proves that not all genes are affected at one alcohol concentration, but rather different alcohol concentrations affect some genes more than others.

Future research needs to be continued to see how other imprinted genes are affected during development. There may be other imprinted genes that have not been found that may also hold some answers to understanding how the different syndrome works. The methodology of our experiment could also be improved. There were some chances for human error to skew the data. Instead of using the densitometry program to measure the expression of the band brightness, using a qPCR (quantitative real time polymerase chain reaction) to analyze gene expression may prove to be more effective. Another way to validate and prove the reliability of our result would be to replicate the entire process starting from the RT (reverse transcriptase) step several times using the same cells to ensure that the changes are consistent.

Conclusion

FASD affects 12,000 children each year. Being able to understand how our genetic recipe is affected by toxins such as alcohol could help in better educating pregnant women and the public. We could help doctors by providing actual proof to support or disapprove the statement that some doctors give to women about drinking wine while pregnant. It could also lead to possible treatment of defects or prevention if the baby has been affected. But in order for these outcomes to occur future research needs to continue. Imprinting is a new frontier and by studying this will help better understand how imprinting plays a role in our development. The results show that alcohol does have an effect at different alcohol concentrations in some genes. Future work needs to be continued because there are clear similarities between FASD and disorders involved for these genes.

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