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Role of FOX01 in Endothelial Cell and Cardiomyocyte Signaling

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ROLE OF FOXO1 IN ENDOTHELIAL CELL AND CARDIOMYOCYTE SIGNALING

A Thesis

Presented to the Faculty

Of the

College of Arts and Sciences

In Partial Fulfillment

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ABSTRACT

The regulation of heart development is an area of research that has important implications for the future treatments of heart injuries. Cardiomyocytes, or cardiac muscle cells, stop proliferating after birth, which limits the adult heart in its ability to repair damaged tissue after injury. The use of targeted therapies to treat heart injury through regenerative mechanisms requires an extremely detailed understanding of the regulatory pathways responsible for directing proliferation of cardiomyocytes during heart development. Interactions between cardiomyocytes in the myocardium with endothelial cells of the endocardium during development are known to occur through cell-cell interactions, including the action of diffusible cell signaling factors. The loss of FoxO1 expression in endothelial cells was shown to disrupt the signaling interactions of endothelial cells and cardiomyocytes (Sengupta et al., 2012) indicating that FoxO1 may be involved in the regulated the signaling pathways between these two cell types. In addition, the growth factors Neuregulin (NRG-1) and IGF-1 are known to mediate the proliferation of cardiomyocytes during heart development (Tian & Morrisey, 2012). FoxO1 has also been shown to regulate many signaling pathways that are involved in controlling cell proliferation, including other growth factor pathways. **The purpose of this study was to investigate the role of FoxO1 in endothelial-cardiomyocyte interactions in an *in vitro* cell co-culture model system to determine if FoxO1 has regulatory roles in cardiomyocyte proliferation via endothelial-myocardial signaling.** In this study, we focused on the regulatory role of FoxO1 in NRG-1 expression and the effects of altered NRG-1 expression on the interactions between endothelial cells and cardiomyocytes in the co-culture model. We found that a lack of FoxO1 expression did affect the gene expression of NRG-1, as well as IGF-1 and their respective receptors, ErbB2/ErbB4 and IGF-1R. Interestingly, we also found a difference in the gene expression of our control, depending on whether they were treated with a control siRNA scramble sequence or if they were untreated. A clear understanding of endothelial-cardiomyocyte signaling is essential to further development of therapeutic treatments for cardiovascular defects and disease.

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1. INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death, not only in the United States, but worldwide. According to data presented by the American Heart Association from 2013, CVD results in 2200 deaths per day in the United States. CVD was responsible for more than 17.3 million deaths per year worldwide and is estimated to grow to more than 23.6 million by 2030 (Mozaffarian et al., 2014). Congenital birth defects are the leading cause of deaths in infants and the most common types seen in newborns are heart defects. Congenital heart defects occur in about 9 in every 1000 births (Schleich et al., 2013). Diseases and congenital defects of the heart share the characteristics of structural and functional malformations of the myocardium. Clinical treatments of cardiovascular injury are challenging due to the complexity of the cardiovascular system. There are several specialized cell types in the cardiovascular system but there is one cell type in particular that plays a critical role in the development and proper functioning of the heart, itself. Cardiomyocytes are the cells responsible for forming the heart muscle, or myocardium, and are specified as atrial or ventricular myocytes (Epstein, 2010). Though cardiomyocytes are just one example of the specialized cells found in the cardiovascular system, they are particularly notable for their role in myocardial development as a critical component of proper heart functioning, as well as for their significant implications in diseases and defects of the heart.

In humans, cardiomyocytes proliferate throughout embryogenesis then withdraw from the cell cycle after birth, completely losing their proliferative abilities. When cardiomyocytes stop proliferating, growth of the myocardium stops as well. This

cessation of growth results in the heart's inability to repair any damaged tissue sustained during injury, which often leads to loss of function. Importantly, loss of function in the myocardium is associated with several types of heart disease and congenital heart defects. Therefore, it is no wonder that studies seeking a better understanding of myocardial proliferation and the possible regeneration of a functional myocardium receive a great deal of attention (Xin et al., 2013).

1.1 Heart Development

The heart is the first organ to develop and begin functioning in vertebrate embryogenesis. The developmental processes responsible for the formation of the heart are referred to as cardiogenesis. Cardiogenesis is mediated by strict transcriptional gene regulation and elaborate cellular signaling pathways that result in embryonic heart formation. (Xin et al., 2013). Cardiogenesis begins about day 16 following the formation of the three embryonic germ layers: endoderm, mesoderm and ectoderm. The heart is formed from the anterior mesoderm, which gives rise to precardiac cells. Precardiac cells are multipotent and have the ability to differentiate into the various cell types observed in the mature heart. Precardiac cells also form the cardiogenic crescent, or the first heart field. The cells of the first heart will migrate anteriorly then fuse at the midline of the embryo, forming a linear primitive heart tube (Xin et al., 2013).

The primitive heart tube is comprised of three cardiac layers: the myocardium, the endocardium and the epicardium. The outer layer is formed by epicardial cells, the myocardium forms the bulk of the heart, and a layer of cardiac jelly exists between the endocardial layer that forms the lumen of the heart. The heart tube will undergo a process

called looping to spatially position the future chambers of the heart. During this process, the myocardium induces the cells of the endocardium to detach and migrate into the cardiac jelly. These cells will migrate into the cardiac cushions to undergo differentiation and become parts of the heart valves (Schleich, 2013). As looping continues, the epithelial proepicardium will migrate to the myocardium's surface. Through rapid proliferation, proepicardial cells will cover the surface of the myocardium. These cells will make up the epicardium and pericardium. Some epicardial cells are able to delaminate and undergo epithelial-to-mesenchymal transitions to form mesenchymal cells. These mesenchymal cells are able to give rise to smooth muscle , vascular endothelial cells and fibroblasts involved in the coronary vasculature (Smith, Bader, 2007). After looping occurs, the heart tube will undergo convergence and wedging to produce a fully septated heart. A second population of cardiac cells, known as the second heart field, will migrate into the pharyngeal regions of the embryo. Cells derived from the second heart field contribute to the walls of the atria and atrial septum, muscular portions of the systemic and pulmonary veins, the outflow tract and the muscular base of the aorta and pulmonary arteries (Schleich, 2013).

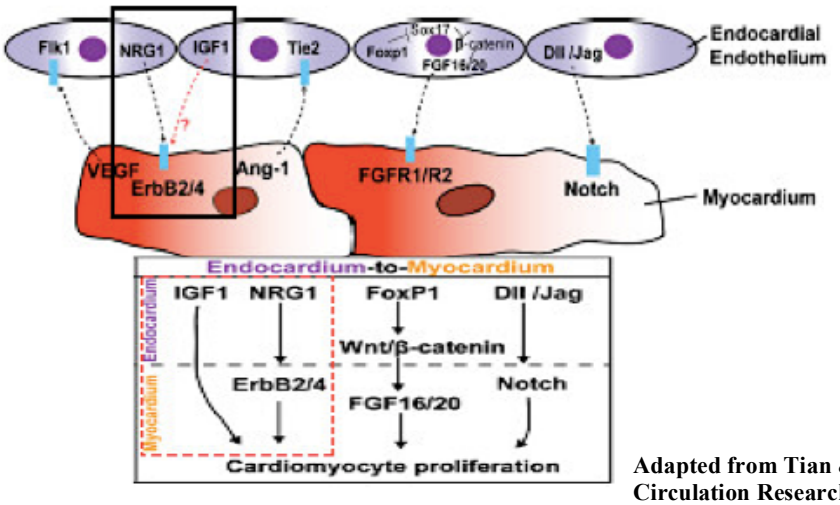
There are three distinct developmental steps required for the proper maturation of a four-chambered heart. The first step is the trabeculation of the myocardium, which forms the defined trabeculae of the ventricle. Trabeculation is responsible for maintaining embryonic blood flow during the early stages of cardiogenesis. The second step is the formation of endocardial cushions, valves and septa. Endocardial cushions are formed from cells of the endocardium that undergo endocardial-mesenchymal transition to

become mesenchymal cells. The mesenchymal cells migrate into the cardiac jelly and push the endocardium into the cardiac lumen. The resulting protrusions give rise to the endocardial cushions. These cushions will eventually result in the formation of the cardiac valves and septa. The third step is myocardial compaction accompanied with coronary vasculature formation. The outer myocardial layers become more compact during later stages of development. During the process of compaction, this compact zone will go from a thickness of about two myocardial cells to a multilayered wall of cardiac cells. The compact zone will also go from being avascular to having angioblasts form vascular tubes from the epicardium. Thickening of the myocardium is the first sign of vascularization in the embryonic heart of vertebrates. The epicardium's ability to undergo epithelial-mesenchymal transition is thought to be the heart's major source of fibroblasts, coronary endothelium as well as vascular smooth muscle cells (VSMC). In addition, the proepicardium, the embryonic structure giving rising to the epicardium, has proved to have critical roles in paracrine signaling for development of the myocardium. Among these paracrine signals, the epicardium secretes growth factors to stimulate the myocardium to proliferate myocytes in the compact myocardial wall (Tian & Morrisey, 2012). Trabeculation, endocardial cushion formation, and myocardial compaction are all required for proper formation of the four-chambered heart and importantly, each step requires endocardial and myocardial signaling.

