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Reliable Detection of WNT7A Protein in Transfected Human Embryonic Kidney 293 Cells

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RELIABLE DETECTION OF WNT7A PROTEIN IN TRANSFECTED HUMAN EMBRYONIC KIDNEY 293 CELLS

by

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ABSTRACT

Fetal Alcohol Spectrum Disorders (FASDs) refer to a set of development abnormalities affecting a fetus that can result from prenatal alcohol exposure (PAE). Studies performed by the National Institute of Health estimate that the pervasiveness of FASDs may number as high as 1 to 5 per 100 school children. Congenital heart defects (CHDs) are a subset of these abnormalities and have been observed to occur in 38% of children with FASDs. While there is an association between PAE and CHDs, the exact molecular mechanism as to how it occurs remains unclear. A 2022 RNA sequencing study points to the *Wnt7a* gene as one of several others to have its mRNA expression significantly decrease in the heart in response to PAE at a critical developmental time point. This gene codes for the Wnt7a protein that can play a role in activating the Wnt signaling pathways. This critical pathway plays a role in cell differentiation, cell proliferation, morphogenesis, embryonic development, and adult tissue homeostasis. While mRNA expression in response to alcohol has been studied, the change in protein expression of Wnt7a is yet to be determined. This thesis serves to demonstrate assay techniques and antibodies that can be used to reliably detect Wnt7a protein. We hypothesize that Wnt7a protein expression can be reliably detected in Human Embryonic Kidney 293 (HEK 293) cells through Western blot and immunofluorescence assays when the protein is overexpressed. Fluorescent, and more effectively, chemiluminescent western blot procedures produced a positive signal for detection of Wnt7a protein at the expected 40-42 kDA molecular weight range. Through Immunofluorescence, the

Wnt7a+ HEK cells were confirmed to express the protein through Alexa Fluor probing of an anti-Wnt7a antibody, and the Dyk HEK cells failed to produce a signal for expression of the protein as expected. The methods and techniques used in this study can be used to detect Wnt7a protein in embryonic hearts and determine how much it is affected by alcohol exposure. This serves toward the larger goal of identifying Wnt7a as a potential biomarker for helping to diagnose alcohol-induced CHDs. Additionally, these future directions can help direct attention to this gene as a useful therapeutic target for preventing and treating CHD formation.

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INTRODUCTION AND BACKGROUND

Fetal Alcohol Spectrum Disorders and Congenital Heart Defects

Fetal Alcohol Spectrum Disorders (FASDs) refer to a set of development abnormalities affecting a fetus that can result from prenatal alcohol exposure (PAE). FASDs include a wide variety of structural and neurological developmental problems, including defects in the development in the limbs and craniofacial skeleton [1] Increased duration or frequency of alcohol exposure can increase the severity of these defects, and they are especially more severe when exposure occurs during the embryonic stage of development [2]. Since 2012, the percentage of women who drink or binge drink have increased, making these disorders ever more prevalent [3]. Studies performed by the National Institute of Health estimate that the pervasiveness of FASDs may number as high as 1 to 5 per 100 school children [4].

Congenital Heart Defects

Congenital heart defects (CHDs) are a subset of the abnormalities observed in FASDs. Alcohol-induced congenital heart defects are present in 50% of children born with FAS and in 38% of children with FASD [5]. The defects form while the fetus is developing in the uterus during pregnancy. They range from atrial or ventricular septal defects, aortic malformation, and valvular defects and vary in severity from small holes to abnormal or even missing parts from the heart [5] They are the most common congenital malformation and occur affect about 1% or 40,000 births per year in the U.S

[6]. Currently, detected cases of CHDs are treated using medications, catheter methods, or surgery during infancy or childhood. However, some cases go undiagnosed or symptoms may linger, leaving an estimated over 1.4 million adults in the U.S living with CHDs [7]. While there is an association between PAE and CHDs, the exact molecular mechanism as to how it occurs remains unclear.

Previous Alcohol-Induced CHD Study

The consumption of alcohol by pregnant mice causes an increase in CHDs when occurring at certain gestational ages. A study performed in 1984 exposed mice to either a single or double dose of alcohol though intraperitoneal injections or oral gavage to analyze its effects on CHDs [8]. The mice were sacrificed on the day before term, and the hearts of the fetuses were examined. Alcohol exposure on day 8, 9 or 10 of gestation lead to a high incidence of ventricular septal defects (60%, 75% and 15% respectively) [8]. Exposure on day 7 did not result in the manifestation of defects down the line, indicating that embryonic days 8-10 are critical for the development of the heart. This contributed to setting the foundation for some of the embryonic ages in which this study and others have focused on.

Analysis of embryonic mice heart gene expression profiles in response to maternal binge alcohol consumption

An RNA- Sequencing (RNA-Seq) study looked at the expression level changes of certain genes in the embryonic mice heart after exposure to alcohol [9]. After mice were exposed to binge ethanol or control saline oral gavage at embryonic day 9.5 (E9.5),

embryonic hearts were collected after 24h (E10.5), 48h (E11.5), and 72h (E12.5). After RNA extraction, an RNA-Sequencing analysis was performed to assess measurable changes in genetic expression for thousands of genes expressed in the embryonic heart. The minimum threshold for differential expression was set at 2-fold with $p < 0.05$ necessary to show significance. The results are shown in Figure 1. The heat map displays the 14 different embryonic mice heart genes that had significant changes in expression at E10.5. Only genes whose expression was significantly different at magnitude 2-fold or greater are shown. The genes also had to show measurable expression above baseline in two or more samples from each age group to be included in the results. Each box represents one sample, and the left and right side of the map represent the control and treatment group, respectively. Wnt7a can be seen to have decreased mRNA expression in E10.5 the treatment group compared to the control group. However, the Wnt7a expression differences between groups was not notable in the E11.5 condition, as observed in figure 2. Figure 3 provides a clear comparison of the expression changes at the 3 different time points for Wnt7a.

