THE ABUNDANCE OF THE LONG LSD1/KDM1A ISOFORM IN ALCOHOL TREATED BRAINS

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

The Abundance of the Long LSD1/Kdm1a Isoform in Alcohol Treated Brains

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Alternative splicing is a eukaryotic mRNA processing mechanism in which exons within a pre-RNA transcript are joined differently or skipped entirely, yielding multiple protein isoforms from a single gene. This project studied the effect of alcohol treated brains in regards to alternative splicing of exon 8a in the Lysine-specific histone demethylase 1A (LSD1) gene. LSD1 is a flavin-dependent enzyme that demethylates mono or dimethylated lysines, and more specifically it removes histone H3K4me2 and changes it to either H3K4me1 or H3K4me0. The inclusion of exon 8a has been noted to create a docking site that helps the conversion of LSD1 into H3K9 demethylase during neuronal differentiation. The expected outcome was that alcohol exposure alters the splicing of LSD1 and, thus, decreasing H3K9me2 activity. In this study, ethanol exposure was examined on neurospheres, which are culture systems clusters of neural stem cells, under control, 0.16 g/dL, and 0.24 g/dL alcohol conditions. Based off of the obtained data there was no clear indication that the inclusion or exclusion of Exon 8a affected the intensity of LSD1 gene expression with increasing levels of alcohol concentration. Rather, it seems that the increasing alcohol concentration overall correlated with a decreasing number of copies amplified of the LSD1 gene during PCR, regardless of the inclusion or exclusion of Exon 8a.

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NOMENCLATURE

LSD1	Lysine-specific histone demethylase 1a
KDM1	Lysine (K)-specific demethylase 1a
PCR	Polymerase Chain Reaction
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary DNA
EtOH	Ethanol
H3K9me2	Histone 3 Lysine 9 dimethylated

CHAPTER I

INTRODUCTION

Lysine-specific histone demethylase 1A (LSD1), also known as lysine (K)-specific demethylase 1A (KDM1a) in *Mus musculus*, is a flavin-dependent enzyme that demethylates mono or dimethylated lysines. LSD1 specifically demethylates histone H3K4me2 and changes it to either H3K4me1 or H3K4me0. LSD1 has been noted to have critical roles in embryogenesis and oocyte growth, as well as an important role in the epigenetic reprogramming that happens when sperm and an egg combine to form a zygote. Deletion of the gene for LSD1 can have effects on the growth and differentiation of embryonic stem cells. There is also some thought that high LSD1 levels are noted in relevance with cancer, so this may be a possible treatment outlook for cancer.

Exon 8a of LSD1

This project will be looking specifically at exon 8a of the LSD1/KDM1a gene. The inclusion of exon 8a has been noted to create a docking site that helps the conversion of LSD1 into H3K9 demethylase during neuronal differentiation. Another study has reported that exon 8a functions as a H4K20 demethaylase (not H3K9), and it promotes transcriptional and elongation of neuronal activity-related genes by removing H4K20. This function of LSD1 is required for spatial learning and memory.

What I Will Be Looking At

My project will be looking at the effect of inclusion and exclusion of Exon 8a in the LSD1 gene on alcohol-treated neurospheres. A neurosphere is a culture system of clusters of neural stem cells; these will be used as the test cells under control, 0.16 g/dL, and 0.24 g/dL alcohol

conditions. I will be using the NCBI website to obtain the sequence of LSD1 and determine the position of exon 8a. From there, I will create two Common (forward) primers and will develop two reverse primers (one including exon 8a, one excluding). Then, I will perform a series of control and experimental samples in different combinations of the forward and reverse primers via Polymerase Chain Reaction (PCR) and Gel Electrophoresis. Finally, I will use ImageJ to collect data from the gel images of said samples and will analyze this data using GraphPad and the Sidak comparison test.