1.2 Endocardial-Myocardial Signaling

Signaling interactions between the endocardium and the myocardium have been implicated in several critical processes in cardiogenesis. Similar to the myocardium, the

endocardium is derived from cardiac mesoderm, separating itself out from the linear heart tube. The endocardium serves as an important source of mesenchymal cells for the development of cardiac valves due to its ability to undergo endothelial-mesenchymal transition. The endocardium has many essential roles throughout heart development, including one of the earliest critical processes of heart trabeculation. After the cardiac linear heart tube develops and undergoes looping to align the chambers of the heart, the heart will undergo trabeculation which helps direct blood flow and increase contractility. The significance of trabeculation as a result of myocardial and endocardial signaling can be demonstrated by a specific zebrafish mutation, *Cloche*, which affect both endothelial and hematopoietic lineages. *Cloche* mutant zebrafish lack the endocardium and many other endothelial cells, making them a model organism to study the endocardium's role in trabeculation. *Cloche* mutants failed to develop ventricular trabeculi, which caused reduced contractility, distended atria and collapsed ventricles (Stainer et al., 1995). Therefore, signaling between the myocardium and endocardium is an absolute requirement for proper formation and functioning of the heart.



Adapted from Tian & Morrisey, Circulation Research, 2012

Figure 1: Cell signaling between endothelial cells of the endocardium and cardiomyocytes of the myocardium. Boxes highlight the pathways focused on in this paper.

There are several important gene networks and signaling pathways involved in the cross-talk between endothelial cells of the endocardium and cardiomyocytes of the myocardium (**Figure 1**). While some of the signaling components have been identified, many details regarding the exact signals involved in heart development are not yet known. Neuregulin (NRG)-1/ErbB2/B4, fibroblast growth factor (FGF)-9, -16, -29, Notch, vascular endothelial growth factor (VEGF) and Angiopoietin-1 (Ang-1) are all known to have essential roles in the maturation and function of myocardial cells (Smith & Bader, 2007).

Neuregulin 1 (NRG-1) has been shown to have particularly critical roles in the development of the myocardium. Endocardial cells synthesize NRG-1 to promote myocyte survival and proliferation by stimulating co-receptors, ErbB4 and ErbB2, expressed on adjacent cardiomyocytes. During development, it has been shown that NRG-1 activation of the ErbB2/ErbB4 complex is required for the process of

trabeculation. It is also known that NRG-1/ErbB4 interactions promote differentiated cardiomyocytes proliferation in the mature heart (Lee et al., 1995). Increased expression of ErbB4 was shown to enhance proliferation of cardiomyocytes, while a global loss of ErbB4 receptor caused a decrease in proliferation in cardiomyocytes. Studies have also demonstrated the importance of NRG-1 and the ErbB2/ErbB4 complex, each as individual components. For example, the global loss of NRG-1 in mice results in absence of trabeculation, failure of myocardial maturation and embryonic lethality, as seen with *Cloche* mutants (Lai, et al., 2010). In addition, null alleles for either the ErbB2 or ErbB4 receptors resulted in normal heart tube formation followed by normal looping but no trabeculation.

It is important to note that NRG-1 is only able to induce proliferation in differentiated, mononucleated cardiomyocytes, not binucleated cardiomyocytes. After birth, cardiomyocytes become binucleated and withdraw from the cell cycle. This led to the thought that adult cardiomyocytes are incapable of proliferating due to a specific inability to perform cytokinesis, which is a key step in the mitotic cell cycle (Bersell et al., 2009). The presence of NRG-1 induces the disassembly of sarcomeres to facilitate cytokinesis, which is similar to the induction of proliferation through p38 inhibition by FGF-1. The activation of PI3K is a common mechanism by which NRG-1, FGF-1 and a third growth factor, Insulin-like growth factor 1 (IGF-1) are able to stimulate cardiomyocyte proliferation. Activation of PI3K leads to the activation of PDK1 and Akt, which promotes cell cycle entry, DNA synthesis and cytokinesis. The PI3K pathway is also known to regulate FoxO1 transcription factor. FOXO proteins are a family within the

Forkhead Transcription factors family. They have various kinds of roles but many are found to control essential cellular processes including proliferation, apoptosis, metabolism and differentiation (Wang et al., 2014). FoxO1 has also been shown to induce expression CKIs, including $p21^{cip1}$ and $p27^{kip1}$, to inhibit cell proliferation (Sengupta et al., 2013). PI3K activates AKT, which is then able to directly phosphorylate FoxO1 and cause translocation of FoxO1 from the nucleus to the cytoplasm, thus inhibiting gene transcription (Xin, 2013). This pathway is summarized in **Figure 2**.

IGF-1 has been shown to have synergistic effects with NRG-1 in the heart. IGF-1 is synthesized by endothelial cells and binds to IGF-1 receptor (IGF-1R) on cardiomyocytes, though significant effects on cardiomyocyte development have not been observed by IGF-1 alone (Bersell et al., 2009). When IGF-1 interacts with NRG-1, they synergistically promote cardiomyocyte proliferation in the ventricles. These two growth factors simultaneously increase the amount of DNA synthesis in cardiomyocytes while an increase in the endocardial cushions and atrioventricular cushions are observed as well (Smith & Bader, 2007).

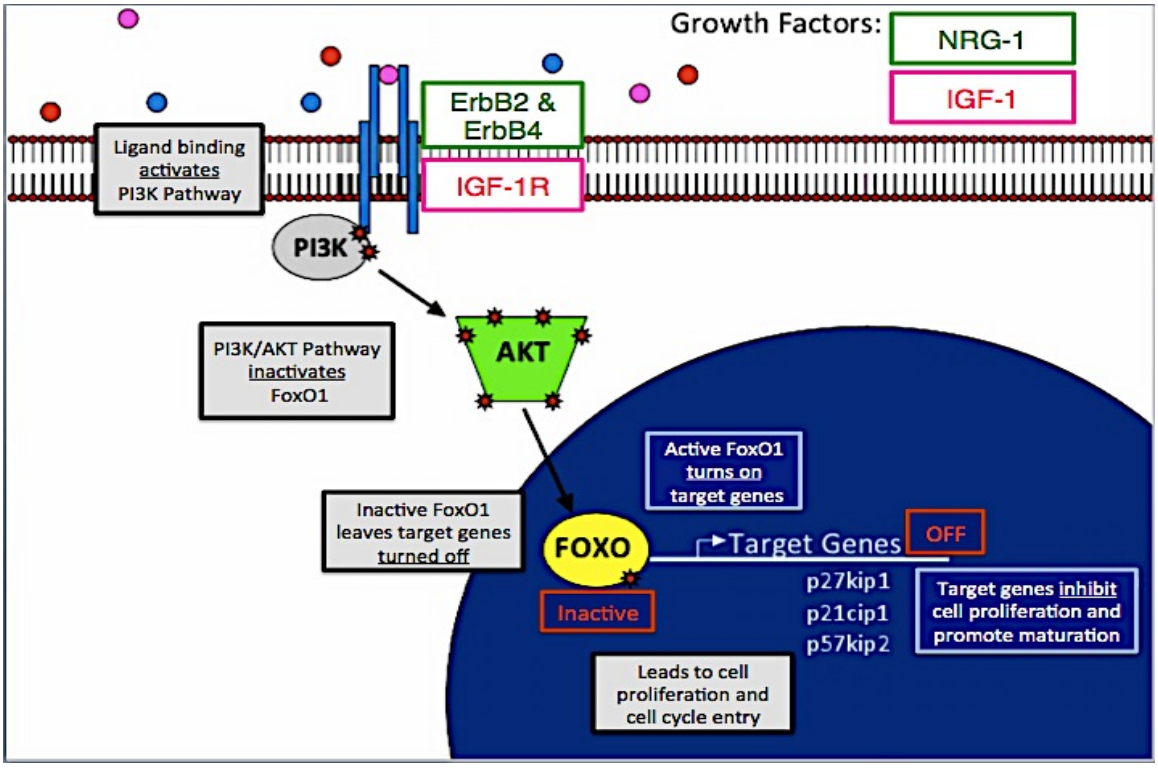


Figure 2: Schematic of PI3K/AKT Pathway. Growth factors like NRG-1 and IGF-1, bind to their receptors on the cell membrane of cardiomyocytes to activate PI3K/AKT pathway, thereby inactivating FOXO transfection factors. FOXO transcription factors, including FoxO1, regulate the expression of target genes, such as p27kip1, p21cip and p57kip2, in turn regulating cell proliferation and maturation.