Upon further investigation, the RNA-Sequencing was followed up with qPCR, which confirmed a significant decrease in Wnt7a expression at E10.5 and revealed a significant decrease at E11.5 as well. Those results can be seen in Figure 4.

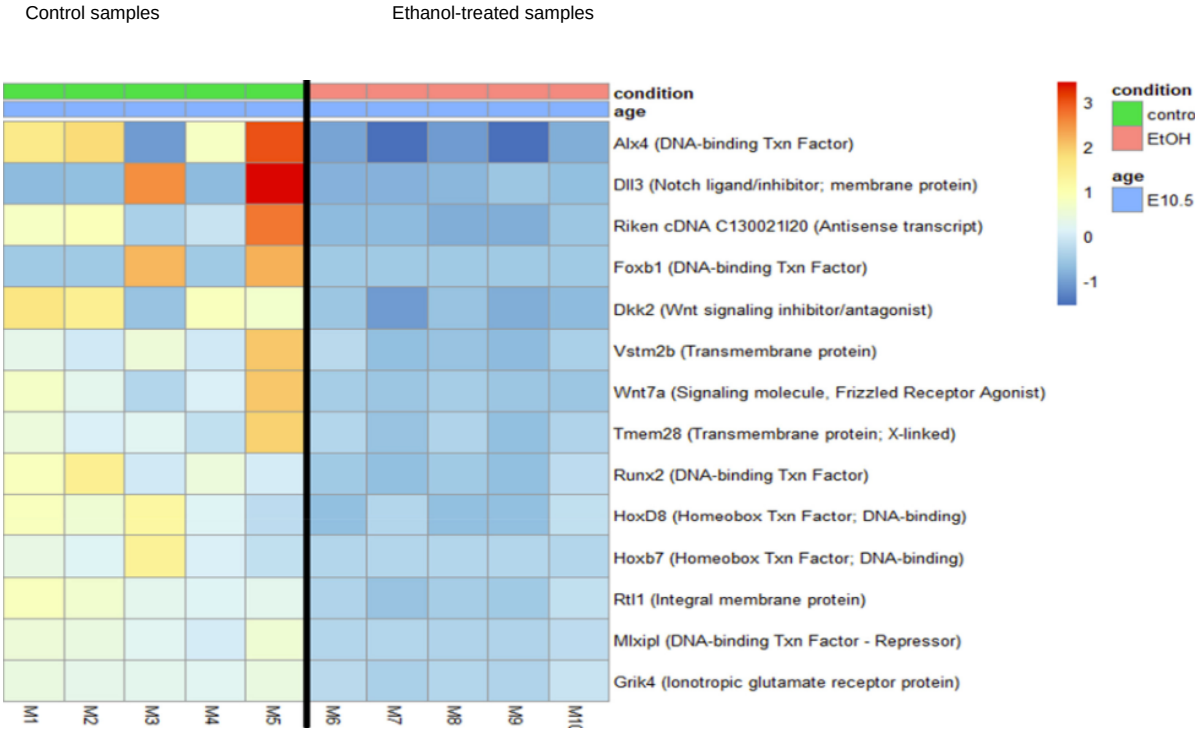

Figure 1: RNA Sequencing data showing several significantly depressed genes at E10.5, including the Wnt7a gene.

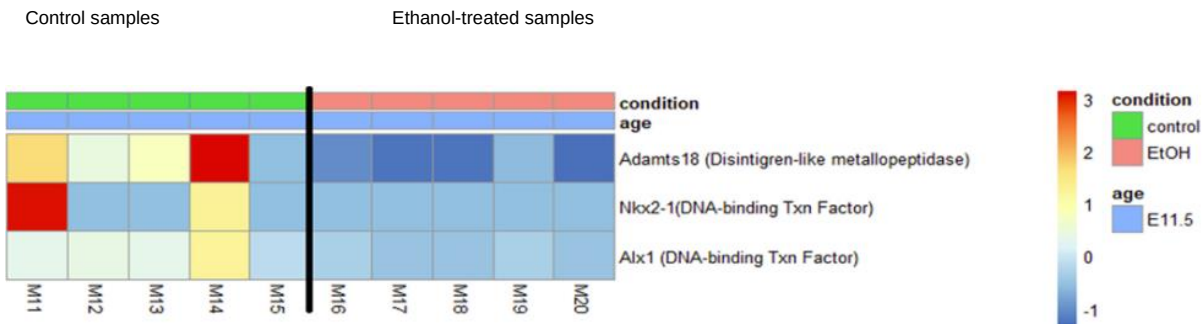

Figure 2:RNA Sequencing data showing three depressed genes at E11.5

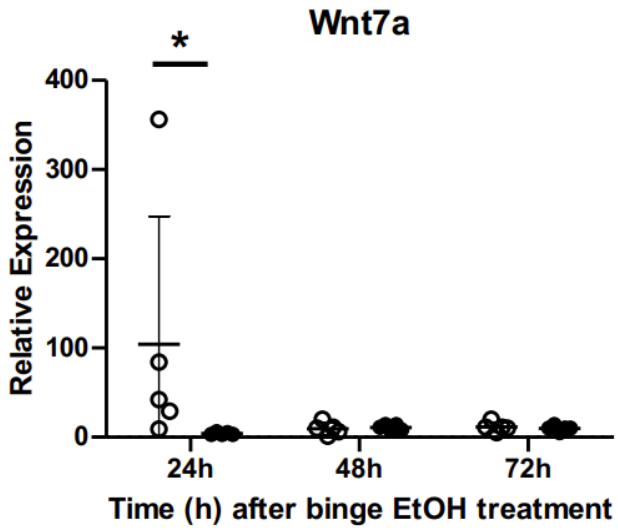

Figure 3: Wnt7a expression difference between saline (open circles) and alcohol treatment (filled circles) groups

