Determining the Role of SOX17 and Hypoxia in Coronary Vessel Development

An Honors Thesis (HONR 499)

by

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Abstract

SOX17 is a gene that is involved in the regulation of angiogenesis, a process by which new blood vessels are formed. It is unclear whether SOX17 in these coronary progenitors is activated by hypoxia-inducible cues. To test whether Sox17 is important in coronary vessel growth, we analyzed coronary vessel growth in embryonic hearts that is lacking Sox17 and compared it to control hearts with normal Sox17 activity. We found that coronary vessel development was stunted when Sox17 was depleted from the heart suggesting that Sox17 is required for normal coronary vessel development. Next, we wanted to test whether Sox17 activity is hypoxia dependent. We tested this in HUVECs cell culture by culturing the control group in normoxic (21% oxygen) and experiment group in hypoxia (1% oxygen) and analyzing Sox17 expression. We did not find conclusive results from our HUVECs experiment and need further quantitative analysis to determine whether Sox17 is activated by hypoxia.

Acknowledgements

I would like to thank Dr. Bikram Sharma, who served as my PI for the last year and a half. He has given me a lot of guidance and has been very helpful and supportive. I also want to thank everyone in our lab. In addition, I want to recognize my research funding by the BSU Provost Start Up Package to B.S.; NIH R15 AREA to B.S., and the Louis Stokes Alliances for Minority Participation (grant number HRD 1618408, 2016-2021).

Process Analysis Statement

The research conducted in this thesis has been done continually over the last two years. The significance of this research comes from the fact that it will add to the scientific literature on the types and functions of proteins that make up the biological cascade of cardiovascular development. In turn, the knowledge gained from this study can be utilized to develop better medicine for individuals who suffer from myocardial infarctions (heart attacks). When the embryonic heart is developing, there are a multitude of complex steps that occur in order for development to move on to different stages. While this is occurring, the physiological and genetic program can influence the developmental stages it is undergoing. Our focus in this study is to understand how the vasculature of the heart commonly known as coronary vessels develop. In this study, we are investigating the potential role of SOX17, a transcription factor that is important for gene expression, in the regulation of coronary vessel development in the embryonic heart. In addition, we are also testing whether Sox17 is active under hypoxic conditions since hypoxia is implicated to be involved in coronary vessel development. To investigate whether Sox17 is important in coronary vessel development, we analyzed coronary vessel development in genetically modified embryos where Sox17 is depleted from stem cells that give rise to coronary vessels.

This study utilized the Cre-LoxP system to generate inducible Sox17 knockout mice to compare coronary vessel development in wild-type embryos compared to those with SOX17 inactivated. We then studied the role of hypoxia in SOX17 regulation by culturing Human umbilical vein endothelial cells (HUVECs) in hypoxia (1% oxygen) and normoxia (21% oxygen level). Embryonic hearts and cultured cells were analyzed by immunostaining analysis to detect

coronary vessel growth in Sox17 depleted hearts and Sox17 expression in cultured HUVECs. Overall, my results show that Sox17 depletion stunted coronary vessel growth suggesting that Sox17 activity is required for normal coronary vessel growth. My results from HUVECs culture were inconclusive in establishing whether Hypoxia stimulated Sox17 expression in endothelial cells. Additional quantitative analysis is necessary to establish this.

The research in the lab took a lot of hours of work per week to complete. During most of the school year, I dedicated 15-20 hours a week of preparatory work including, mice breeding, staining, and cell culturing. In addition, I worked over the summer conducting research. Overall, I was able to learn and grow a lot from this project. I mainly gained confidence in myself as a scientist. Coming in, I had very little research experience outside of class. Now, I not only am more confident in my research skills, but I am also better at giving formal presentations on my research. I presented my research work at three conferences: Louis Stokes Midwest Regional Center of Excellence, Indiana Physiological Society, and Indiana Academy of Sciences meetings. I have been challenged to grow as both a researcher and as a person and will continue to use the skills I have fostered in my personal and professional life.

