REQUIREMENT OF OSR1 IN SECOND HEART FIELD (SHF) FOR PROPER OUTFLOW TRACT (OFT) ALIGNMENT

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by

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ABSTRACT

Requirement of *Osr1* in Second Heart Field (SHF) for Proper Outflow Tract (OFT) Alignment

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Congenital heart disease (CHD) is current in 1/3 of all birth defects and has the prevalence of 1 in 1000 births.[1] 1/3 of all CHDs consist of cardiac outflow tract (OFT) defects.[1] Despite its high prevalence, the genetic ontogeny of CHD has much to be studied. Knocking out *Osr1*, a gene encoding for a putative transcription factor containing four C2H2-type zinc finger motifs, is reported to cause heart defects including atrioventricular septal defects (AVSDs) in mice[2-4] while its involvement in OFT development has yet to be studied. *Osr1* is expressed in the second heart field (SHF), overlapping the expression of *Gli1*, an important modulator of sonic hedgehog (*Shh*) signaling pathway.[4] *Shh*-signaling has been reported to contribute to the OFT development.[5] Our preliminary study has shown that *Osr1* deletion causes OFT defects including DORV and OA—suggesting its role in OFT alignment. We hypothesize that *Osr1* is required in the SHF and *Shh*-signaling for proper OFT alignment. We will use a Cre-lox cell-specific KO technique to create embryos with *Osr1* specific KO in SHF cells and *Shh*-receiving cells. We expect to observe high percentages of OFT misalignment including DORV and OA in embryos with *Osr1* specific KO in SHF cells and *Shh*-receiving cells. This study will provide information which cell lineage is required for *Osr1* in OFT development.

DEDICATION

This thesis is dedicated to my late grandmother, Sumiko Ishiguro, whose courage and determination, and love has always been guiding, encouraging, and protecting my family and me. After her disease took over her breathing, she fought back for four years, treasuring every moment that she spent with her grandchildren. Naturally, my life goal was to become part of a healthcare team that can make a huge impact on a person's life, such as the nurses and doctors that treated and fought alongside my grandmother –one day by day. Her grit to fight against her disease and the love she brought to our family and the hospital community rooted an aspiring healthcare worker. My grandmother has given me a challenge and a dream, but a challenge and journey I hope to lead. The day when I walk across the graduation stage, she will be there with me as she has been every day.

.

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I would like to thank my faculty mentor, Dr. Linglin Xie for welcoming me as part of her research team and allowing me to have the opportunity for this research. Dr. Xie has given me immense support throughout this project. I would also like to thank the postdoctoral researchers—Dr. Jimmy Liu, Dr. Hui Peng, Dr. Henghui Cheng, and Mr. Zehuan Ding, Mr. Jeilin Liu, Ms. Douhua Chen, and Ms. Michelle Summerfield for their guidance and support throughout the course of this research. From bench side assistance, much encouragement, and friendship, my experience at the Dr. Xie's lab has been full of productive and entertaining days.

Great thanks also go to my friends, peer lab mates, and the department faculty and staff for making my time at Texas A&M University rich with new knowledge, discoveries, and meaningfulness.

Finally, thanks to my mother, Harumi McCauley, my father, Mark McCauley, and my dearest friend, Li Wang, for their encouragement, support, patience, and love.

NOMENCLATURE

CHD	Congenital heart disease
OFT	Outflow tract
AVSD	Atrioventricular septal defects
SHF	Second heart field
Shh	Sonic hedgehog
DORV	Double outlet right ventricle
OA	Overriding aorta
VSD	Ventricular septal defect
CNC	Cardiac neural crest
PHF	Primary heart field
NCC	Neural crest cells
EMT	Epithelial-mesenchymal transformation
E#	Embryonic stage #
Hh	Hedgehog
GIFM	Genetic inducible fate mapping
KO	Knockout
SpM	Splanchnic mesoderm
ASD	Atrial septal defect
TM	Tamoxifen
ER	Estrogen receptor

CHAPTER I

INTRODUCTION

Background and Significance

Congenital Heart Disease (CHD)[1] is one of the most common birth defects as it constitutes 1/3 of all birth defects with an occurrence of nearly 1 in 1000 live births.[1] Cardiac outflow tract (OFT) defects account for nearly 1/3 of CHDs and causes significant morbidity and mortality of children and adults.[1] Despite its common occurrence, the genetic ontogeny of OFT has yet to be fully elucidated. The cardiac OFT is formed through complex interactions of multiple cell types within the looped heart and populations of invading extra-cardiac cells. A variety of OFT defects result from the incorrect morphogenetic patterning occurring at the arterial extremity of the heart which is known to be essential to the formation of independent pulmonary and systemic circulations in vertebrates.[1] Because of the complex coordination of extra-cardiac progenitor populations in location and timeframe, OFT is highly susceptible to congenital defects.

Variations of OFT Defects

Double outlet right ventricle (DORV) and overriding aorta (OA) (Figure 1) are both common types of OFT anomalies. In a normal heart and OFT, the aorta and pulmonary trucks are separate and exit fully divided left and right ventricles respectfully.[6] In DORV, the aorta and pulmonary trunk exit the right ventricle together and the interventricular septum remains open resulting in interventricular septal defect (VSD). In OA the aorta is located on top of the VSD.

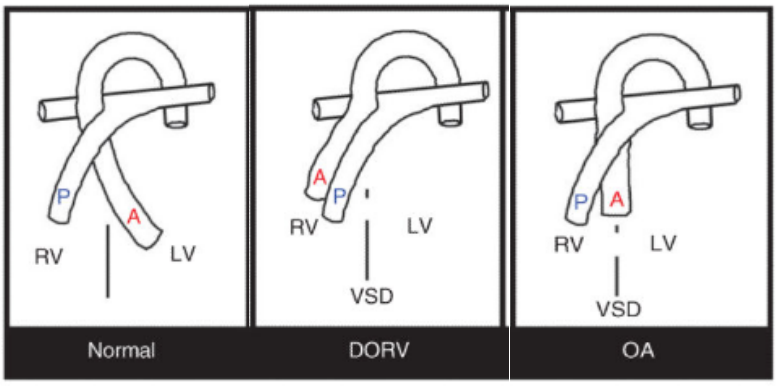

Figure 1.