The genomic loss of FoxO1 expression in mice (*Tie2/Cre/FoxO1*$^{-/-}$) results in heart malformations that lead to embryonic lethality at embryonic day 10.5 (E10.5) due to cardiovascular failure (**Figure 3**). Recent studies using cell-type specific loss of FoxO1 expression have indicated that FoxO1 is required in endothelial cells for proper heart development but it is not required in cardiomyocyte cells (Sengupta et al., 2012). Further research must be done to determine the signaling pathways that are involved in FoxO1 regulation of cardiomyocyte proliferation.

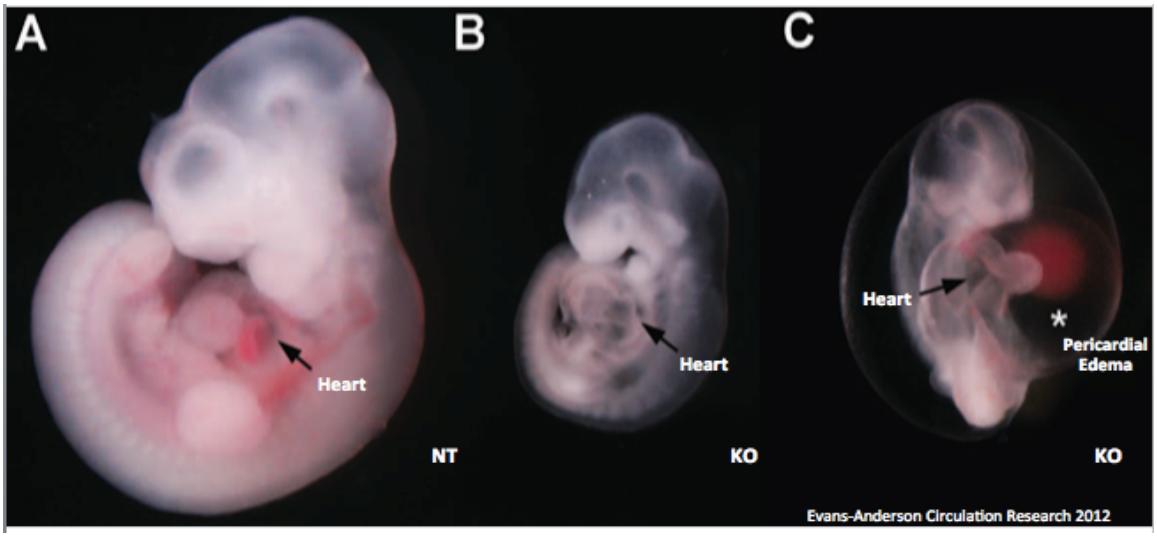


Figure 3: Embryonic lethality demonstrated by knockout of FoxO1 due to cardiovascular failure. NT: no treatment, KO: FoxO1 knock out a) Normal murine embryo at E10.5. Arrow shows normal heart development b) Growth retardation and heart malformation (indicated by arrow) in *Tie2/Cre/FoxO1*$^{-/-}$ KO embryo at E10.5. c) Pericardial edema (indicated by asterisk) demonstrated in *Tie2/Cre/FoxO1*$^{-/-}$ KO embryonic lethality at E10.5. Arrow shows malformation of heart.

1.3 Rationale

It is known that loss of FoxO1 in endothelial cells results in extreme morphological defects in embryos, growth retardation in overall embryonic development, heart failure and embryonic lethality by E11. FoxO1 mutant embryos also lack trabeculation of myocardium, have hypoplastic endocardial cushion formation and a very thin compact layer of myocardium. However, in contrast, specific loss of FoxO1 in cardiomyocytes does not have any phenotypic effects on myocardial development (Sengupta et al., 2012). Therefore, it can be concluded that FoxO1 is required in endothelial cells for normal endocardial signaling to adjacent cardiomyocytes to maintain proper cardiovascular function but FoxO1 is not required by cardiomyocytes. It is thought that growth factors including NRG-1 and IGF-1 may have roles in activating the

PI3K/AKT signaling pathway, which is thought to deactivate FoxO1 by inducing translocation from the nucleus to the cytoplasm. However, the exact signaling pathways that result in the cardiovascular defects observed in endothelial-specific loss of FoxO1 are not yet known.

In my project, I aimed to further investigate the role of FoxO1 during cardiovascular development by observing the effects of loss of FoxO1 expression on the signaling interactions between cardiomyocytes and endothelial cells. Though it is known that FoxO1 in endothelial cells is required for proper signaling, though the specific mechanism responsible for these interactions are not known. **The purpose of this study was to investigate the role of FoxO1 in endothelial-cardiomyocyte interactions in an *in vitro* cell co-culture model system to determine if FoxO1 has regulatory roles in cardiomyocyte proliferation via endothelial-myocardial signaling.** I used siRNA (small interfering RNA) for a cell type-specific silencing of FoxO1 expression in endothelial cells to study the implications that endothelial-specific loss of FoxO1 has on cardiomyocyte-endothelial cell signaling. Transfection of FoxO1 siRNA allowed for the cell-type specific removal of FoxO1 expression in an in vitro model system, similar to the in vivo study conducted in the paper '*FoxO1 is required in endothelial but not myocardial cell lineages during cardiovascular development',* with the use of *Tie2Cre* transgenic mice (Sengupta et al., 2012).

I also investigated the signaling of NRG-1 between cardiomyocytes and endothelial cells to determine if cardiomyocyte proliferation and heart development could possibly be mediated through the PI3K/AKT signaling pathway. Recent studies have also

shown that NRG-1 has the ability to promote cardiac regeneration after ischemic injury by inducing proliferation via ErbB4 receptor stimulation. Therefore, NRG-1 and its ErbB4 receptors may be potential targets for therapeutic techniques to repair heart damage following cardiac injury (Tian & Morrisey, 2012). I investigated the gene expression of IGF-1 and its receptor IGF-1R, since studies have indicated that IGF-1 works synergistically with NRG-1 in heart development (Evans-Anderson, et al., 2008).

There is a tremendous amount of clinical significance in studying the interactions between endothelial cells and cardiomyocytes. Cardiovascular disease and congenital heart defects have extensive effects across the United States and communities around the world. Cell-based regenerative approaches in therapeutic techniques point to promising new clinical treatments of cardiac injury (Alexander & Bruneau, 2010). These therapies aim to transplant stem cells into injured hearts to repair the cardiac tissue damage associated with heart disease or congenital heart defects with new, functional cardiac cells. Therefore, the key to discovering therapeutic methods of regenerating the heart is through a deeper understanding of the specific cell signaling that occurs between the myocardium and the endocardium during heart development.

1.4 Specific Aims

1. To develop a co-culture model system using cardiomyocytes and endothelial cells, creating an *in vitro* environment that emulates the conditions of early cardiovascular development *in vivo*.

I developed a co-culture model of cardiomyocytes and endothelial cells to study the cell signaling interactions that occur between the two cell types (**Figure 4**). Co-culture model systems create a 3D cellular environment for the cultures to grow and interact with their surroundings. 2D cell culture models are inadequate when compared to 3D culture models in the way they allow cells to attach, grow, affect their morphology and overall function. By using a 3D culture model via Transwell inserts, the cardiomyocytes and the endothelial cells were able to communicate in similar ways as they would *in vivo.* The Transwell insert provide permeable support for the cell cultures and allow for the analysis of the individual cell types, the cell matrix media and the cell lysates that accumulated over the duration of the co-culture (Sanyal, 2014).