Introduction

Myocardial infarctions, commonly known as heart attacks, are under the category of heart disease which is the leading cause of death in the United States of America [1]. A heart attack is when the heart muscles begin to die because there is a blockage of blood flow due to plaque that has built up in the arteries that feed the heart muscles. If this blood flow is not restored quickly, there can be permanent heart damage and can also lead to death [2], [3]. Unfortunately, the ability of the heart muscle to regenerate is very limited and hence there is a need for regenerative therapy to restore damaged blood vessels [4]. The process of growing new blood vessels occurs through a process called angiogenesis. During embryonic development, new blood vessels are formed to transport oxygen and blood to the heart. Coronary vessels develop through angiogenesis from pre-existing coronary endothelial cell precursors that reside in the endocardium of sinus venosus and endocardium [5]. Several genes encode for proteins that specifically regulate coronary angiogenesis. *Sox17* is among such genes, which encode for a protein, SOX17, which is a transcription factor that activates gene expression and is shown to regulate coronary angiogenesis. While the heart is forming, various cellular layers are developing around the heart making diffusion of oxygen more difficult. During these times, angiogenesis is needed to increase the flow of blood and oxygen to the heart. It has been shown that SOX17 has increased expression in areas of the heart that have low oxygen levels (hypoxia) and active coronary angiogenesis [6]. It is predicted that SOX17 is activated due to hypoxia to initiate coronary angiogenesis.

This study aims to determine the role that hypoxia has on SOX17 and whether that then regulates the process of angiogenesis during the embryonic stages of development. To study this,

we used a genetic tool known as Cre-LoxP system (see information on this system below), which allows us to selectively delete a portion of the desired gene to deplete its function and observe the impact of its loss-of-function. Such a Cre-LoxP system is utilized in mice to generate SOX17 depleted mice [7]. We hypothesize that the cells in hypoxic conditions will have increased expression of SOX17 which will lead to increased myocardial angiogenesis. A better understanding of this process can result in treatments for individuals suffering from damaged coronary vessels. In addition, we also utilized a cell culture model to ascertain whether exposure of endothelial cells to hypoxic conditions would activate SOX17 expression from its gene in the cells that give rise to coronary vessels. We hypothesize that the cells in hypoxic conditions will have increased expression (production from genetic instruction) of SOX17, which will lead to increased coronary angiogenesis in the myocardium. A better understanding of this process can result in the discovery of therapy and treatments to regenerate coronary vessels for individuals suffering from damaged coronary vessels.

Background on Cre-LoxP genetic system used to obtain conditional knockout transgenic mouse

The Cre protein is a site-specific DNA recombinase that can catalyze the recombination of DNA between specific sites in a DNA molecule. Locus of X-over P1 (LoxP) is a site on the bacteriophage P1 consisting of a 34-sequence which has two 13 bp inverted and palindromic (reads the same forward and backward) repeats and 8 bp core sequences. Cre recombinase can be genetically modified to make it available only in desired tissue by controlling its expression under tissue-specific promoter. Additionally, the Cre-LoxP system can be temporally induced by an exogenous inducer such as tamoxifen, an estrogen receptor modulator (synthetic ligand). These modified variants of Cre recombinase have been fused to a mutated ligand-binding domain of the estrogen receptor. Cre-ER proteins are kept in the cytoplasm via association with

the HSP90 chaperone (recognizes it is nonnative). Upon addition of tamoxifen, tamoxifen-bound Cre-ER dissociates from HSP90, translocates into the nucleus, and carries out site-specific recombination between flanking LoxP sites (**Figure 1**).