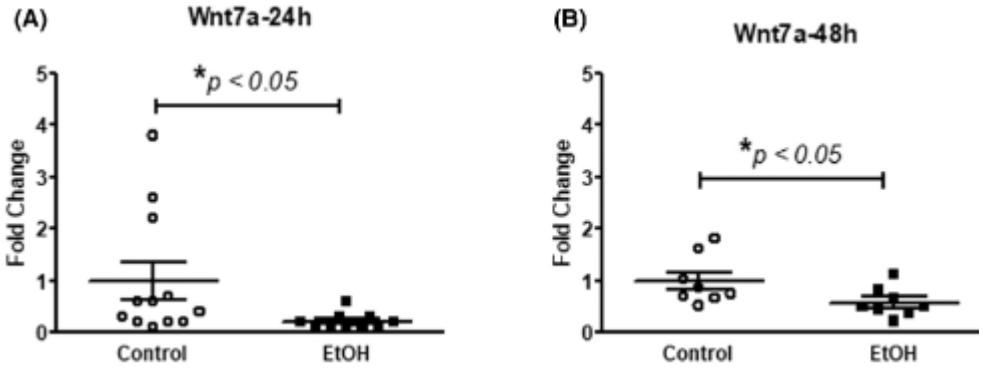

Figure 4: Follow up q-PCR highlighting consistent depression of Wnt7a at 10.5 and 11.5 following ethanol administration at E9.5

This study highlights the relationship between PAE and Wnt7a mRNA expression and further highlights the sensitivity of certain embryonic time periods. It calls for further

investigation into the protein expression in the embryonic hearts at these time periods, which will be the focus of this thesis.

Wnt Signaling Pathway

The Wnt signaling pathway is an evolutionarily conserved, ancient pathway that regulates critical aspects of embryonic development, including cell fate determination, cell migration, cell polarity, neural patterning, and organogenesis [10]. Improper regulation of this pathway can lead to many defects in the developing embryo and different steps appear to be targets for the induction of cardiac defects [11].

The Wnt signaling pathway is hypothesized to influence cardiogenesis through different modes of action and in a time-dependent manner. In zebrafish, Wnt/B-catenin promoted cardiogenesis prior to Nkx2.5 and GATA4 expression but inhibited this development when activated in later stages [12]. The pathway's activity has been observed to be present in various areas of the heart. Utilizing a LEF/TCF transgenic reporter line, pathway activity was observed in the endocardial cushions, the pericardium, the adjacent cardiac mesoderm, and the out-flow tract [13].

Various studies have indicated the relevance of the different Wnt isoforms in cardiac development. To name a few, Wnt2, Wnt2b, Wnt11, and Wnt8a have demonstrated a high expression level in the early heart [13]. These isoforms have the capability to act through two recognized categories of Wnt signaling pathways: the canonical or noncanonical pathways [13].

Canonical Pathway

The Wnt/β-catenin signaling pathway is necessary for embryonic development and homeostatic maintenance of various tissues in the body. It comprises four segments: the extracellular signal, membrane segment, cytoplasmic segment, and nuclear segment [14]. Extracellular signals are mediated by different Wnt proteins. The membrane segment consists of the Frizzled receptor, and uniquely, the LRP5/6 receptor. The cytoplasmic segment consists of effector proteins like GSK-3β, β-Catenin, and AXIN [14]. The pathway is activated when a Wnt signaling protein binds to the LRP5/6 and Frizzled receptors, leading to downstream stabilization and accumulation of β-Catenin. This downstream effector protein translocates to the nucleus to act as a transcriptional coactivator. This pathway plays a role in cell differentiation, cell proliferation, morphogenesis, embryonic development, and adult tissue homeostasis [14]

Noncanonical Pathway

The noncanonical Wnt signaling pathway is independent of β-Catenin and uses other intracellular mediators. One of its main functions is to regulate cell polarity and migration [12]. It can be categorized as the Wnt/jun N-terminal kinase (JNK) or Wnt/calcium pathway based on the major intracellular mediators used [15]. These pathways have been linked to cardiac development in a number of studies. For example, one study analyzed *Xenopus laevis* embryos and the effect of Wnt11 presence on the phenotype of the heart [16]. Administration of Wnt11 promoted cardiogenesis through the noncanonical JNK pathway.

Wnt7a

Wnt7a was discovered to play a crucial role in a number of different body systems during embryonic development. This includes development of the cerebral cortex, synapse formation, CNS vasculature formation and maintenance, limb development, and female reproductive system development [17]. *Wnt7a* was also discovered to play a role in homeostatic maintenance and self-renewal of adult neural stem cells, skeletal muscle, cornea, hair follicle, and cartilage. The gene was also linked to a number of human diseases and defects [17] [18]. Mutations in the gene were correlated to limb malformations and female reproductive system defects [1]. Wnt7a has also acknowledged as dual role player in human tumors, acting as both a tumor suppressor and a tumor promoter in different tumor types [17]. Its expression was also found to be positively correlated with metastasis of UBC and other clinical outcomes [19].