Schematics of normal and two types of OFT defects, DORV and OA are shown.[1]

DORV and OA differ from other OFT defects in that the aorta and pulmonary trunk are fully separated. However, the abnormality is prevalent in the incorrect alignment over the right ventricle. Although the cellular, molecular, and genetic basis of OFT development is fully elucidated, the ontology of proper OFT alignment is still unclear. This study is significant as it will be investigating the genetic elements involved in proper OFT alignment.

Genesis of OFT

Mammalian OFT conduit is consisting of migratory progenitor cells, such as cardiac neural crest (CNC), primary heart field (PHF), and second heart field (SHF) cells. These progenitor cells generate endocardial and myocardial cells. Precise spatiotemporal regulation and multiple lineage signaling are required during extra-cardiac colonization of the of the OFT from distinct embryonic regions take place for normal OFT development.[1]

PHF Lineage

The very first form the primeval heart can be described as a single linear heart tube made of paired lateral pre-cardiac mesoderm of the PHF.[1] The paired lateral pre-cardiac mesoderm of PHF later contributes to the formation of the left ventricle and portions of the atria.

CNC Lineage

Neural crest cell (NCC) derivatives are indispensable in OFT remodeling,[1] septation, and arrangement of the trunks. CNC is a subset of neural crest that originate from the lower hindbrain and plays a crucial role in normal heart development.[1] Epithelial-mesenchymal transformation (EMT) allows CNC to migrate to the aortic sac and OFT truncal cushions around embryonic stage 9 (E9) in mice (days 20-22 in human) and onwards until E11 giving rise to the aorticopulmonary septa.[1] The aortic sac is a dilated, non-muscular, vascular channel lined by endothelial cells connecting the primitive OFT and arch arteries. The OFT septum separates the systemic and pulmonary circulation. This structure is originally like an extending shelf, then goes through OFT elongation and grows its way to meet the OFT cushions.[1] OFT rotation occurs with simultaneous shortening and shift to a medial position above the ventricular septum, meaning, the septation of OFT occur as the aorticopulmonary septum and growing endocardial cushions fuse due to increased CNC colonization of truncal cushions. Failure of proper OFT rotation while OFT elongation takes place can lead to OFT defects, such as DORV and OA. [1] CNC also play a role in the development of the aortic arch and atrial wall by contributing to the vascular smooth muscle cells of those structures.

SHF Lineage

The right ventricle, ventricular septum, and OFT precursor is then derived from cardiomyocytes from the SHF pharyngeal mesoderm that migrate into the heart at E8 in mice,[1] while third week in humans.[1] Different segments of the OFT is formed by different progenitor cells –from within the loop shaped heart and from extra-cardiac cells, such as CNC and SHF cells, that migrate from outside the heart to within. Part of the OFT precursor is formed by the SHF that grows out of the cardiac crescent to the arterial end of the developing left ventricle. [1]

Rightward looping and elongation of the OFT occur with the addition of SHF-derived myocardial precursors from pharyngeal mesoderm.[1] As this occurs, simultaneously, the SHF progenitor proliferation is progressively inhibited while the cardiomyocyte lineages are activated. Impaired SHF proliferation, primarily caused by the failure of SHF to colonize the arterial extremity of the heart tube, can lead to underdevelopment of the OFT. Gene deletions that are highly expressed in SHF, such as *Mef2c*,[1] has shown to result in abnormalities during heart development, such as failure in elongation and looping of the heart tube and underdevelopment of the right side of the heart, atria, and OFT.

Endocardial Cell Lineage

Derived from cardiac mesoderm, endocardial cells are endothelial cells that line the myocardium of the heart.[1] The endocardial cell lineage plays two important roles in OFT morphogenesis: one in endocardial cushion development and second as a regulator of OFT myocardial proliferation.[1] The endocardium goes through EMT to produce mesenchymal cells that migrate for acellular endocardial cushion to prevent retrograde blood flow.[1] After the cushion and septation of the OFT fuse, the truncal OFT endocardial cushion further transforms into aortic and pulmonary valves.[1] Here, truncal OFT (CNC-derived) refers to the portion of the OFT distant from the heart making it closer to the pharyngeal arches.[1]

Hh Signaling

Hedgehog (*Hh*)-signaling has shown to have an important role in multiple aspects of the heart development. While critical in left-right symmetry in mice,[7-9] *Hh*-signaling is also required in CNC survival and pharyngeal endoderm. A study that disrupted the the *Hh*-signaling in CNC by crossing *Wnt-cre* line with *Smo-flox* line producing mutant embryos all showed OFT defects, such as DORV.[10,11] Additionally, but no less, *Hh*-signaling was shown to mark a subset of

cardiac progenitors specific for atrial septum and pulmonary trunk in the SHF of the mouse heart.[11] *Hh*-receiving progenitor cells were marked in SHF between E8 and E10,[11] using genetic inducible fate mapping (GIFM) with *Gli1: CreERT2*. Atrial septum and pulmonary trunk progenitors migrate into the heart after receiving *Hh* signals outside of the actual developing heart.[11] The atrial septum progenitor cells receive *Hh* signaling in the posterior SHF mesoderm and migrate to the atrial septum at E9.5 and E11.5. [11] As for the cardiac OFT, the progenitor cells receive *Hh*-signaling in the anterior SHF mesoderm around E8 and E10 in the pharyngeal mesoderm and migrate into the pulmonary artery between E9.5 and E11.5.[11] Sonic hedgehog (*Shh*) signaling in the SHF, was shown to be required in the pulmonary endoderm for atrioventricular septation. While *Hh*-signaling is known for differentiating atrial septum and pulmonary trunk cardiac progenitors from posterior and anterior SHF respectively, *Shh*-signaling is required to induce *Hh*-signaling, which in turn, specifies atrial septum progenitor cells from a subset of atrial progenitors. This showed that *Hh*-signaling is required in the SHF during a critical time window for proper OFT development while *Shh*-signaling being crucial to initiating the *Hh*-signaling itself.

Osr1

Osr1, a gene encoding for a putative transcription factor containing four C2H2-type zinc finger motifs, when knocked out are reported to cause atrioventricular septal defects (AVSDs) in mice[2-4]. Previous studies have shown that *Osr1* is a downstream target of Tbx5, creating the Tbx5-Osr1 complex, working simultaneously with Tbx5-*Hh*-signaling, regulates the cell cycle progression and proliferation of SHF cardiac progenitor cells that take role in cardiac septation. [2,3,12] While *Osr1* is known to contribute in regulating atrial septation, *Osr1* involvement in OFT development is yet to be fully elucidated.