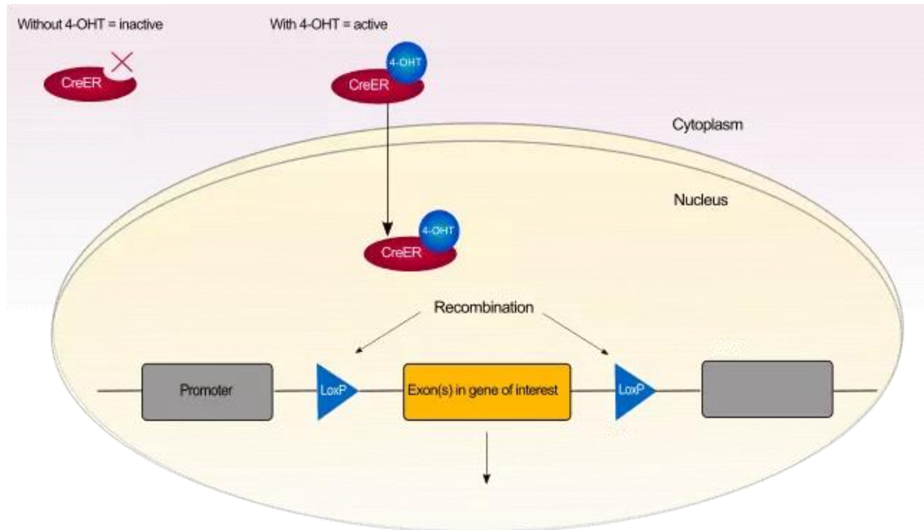

Figure 1: Basics of the Cre-Lox P System in the Cytoplasm and Nucleus of the Cell. Afimoxifene, 4-OHT, is the active metabolite of tamoxifen. Tamoxifen is utilized in the study to induce the Cre-LoxP system to excise out the SOX17 gene at e14.5 (embryonic day) in the embryonic mice hearts.

The operon displayed is inactive due to the stop codon but has two LoxP sites for genetic modification. If the two LoxP sites are in the same orientation, the floxed sequence (sequence flanked by two LoxP sites) is either excised, translocated, or inverted by Cre recombinase

depending on the orientation of the LoxP site (Figure 2). The newly formed strand of DNA now has the gene downstream of where the stop codon is being expressed. We breed BmxCreER and Sox17 flox mouse lines to achieve endocardium-specific deletion of Sox17 in the embryos.

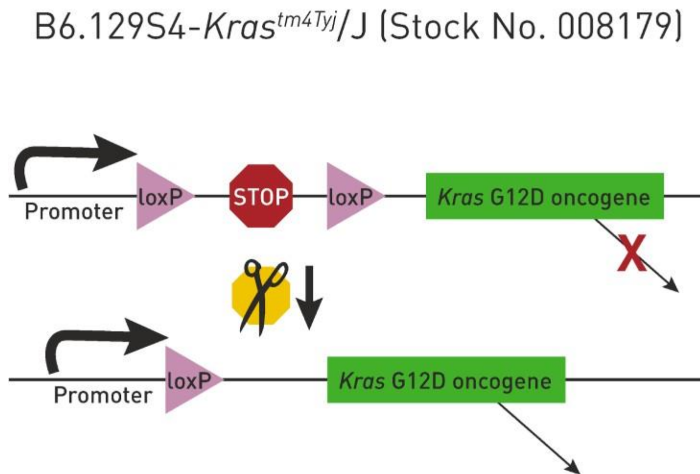

Figure 2: LoxP Placement Influences Regulation of Upstream Genes. The location (which genes it flanks) and the orientation of the LoxP sequences determine where and type of editing the Cre recombinase will do.

Materials and Methods

<u>HUVECs culture Experiments</u>

HUVECS cells were acquired from ATCC, and a cell culture was conducted. Cells were initially cultured from a frozen sample before they were subjected to cell culture for the experiment. For experiments, the cells were plated in separate 24-well plates at 50% confluency and were incubated either in normoxic (21% oxygen) or hypoxic (1% oxygen) conditioned CO_2 incubator. After 24 hours of culture, immunohistochemistry was performed using an anti-hif-1alpha antibody (1:100) (and the samples were co-stained with an anti- Sox17 antibody (1:500). After primary antibody staining overnight, cultures were subjected to wash steps. For this, the primary staining was removed, and it was washed with 1X PBS three times for five minutes each time on the rocker. After washing the primary antibody, the secondary antibody staining was performed. We used fluorescently labeled secondary antibody mixes including donkey anti-rabbit antibody 488 (to label rabbit anti-hif-1alpha primary antibody) with a working dilution of 1:250, and donkey anti-goat antibody 555 (to label goat anti-Sox17 primary antibody) with a working dilution of 1: 250. Antibody mixes were prepared in 0.5% PBST solution. The secondary staining was performed on a rocker at room temperature for two hours by covering the plate with aluminum foil to avoid light bleaching. Then the secondary staining was removed, and it was washed with 1X PBS three times for five minutes each time on the rocker. The cells in cover glass were then mounted onto the slides using fluorescent mounting media containing nuclear staining DAPI. Samples for normoxia and hypoxia were mounted into two separate slides. SOX17, HIF-1A, and DAPI staining were analyzed from collected micrograph images taken in either the confocal microscope or upright fluorescent microscopes. Data were collected by

counting the number of Hif-1alpha +, Sox17+ and Dapi+ cells in 5 fields of view (FOV) and comparing the averages in normoxic and hypoxic conditions.