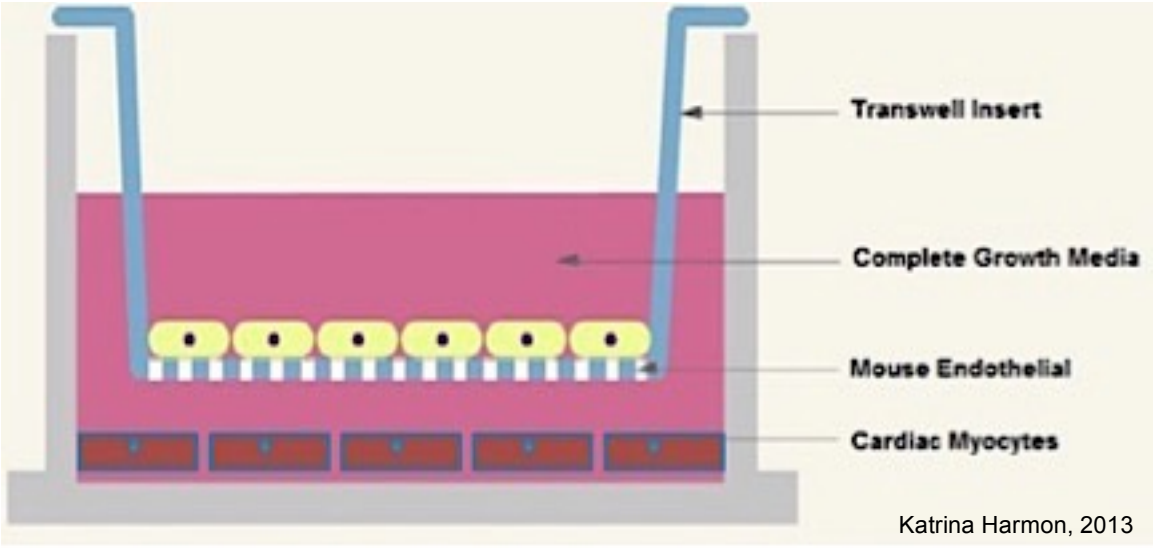


Figure 4: Cardiomyocyte and Endothelial Cell Co-Culture Schematic.

2. To examine the activity of growth factor Neuregulin in co-cultures that contain control endothelial cells and cardiomyocytes in comparison to the co-cultures that contain treated endothelial cells that lack FoxO1 expression.

I analyzed the expression of NRG-1 in endothelial cells and ErbB2/ErbB4 receptors in cardiomyocyte cells in both co-cultures containing treated endothelial cells with silenced FoxO1 expression as well as control endothelial cells with normal FoxO1 expression. Quantitative analysis of the various gene expression levels was performed by real time Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR). I also analyzed the gene expression of IGF-1 and IGF-1 receptor. Altogether, the aim of this analysis was to determine whether FoxO1 expression affects NRG-1 signaling during heart development. I hypothesized that there would be a decrease in the level of NRG-1 expression between cardiomyocytes and endothelial cells in co-cultures with endothelial cells that lack FoxO1 expression compared to co-cultures with control endothelial cells, thus resulting in decreased cardiac myocyte proliferation.

Identification of the effects of FoxO1 expression on cell signaling between endothelial cells and cardiomyocytes would disclose important information about the detailed processes that occur during heart development. Furthermore, the elucidation of specific signaling events involved in the regulation of cardiomyocyte proliferation is necessary to further investigate the induction of cardiomyocyte proliferation in the adult heart as a possible regenerative mechanism in the treatment of heart injury.

2. METHODS

2.1 Cell Culture

HL-1 Cell Line

HL-1 murine cardiomyocyte cell line was previously obtained from Dr. William C. Claycomb's Laboratory at Louisiana State University. A special preparation of growth media is required for the HL-1 cells: Claycomb medium, which was obtained from JRH Biosciences and the bottle must be wrapped in aluminum foil due to its light sensitivity. The media will be supplemented with 10 mL of Fetal bovine serum (FBS), 1 mL of penicillin (100 U/mL), 1 mL of streptomycin (100 μg/mL), 1 mL of Norepinephrine (10mM stock) and 1 mL of L-Glutamine (200 mM stock). I will be following all of the HL-1 Specific Procedures on Cell Culturing, Passage, Freezing and Thawing provided by Dr. Claycomb's Laboratory. See Appendix 1 for detailed description of the procedures (Claycomb, not dated).

C166 Cell Line

C166 murine endothelial cell line was previously purchased from ATCC (#CRL-2581). I followed the cell culturing protocol provided by Dr. Evans-Anderson as well as the guidelines provided by ATCC upon purchase, both of which can be found in Appendix 2. According to these procedures, the following materials are required for proper C166 cell culturing and were purchased from ATCC: one bottle Trypsin-EDTA solution 1X, five bottles of Dulbecco's Phosphate Buffered Saline (D-PBS), one bottle

Fetal Bovine Serum, five bottles of Dulbecco's Modified Eagle's Medium (DMEM) and 1 bottle of Dimethylsulfoxide (DMSO).

C166 cells and HL-1 cells were cultured individually to increase the population of each cell type. Cell cultures were grown to confluence and then split until we obtained a sufficient amount of cells to implant into each co-culture model.

2.2 Co-Cultures Models

Polyethylene Transwell-Clear 0.4 μm pore size inserts (Corning; catalog# 3450) were used to construct the 3D cell culture model. HL-1 cells were placed on the bottom of the culture model, on the 6-well plate surface. The C166 cells were suspended in the wells, on the surface of the inserts. This cell culture set up enables cell-cell communication between the HL-1 and C166 cells, while allowing the separation of the two cell lines for analysis. The cells were placed into the co-culture model following C166 siRNA transfection, which is explained in the following section.

Insert Pore Size Optimization

Optimal Transwell insert pore size was determined by culturing C166 cells on 0.4 μL pore size inserts and 3 μL pore size inserts, then comparing cell growth between the two sizes. Inserts were also removed to evaluate if cells were able to move past the insert and into the bottom of the 6-well plate.

Cell Culture Media Optimization

Cell media optimization experiments were performed to evaluate the response of HL-1 cells and C166 cells when exposed to Supplemented DMEM and Supplemented

Claycomb Media, respectively. Cell growth was evaluated at different media volumes for the HL-1 and C166 co-culture design. Based on Transwell's recommended volumes for the 6-well plates as well as the inserts, 1 mL, 1.5 mL and 2 mL of media were added to three different wells containing HL-1 cells on 6-well plate and C166 cells on the inserts. After 24-48 hours, inserts were placed into empty wells to evaluate the growth of both cell types individually.

Cell Volume Optimization

Cell growth was evaluated at different cell volumes for both HL-1 and C166 cells. Based on Transwell's recommended volumes for the 6-well plates as well as the inserts, 250 μL, 500 μL and 1 mL volumes of HL-1 cells were added to the 6-well plate, as well as C166 cells to the inserts. After 24-48 hours, the inserts were placed into empty wells to evaluate the growth of both cell types individually.

2.3 siRNA silencing of FoxO1 Expression

SignalSilence FoxO1 siRNA II from Cell Signaling was used to reduce or remove FoxO1 signaling from the endothelial cells. This allowed for the observation of changes in cell-cell communication between cardiomyocytes and endothelial cells, depending on endothelial cell treatment. The first control group of endothelial cells that did not undergo transfection at all (E4-NTC). The second control group of endothelial cells did undergo transfection but received a control scramble siRNA that did not induce a change in FoxO1 expression (E3-SiC). Two treatment groups of endothelial cells were transfected and received the FoxO1 siRNA to reduce or remove the proteins expression (E1-SiF and

E2-SiF). 100nM of FoxO1 siRNA was used to transfect the endothelial cells using Lipofectamine 2000 from Invitrogen. The cardiomyocytes did not receive any transfection. The three experimental groups were then cultured under normal cell culturing conditions for 24 hours post-transfection before they were transferred to the co-culture model, where all cells were treated with Claycomb media and incubated for 48 hours. Detailed transfection procedures were provided by Dr. Evans-Anderson and can be found in the Appendix 2.

Sample Abbreviations	Cell Type	Treatment
E1-SiFx	Endothelial	FoxO1 siRNA to silence FoxO1 gene expression; Group 1
E2-SiFx	Endothelial	FoxO1 siRNA to silence FoxO1 gene expression; Group 2
E3-SiC	Endothelial	**Si**RNA **C**ontrol
E4-NT	Endothelial	**N**o **T**reatment Control
C1+E1-SiFx	Cardiomyocyte	Co-cultured with Group 1 Endothelial cell + FoxO1 siRNA
C2+E2-SiFx	Cardiomyocyte	Co-cultured with Group 2 Endothelial cell + FoxO1 siRNA
C3+E3-SiC	Cardiomyocyte	Co-cultured with Endothelial cell + Control siRNA
C4+E4-NT	Cardiomyocyte	Co-cultured with Endothelial cells with No Treatment

Table 1: Experimental Group Identification. Details for the experimental groups, indicating sample abbreviation, cell type and specific treatment.