Preliminary Studies on *Osr1*

Previous studies that used in-situ hybridization and immunohistochemistry showed high expression of *Osr1* at E9.5 and E10.5 within the dorsal mesocardium, but not in the endothelium.[2,12] Using GIFM, a previous study showed that *Osr1* also contributes to atrial septum progenitors between E8.0 and E11.0.[2] *Osr1* knockout (KO) resulted in high occurrences of AVSDs and OFT misalignment, including DORV and OA, suggesting that *Osr1* contributes to OFT alignment.[2,3] Refer to Figure 2, where mice embryos at E14.5 showed a normal phenotype with *Osr1* knockdown and showed abnormalities when *Osr1* was knocked out. When OFT misalignment was further investigated at E11.5, at which OFT is yet to be separated but has begun its rotation for two days, OFT in *Osr1* KO embryos had abnormal rotation, suggesting that *Osr1* is involved in OFT alignment through the process of its rotation.[2,3] *Osr1*deletion also caused a decrease in number of proliferating cells in the SHF at E10.5, resulting in shortened SHF splanchnic mesoderm (SpM) between the outflow and inflow tract;[2] suggesting its involvement in SHF patterning. Evaluation of SHF cells using IHC staining showed that *Osr1* KO mice embryos had 53.7% fewer mitotic cells, suggesting that *Osr1* is involved in SHF cell proliferation.[2] A significant decrease in *Smo* expression, a *Shh*-signaling pathway modulator, in the pSHF of mice embryos with *Osr1* KO ate E9.5 was found, in turn suggesting the role of *Osr1* in *Shh*-signaling regulations.[2] The dosage of Osr1expression effected the *Hh*-signaling pathway positively during E8 to E10.5.[3]

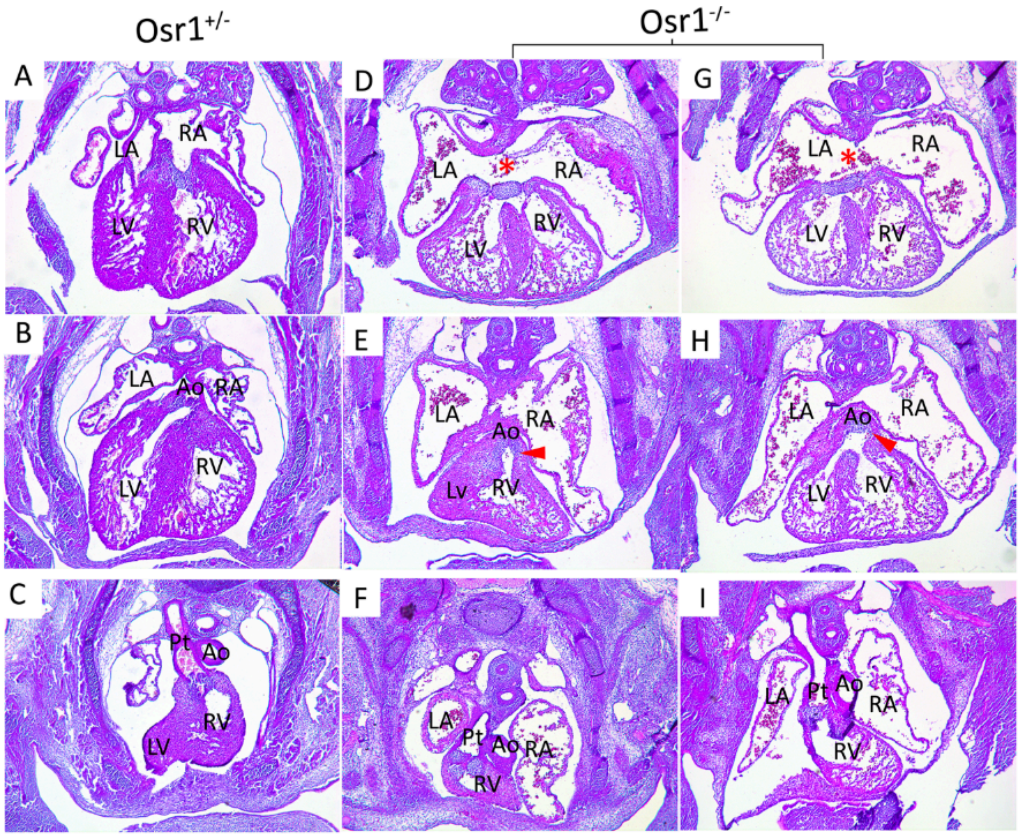

Figure 2. Image of normal phenotype with *Osr1* knockdown and abnormalities with *Osr1* KO. Picture A, B, C show normal phenotypes when *Osr1* is knocked down. In picture A, the interatrial septum fully separates the left and right atrium. In picture B, the aorta is correctly connected to the left ventricle. In picture C, the aorta is completely disconnected from the right ventricle when the pulmonary trunk is connected to the right ventricle. Pictures D through I show abnormalities that occurred when *Osr1* was knocked out. In picture D and G, the interatrial septum fails to fully separate the left atrium and the right atrium resulting in an atrial septal defect (ASD). In picture E, the aorta is incorrectly positioned over the right ventricle, resulting in a DORV. In picture H, the aorta is located above a VSD, resulting in a OA. In picture F and I, the aorta is fails to fully separate from the right ventricle when the pulmonary trunk is visualized to be leaving the right ventricle; which is also a characteristic for both DORV and OA.

Rationale

Beginning around E8, additional mesenchymal cells migrate to the anterior side of the heart from the SHF[15-19], and populate the OFT. Subsequently, these endocardial cells and endothelial cells that line the interior of OFT and go through EMT to make the endocardial cushions. In turn, the endocardial cushions form the OFT septum with the migrating CNC. Failure of SHF cell migration to the arterial pole of the heart commonly results in shortened OFT that leads to arterial pole misalignment[1,20,21]. Previous studies have shown that *Osr1* is strongly expressed in the SHF and contribute to the pulmonary trunk between development stages E9.5 and E10.5.[2,12] KO of *Osr1* also resulted in SHF proliferation defects, shortened SHF SpM, and OFT misalignment: which all suggested that *Osr1* plays an important role in SHF in turn effecting OFT development.