<u>Mice Breeding strategy to obtain Sox17 conditional deletion embryos</u>

We bred a mouse line containing two copies of LoxP allele (flox/flox, abbreviated as fl/fl) with another mouse line that has a one copy of Bmx-CreER allele. Bmx is a promoter for endocardium. When these two mouse lines are crossed, it will yield embryos with the following genotypes: BmxCreER-; Sox17fl/fl (no deletion of Sox17, control group) and BmxCreER+; Sox17fl/fl (deletion of both Sox17 copy of gene, conditional knockout, cKO group). The DNA of the mice were tested to ensure their genotypes for the presence and absence of Cre and LoxP allele **(Figure 3)**.

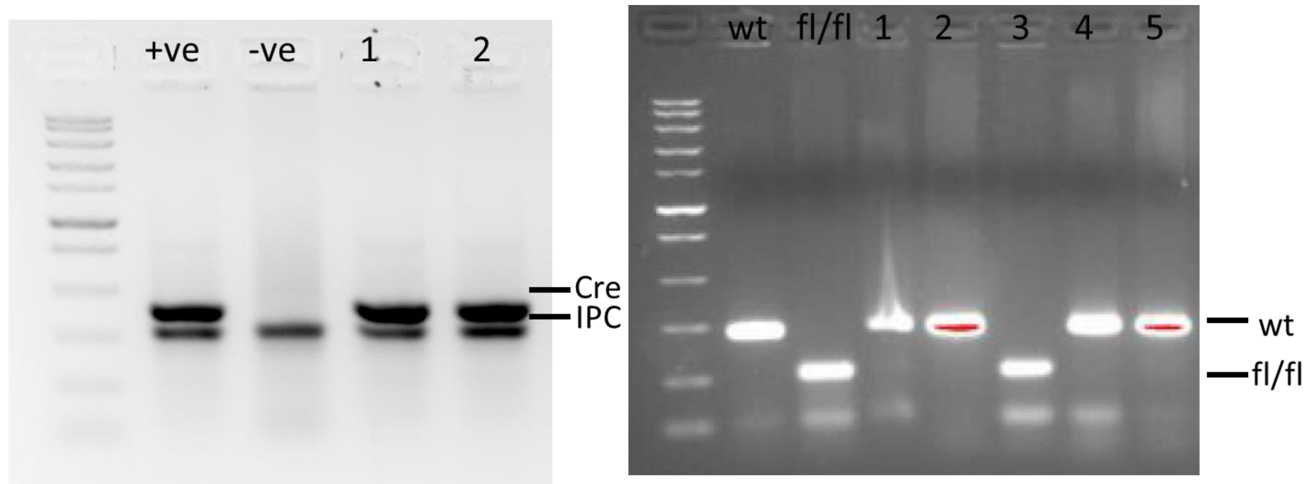

Figure 3. Micrograph of Gel electrophoresis showing genotyping for BmxCreER allele (left Panel) and Sox17 flox allele (right panel). For Cre genotyping, internal positive control (approx. 300 bp); Cre (approx. 350bp). For Sox17 flox genotyping, wildtype allele (approx. 278bp) and flox allele (approx. 155 bp). +ve, positive control, -ve, negative control. Wt, wildtype, fl/fl – homozygous floxed allele. 1-5 indicate sample numbers.