CHAPTER II

METHODS

All animal procedures were approved and conducted in accordance with the Institutional Animal Care and Use Committee at the Texas A&M College of Veterinary Medicine (protocol number 201-0308) and the Texas A&M University Institutional Biosafety Committee (protocol number 2015-100). Groups of *Mus musculus* (house mice) were monitored and used to obtain samples for this experiment. Cells from these mice were cultured as neurospheres to be used for the experiment and were preserved and incubated at 37°C. The treatment groups were treated in mediums containing: 0 g/dL, 0.16 g/dL, and 0.24 g/dL ethanol concentrations; 0 g/dL being the control treatment group, while 0.16 g/dL and 0.24 g/dL were used for the alcohol treatment groups. These samples were treated for up to 7 days, while obtaining samples at Day 3 and Day 7. Aliquots were taken from these samples after the treatments were complete. These samples were set aside to be used later, with the designed forward and reverse primers, as the complementary-DNA (cDNA) in the Polymerase Chain Reaction procedures.

Designing the Primers

After the samples were treated and obtained for later experiments, the forward and reverse primers were designed. Using the National Center of Biotechnology Information's Gene Website [4], two isoform nucleotide sequences of the Lysine (K)-specific demethylase 1a (Kdm1a) gene were obtained. After the two isoform nucleotide sequences were obtained, the National Center of Biotechnology Information's Basic Local Alignment Search Tool (BLAST) [5] was used. This tool finds regions of similarity between multiple biological sequences and will compare these sequences with other sequence databases. The nucleotide sequences of the two

isoforms yielded a long, singular nucleotide sequence after being aligned. From this singular nucleotide sequence, the exact location of Exon E8a was determined. Exon E8a was determined to be approximately twelve nucleotides long. Based off of this location and information, a forward primer nucleotide sequence was determined: TGGAAGCCAGGGATCGAGTA. This forward primer was determined to be a common forward primer, which would be used in conjunction with both reverse primers that were designed. Using the nucleotide sequence, two reverse primers were designed around the position of Exon E8a, as shown in Figure 1. The first was a 22 nucleotide long sequence that contained Exon E8a (12 nucleotides long). This primer was designed to have the sequence: GGAACCTTGACAGTGTCAGCTT. The second reverse primer was a 24 nucleotide long sequence that did contain Exon E8a. This primer spanned the splice junction between Exons 7 and 9. This primer was designed to have the sequence: TCTTTTGGAACAGCTTGTCCATTG. These primers were ordered from Invitrogen to be tested.