2.4 Western Blot Analysis

Western Blot Analysis is used to detect the presence of a specific protein in a sample by using gel electrophoresis to separate proteins based on their 3D structures and size (Lenico Technologies). This process is done by using primary antibodies to bind to a target protein, followed by the binding of a secondary antibody, which is conjugated with peroxidase. When the secondary antibody is exposed to the substrate contained in the ECL reagents, the conjugated peroxidase cleaves the substrate to emit light. This light is

detected by the blot reader and creates an image seen in **Figure 6**, with bands indicating presence of target genes.

Western blot analysis was used to confirm that FoxO1 expression had been reduced or removed following siRNA transfection into endothelial cells. Lanes 2-4 contained cell samples isolated 24 hours post-transfection, while Lanes 5-7 contained cell samples isolated 48 hours post-transfection. FoxO1 primary antibody was purchased from Cell Signaling (Product Number-C29H4) to bind to any FoxO1 proteins that was present in the sample. A 1:1000 dilution was prepared for FoxO1 primary antibody in 1% milk/TBS , as the manufacturer's suggested dilution range was 1:1000 to 1:50,000 dilution range from a 1 mg/mL stock. Anti-Rabbit HRP conjugated secondary antibody was purchased from Santa Cruz Biotechnology. A 1:10000 dilution was prepared for the secondary antibody in 1% milk/TBS, as the manufacturer's suggested dilution range was 1:50,000 to 1:250,000 from a 1 mg/mL stock. Pierce^{TM} Enhanced Chemiluminence (ECL) Western Blotting substrate from ThermoFischer was used for protein detection, followed by blot imaging using the Bio-Rad's Gel-Doc EZ imaging system. The detailed Western Blot Analysis protocol followed in this study was provided by Dr. Glasscock and can be found in Appendix 3.

2.5 RNA Isolation and Conversion

The HL-1 and C166 cells were co-cultured together for 48 hours before they were removed for analysis. Treatment groups were pooled together from the three plates, respectively. RNA was isolated using Trizol RNA purification kit from Invitrogen. Thermo Fischer's NanoDrop2000 spectrophotometer was used to determine the

concentration of RNA present in each sample (in ng/μL), which not only confirms whether the RNA isolation was successful but it also provides dilution information required for the conversion of RNA to cDNA. Quality of RNA can also be determined by comparing the 260/280 absorbance ratios, which indicates the amount of 'pure' RNA. The optimal 260/280 ratio is 2.0 for RNA. A second measurement for RNA quality is determined by comparing the 230/260 absorbance ratios, which indicates how much of the sample is purely nucleic acid. The optimal 230/260 ratio is also around 2.0.
RNA was converted to cDNA using iScript™ cDNA Synthesis Kit from Bio-Rad, following manufacturers protocol, then stored at -20°C until RT-PCR analysis.

Sample	Nucleic Acid (ng/uL)	260/280	260/230
E1-SiFx	306.4	1.92	1.24
E2-SiFx	1129.3	1.95	1.45
E3-SiFx	1130.1	1.94	1.19
E4-NT	917.3	1.95	1.74
C1+E1-SiFx	530.2	1.88	0.85
C2+E2-SiFx	331.6	1.90	1.17
C3+E3-SiC	217.6	1.80	0.77
C4+E4-NT	527.9	1.87	1.25

Table 2: RNA Isolation Spectrophotometry. Data collected from spectrophotometer from RNA isolated from each sample, indicating RNA concentration, 260/280 and 260/230 ratios (2.0 is ideal for 260/280 and 260/230 ratios)

2.6 RT-PCR Analysis

cDNA samples were combined with SsoUniversal Advanced SYBR Green Supermix, Reverse Transcriptase and nuclease-free water in preparation for RT-PCR analysis according to Bio-Rad's protocol for customized PrimePCR Plates (**Figure 5**). Samples were loaded in triplicate for each gene assay. PrimePCR plates were run using

the CFX96 Touch Real-Time PCR Detection System. Data was analyzed and exported from CFX Manager Software.

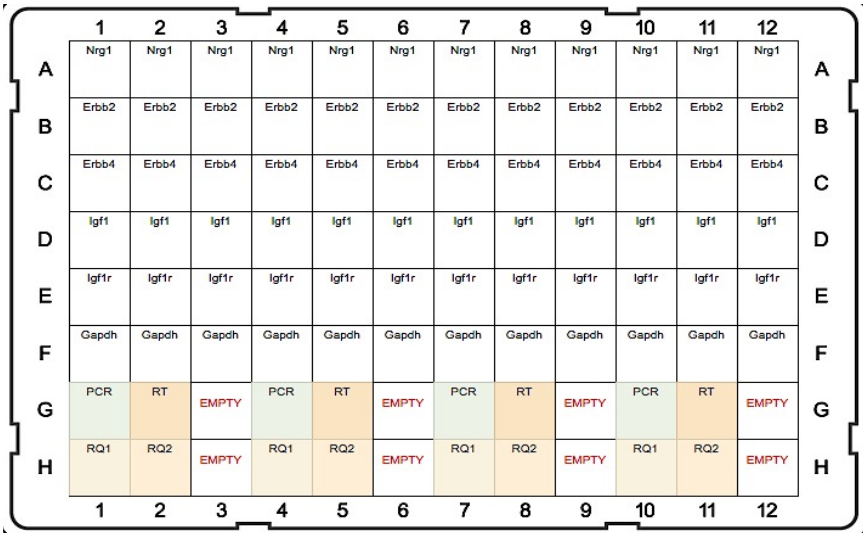


Figure 5: PrimePCR Plate Design. Plates contained 5 unique gene assays, 1 reference gene and controls, including: PCR, Reverse transcriptase, RNA Quality; Empty: positive and negative controls

3. RESULTS

3.1 Co-Culture Optimization

Optimization experiments were performed to determine optimal pore size for the Transwell culture plates by comparing C166 cell growth on 0.4 μm and 3 μm insert pore sizes. The insert pore size of 0.4 μm showed superior C166 cell growth compared to the 3 μm pore size. The 3 μm pores appeared to be too large to cultivate sufficient C166 cell growth, due to the reduced number of endothelial cells present on the 3 μm insert when compared to the 0.4 μm insert. In addition, C166 cells were found on the bottom of the 6-well plate, which indicated that the 3 μm pores were large enough to allow C166 cells to pass through, which defeats the purpose of our co-culture design.

Optimization experiments were performed to observe both HL-1 and C166 cells in response to DMEM media and Claycomb media, respectively. Resulting cell growth indicated that Claycomb media supported the growth of both HL-1 and C166 cells. Interestingly, I observed increased growth of C166 in Claycomb media when compared to DMEM media, which is normally used in C166 cell culture. The increased C166 growth could be attributed to the increased amount concentration of growth factors like epinephrine and FBS contained in supplemented Claycomb media as compared to supplemented DMEM.

Following cell media optimization, optimal cell and media volume were determined for the co-culture system to support both HL-1 and C166 cell growth. I found

the optimal cell volume to be 0.5 mL of cells and the optimal media volume to be 1 mL of media, based on cell growth comparisons for each cell type in varying conditions.