Whole mount immunostaining of embryonic hearts

Each pregnant mouse was injected with 20 mg/ml of tamoxifen at e14.5 and the embryos were harvested at e17.5. Embryos were fixed at 4% PFA. Hearts were isolated from fixed embryos and were subjected to whole-mount immunostaining. Primary antibodies used were rabbit anti-Dach1 (used at 1:1000 dilution) and goat anti-Sox17 (used at 1:500 dilution). Primary antibodies were diluted in 0.5% PBT solution. The hearts were stained in primary antibody mix overnight at 4 °C. The next day, after several wash steps, hearts were subjected to secondary antibody staining. Secondary antibodies used were Alexa fluor donkey anti-rabbit 488 (used at dilution 1:250) and Alexa fluor donkey anti-goat 555 (used at dilution of 1:250). The hearts were stained in secondary antibody mix overnight at 4oC. The next day, after several wash steps, samples were mounted in fluorescent mounting media and were imaged in a confocal microscope. Images were used to compare and analyze the KO and wild-type SOX17 embryos. Data were collected by counting the cells across the septum and measuring the left and right ventricle width using *ImageJ*.

Results

Sox17 expression did not change under hypoxic conditions.

We used HUVECs cell cultures to study Sox17 expression when the cells are incubated in normoxia vs hypoxia. Hypoxic incubation did not positively stimulate Sox17 expression in the HUVECs cell cultures. Sox17 was visualized by immunostaining using anti-hif-1alpha antibody and the samples were co-stained with anti- Sox17 antibody. The HUVECs in normoxic conditions (21% oxygen) and hypoxic conditions (1% oxygen), did not show a statistically significant difference in Sox17 expression (**Figure 4**).

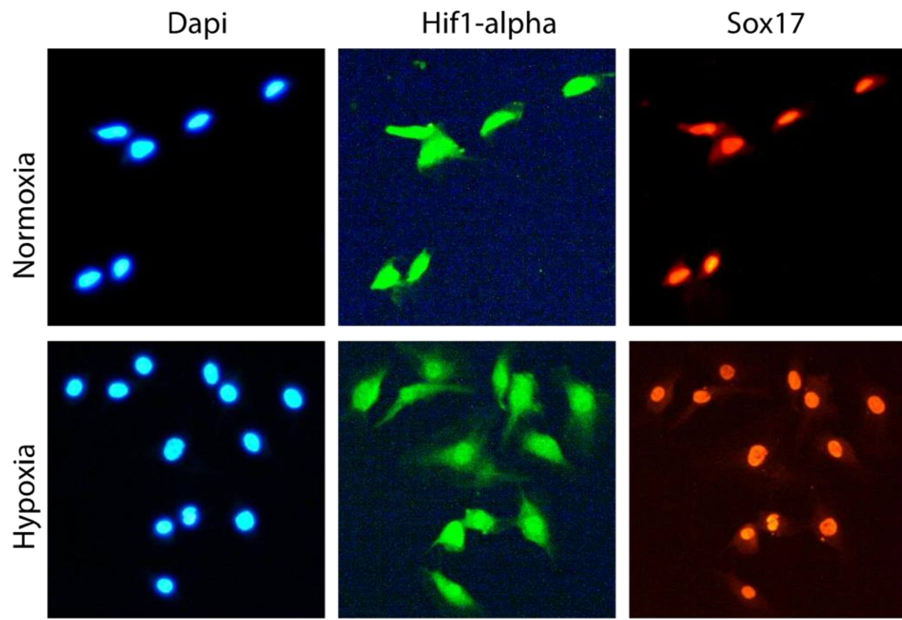

Figure 4. Confocal Imaging of Sox17 expression in HUVECs cell culture. Qualitative analysis of HUVECs cell culture showed that normoxia (top), 21% oxygen, and hypoxia (bottom), 1% oxygen, incubation did not result in significant difference in Sox17 expression.

Sox17 depletion stunted growth of coronary vessel development

We have utilized BmxCreER deletor line, which is known to be active only in the coronary endothelial progenitors arising from endocardium as revealed by lineage tracing experiments. We bred BmxCreER deletor line with Sox17 flox line to produce control (BmxCreEr-; $Sox17^{flox/flox}$) and conditional knockout (BmxCreEr+; $Sox17^{flox/flox}$) embryos. BmxCreEr+; $Sox17^{flox/flox}$ hearts showed significant absence of Sox17 expression compared to control (**Figure 5,** Sox17 panel).