Wnt7a's role in the heart has been studied through the screening of an in vitro system of conduction cell differentiation from embryonic chick myocytes. These myocytes were treated with Endothelin-1 to mimic Purkinje Fiber formation. Through RT-qPCR, significant amounts of Wnt7a expression were found in the Purkinje Fiber-like tissue. This study highlights Wnt7a's involvement in the development of the conduction system of the heart and other parts like the outflow tract and the trabeculae of the ventricles [20].

Hypothesis

Wnt7a protein expression can be reliably detected in Human Embryonic Kidney 293 (HEK 293) cells through Western blot and immunofluorescence assays when the protein is overexpressed.

Significance

The existing literature demonstrates a gap in knowledge on Wnt7a's protein expression change in response to PAE during E9.5. Prior to treating mice with ethanol and comparing Wnt7a protein expression between those mice and a control group, this study was conducted to confirm a few things. First, the Wnt7a protein can be detected on a Western blot assay near the expected 40-42 kDA band range using our anti-Wnt7a primary antibody from Abcam. Second, chemiluminescence will be more effective than fluorescence at visualizing a more intense band at lower concentrations of Wnt7a proteins. Third, the antibody can effectively detect Wnt7a using Immunofluorescence staining. This study sets a foundation for the assays and reagents that can be used in future studies to effectively compare Wnt7a protein expression in alcohol-treated and control embryonic hearts. The future goal of this work is to determine if Wnt7a is a useful biomarker for helping to diagnose and possibly prevent or treat alcohol-induced CHDs.

EXPERIMENTAL METHODS

Transformation of Escherichia Coli (E. Coli) on Agar plates

Agar plates were produced using LB-Broth, bacto-agar, and a 50 µg/mL Ampicillin concentration. Afterwards, JM109 High Efficiency Competent E. Coli Cells were plated using Promega's Standard Transformation Protocol for Single-Use Cells and Multi-Use Cells. The bacteria were plated and grown using 3 different plasmid conditions: *Wnt7a*- plasmid (DYK), *Wnt7a*+, and no plasmid. An image of the vector maps can be seen in Figure 5. 0.1 ng of plasmid DNA per 100 µl of E. Coli cell solution was used. There were a *Wnt7a* vector, two positive controls with just the vector, and a negative control condition with no vector.

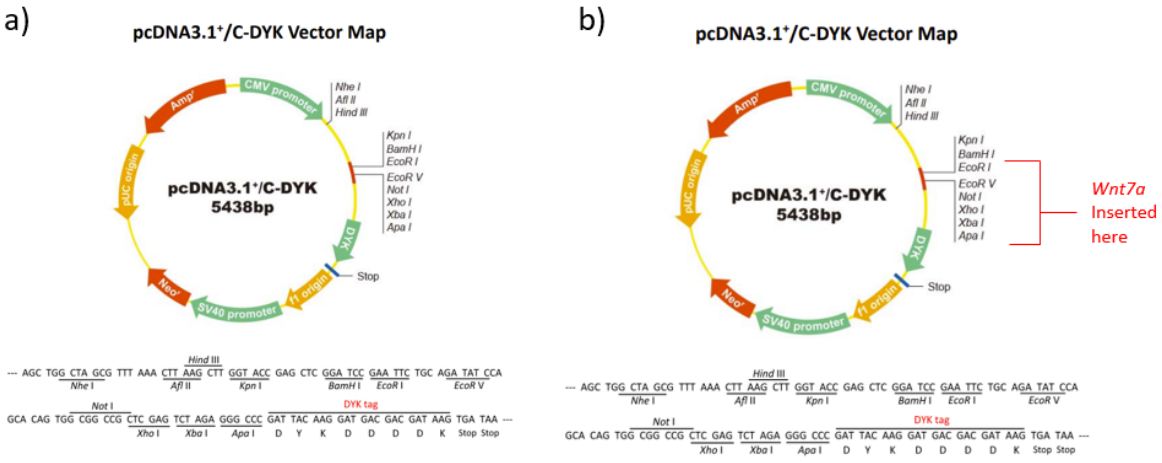

Figure 5: Images from Genscript of the pcDNA3.1+/C-DYK 5438bp plasmid vector map for a) DYK and b) plasmid with Wnt7a insertion.

Growth of Bacteria

Afterwards, colonies of bacteria with each plasmid were selected for and inoculated into an LB-Broth Growth medium with a 50 µg/mL Ampicillin concentration and grown for expression vector amplification.

Restriction Enzyme Digestion and Gel Electrophoresis

The Wnt7a+ and DYK plasmids were digested and analyzed using gel electrophoresis to check for correct plasmid size and orientation to ensure the *Wnt7a* gene was inserted correctly. The restriction enzymes used for digestion were Hind III and Pvu.

Extraction of Plasmid DNA and Transfection

Subsequently, the DNA was extracted using a QIAprep Spin Miniprep Kit. The purified plasmid DNA was utilized to transfect Human Embryonic Kidney (HEK) cells through a lipofectamine mediated process.

Protein Extraction and Quantification

The HEK cells then were lysed an electric homogenizer and RIPA lysis buffer with 1x Halt™ Protease Inhibitor Cocktail. They were incubated at 4°C and centrifuged for 20 minutes at 12000 rpm in 4ºC. The pellet was then discarded. The protein extracts were quantified using a Bio Rad Bradford Assay protocol and their Bio-Rad Protein Assay Dye Reagent Concentrate.