3.2 Western Blot Analysis

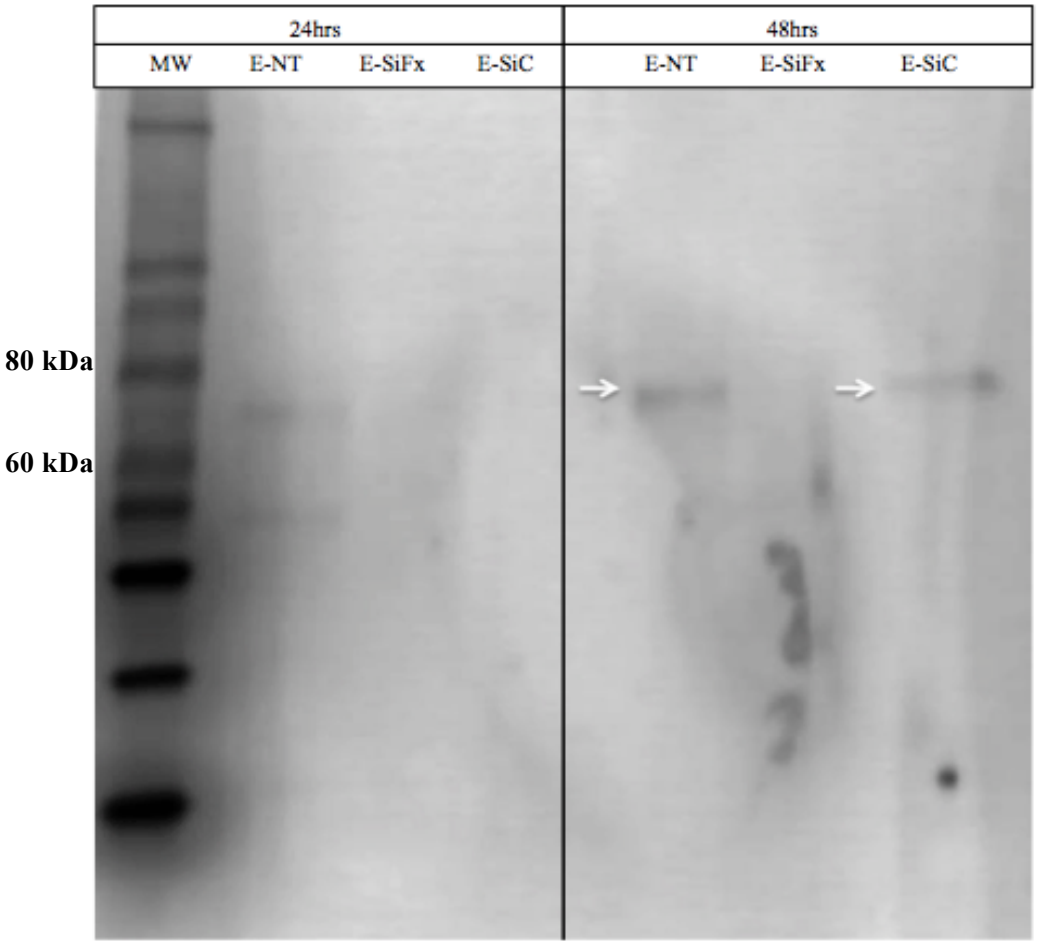


Figure 6: Western Blot Analysis of FoxO1 Expression in C166 Endothelial cells. White arrows show the bands at approximately 78 kDa, indicating presence of FoxO1 expression.

As shown in **Figure 6**, western blot analysis showed loss of FoxO1 protein from endothelial cell cultures. Lanes containing samples from non-transfected endothelial cells (E-NT) showed a band near 80 kDa in 24 hour-post-transfection time point samples, which indicates normal FoxO1 expression. Endothelial cells treated with SignalSilence

for FoxO1 (E-SiFx) or the scrambled sequence (E-SiC) did not have bands in samples taken 24 hours post transfection, which indicates loss of FoxO1 protein. However, there was a band in E-SiC samples in 48 hour-post-transfection time point samples, which indicates FoxO1 expression. There was not a band in E-SiFx samples in 48 hour post transfection samples, which indicates loss of FoxO1 protein. Thus, 48 hours post transfection was previously determined as the optimal time point to determine reduced or removed gene expression of FoxO1 following siRNA transfection of C166 cells. The absence of bands at approximately 78 kDa in Lane 6 (E-SiFx) indicates FoxO1 expression was silenced and that transfection was successful.

3.3 RNA Isolation

I used spectrophotometry following the RNA isolation to determine the concentration of RNA in each sample, as well as the quality of the RNA by comparing 260/280 and 230/260 absorbance ratios. With the exception of E1-SiFx, there was a significant amount of RNA isolated from each endothelial samples. The 260/280 ratios in the endothelial samples were all very close to 2.0, which indicates high quality of isolated RNA. The 230/280 ratios in the endothelial samples were not very close to the 2.0 ratio, ranging from 1.19 to 1.74 (**Table 1**), which indicates ethanol contamination. The cardiomyocyte samples had considerably lower concentrations of RNA compared to the endothelial samples. The 260/280 ratio in the cardiomyocyte samples were relatively close to 2.0, but not as close as the endothelial samples. However, the 230/260 ratios in the cardiomyocytes were not close to 2.0 and varied greatly from one another, indicating a low quality of pure nucleic acid in these samples (**Table 2**). Residual ethanol that was

not completely removed during RNA isolation is a possible explanation for the poor 230/260 ratios measured in the cardiomyocyte samples and the slightly decreased 230/260 ratios measured in the endothelial samples.

3.4 RT-PCR Analysis

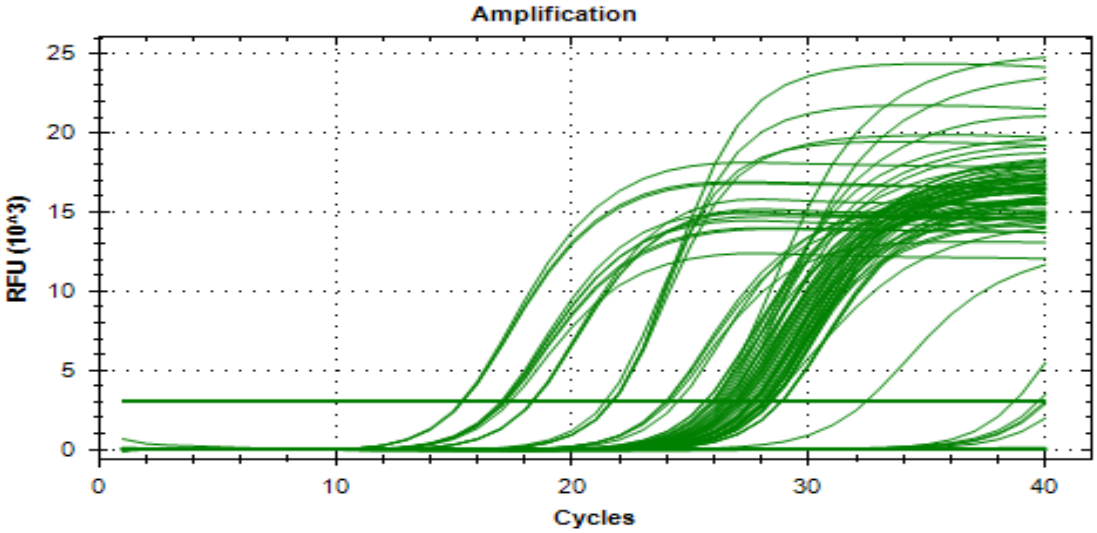


Figure 7: PCR Amplification Curve for Cardiomyocyte samples. RFU: Relative fluorescence units. Green line represents threshold for determining relative gene expression.

Real time RT-PCR was conducted using the PrimePCR plates. The results of the amplification curves can be seen in **Figure 7**. Using the BioRad CFX analysis software, relative normalized gene expression values were reported for NRG-1, ErbB2, ErbB4, IGF-1 and IGF-1 in all samples. All triplicate values were averaged for each respective sample. I transformed the average relative normalized gene expression values for each sample into fold-change differences by normalizing the treatment groups with a control group. Since there were two control groups for each cell type (siRNA controls- E3-SiC and C3+E3-SiC and no treatment control- E4-NT and C4+E4-NT), I calculated two sets

fold-change values:

1) Fold-change differences observed in treatment groups when normalized against siRNA control groups, E3-SiC and C3+E3-SiC (**Table 4**),

2) Fold-change differences observed in treatment groups when normalized against no treatment control groups, E4-NT and C4+E4-NT (**Table 5**).

Fold-change differences were graphed to represent gene expression changes in treatment samples in **Figure 8** and **Figure 9** (corresponding to **Table 3** and **Table 4**, respectively.)

	Normalized against E3-SiC		Normalized against C3+E3-SiC	
	E1-SiFx	E2-SiFx	C1+E1-SiFx	C2+E2-SiFx
NRG-1	-1.16	-2.51	-1.23	-1.04
ErbB2	-1.11	-2.28	-1.34	-1.22
ErbB4	1.62	-1.20	0	0
IGF-1	-1.08	-2.36	-2.42	-3.32
IGF-1R	1.05	-1.56	1.02	-1.64

Table 3: Fold-Change Differences for Treatments. Fold-change differences observed in treatment samples normalized by their respective siRNA control groups (E3-SiC or C3+E3-SiC). Green: up-regulated fold-change. Red: down-regulated fold-change.