Previous studies have indicated *Osr1* to be essential to the collaborative work of Tbx5-*Osr1* complex and Tbx5-*Hh*-signaling in regulating the cell cycle progression and proliferation of SHF cardiac progenitor cells that take role in cardiac septation.[2,3,12] The knockdown of *Osr1* resulted in decreased *Smo* expression, a *Shh*-signaling pathway modulator, in the pSHF. This suggested that *Osr1* might have a role in *Hh*-signaling within SHF.[3]

Innovation

Despite the high prevalence of OFT anomalies, little is known about the OFT alignment and DORV. The preliminary data show that *Osr1* is involved in OFT alignment; and this proposed study aims to clarify in which cell lineage *Osr1* is required for normal OFT alignment, specifically, by looking into the role of *Osr1* in SHF cell lineage and *Hh*-signaling during OFT development. This study will add new details to the multi-gene, lineage, time-point regulated process of the morphogenesis of OFT alignment using Cre-lox recombinase technique, allowing

for tissue and time specific KO of *Osr1* in SHF cells (*Mef2cAHF: Cre*) and *Shh*-receiving cells (*Gli1: Cre*) in mice embryo hearts.

CHAPTER II

METHODS

Study Design

In order to define a clear map to determine if *Osr1* is required in SHF for OFT alignment, a cre-lox recombinase technique will be utilized to achieve a tissue specific KO of the *Osr1* in SHF and *Shh*-receiving cells. Mice embryos with *Osr1* deletion in *Shh*-receiving cells (*Gli1: Cre*) and SHF cells (*Mef2cAHF: Cre*) will be created using Cre-lox recombinase technique. The overall timeline of the mice treatments and processing can be seen in the Figure 3.

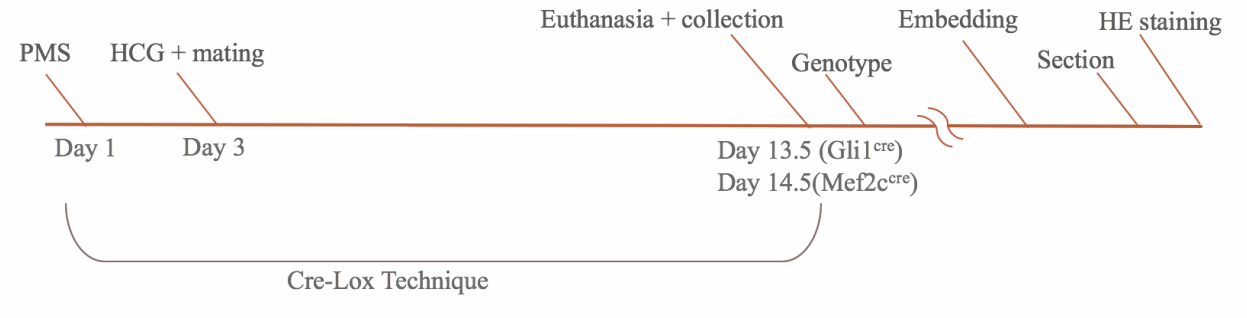

Figure 3. Timeline of mice treatments and processing.

Mouse Husbandry

General Mouse Care

Full length of mice gestation is about 18.5 to 21 days with its litter size ranging from 2 to 12 or more.[22] We will be weaning mice at approximately 21 days after birth. Gender separation, tagging of the ear, and tail sample collection is conducted for genotyping at the time of weaning. Productive breeding of mice begins between the 5th to 8th week until 7 or 8 months of age.[22] To

optimize the breeding conditions, female mice that are raised for the purpose will be mated starting 6 weeks old while male mice will be mated starting 8 weeks.

Hormone Priming

Hormone priming for female mice will induce superovulation which will increase the number of fertilized embryos for analyzing. Female mice (6 weeks old ~), are injected intraperitoneally with 0.1 mL (50 IU/ 1mL) of pregnant mare serum, and with 0.1 mL (50 IU/ 1 mL) of human chorionic gonadotropin three days later, then immediately placed in a breeding cage with the appropriate stud male (8 weeks old~). Fertilization will take place approximately 12 hours later when the ovulation occurs.

Cre-lox Recombinase

Cre-lox technique is a two component system consisting of Cre recombinase and 2 loxP sites. LoxP sites are 34 base pairs within the DNA that sandwich a specific sequence. Cre recombinase is a site-specific enzyme that catalyzes recombination of the specific sequence between the two loxP sites. The sequence flanked by the two loxP sites is often referred to as the "floxed" sequence. The location and orientation of the loxP site determines the type of recombination that takes place – deletion (when the two loxPs are in series in the same direction), inversion (when the two loxPs are in series in the opposite direction) or translocation (when the two loxPs are parallel). In this experiment we will be deleting the *Osr1* sequence in SHF and atrial septum progenitors.

Optimal Breeding Scheme for Breeding Target Genotype Mice

In order to breed mice for tissue-specific deletion, two generations of breeding (25% homozygous for targeted gene KO) is recommended to optimize chances of target genotyped

mice. Genotype for a *Osr1* knockdown mouse will be heterozygous flox; Cre. Tissue-specific deletion or KO of a genotype will be homozygous lox-deleted; Cre.

Tamoxifen Induction

The type of Cre-recombinase used for *Shh*-receiving cells (*Gli1: Cre*) is tamoxifen (TM) dependent. This is because the Cre recombinase is combined with a gene encoding the estrogen receptor (ER). Thus, this particular Cre-recombinase is called CreER. The ER and Cre fuse together to bind the chemical TM, a type of estrogen antagonist, instead of the endogenous estrogen present in the mice.[23] Before binding to its ligand, CreER is in the cytoplasm unable to enter the cell nucleus where the loxP sites present.[23] Upon injection of the TM, the CreER then crosses over the nucleus membrane to catalyze recombination of the DNA between the two loxP sites.[23] Therefore, CreER allows control of time, and tissue specific gene intervention.[23] TM activates expressions 12 hours after injections while its effect lasts 36 hours.[13,14] In this experiment, *Osr1* KO in *Shh*-receiving cells (*Gli1: Cre*), TM will be injected at E8.5 and E9.5 as *Hh*-signaling is known to take place between E8 and E10. By inducing the KO of *Osr1* during known expression time-frames of SHF cell proliferation and *Hh*-signaling, time and cell-line specific involvement of *Osr1* can be investigated.

