THE INFLUENCE OF ALCOHOL EXPOSURE ON ALTERNATIVE SPLICING OF THE G9a HISTONE METHYLTRANSFERASE

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

The Influence of Alcohol Exposure on the Alternative Splicing of the G9a Histone Methyltransferase

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Alternative splicing is a mechanism that leads to the generation of multiple mature mRNA products from a single gene. This process can produce isoforms, resulting in proteome diversity. In this study, we explore how EtOH exposure affects the alternative splicing of the G9a histone methyltransferase. G9a is an enzyme responsible for histone H3 lysine 9 dimethylation. The inclusion of G9a exon 10 is essential for neuron differentiation. The inclusion of the alternatively spliced exon 10 increases the nuclear localization of this histone methyltransferase and promotes a positive feedback loop that reinforces cellular commitment to differentiation. Studies in our laboratory have shown that EtOH exposure disturbs histone H3 lysine 9 dimethylation; therefore, this thesis describes the effect of EtOH exposure on alternative splicing on G9a histone methyltransferase. We examine the specific effects of EtOH exposure on inclusion or exclusion of exon 10 using a neural stem culture model. Here, cells were treated with 160, and 240 mg/dL EtOH for 72 hours, then allowed to recover for 4 days. We found that alcohol promoted the inclusion of exon 10, which may explain the increased levels of histone H3 lysine 9 dimethylation observed in alcohol-exposed cells.

DEDICATION

This project is dedicated to my parents. Thank you for all your support and for giving me the opportunity to pursue my dreams.

ACKNOWLEDGEMENTS

I would like to thank Dr. Michael Golding who has given me the opportunity to work in his laboratory for the past three years, as well as his guidance for this project and my future goals. I would also like to thank Richard Chang for being a mentor to me these past three years. His guidance and patience have been crucial to my achievements as well as my development into a scientist.

NOMENCLATURE

cDNA	complementary deoxyribonucleic acid
EtOH	ethanol
H3K9me2	histone H3 lysine 9 dimethylation
PCR	polymerase chain reaction

CHAPTER I

INTRODUCTION

Fetal Alcohol Spectrum disorder (FASD) is a well-known term that encompasses a range of possible disorders from prenatal alcohol exposure. This study focuses on the effects of alcohol exposure on alternative splicing of G9a histone methyltransferase (Figure 1). Alternative splicing is a mechanism that leads to the generation of various mature mRNA products from a single gene. We examine the specific effects of ethanol (EtOH) exposure on the inclusion or exclusion of G9a exon 10 using a neural stem cell culture model.

Multiple studies have supported the idea that prenatal alcohol exposure can change histone epigenetic marks in a developing fetus [3]. Previous studies have suggested that EtOH can alter histone modifications, specifically methylation, and influence histone methylation marks [1,3]. G9a histone methyltransferase is an enzyme responsible for histone H3 lysing 9 dimethylation (H3K9me2) [2]. The splicing of G9a results in two isoforms, one isoform including exon 10 and the other isoform lacking exon 10.

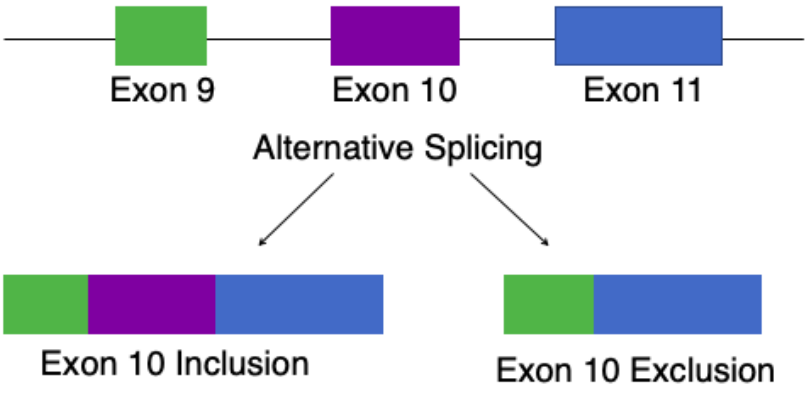

Figure 1. Influence of EtOH exposure on the alternative splicing mechanism of the G9a gene.

The inclusion of alternatively G9a exon 10 increases H3K9me2 levels due to higher nuclear localization [2]. G9a-exon 10 inclusion results in the appearance of increased H3K9me2 on the G9a exon 10 within the genome [2]. This suggests a positive feedback loop where G9a regulates its own alternative splicing and reinforces cellular commitment to differentiation [2]. Moreover, studies in our laboratory have shown that EtOH exposure disturbs H3K9me2; therefore, we hypothesize that alcohol exposure will influence the alternative splicing of G9a histone methyltransferase by promoting inclusion of exon 10.