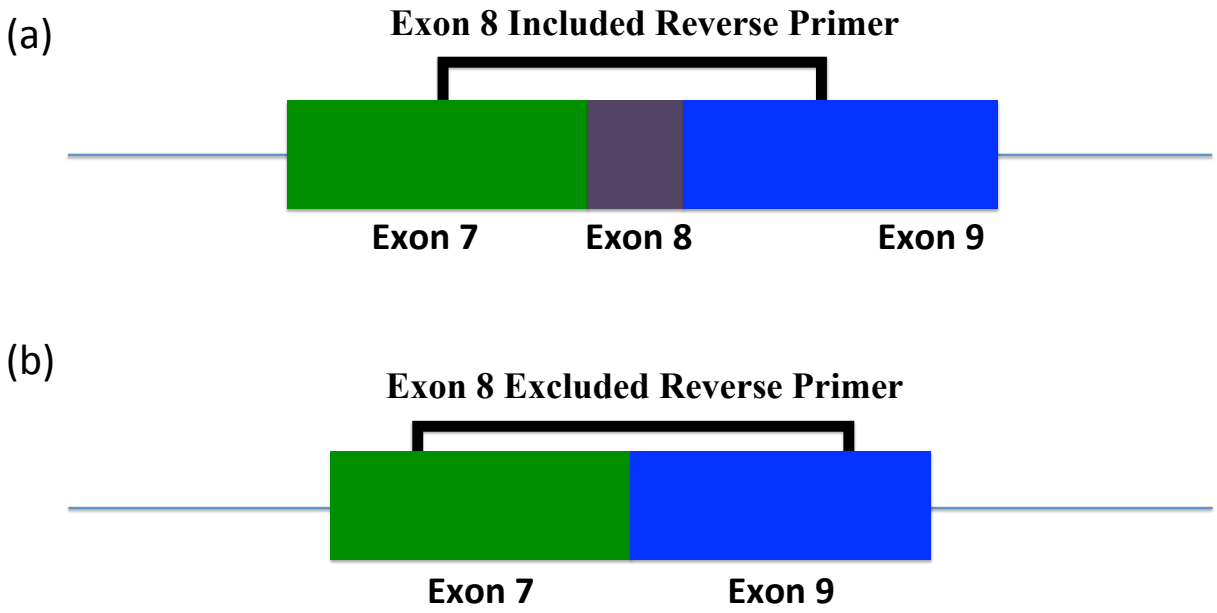

Figure 1. (a) Exon 8 Included Reverse Primer Design. 22 nucleotide long reverse primer (GGAACCTTGACAGTGTCAGCTT) designed to be paired with a common forward primer (TGGAAGCCAGGGATCGAGTA). Used to measure the intensity of increasing alcohol exposure, with the inclusion of LSD1 gene Exon 8a, on neurosphere cells. **(b) Exon 8 Excluded Reverse Primer Design.** 24 nucleotide long reverse primer (TCTTTTGGAACAGCTTGTCCATTG) designed to be paired with a common forward primer (TGGAAGCCAGGGATCGAGTA). Used to measure the intensity of increasing alcohol exposure, with the exclusion of LSD1 gene Exon 8a on neurosphere cells.

Polymerase Chain Reaction (PCR)

After the designed primers arrived to the lab, they were ready to be tested on the cDNA samples that were obtained from the alcohol treated neurospheres. It was determined that a PCR would help amplify the interaction between the cDNA and the forward/reverse primers. Firstly, the cDNA aliquots were diluted in a mixture with water. 3 uL of cDNa were added into 69 uL of distilled H_2O, yielding a concentration of 0.625 ng/uL. Using this diluted cDNA, a 20 uL PCR reaction sample was created containing: 10 uL of PCR Master Mix (PCR buffer, dNTP, PCR enzyme, and a salt compound), 1 uL forward primer (common), 1 uL reverse primer (including or excluding Exon E8a), and 8 uL premade H_2O and cDNA mix (0.35 uL cDNA, 7.65 uL H_2O). The PCR experiments were run at 60°C for 36 cycles. The PCR experiments were conducted for

each combination of common forward primer with the reverse primer including Exon E8a and common forward primer with the reverse primer excluding Exon E8a. These experiments were repeated due to the initial gel images not being clear.

Gel Electrophoresis

After the PCR experiments were completed at 60°C and 36 cycles, the obtained samples needed to be visualized to see the final results. It was determined that gel electrophoresis would provide a good visualization of the PCR experiments. Gel electrophoresis was performed at 90 volts for approximately 27 minutes, and the resulting gel was visualized using a UV-based imaging system under the ethidium bromide imaging tag.

Data Collection

After all PCR experiments were visualized using gel electrophoresis and the UV-based imaging system, the obtained pictures were copied into a flash drive. These pictures were then loaded up on a computer containing the program ImageJ. It was determined that this program would help collect data from these images. Using basic measurement setting on ImageJ, the intensity of each gel background was measured as a control average. Then, the intensity of each well was determined. The average of the background intensity was subtracted from each specific well's intensity to determine its intensity ratios. These numbers were collected in an excel spreadsheet for further analysis. Finally, the program GraphPad was used to import all numbers regarding the intensity of the control and alcohol treatment samples. These results were graphed and were used in a Sidak comparison test: control vs. alcohol (0.16 g/dL), control vs. alcohol (0.24 g/dL), and alcohol (0.16 g/dL) vs. alcohol (0.24 g/dL). The results between these were charted and were determined to be either significant or not at an alpha = 0.05 significance level.

CHAPTER III

RESULTS

Using the aforementioned methods, polymerase chain reaction was performed for two samples: common primer + included exon 8a reverse primer and common primer + excluded exon 8a reverse primer. The obtained samples from the PCR were then visualized using gel electrophoresis, as shown in Figure 2 below.