Euthanasia

The pregnant female mice will then be sacrificed using a CO_2 chamber between 13:00 and 15:00 of the 14th day of pregnancy to collect the embryos at embryonic stage E14.5. CO2 will be administered until 1 minute after the breathing has stopped continued with cervical dislocation to confirm euthanasia is successful and assuring that the IACUC guidelines are followed.

Microdissection of Embryos

After successful euthanasia of the pregnant mice, the peritoneum is snipped at the lower abdominal area and pulled back. The embryos will be extracted from the abdominal cavity and inserted into a tube of PBS for preservation until further processing takes place. After assuring that there are no more embryos present in the mother mice, the corpse is discarded properly following IACUC guidelines.

Genotyping

After embryos are collected, genotyping will take place in a timely manner. The tails of the embryos will be used for genotyping to identify the two experimental groups, *Osr1* KO genotype embryos and *Osr1* knockdown genotype embryos, and controls for this experiment, $Osr1^{+/-}$ genotype embryos. After the tails are removed, the embryos will be preserved in formalin.

Tissue Processing

All of the embryos will be processed and embedded in paraffin wax to later be serial-tissue sectioned using a microtome. These slides will then be dyed with hematoxylin and eosin (HE staining) and analyzed for detection of any OFT alignment defects such as DORV and OA. Occurrence of defect will be calculated individually - within controls and within experimental mice that have Osr1 KO in their respective tissues.

Statistics

At least 3 embryos for each cell-specific *Osr1* KO mice are needed to identify the presence of OFT anomalies, including DORV and OA. Other heart defects such as ASD, VSD, and AVSD will also be identified and noted. The incidence of abnormalities will be summed by

each group, *Osr1* knockdown and KO each in the SHF cells and *Shh*-receiving cells and control. The chi-square test will be used to investigate the statistical significance of the data.

Expected Results

High prevalence of OFT misalignment, including DORV and OA, in mice embryos with *Osr1* KO in SHF (*Mef2cAHF : Cre)* and embryos with *Osr1* KO in *Shh*-receiving cells (*Gli1: Cre*) is expected. This is highly likely to happen due to high expression of *Osr1* in SHF and the effect of *Osr1* dosage on *Hh*-signaling.

Compliance

I have already worked with Research Compliance & Biosafety to get approval for the project. I have completed the CITI training course & "Working with the IACUC". The course completion record can be seen in the appendix section. I have started mouse work in Dr. Linglin Xie's lab since June, 2017 and gained training and experience on mouse manipulation.

CHAPTER III

RESULTS

Effect of *Osr1* Deletion in SHF cells (*Mef2cAHF : Cre*)

Mice embryos were collected at E14.5 and genotyped immediately using the tails for genotype identification of each embryo. Each embryo was then categorized into the control for genotype $Osr1^{fl/+}$, or experimental mice with the genotypes $Osr1^{fl/+}Mef2c^{cre}$ and $Osr1^{fl/fl}Mef2c^{cre}$ for *Osr1* knock down and knock out respectfully. Each slide was then examined then noted for identified CHD defects, such as ASD, VSD, AVSD, OA, and DORV. Refer to Figure 4 to see the contrast between normal phenotypes in control mice embryos and abnormalities that occurred in *Osr1* KO mice embryos.

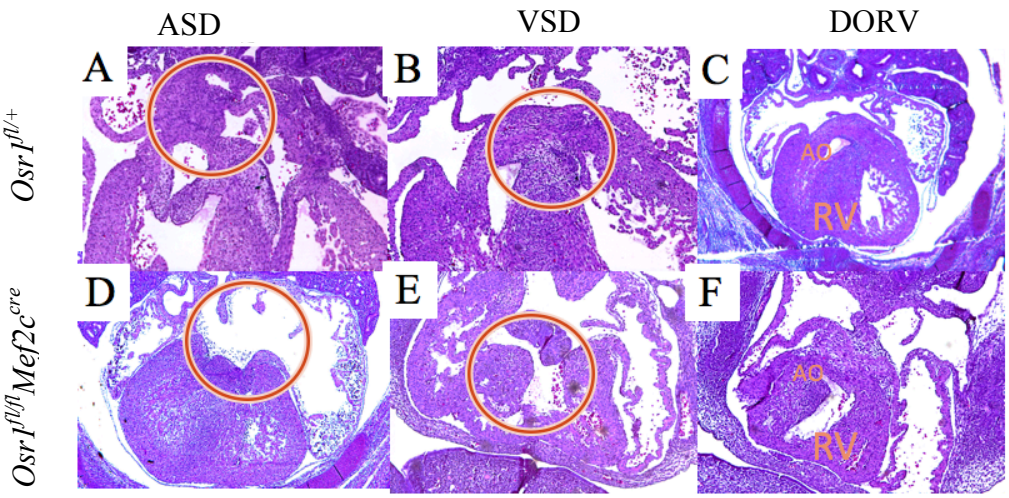

Figure 4. Identified Defects in Embryos with *Osr1* KO in SHF cells (*Mef2cAHF : Cre*).

In the control (Figure 4.A) you can see that the atrial septum fully elongated to separate the left atrium and right atrium. However, in the ASD identified in the *Osr1* KO in SHF (Figure 4.D), the atrial septum does not fully separate the two atriums making a hole present. In the control (Figure 4.B) you can see that the ventricular septum fully separates the two ventricles. However,

in *Osr1* KO in SHF (Figure 4.E), the ventricular septum fails to separate the two ventricles leaving a hole. In the control (Figure 4.C), aorta is leaving the left ventricle. However, in the *Osr1* KO in SHF (Figure 4.F), shows the aorta leaving the right ventricle resulting in a DORV. Refer to Table 1 for the raw numbers of heart defects identified among experimental mouse embryos with $Osr1^{fl/+}Mef2c^{cre}$ and $Osr1^{fl/fl}Mef2c^{cre}$ for *Osr1* knock down and knock out respectively in SHF cells (*Mef2cAHF : Cre*) and control embryos.