Western Blot

SDS Page and Transfer

Western Blot was intended to be used to ensure that our primary Wnt7a antibody could detect the Wnt7a protein and produce bands on a gel. The protein extracts were then prepared by normalizing the volumes to load 50 µg/lane, adding Laemmli Buffer to a 1x final concentration, then denaturing the protein in boiling water for 5 minutes. 5µl PageRuler Prestained Protein Ladder was used. The samples were then loaded on a 4-12% Bis-Tris Protein Gel gel using MOPS Running Buffer at 200V for 55 minutes. Afterward, the samples were then transferred onto a PVDF membrane using a Tris-Glycine Transfer Buffer, composed of 2.5mM Tris-Base, 19.2 Glycine, and 12% Methanol. The wet transfer was run for 60 min at 35 V. The membrane was then treated with either of the two conditions primary conditions listed below.

Fluorescent Primary and Secondary Antibody Application

After SDS Page and transfer, the PVDF membrane was washed in PBS-T twice and then washed in PBS, 10 minutes each. The membrane was blocked in 10 mL Intercept Blocking Buffer for 1 hour at 4°C. Afterward, washed in PBS-T for 5 minutes. A 1:2000 dilution of anti-Wnt7a monoclonal rabbit primary antibody in 10 mL of Intercept Blocking Buffer was applied and the membrane incubated with agitation in 4°C overnight. The next day, it was rinsed with PBS-T 3 times for 5 minutes. A 10mL solution of Intercept Blocking Buffer, 0.2% Tween-20 and a 1:2000 dilution of IRDye™ 680RD goat anti-rabbit was applied. The membrane was then rinsed in PBS-T 3 times, for 5 minutes each at room temperature with agitation, and then finally with PBS for 5

minutes. It was then imaged using Chemi-Doc™ MP's Colorimetric, IRDye 680 and IRDye 800 filter. Afterward, the membrane was stripped of antibodies using Restore™ PLUS Stripping Buffer overnight at 37°C. It was then probed using the same procedure but with a beta Actin Loading Control Antibody (1:1000 dilution) and then an IRDye™ 800RD goat anti-mouse IgG Secondary Antibody (1:2000 dilution).

Chemiluminescent Primary and Secondary Antibody Application

After SDS Page and transfer, the PVDF membrane was washed in PBS-T twice and then washed in PBS, 10 minutes each. The membrane was blocked in 10 mL Intercept Blocking Buffer for 1 hour at 4°C. Afterward, washed in PBS-T for 5 minutes. A 1 ug/ml final concentration of anti-Wnt7a recombinant rabbit antibody (1µg/ml) in 10 mL of blocking buffer was applied and the membrane incubated with agitation in 4°C overnight. The next day, it was rinsed with PBS-T 3 times for 5 minutes. A 10mL solution of blocking buffer, 0.2% Tween-20 and a final concentration of 10 ng/ml was applied. The membrane was then rinsed in PBS-T 2 times, for 10 minutes each at room temperature with agitation. Afterward, it incubated for 5 minutes in SuperSignal™ West Atto Ultimate Sensitivity Substrate and then imaged using Chemi-Doc™ MP's Chemiluminescence and Colorimetric filter. The latter of which was used to visualize the ladder so it can be spliced onto the chemiluminescence filter. Afterward, the membrane was stripped of antibodies using Restore™ PLUS Stripping Buffer overnight at 37°C. It was then probed using the same procedure but with a beta Actin Loading Control Antibody (1:1000 dilution) and then a Goat Anti-Mouse IgG H&L (HRP) secondary antibody (10 ng/ml dilution).

Immunofluorescence (IF)

Wnt7a+ and Dyk HEK cells were fixed onto slides. A PAP pen was used to create a hydrophobic barrier around each section of cells. Each section was rehydrated through a 20 min PBS wash then blocked for one hour at room temperature in BLOTTO. Afterward, the BLOTTO was removed and they were incubated in a 1:1200 anti-Wnt7a antibody solution for 1 hour at room temperature. Each section had the primary solution removed and was then washed using PBS. After, each section incubated for one hour with a secondary solution consisting of Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor™ 488 or Donkey anti-Rabbit IgG (H+L), Alexa Fluor™ 594. Afterwards, the slides were washed in PBS, dried, and covered with a coverslip using a DAPI medium.

Table 1: Antibodies used throughout this study.

Antibody	**Manufacturer, Catalog #**
Recombinant Anti-Wnt7a antibody	Abcam, ab274321
beta Actin Loading Control Monoclonal Antibody (BA3R)	Thermo Fisher, MA5-15739
IRDye™ 800RD goat anti-mouse IgG Secondary Antibody	LICOR, 926-32210
IRDye™ 680RD goat anti-rabbit IgG Secondary Antibody	LICOR, 926-68071
Goat Anti-Rabbit IgG H&L (HRP)	Abcam, ab205718
Goat Anti-Mouse IgG H&L (HRP)	Abcam, ab205719

Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	Thermo Fisher, A11034
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594	Thermo Fisher, A21207

Table 2: Other products used in this study.

Product	**Manufacturer, Catalog #**
QIAprep Spin Miniprep Kit	Qiagen, 27104
JM109 High Efficiency Competent E. Coli Cells	Promega, L20005
RIPA lysis buffer	Thermo Fisher, 89901
Halt™ Protease Inhibitor Cocktail	Thermo Fisher, 87786
PageRuler Prestained Protein Ladder was used	Thermo Fisher, 26616
4-12% Bis-Tris Protein Gel	Thermo Fisher, NP0322BOX
PVDF membrane	NEF-1000
Intercept Blocking Buffer	LICOR, 9270700001
SuperSignal™ West Atto Ultimate Sensitivity Substrate	Thermo Fisher, A38555
VECTASHIELD HardSet Antifade Mounting Medium With DAPI	Vector Laboratories, H-1500
Restore™ PLUS Western Blot Stripping Buffer	Thermo Scientific, 46430
293 [HEK-293]	ATCC, CRL-1573

Lipofectamine® Reagent	Thermo Fisher, 18324-012
Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad, 500-0006
pcDNA3.1+/c-(k)dyk expression vector	Genscript

RESULTS

Transformation of *E. coli*

After the *E. coli* were transformed and grown with the Wnt7a+ and DYK plasmid, they were digested using the Hind III and Pvu restriction enzymes. In reference to Figure 6, it can be seen that the DYK digested with Hind III is around the expected 5444 bp molecular weight. The heavier Wnt7a+ plasmid digested with Hind III is near a larger molecular weight marker at around 6491 bp. This shows that the bacteria were transformed with the correct plasmids inserted with the correct orientation.