There are two potential outcomes from this experiment concerning the effects of alcohol exposure on the inclusion or exclusion of G9a exon 10. One can postulate that if alcohol promotes the exclusion of G9a exon 10 then H3K9me2 levels would be lower since there will be less nuclear localizations of G9a. Consequently, this may lead to inadequate differentiation, which may result in birth defects. Conversely, if alcohol promotes the inclusion of G9a exon 10 this would promote premature differentiation.

CHAPTER II

MATERIALS AND METHODS

Neural stem cell culture and EtOH exposure

Obtaining the primary mouse fetal cerebral cortical neuroepithelial stem cells and culturing have been detailed in the previous study by Veazey, K.J., et al [1]. The neuroepithelial stem cells used in this study were cultured in mediums containing 160 and 240 mg/dL EtOH or control cultures containing no EtOH [1]. Cells were harvested at days-3 and 7. Day-3 cells were maintained in stem cell state and exposed to EtOH for three days [1]. Day-7 cells were maintained in stem cell state and exposed to EtOH for three days; then allowed a 4-day recovery period [1].

RNA analysis and reverse transcription

The cultured cells were spun down, washed once in cold PBS, and RNA isolated using Trizol (Cat# 15596026; Invitrogen) according to the manufacturer's protocol [1]. Spectrophotometric analysis was used to determine the RNA nucleic acid concentrations. The mRNA was reverse-transcribed into complementary deoxyribonucleic acid (cDNA) using the High Capacity RNA-to-cDNA Kit from Thermo Fisher Scientific (Thermo). A programmable thermal cycler, 2 μg of total RNA per 20-μL reactions, reverse transcriptase, and random primers were used according to the provided instructions (CAT# 4387406; Thermo).

Primer Design, PCR, and Gel Electrophoresis

Three primer pairs were designed using NetPrimer. Primer pair one spanning a 331-nucleotide amplicon (Figure 2A), primer pair two spanning a 155-nucleotide amplicon (figure

2B), and primer pair three spanning a 146-nucleotide amplicon (Figure 2C). The three primer pairs were ordered from Invitrogen and were re-suspended in water.

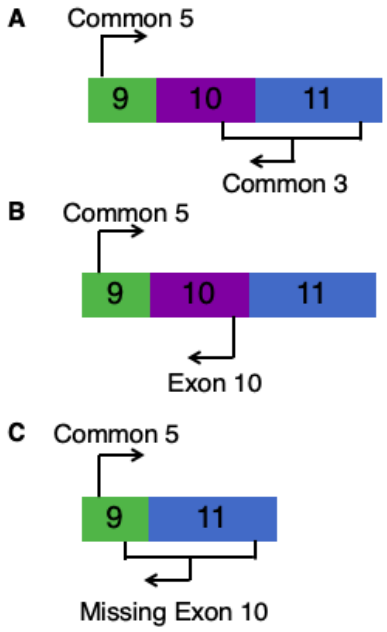

Figure 2. (A) Primer pair one, Common 5 + Common 3, was used as a positive control on a known sequence of G9a gene. (B) Primer pair two, Common 5 + Exon 10, was used to amplify the inclusion of Exon 10. (C) Primer pair three, Common 5 + Missing Exon 10, was used to amplify the exclusion of Exon 10.

Polymerase chain reaction (PCR) was preformed using GoTaq Green Master Mix 2X (Promega), and the products were visualized using 2% Agarose (Invitrogen) gel stained with 5 μL of 0.5 ml GelRed Nucleic Acid Stain (Phenix Research Products).

Densitometry

ImageJ was used to analyze the density of bands on the agar gel. Each band was measured for their individual density. Each individual density value was subtracted from the average background density to normalize the data.

Statistical analysis

All statistical analysis was prepared for presentation using Prism GraphPad. Holm-Sidak's multiple comparisons test was used to compare the data generated from ImageJ. Statistical significance was set at alpha = 0.05 for this experiment.

CHAPTER III

RESULTS

This experiment sought to determine if alcohol exposure influences the alternative splicing of G9a histone methyltransferase by promoting inclusion of exon 10. All data was normalized to the measurements of total G9a, and measured using primers (Figure 2A) that amplify a unique region of G9a. Significance was determined using Holm-Sidak's multiple comparison test.