	Normalized against E4-NT		Normalized against C4+E4-NT	
	E1-SiFx	E2-SiFx	C1+E1-SiFx	C2+E2-SiFx
NRG-1	4.42	2.05	6.27	7.42
ErbB2	30.0	14.64	1.852	-1.22
ErbB4	135.37	69.68	0	0
IGF-1	6.82	3.13	1.05	-1.31
IGF-1R	1.06	-1.54	5.32	3.20

Table 4: Fold-Change Differences normalized by E4-NT. Fold-change differences observed in treatment samples normalized by their respective no treatment control groups (E4-NT or C4+E4-NT). Green: up-regulated fold-change. Red: down-regulated fold-change.

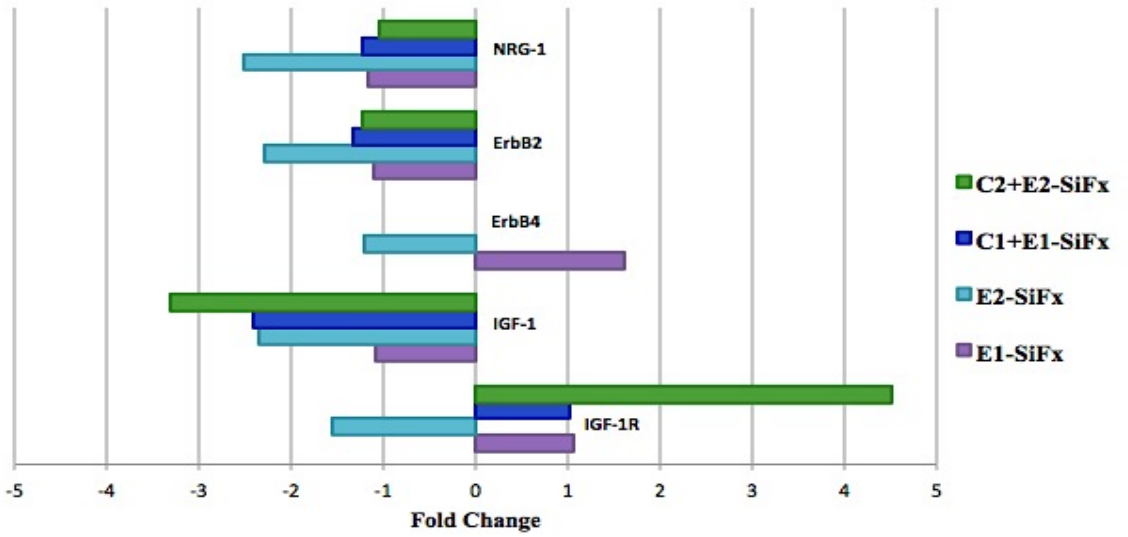


Figure 8: Gene expression of treatment samples when normalized by E3-SiC. Gene expression difference in treatment samples by fold-change difference when normalized by their respective siRNA control groups (E3-SiC or C3+E3-SiC). Based on the data given in **Table 3**. Negative values indicate down-regulated.

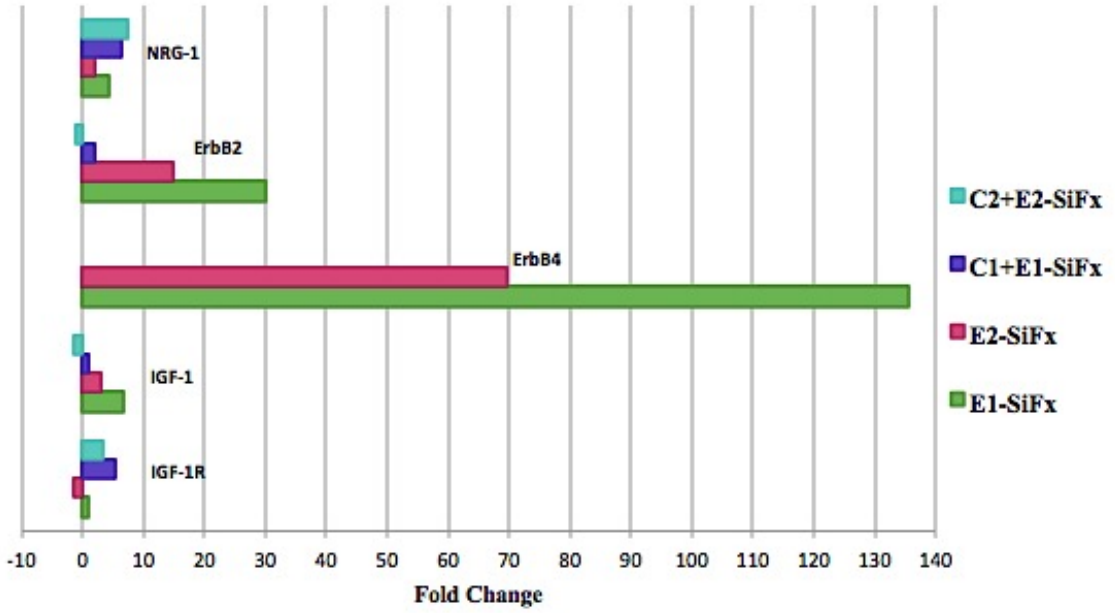


Figure 9: Gene expression of treatment samples when normalized by E4-NT. Gene expression difference in treatment samples represented by fold-change difference when normalized by their respective no treatment control groups (E4-NT or C4+E4-NT). Based on the data in **Table 4**. Negative values indicate down-regulation.

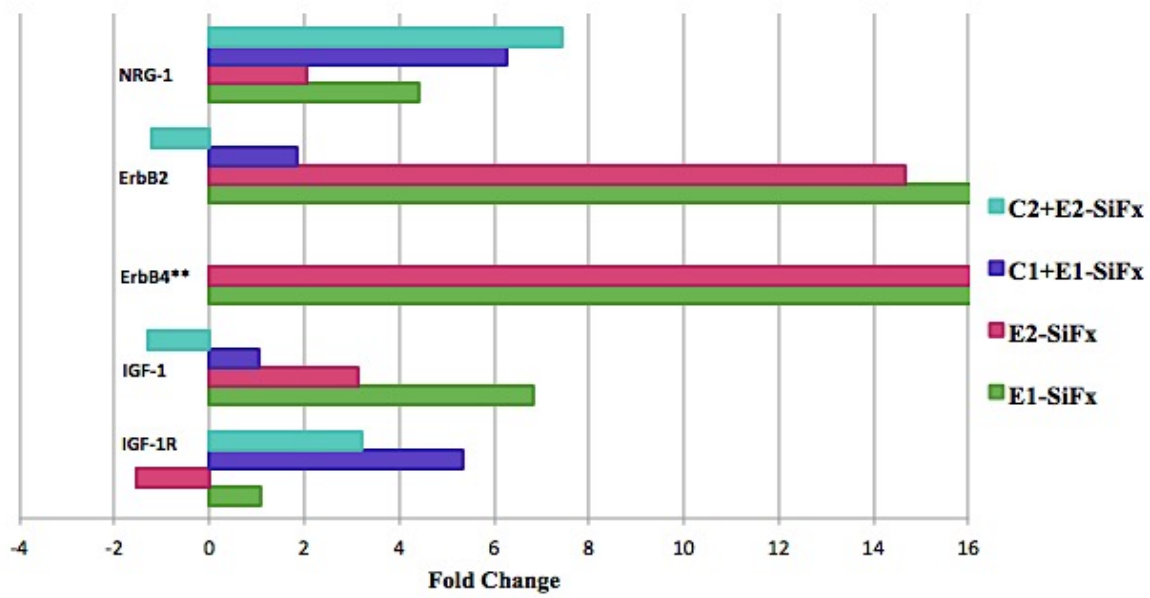


Figure 10: Magnified view of the E4-NT normalized gene expression. Fold change difference in treatment samples when normalized by no treatment control groups (E4-NT or C4+E4-NT) as seen in **Figure 10**, graphed on using a smaller scale on the horizontal axis. **ErbB4****: upper range of fold change differences in E1-SiFx (135.27) and E2-SiFx (69.68) not shown on graph.