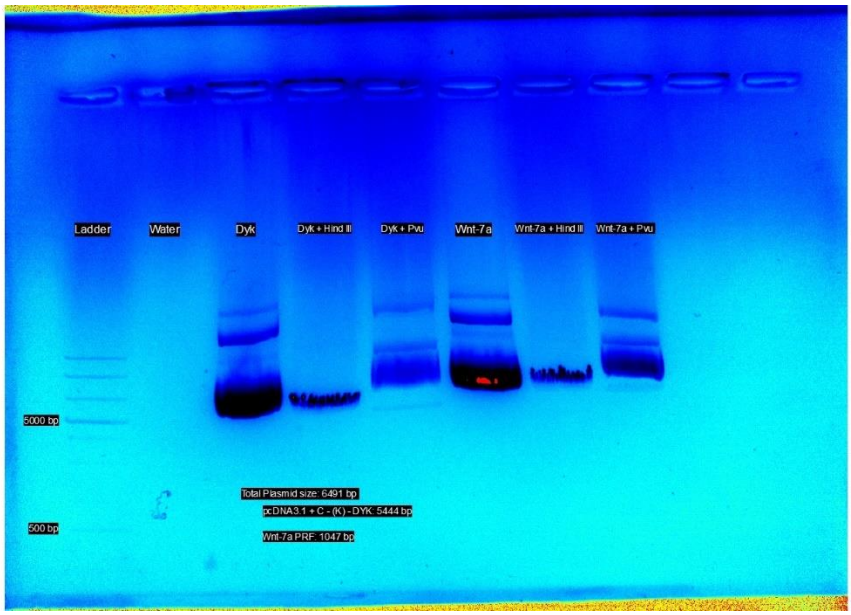

Figure 6:Image of Gel Electrophoresis ran with Wnt7a+ and DYK Plasmids and digested with the Hind III and Pvu Restriction Enzymes

Western Blot

Western Blot was used for analysis of these HEK Cell samples with two goals in mind. The first one being to further confirm that the transfection was successful and that

Wnt7a is expressed in the Wnt7a+ HEK cells, and not expressed in the DYK HEK cells. The second goal being to confirm that our anti-Wnt7a primary antibody can successfully detect Wnt7a. Both goals were met, as seen in Figure 7. The Wnt7a+ HEK cell lane gives a positive signal when probed with our anti-Wnt7a primary antibody. The intensity of the signal was also quantified using Bio Rad Image Analyzer to determine the amount of Wnt7a, in each sample. The results show the presence of Wnt7a expression in the Wnt7a+ HEK cells and the lack of it in the DYK cells.

Moreover, the membrane images prove the effectiveness of using chemiluminescence to detect Wnt7a, due to its stronger signal detection. As seen in Figure 7, probing with a chemiluminescent secondary allows for detection of Wnt7a in as small as 5g of protein extract. In contrast, fluorescent probing only detected proteins in as small as 20 µg of protein extract, and less precisely.

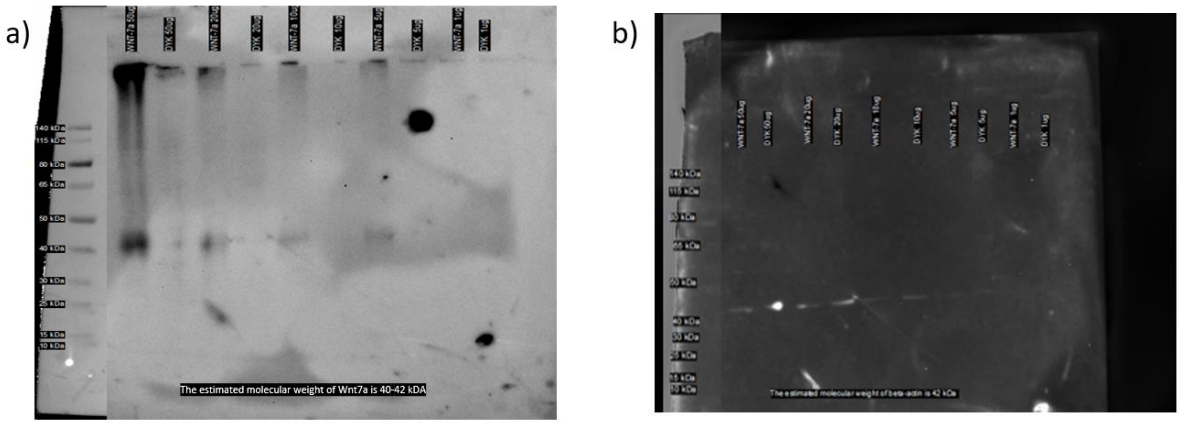

Figure 7:Chemiluminescence Western Blot images probed with a) anti-Wnt7a primary and b) anti-beta actin primary.