The first set of data (Figure 3A) compare the inclusion of G9a exon 10 between the control and alcohol treated (160 mg/dL and 240 mg/dL) neural stem cells. There is no significant increase in G9a exon 10 inclusion when neural stem cells are exposed to 160 mg/dL EtOH for a 3-day exposure period ($p = 0.2237$). There is a positive trend towards significance when neural stem cells are exposed to 240 mg/dL for a 3-days period ($p = 0.0559$). Neural stem cells exposed to 160 mg/dL EtOH for a 3-day period and a 4-day recovery period exhibit no significant increase of G9a exon 10 inclusion ($p = 0.5597$), however, neural stem cells exposed to 240 mg/dL EtOH exhibit a significant increase of G9a exon 10 inclusion ($p = 0.0179$). Comparison of neural stem cells exposed to 160 mg/dL and 240 mg/dL EtOH for 3-day period and a 4-day recovery period exhibit a significant increase in the inclusion of G9a exon 10 ($p = 0.0245$).

The second set of data (Figure 3B) compare the exclusion of G9a exon 10 between the control and alcohol treated (160 mg/dL and 240 mg/dL) neural stem cells. There is no significant increase of G9a exon 10 exclusion when neural stem cells are exposed to 160 mg/dL and 240 mg/dL EtOH for a 3-day period ($p = 0.4252$, $p = 0.9550$). There is no significant increase of G9a

exon 10 exclusion when neural stem cells are exposed to 160 mg/dL and 240 mg/dL EtOH for a 3-day period and 4-day recovery period ($p = 0.8144$, $p = 0.9949$).

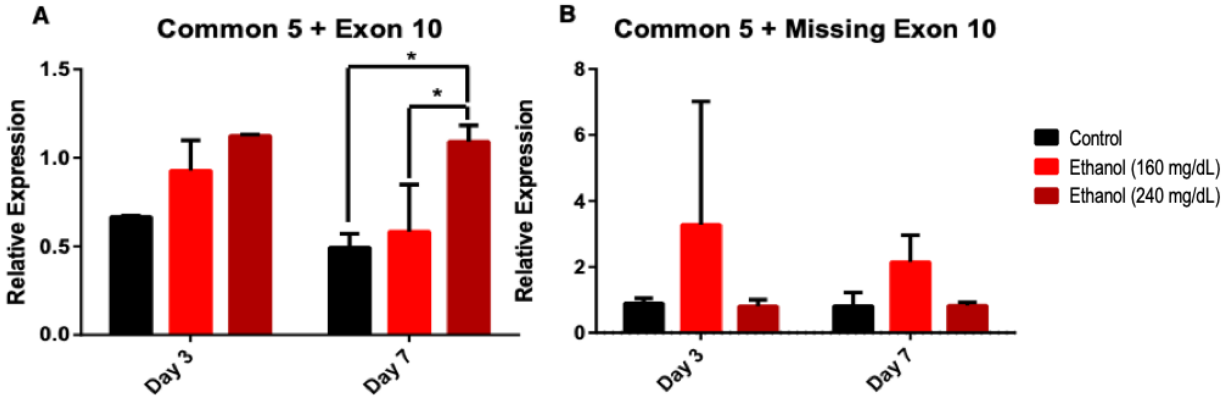

Figure 3. Comparison of G9a Exon 10 inclusion and exclusion between Control and Alcohol treated (160 mg/dL and 240 mg/dL) neural stem cells, both during the exposure period and after a 4-day recovery. Data was normalized to measurements of total G9a, measured using primers amplifying a unique region of G9a. (A) Relative expression of G9a mRNAs including exon 10 during the period of exposure. (B) Relative expression of G9a mRNAs excluding exon 10 during the period of exposure.

CHAPTER IV

CONCLUSION

Prenatal alcohol exposure has been reported to cause alterations in the enzymes regulating histone methylation [1,4]. Previous studies show that EtOH exposure is linked to alteration in repressive chromatin structure, specifically H3K9me2, and chromatin modifications can affect alternative splicing [1]. In addition, the inclusion of G9a exon 10 correlates with increased nuclear localization of G9a and H3K9me2 levels, but not G9a catalytic activity [2]. The increased levels of H3K9me2 observed in alcohol-exposed neurosphere cultures may be the consequence of increased nuclear localization of the enzyme, thereby creating a positive feedback loop where G9a regulates its own alternative splicing [2].

Thus far, this study has demonstrated that alcohol exposure influences the alternative splicing of G9a histone methyltransferase by promoting the inclusion of G9a exon 10, which may explain the increased levels of H3K9me2 observed in alcohol-exposed cells.

This study also reveals that there may be an amount of alcohol exposure that is required to be exceeded for there to be an increase in the inclusion of G9a exon 10. Looking at the first set of data (Figure 3A), there is a significant increase in the inclusion of G9a exon 10 when 240 mg/dL EtOH is exposed to neural stem cells for a 3-day period and a 4-day recovery period, but not when exposed to 160 mg/dL (p=0.0179, p=0.5597). This is further supported by a positive trend towards significance when neural stem cells are exposed to 240 mg/dL for a 3-days period, but not 160 mg/dL (p = 0.0559, p=0.2237).

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