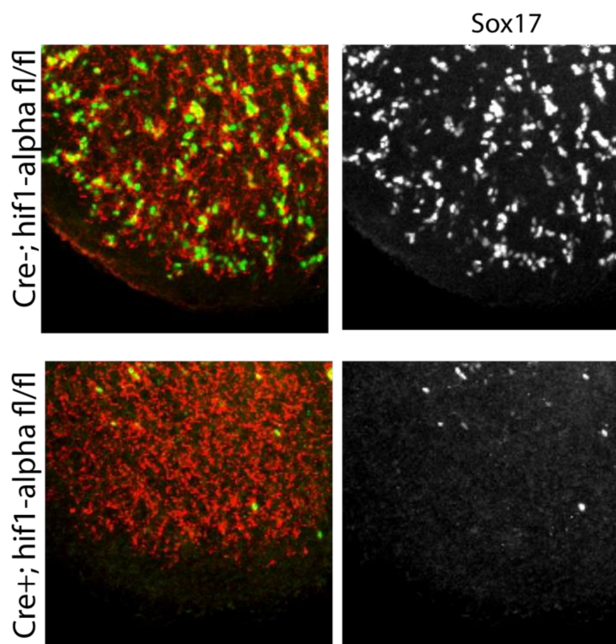

Figure 5. Depletion of hif1-alpha in myocardium resulted in downregulation of Sox17 expression. Confocal images of e12.5 hearts from control (top panel) and hif1-alpha knockout mouse embryos immunostained for Sox17 (green) and VE-Cadherin (red).

We analyzed hearts from control and knockout e16.5 embryos by immunostaining to visualize coronary vessel sprouts (anti-VE-cadherin staining) and coronary endothelial cell nuclei (anti-Dach1 staining). From the qualitative analysis of confocal micrograph of control and Sox17 conditional knockout (cKO) hearts, we found that the depletion of Sox17 from endocardial progenitor stem cells resulted in stunted coronary vessel development in the RV compared to control **(Figure 6)**. While we observed normal coronary capillary plexus in control,

we observed rounded (blood islands) premature coronary plexus in the knockout. This indicates that in the absence of Sox17 coronary vessel growth was developmentally stunted (**Figure 6**).