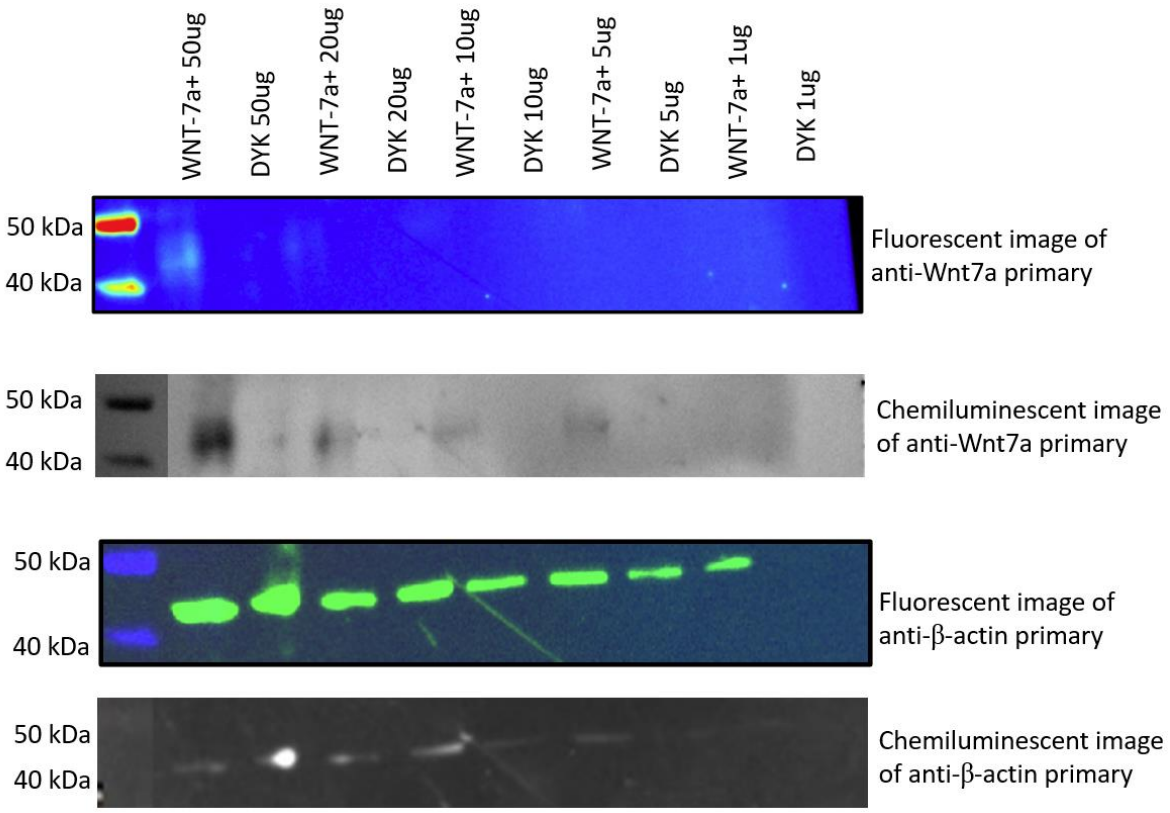

Figure 8: Comparison of chemiluminescent and fluorescent imaging of the HEK cell Western Blots

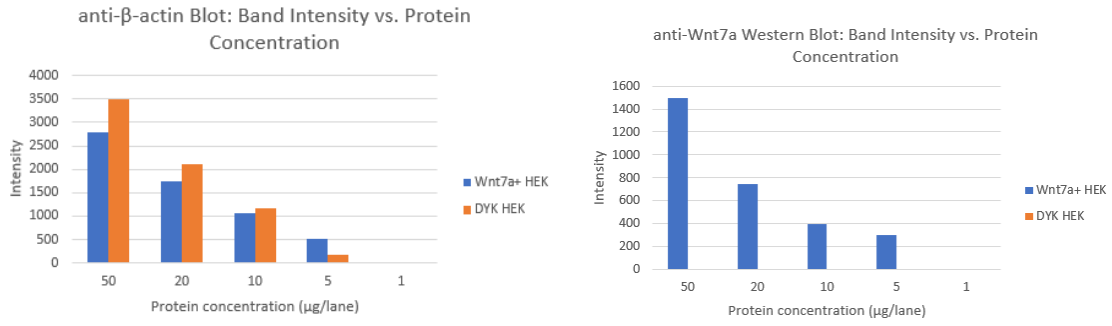

Figure 9: Western Blot band intensities for the Wnt7a+ and DYK HEK cell protein extracts probed using chemiluminescence.

Immunofluorescence

IF was used as another means of analyzing the presence of Wnt7a protein expression in the Wnt7a+ and DYK HEK cells. There was positive detection of Wnt7a in the Wnt7a+ HEK cell, and negative detection of it in the DYL HEK cells. This clearly confirms that the former can serve as a positive control, and the latter can serve as a negative control.