Table 1. Raw numbers of heart defects in mice with *Osr1* KO in SHF cells (*Mef2cAHF : Cre*) and control mice.

Groups	Genotype	Total #	ASD	AVSD	OA	DORV
Control	$Osr1^{fl/+}$	18	0	0	2	0
Experimental	$Osr1^{fl/+}Mef2c^{cre}$	6	0	0	1	0
	$Osr1^{fl/fl}Mef2c^{cre}$	4	1	0	0	1

Note: $Osr1^{fl/fl}$ is not included as a viable control due to the both copies of *Osr1* being modified.

The raw data from Table 1 was then analyzed using comparing the total number of OFT misalignments, including OA and DORV, between the control and experimental group. The % penetrance was calculated using Equation 1:

$$\%\ penetrance = \frac{Total\ \#\ of\ OFT\ misalignment}{Total\ \#\ of\ samples\ in\ group} * 100 \qquad \text{(Eq.1)}$$

Refer to Table 2 for the total number of samples and OFT misalignment for each category and calculated percent penetrance.

Table 2. Percent Penetrance of OFT Alignment Defects across mice with *Osr1* KO in SHF cells (*Mef2cAHF : Cre*) and control mice.

Groups	Genotype	Total #	OFT Alignment Defects	% Penetrance
Control	$Osr1^{fl/+}$	18	2	11.11%
Experimental	$Osr1^{fl/+}Mef2c^{cre}$	6	1	16.67%
	$Osr1^{fl/fl}Mef2c^{cre}$	4	1	25.00%

From these results, we found that *Osr1* knockdown in SHF cells resulted in 150.0% higher occurrence of OFT misalignment defects compared to the study control and *Osr1* KO in SHF cells resulted in 225.0% higher occurrence of OFT misalignment defects compared to the study control using equation 2.

$$\%\ Difference = \frac{\%\ penetrance\ of\ OFT\ alignment\ defects\ in\ experimental\ mice}{\%\ penetranc\ of\ OFT\ alignment\ defects\ in\ control\ mcie} * 100 \quad \text{(Eq. 2)}$$

From percent differences in OFT misalignment penetrance in *Osr1* knockdown and KO compared to the control, you can see that depending on the degree of *Osr1* expression, the % penetrance of OFT misalignment is affected. The less *Osr1* is expressed the % penetrance of OFT misalignment increases. To determine this trend's statistical significance a Chi-square test was utilized. Our null hypothesis (H_0) and hypothesis (H_1) for this X^2 test were:

H_0: There are no statistical significant difference between the observed OFT misalignment penetrance between the control and experimental groups.

H_1: H_0 is false.

Due to small sample size we set the significance level as $\alpha = 0.1$. The X^2 value was calculated using Equation 3. The subscript "c" represents the degrees of freedom calculated by subtracting 1 from the total number of samples.

$$X^2c = \Sigma \frac{(Observed - Expected)^2}{Expected} \quad \text{(Eq. 3)}$$

For *Osr1*$^{fl/+}$*Mef2c*^{cre} *and Osr1*$^{fl/fl}$*Mef2c*^{cre} the X^2 value came out to be 0.127 and 0.5361 respectfully. The calculated X^2 value was then used to get the p-value. For *Osr1*$^{fl/+}$*Mef2c*^{cre} *and Osr1*$^{fl/fl}$*Mef2c*^{cre} the p-value were 0.72158 and 0.464069 respectfully. For both *Osr1* KO and knockdown, the p-values were greater than $\alpha = 0.1$, failing to reject the null hypothesis. This indicates that the trend observed for *Osr1* knockdown and *Osr1* KO is not statistically significant as it has a high possibility it just happened due to chance and not due to the treatments. Overall, the experimental data show a trend of higher penetrance of OFT misalignments with the degree of *Osr1* expression but the X^2 test indicated that the observed trend was statistically insignificant. However, the small sample size for the experimental mice *Osr1*$^{fl/+}$*Mef2c*^{cre} *and Osr1*$^{fl/fl}$*Mef2c*^{cre} should be noted from the table 2. Due to small sample size we cannot make a strong definitive conclusion.

Effect of *Osr1* Deletion in *Shh*-receiving cells (*Gli1: Cre*)

TM induction of Cre-lox KO of *Osr1* from *Shh*-receiving cells was conducted at E8.5 and E9.5, and the embryo was collected at E13.5 for data analysis. After genotyping, the embryos were categorized into two controls and two experimental groups, *Osr1*$^{fl/+}$*Gli1*^{cre} and *Osr1*$^{fl/fl}$*Gli1*^{cre} for *Osr1* knockdown and KO respectively. In Figure 5, normal phenotypes of the control mice with genotype *Osr1*$^{fl/+}$ can be seen in the top row in comparison to the abnormalities identified in *Osr1* KO embryos with genotype *Osr1*$^{fl/fl}$*Gli1*^{cre} can be seen.

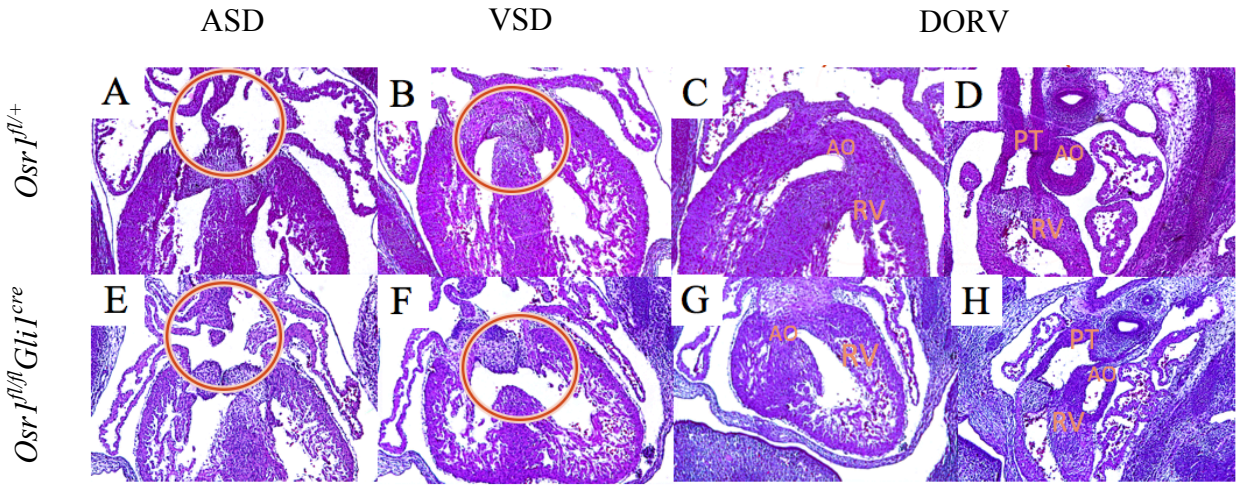

Figure 5. Identified defects in embryos with *Osr1* KO in in *Shh*-receiving cells (*Gli1: Cre*).