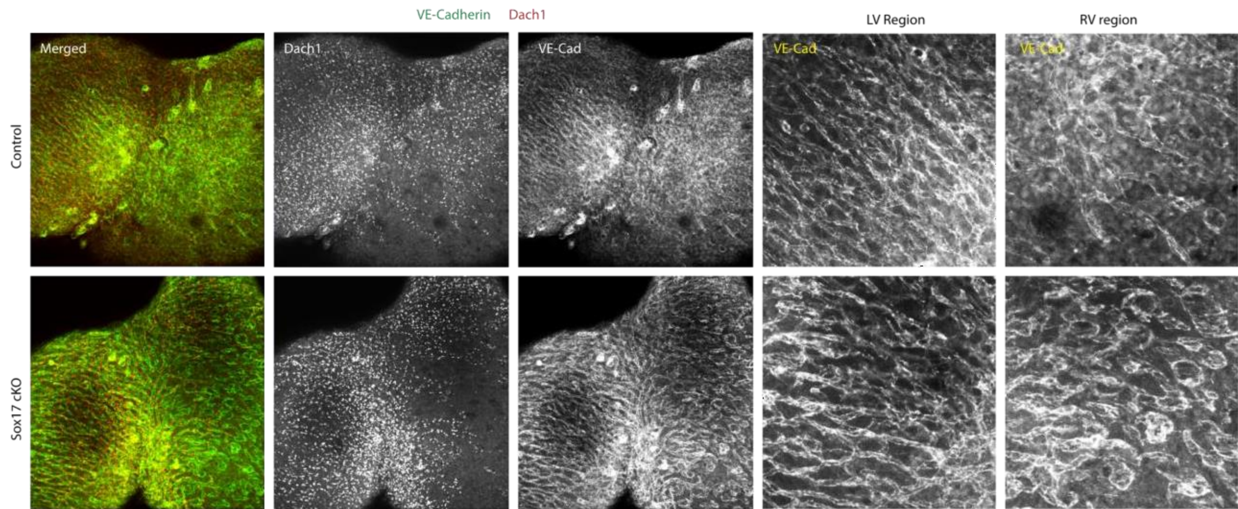

Figure 6: Depletion of Sox17 from endocardial progenitor shows stunted coronary vessel growth phenotypes. Whole-mount confocal micrograph of e14.4 control (BmxCreER-; $Sox17^{fl/fl}$) and Sox17cKO (BmxCreER-; $Sox17^{fl/fl}$) mouse hearts immunostained with antibodies for VE-Cadherin (in green) to visualize coronary endothelial cell membrane and Dach1 (in red) to visualize coronary endothelial cells nuclei. The top panel is the control, the bottom panel is Sox17 cKO (Sox17 conditional knockout). LV, left ventricle; RV, Right ventricle.

Furthermore**,** analysis of confocal micrograph of the cross-section immunostained hearts revealed that the left ventricle (LV) and Right ventricle (RV) of the SOX17 KO did not show a statistically significant difference in the myocardium width compared to the control ($p = 0.901$, LV; ($p = 0.435$, RV) **(Figure 7 and 8)**.

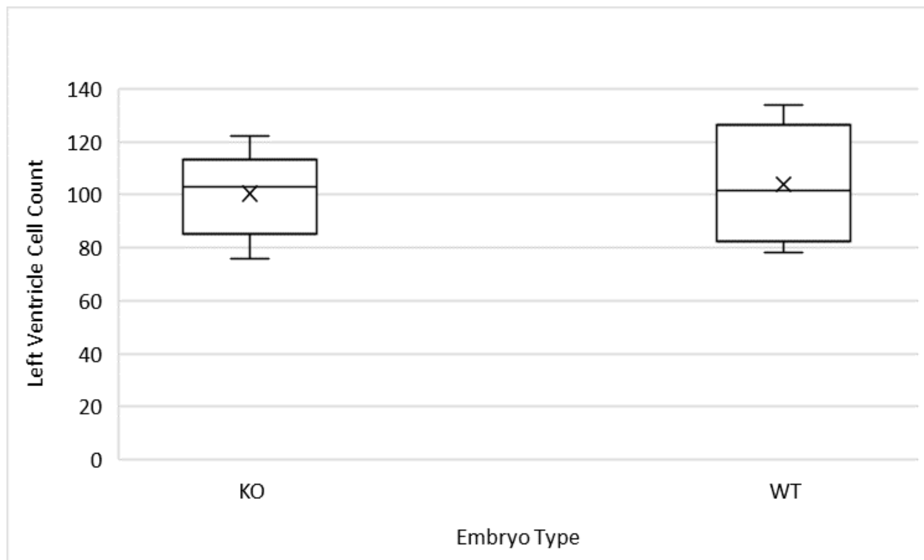

Figure 7: SOX17 WT and KO Left Ventricle Width Measurements. The differences between the means of the left ventricle width measurements were shown to not be statistically significant.

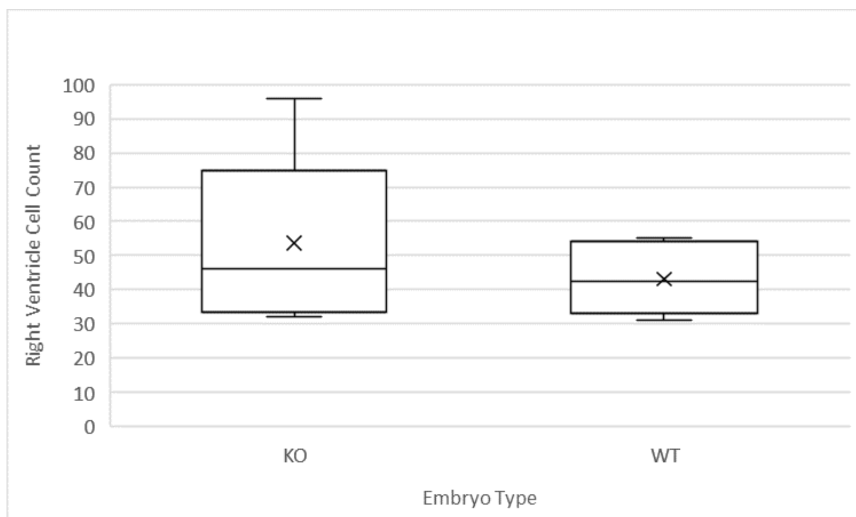

Figure 8: SOX17 WT and KO Right Ventricle Width Measurements. The differences between the means of the right ventricle width were shown to be statistically significant.