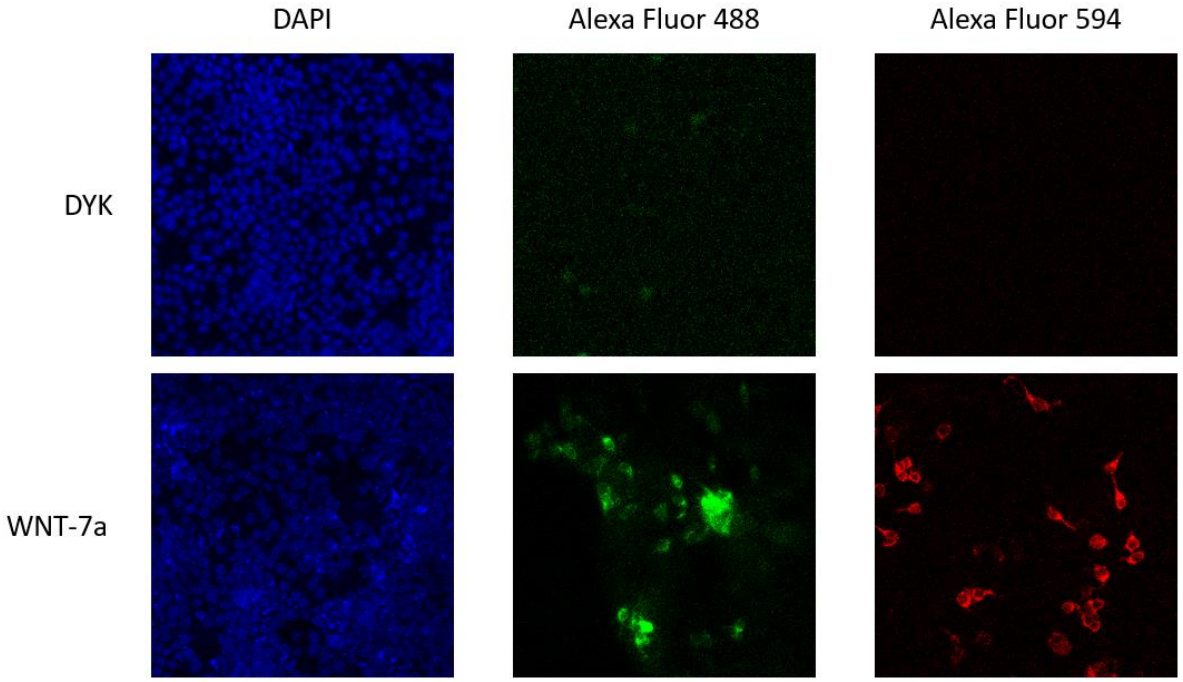

Figure 10: Confocal 20x Magnification images of HEK cells probed with an anti-Wnt7a primary antibody and fluorescent secondary antibodies.

DISCUSSION

Wnt7a was previously identified as one of 14 genes to experience a negative change in regulation at E10.5 in response to maternal binge alcohol consumption at E9.5 [9]. This study among others was key in directing us towards studying *Wnt7a*. While the change in Wnt7a mRNA expression has been studied, it is yet to be determined how this manifest in its protein expression profile.

Wnt7a has been reported to play roles in the cortex, lungs, testes, and liver [17]. As a result, adult mice tissues from these organs were extracted and treated with a WB to test our anti-Wnt7a antibody. Despite the expectation that these tissues would express Wnt7a and serve as a positive control, there was a negative signal on their blot. Consequently, prior to utilizing western blotting to analyze the changes in WNt7a protein expression in embryonic hearts, an effective positive and negative control needed to be prepared to ensure that our anti-Wnt7a primary antibodies and assay techniques detect the protein effectively. As seen in figures 5-7, our efforts to do so were successful. The Wnt7a+ HEK cell had a positive signal ~40-42 kDA, while the Dyk cells had a negative signal. There were also the expected positive and negative signals on IF images. This successfully established the HEK cell protein extracts as positive and negative controls that can be further used as control in embryonic heart analyses. This also showed that our anti-Wnt7a primary antibody can adequately detect the protein.

Due to the small size of the embryonic heart between embryonic days 10.5-12.5, concerns were brought up on whether one would be able to detect Wnt7a in such a small tissue. As a precautious measure, we employed chemiluminescent procedures in

our WB to compare it to the detection seen using fluorescent procedures. As seen in Figure 7, chemiluminescence proved to be the more sensitive technique, identifying Wnt7a in protein concentrations as small as 5 µg/lane. This highlights it as the more promising visualization method for analyzing Wnt7a protein in embryonic heart protein extracts.

Limitations

Optimizing our Western blot assay for clear detection of Wnt7a protein was one of the goals of this study. There is still room for better optimization, and there are a few areas in our protocol that could be contributing to this. For example, we have been using a Tris-Glycine based buffer, though according to Thermo Fisher, this is not the preferred transfer buffer for our Bis-Tris Gels. This could be contributing to sample smearing or incomplete transferring of protein.

The re-probing of some of our blots with β-actin following Wnt7a probing and stripping had been done after a number of days. There is a possibility that this delay has given room for protein degradation, resulting in less intense β-actin bands than there should be, relative to Wnt7a bands. This could affect the interpretation of how much β-actin there is in the protein samples compared to Wnt7a.

Future directions

This study has set the foundation of antibodies, reagents, and assays that can be used to quantitatively analyze Wnt7a protein expression in samples. This knowledge can be used to continue studying the differences in expression in control and ethanol

treated embryonic hearts from E10.5 to E12.5 using chemiluminescent WB and IF. We are currently in the process of identifying Wnt7a protein in wild-type embryonic mice hearts at 10.5, through chemiluminescent western blotting. The initial results look promising, but the protocol still needs optimization.

If our hypothesis proves correct, it could be followed up by a study looking at the downstream effects of reduced Wnt7a protein expression. Protein expression of downstream compounds like β-Catenin can be studied. This can help to determine the type of Wnt pathway that is affected and how it is contributing to the phenotypes exhibited in CHDs [6].

If no effect on protein expression of Wnt7a is observed, this could call for investigating the protein expression of the other 16 genes that were observed to have downregulated mRNA expression [9]. Adamts18 is one of those downregulated genes that can be studied. There has already been a report of it being connected to aortic arch development during E11.5-E14.5 [21].

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