Note: In Figures 5A, 5B, and 5C normal phenotypes of the atrial septum, ventricular septum, and the aorta positioning respectfully in control embryos with the genotype $Osr1^{fl/+}$. In Figures 5E, 5F, 5G, there is ASD, VSD, and DORV present respectfully in experimental embryos with Osr1 KO with the genotype $Osr1^{fl/fl}Gli1^{cre}$. In Figure 5D, a normal phenotype of the aorta being fully disconnected from the right ventricle when the Pulmonary trunk is connected to the right ventricle can be seen. However, in Figure 5H, a different view of DORV presented in Figure 5G can be seen from the aorta being connected to the right ventricle when the pulmonary trunk is leaving the right ventricle.

Refer to Table 3 for the raw numbers of heart defects identified among experimental mice with $Osr1^{fl/+}Gli1^{cre}$ and $Osr1^{fl/fl}Gli1^{cre}$ for *Osr1* knock down and knock out respectively in *Shh*-receiving cells (*Gli1: Cre*) and control embryos.

Table 3. Raw numbers of heart defects in mice with *Osr1* KO in *Shh*-receiving cells (*Gli1: Cre*) and control mice.

Groups	Genotype	Total #	ASD	AVSD	OA	DORV
Control	$Osr1^{fl/+}$	18	0	0	2	0
Experimental	$Osr1^{fl/+}Gli1^{cre}$	5	0	0	0	1
	$Osr1^{fl/fl}Gli1^{cre}$	6	0	0	0	3

Note: $Osr1^{fl/fl}$ was not included in the control category due to the both alleles of *Osr1* being modified.

The raw data from Table 3 was then analyzed by comparing the total number of OFT misalignments, including OA and DORV, between the control and two experimental groups. The % penetrance was calculated using Equation 1. Refer to Table 4 for the total number of samples and OFT misalignment for each category and calculated percent penetrance.

Table 4. Percent Penetrance of OFT alignment defects across mice with *Osr1* KO in *Shh*-receiving cells (*Gli1: Cre*) and control mice.

Groups	Genotype	Total #	OFT Alignment Defects	% Penetrance
Control	$Osr1^{fl/+}$	18	2	11.11%
Experimental	$Osr1^{fl/+}Gli1^{cre}$	5	1	20.00%
	$Osr1^{fl/fl}Gli1^{cre}$	6	3	50.00%

From these results, we calculated that *Osr1* knockdown in *Shh*-receiving cell had 180% higher occurrence of OFT misalignment defects compared to control and *Osr1* KO in *Shh*-receiving cells had 200% higher occurrence of OFT misalignment defects compared to the study control using equation 2. From percent differences in OFT misalignment penetrance in *Osr1* knockdown and KO compared to the control, you can see the that depending on the degree of *Osr1* expression, the % penetrance of OFT misalignment is affected. The less *Osr1* is expressed the % penetrance of OFT misalignment increases. To determine this trend's statistical significance a Chi-square test was utilized. The null hypothesis (H_0) and hypothesis (H_1) for this test are the same as the ones used in the X^2 test performed earlier. Due to small sample size we set the significance level as $\alpha = 0.1$. The X^2 value was calculated using Equation 3. For $Osr1^{fl/+}Gli1^{cre}$ *and* $Osr1^{fl/fl}Gil1^{cre}$ the X^2 value came out to be 0.2726 and 4.1263 respectfully. The calculated X^2 value was then used to get the p-value. For $Osr1^{fl/+}Gli1^{cre}$ *and* $Osr1^{fl/fl}Gli1^{cre}$ the p-values were 0.601598 and 0.042221 respectfully. For $Osr1^{fl/+}Gli1^{cre}$ the p-value was greater than $\alpha = 0.1$ thus, failed to reject the null hypothesis making the trend observed insignificant. For $Osr1^{fl/fl}Gli1^{cre}$ the p-value was less than $\alpha = 0.1$, making the trend observed statistically significant. In summary, both $Osr1^{fl/+}Gli1^{cre}$ *and* $Osr1^{fl/fl}Gil1^{cre}$ had higher penetrance of OFT misalignments in relation to decreasing *Osr1* expression, but only the *Osr1* KO with the genotype $Osr1^{fl/fl}Gli1^{cre}$ had statistical significance. Due to small sample size we cannot make a strong definitive conclusion. However, the results suggest a strong involvement of *Osr1* in *Shh*-signaling in its crucial activation period E8.5 and E9.5 for normal OFT alignment.

CHAPTER IV

CONCLUSION

Major Findings

For both knockdown and KO of *Osr1* in SHF cell and *Shh*-receiving cells, a higher penetrance of OFT misalignment was observed with decreasing *Osr1* expression. However, using the X^2 test it was found that *Osr1* knockdown and KO in SHF cells (*Mef2cAHF : Cre*), and *Osr1* knockdown in *Shh*-receiving cells showed a non-significant difference in occurrence of OFT misalignment for mice compared to the control. However, the X^2 test showed statistical significance in higher penetrance of OFT misalignment, including DORV and OA, in mice embryos with *Osr1* KO in *Shh*-receiving cells (*Gli1: Cre*). Due to small samples size, the current results do not allow for a strong power analysis. Overall, the current results suggest a strong involvement of *Osr1* in *Shh*-signaling at E8.5 and E9.5 for OFT development.