Discussion

The regulation of SOX17 plays a major role in cardiovascular development. SOX17 is known to stimulate coronary vessel formation in hearts through coronary angiogenesis. Knockout studies in mouse models have indicated the importance of SOX17 in cardiovascular development [8],[9]. But it is unclear whether SOX17 in endocardial coronary progenitors is activated by hypoxia-inducible cues. This project aimed to evaluate the role that hypoxia has on SOX17 and whether that then regulates the process of angiogenesis during the embryonic stages of development.

To determine the role of Sox17 and hypoxia in coronary vessel development, HUVECs were incubated in normoxia and hypoxia for 24 hours. After immunostaining, the qualitative analysis found no significant difference in the expression of Sox17 in normoxia vs hypoxia (**Figure 4**). Sox17 has shown upregulation in the EC when tissue hypoxia is induced [10]. Therefore, there may have been an insufficient length of incubation time, or the incubator was not properly administering the oxygen levels.

The effect on coronary development and the depletion of SOX17 on coronary vessel growth in embryonic mice hearts were evaluated. Coronary vessel growth was analyzed in Both SOX17 WT and SOX17 KO hearts. Our data show phenotypic differences in coronary vessel growth. We found a stunted coronary growth phenotype in knockout hearts compared to control (**Figure 6**). Despite defects in coronary growth, the myocardial thickness both in the left and the right ventricle was unaffected **(Figure 7).** Our results show the endothelial cell-specific function of Sox17. Consistent with our data, depletion of Sox17 in the mesoderm has shown cardiac

defects [11]. It is reasonable to believe that more quantitative measures such as quantitative polymerase chain reaction (qPCR) and western blotting will provide a more accurate measurement of Sox17 expression and this might provide us with more quantitative data on Sox17 expression under hypoxia.

Future Directions

We will analyze coronary vessel formation in Sox17 cKO conditions at other earlier and later time points to understand the temporal effect of Sox17 on coronary vessel formation.

In addition, we will repeat culture experiments with human cell lines to assay for Sox17 expression using cardiac microvascular cell lines. Alternatively, we will also use ex vivo explant culture to assay for Sox17 expression under hypoxia gain-of-function conditions. Also, quantitative polymerase chain reaction (qPCR) and western blot methods will be used to evaluate SOX17 expression. It was originally planned to be used but due to the pandemic, all of the mice in the lab were sacked multiple times, limiting the time we could develop mouse lines for various forms of analysis.

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DATE:	May 4, 2021
TO:	Bikram Sharma, PhD
FROM:	Ball State University IACUC
RE:	IACUC Protocol #: 1320488-4
TITLE:	Analysis of cardiovascular development and function
SUBMISSION TYPE:	New Project
ACTION:	**APPROVED**
DECISION DATE:	May 4, 2021
EXPIRATION DATE:	May 4, 2024
REVIEW TYPE:	Full Committee Review

The Institutional Care and Use Committee (IACUC) recently reviewed the protocol listed above. Your protocol was **APPROVED**.

Approval Period: May 4, 2021 through May 4, 2024

Category:

- [X] Laboratory Research
- [ ] Wildlife
- [ ] Non-Research
- [ ] Breeding Colony

Approved Animal Biosafety level (ABSL): [ ] **ABSL 1** [ ] **ABSL 2** [ ] **No Changes**

Biosafety Committee (IBC) approval # *[enter if applicable]*

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Tim Carter, PhD, Associate Professor, Chair
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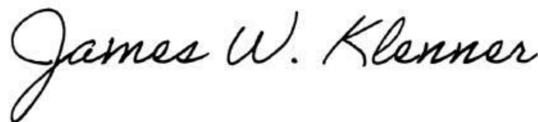

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