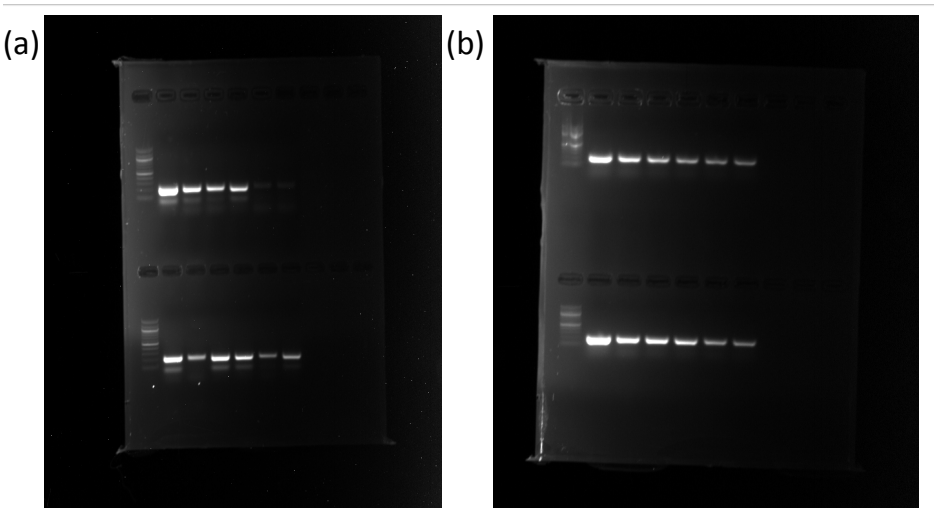

Figure 2. **(a) Exon 8 Included Gel Electrophoresis Result.** Visualization using Ethidium Bromide (EtBr) in UV-based imaging system. Gel Electrophoresis was performed on 2% agarose gel at 90V for approximately 27 minutes. **(b) Exon Excluded Gel Electrophoresis Results.** Same conditions as described in (a). For all gels: the samples on the top row were conducted using Day 3 cDNA PCR product and the samples on the bottom row were conducted using Day 7 cDNA. For all gels, wells 1 & 2 at 0.00 g/dL EtOH concentration, wells 3 & 4 at 0.16 g/dL EtOH concentration, and wells 5 & 6 at 0.24 g/dL EtOH.

Gel Electrophoresis Result

The combination of the common forward primer and the exon 8a included reverse primer is shown in Figure 2a. From the top row (Day 3), it can be seen that the intensity of the wells in the image gradually decreased as the concentration of alcohol treatment increased. There was also no visualization obtained for wells 5 & 6, which denoted the treatment of 0.24 g/dL EtOH.

From the bottom row (Day 7), it can be seen that the intensity of the wells fluctuated; however, with an overall decrease in intensity as concentration of alcohol treatment increased. The combination of the common forward primer and the exon 8a excluded reverse primer is shown in Figure 2b. From the top row (Day 3), it can be seen the intensity of the wells in the image gradually decreased as the concentration of alcohol treatment increased. From the bottom row (Day 7), it can also been seen that the intensity of the wells gradually decreased as the concentration of alcohol treatment increased.

Data Analysis/Densitometry

The obtained gel electrophoresis images were uploaded to a densitometry program, ImageJ, and intensity data of the wells was measured using its measurement tool. The data was further analyzed and significance was determined using Holm-Sidak's multiple comparison tests. The obtained data is shown in Figure 3 below.