Future Approaches

In order to increase the statistical significance of our study, we plan to collect at least 20 embryos for each cell-specific *Osr1* KO mice. Each cell-line will be collected from up to 10 different litters (considering the average size being 8) in order to ensure 90% power analysis. Next, we aim to identify if other lineages such as myocardium myocardium (*Tnt: Cre*), endocardium (*Tie2: Cre, Nfatc1: Cre*), and CNC cells (*Wnt1:Cre*) are affected by *Osr1* for OFT alignment due to their important roles in cardiac development. Ultimately, using the cell-lineages identified to be affected by *Osr1* for OFT alignment, we will investigate the interaction of *Osr1* and Hh-signaling and their functional role in OFT development.

There is still much to be done, but this study adds additional details to the multi-gene, lineage, time-point regulated process of the morphogenesis of OFT alignment which is yet fully mapped. It is also significant as it brings us one step closer to future preventions and interventions that can eliminate and alleviate the suffering of people affected by CHDs.

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APPENDIX

COLLABORATIVE INSTITUTIONAL TRAINING INITIATIVE (CITI PROGRAM)

COMPLETION REPORT - PART 1 OF 2
COURSEWORK REQUIREMENTS*

* NOTE: Scores on this Requirements Report reflect quiz completions at the time all requirements for the course were met. See list below for details. See separate Transcript Report for more recent quiz scores, including those on optional (supplemental) course elements.

- **Name:** Naomi Mccauley (ID: 5702824)
- **Institution Affiliation:** Texas A&M University (ID: 660)
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- **Institution Unit:** 02144006
- **Phone:** 9798454640

- **Curriculum Group:** Working with the IACUC
- **Course Learner Group:** Same as Curriculum Group
- **Stage:** Stage 1 - IACUC Basic Course
- **Description:** The CITI Basic Course in Laboratory Animal Welfare for Investigators, Staff and Students.

- **Record ID:** 21088397
- **Completion Date:** 19-Jun-2017
- **Expiration Date:** N/A
- **Minimum Passing:** 80
- **Reported Score*:** 100

REQUIRED AND ELECTIVE MODULES ONLY	DATE COMPLETED	SCORE
Introduction (ID: 15357)	19-Jun-2017	No Quiz
Working With The IACUC (ID: 15358)	19-Jun-2017	3/3 (100%)
Federal Mandates (ID: 15359)	19-Jun-2017	4/4 (100%)
The Veterinary Consultation (ID: 15360)	19-Jun-2017	1/1 (100%)
Getting Started (ID: 15361)	19-Jun-2017	4/4 (100%)
USDA Pain/Distress Categories (ID: 15362)	19-Jun-2017	8/8 (100%)
Endpoint Criteria (ID: 15363)	19-Jun-2017	2/2 (100%)
Alternatives (ID: 15364)	19-Jun-2017	8/8 (100%)
Avoiding Unnecessary Duplication (ID: 15365)	19-Jun-2017	1/1 (100%)
Surgery (ID: 15366)	19-Jun-2017	11/11 (100%)
Euthanasia (ID: 15367)	19-Jun-2017	5/5 (100%)
Collecting Blood Samples (ID: 15368)	19-Jun-2017	3/3 (100%)
Personnel Training and Experience (ID: 15369)	19-Jun-2017	3/3 (100%)
Occupational Health and Safety (ID: 15370)	19-Jun-2017	1/1 (100%)
Making Changes After Initial Approval (ID: 15371)	19-Jun-2017	1/1 (100%)
Reporting Mistreatment or non-Compliance (ID: 15372)	19-Jun-2017	No Quiz
Final Comments (ID: 15373)	19-Jun-2017	No Quiz

For this Report to be valid, the learner identified above must have had a valid affiliation with the CITI Program subscribing institution identified above or have been a paid Independent Learner.

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COLLABORATIVE INSTITUTIONAL TRAINING INITIATIVE (CITI PROGRAM)

COMPLETION REPORT - PART 2 OF 2
COURSEWORK TRANSCRIPT**

** NOTE: Scores on this Transcript Report reflect the most current quiz completions, including quizzes on optional (supplemental) elements of the course. See list below for details. See separate Requirements Report for the reported scores at the time all requirements for the course were met.

- **Name:** Naomi Mccauley (ID: 5702824)
- **Institution Affiliation:** Texas A&M University (ID: 660)
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- **Phone:** 9798454640

- **Curriculum Group:** Working with the IACUC
- **Course Learner Group:** Same as Curriculum Group
- **Stage:** Stage 1 - IACUC Basic Course
- **Description:** The CITI Basic Course in Laboratory Animal Welfare for Investigators, Staff and Students.

- **Record ID:** 21088397
- **Report Date:** 24-Jul-2017
- **Current Score**:** 100

REQUIRED, ELECTIVE, AND SUPPLEMENTAL MODULES	MOST RECENT	SCORE
Introduction (ID: 15357)	19-Jun-2017	No Quiz
Working With The IACUC (ID: 15358)	19-Jun-2017	3/3 (100%)
Federal Mandates (ID: 15359)	19-Jun-2017	4/4 (100%)
The Veterinary Consultation (ID: 15360)	19-Jun-2017	1/1 (100%)
Getting Started (ID: 15361)	19-Jun-2017	4/4 (100%)
Alternatives (ID: 15364)	19-Jun-2017	8/8 (100%)
Avoiding Unnecessary Duplication (ID: 15365)	19-Jun-2017	1/1 (100%)
USDA Pain/Distress Categories (ID: 15362)	19-Jun-2017	8/8 (100%)
Endpoint Criteria (ID: 15363)	19-Jun-2017	2/2 (100%)
Surgery (ID: 15366)	19-Jun-2017	11/11 (100%)
Collecting Blood Samples (ID: 15368)	19-Jun-2017	3/3 (100%)
Personnel Training and Experience (ID: 15369)	19-Jun-2017	3/3 (100%)
Occupational Health and Safety (ID: 15370)	19-Jun-2017	1/1 (100%)
Euthanasia (ID: 15367)	19-Jun-2017	5/5 (100%)
Making Changes After Initial Approval (ID: 15371)	19-Jun-2017	1/1 (100%)
Reporting Mistreatment or non-Compliance (ID: 15372)	19-Jun-2017	No Quiz
Final Comments (ID: 15373)	19-Jun-2017	No Quiz

For this Report to be valid, the learner identified above must have had a valid affiliation with the CITI Program subscribing institution identified above or have been a paid Independent Learner.

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