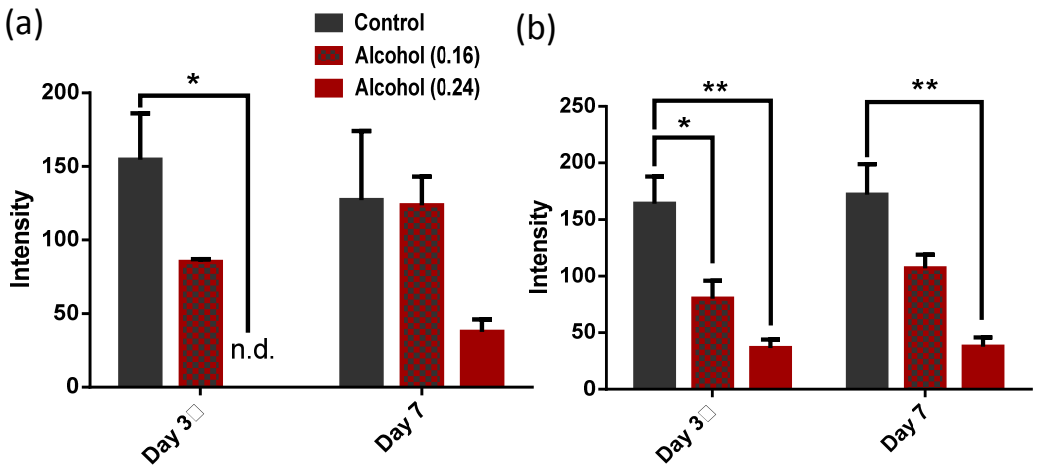

Figure 3. Comparison of LSD1 Exon 8a inclusion and exclusion in Control (0.00 g/dL) and Ethanol (0.16 g/dL and 0.24 g/dL) treated neurospheres, both during the period of exposure (Day 3) and after a 4-day recovery period (Day 7) **(a) Data for increasing alcohol exposure with Exon 8 included.** No statistical significance was determined between Day 3 control vs. 0.16 g/dL EtOH data, Day 3 0.16 g/dL vs. 0.24 g/dL EtOH data, Day 7 control vs. 0.16 g/dL EtOH data, Day 7 control vs. 0.24 g/dL EtOH data, and Day 7 0.16 g/dL vs. 0.24 g/dL EtOH data. There was statistical significance determined between Day 3 control vs. 0.24 g/dL EtOH data. **(b) Data for increasing alcohol exposure with Exon 8 excluded.** No statistical significance was determined between Day 3 0.16 g/dL vs. 0.24 g/dL EtOH data, Day 7 control vs. 0.16 g/dL EtOH data, and Day 7 0.16 g/dL vs. 0.24 g/dL EtOH data. There was statistical significance determined between Day 3 control vs. 0.16 g/dL EtOH data, Day 3 control vs. 0.24 g/dL EtOH data, and Day 7 control vs. 0.24 g/dL EtOH data.

The obtained data for the combination of the common forward primer and the exon 8a included reverse primer are shown in Figure 3a. As seen from the chart, there was an overall decrease in intensity in both Days 3 and 7 as the alcohol concentration increased. As there was no intensity detected for wells 5 & 6 during Day 3, the data was listed as ND or not detectable. There was a statistical significance noted between control vs. 0.24 g/dL EtOH conditions on Day 3. It was determined that the overall drop in intensity passed a certain threshold to be considered significant for that data. There was no statistical significance noted in the following comparisons: Day 3 control vs. 0.16 g/dL EtOH, Day 3 0.16 g/dL vs. 0.24 g/dL, Day 7 control vs. 0.16 g/dL EtOH, Day 7 control vs. 0.24 g/dL EtOH, and Day 7 0.16 g/dL vs. 0.24 g/dL. The obtained data for the combination of the common forward primer and the exon 8a excluded reverse primer are

shown in Figure 3b. As seen from the chart, there was an overall decrease in intensity in both Days 3 and 7 as the alcohol concentration increased. There was statistical significances noted in the following data comparisons: Day 3 control vs. 0.16 g/dL EtOH, Day 3 control vs. 0.24 g/dL EtOH, and Day 7 control vs. 0.24 g/dL EtOH. It was determined that the overall drop in intensity between these sets of data passed a certain threshold to be considered significant. There was no statistical significance noted in the following data comparisons: Day 3 0.16 g/dL vs. 0.24 g/dL EtOH, Day 7 control vs. 0.16 g/dL EtOH, and Day 7 0.16 g/dL vs. 0.24 g/dL EtOH.

CHAPTER IV

CONCLUSION

As mentioned previously, the expected outcome of the project was that alcohol exposure alters the splicing of LSD1 and, thus, decreasing H3K9me2 activity**.** The purpose of the project was to determine whether the inclusion or exclusion of Exon 8a, in the presence of increasing alcohol concentration, alters H3K9me2 activity, positively or negatively. However, based off the results shown in Figure 3, there was no clear indication that the inclusion or exclusion of Exon 8a affected the intensity of LSD1 gene expression with increasing levels of alcohol concentration. Rather, the data shows that the increasing alcohol concentration overall correlated with a decreasing number of copies amplified of the LSD1 gene during PCR, regardless of the inclusion or exclusion of Exon 8a. This means that increasing alcohol concentration directly affects the LSD1 gene amplification during PCR, but the inclusion or exclusion of Exon 8a does not affect the intensity drastically in any way. As mentioned before, there is also some thought that high LSD1 levels are noted in relevance with cancer. This may be a possible treatment outlook for cancer and a further implication of this project.

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