The expression levels of the treatment groups that were normalized against the no treatment control (E4-NT and C4+E4-NT) expression levels had significantly larger fold-changes when compared to the treatment groups normalized with the siRNA control groups (**Figure 8** v. **Figure 9** and **Figure 10**). Specifically, the ErbB2 and ErbB4 expression fold-changes observed in both E1-SiFx and E2-SiFx groups (normalized against E4-NT) each had a significantly higher magnitude than any other expression level fold-change observed. Because of these large fold-change values, the axis scale in **Figure 9** makes it difficult to accurately interpret changes in expression levels for the other genes measured, especially those with much smaller fold-change values. Therefore, I graphed the same fold-change data displayed in **Figure 9** (also found in **Table 4**) using a smaller scale on the horizontal axis to magnify the smaller changes in expression levels observed

in NRG-1, ErbB2, IGF-1 and IGF-1R (**Figure 10**). For endothelial cells, gene expression levels were down-regulated in both samples when normalized against E3-SiC, except ErbB4 and IGF-1R in E1-SiFx, which had 1.62 and 1.05 fold-change differences, respectively. Interestingly, almost all of the gene expression levels were up-regulated in both samples when normalized against E4-NT, except IGF-1R in E2-SiFx, with a fold-change difference of 1.54 (negative; down-regulated).

4. DISCUSSION

In this project, I developed a co-culture model system to study NRG-1 and IGF-1 signaling from endothelial cells to ErbB2/ErbB4 and IGF-1R on cardiomyocytes to emulate signaling events that occur during heart development. As seen in **Figure 2**, NRG-1 and IGF-1 are both able to activate the PI3K/AKT pathway, which is able to directly phosphorylate FoxO1 to induce translocation from the nucleus to the cytoplasm, therefore, making it inactive for transcription of its target genes. Its target genes including cyclin kinase inhibitor, cause cells to withdraw from the cell cycle, leading to the cessation of cell proliferation. When FoxO1 expression is completely silenced in murine embryo, there are extreme malformations of the heart leading to embryonic lethality. Importantly, the phenotype associated with the *FoxO1*$^{-/-}$ embryo shows an absence of trabeculation in the ventricles, endocardial cushion formation and proper myocardial compaction, indicating that endocardial signaling is affected by the global loss of FoxO1 expression.

The co-culture design used in this project (**Figure 4**) provided an *in vitro* representation of signaling interactions between endothelial cells of the endocardium and cardiomyocytes of the myocardium that occur *in vivo*. *In vitro* cell culture systems cannot completely replicate *in vivo* processes; however, a co-culture model system allows for individual treatments as well as a more direct analysis of cells. By culturing the cardiomyocytes on 6-well plates and culturing endothelial cells on the 0.4 μm Transwell co-culture inserts, I used SignalSilence siRNA to reduce or remove FoxO1 expression, thereby 'silencing' it's function in the endothelial cells. I hypothesized that there would

be a decrease in expression for NRG-1, IGF-1 and their receptors, ErbB2, ErbB4 and IGF-1R when FoxO1 expression was silenced.

4.1 Gene expression

Control Samples: SiC v. NT

The gene expression of NRG-1, ErbB2, ErbB4, IGF-1 and IGF-1R each cell sample (8 total; 4 cardiomyocyte samples and 4 endothelial samples). Surprisingly, when I compared the expression levels of corresponding genes in the siRNA control samples vs. the no treatment control samples for both cell types, I observed extreme differences in expression level fold-changes. Ideally, gene expression would be the same in both types of control samples, since the control siRNA contains a scramble sequence, which does not have any function once transfected into the cell. Because of how substantial the fold-change differences were for the siRNA controls v. the NT controls expression level, I created two sets of normalized fold-change values for each cell type, as seen in **Table 4** and **Table 5**. These results could indicate that the process of siRNA transfection into endothelial cells affected gene expression levels. Transfection is a stressful process for cells so it is not unreasonable to think that the siRNA treatment affected expression level by a considerable amount when compared to the no treatment controls. Because of this information, I normalized all of treatment sample expression levels against both the siRNA control and the no treatment control expression levels (**Table 3** and **Table 4**). In this study, the fold-changes in expression normalized against the siRNA controls support the hypothesis while expression changes normalized against the no treatment controls did

not. Further experiments would be needed to draw conclusions regarding these differences.

In the endothelial treatment samples, fold-change differences in gene expression levels of E1-SiFx and E2-SiFx normalized by E3-SiC showed down-regulation of expression of NRG-1, ErbB2 and IGF-1. IFG-1R and ErbB4 were up-regulated only in the E2-SiFx sample, but it is difficult to make conclusions with such limited data.

In the cardiomyocyte treatment samples, fold-change differences in gene expression levels of C1+E1-SiFx and C2+E2-SiFx normalized by C3+E3-SiC both showed down-regulation of expression of NRG-1, ErbB2 and IGF-1, similar to the endothelial cell samples. IGF-1R was up-regulated in C1+E1-SiFx but down-regulated in C2+E2-SiFx. No expression levels were detected for ErbB4 in either C1+E1-SiFx and C2+E2-SiFx samples. Again, additional data from repeated trials would have helped interpret the data from a biological point of view. Quality of RNA (**Table 2**) could have affected the detection of ErbB4. Taken together, the data supports the hypothesis that there is a decrease in NRG-1 and IGF-1 expression when FoxO1 expression is absent.

4.2 Limitations

Due to personnel changes in Dr. William Claycomb's lab, I was unable to obtain any more HL-1 cells during my research. This left our lab with a limited amount of HL-1 cells stored in cryopreservation. The HL-1 cells became contaminated mid-way through my research therefore I was forced to pull from our limited quantity of frozen cells. The HL-1 cells we had in cryopreservation were of a very high passage, with some dating back to the early 2000's; however, these cells were my only option for cell culture at that

point in my research. Cryopreservation is an extremely stressful process for cells to undergo and thousands of cells are killed in the process. In addition, cell lines decrease in stability as well as integrity as their age and passage number increase. Therefore, the chances of successfully culturing the HL-1 cells left in cryopreservation were very slim. Multiple trials of the co-cultures would have been ideal but unfortunately, I was not able to culture the amount of HL-1 cells necessary carry out more than one trial. That being said, the experimental trial I was able to complete was done in triplicate and produced a sufficient amount of RNA and DNA for a significant analysis. In addition, because I was only able to perform one experimental trial, I could not use statistical analysis to compare multiple data sets as I would have preferred.

4.3 Future Directions

I would first recommend future studies complete additional trials of this co-culture system to collect a substantial amount of data to perform statistical analysis. I would also recommend studies investigating the differences in siRNA expression levels compared to no treatment controls, given the significant differences observed in the results of this project.

The immortalized HL-1 cardiomyocyte cell line provides a convenient way to study heart development in a cell culture environment, in addition to serving as a simpler alternative to using primary cardiomyocyte cells. In order to study cardiomyocyte proliferation, the cells must be isolated from an embryo of timed-pregnant mice, which is a more time-consuming and costly way to study cardiomyocyte cell cultures. However, recent advances in stem cell biology and culturing methods have made the use of HL-1

cardiomyocytes a less popular choice in cell culture labs. The use of multipotent stem cells derived from human somatic cells has become extremely popular in cell culture research. Specifically, the ability to induce these derived mesenchymal stem cells into various different cell types has created many new opportunities for cell culture research. For example, human adipose-derived stem cells (ADSCs) can be induced to differentiate into cardiomyocyte cells. Cell cultures using this method of induced differentiation to cardiomyocytes is a much more attractive option than using the immortalized HL-1 cell line. For future cell culture studies in heart development, I would recommend the use of a co-culture model system between cardiomyocytes and endothelial cells to emulate early *in vivo* cellular signaling involved in cardiomyocyte proliferation and heart development. I would also recommend the method of induced differentiation of human ADSC to produce human cardiomyocytes.

4.4 Conclusions

Though my thesis project was limited by my ability to acquire more cardiomyocyte cells, my execution of the cardiomyocyte-endothelial cell co-culture system proved to be a promising technique in studying early cell signaling events that occur during heart development *in vivo*. The successful endothelial-specific FoxO1 knock down allowed me to study the affect of FoxO1 gene expression on the expression of genes known to have significant roles in heart development. In addition, the co-culture design allowed me to further investigate the affect of gene expression levels on *in vitro* cell-cell signaling interactions between cardiomyocytes and endothelial cells to emulate signaling between the myocardium and endocardium *in vivo*. My results provide further

support that FoxO1 expression could be regulated by the PI3K/Akt pathway, which is activated by growth factors like NRG-1 and IGF-1. It is my hope that my project might assist in the pursuit of a deeper understanding of myocardial-endocardial signaling in the developing heart, as these signals remain key to discovering therapeutic methods for heart regeneration.

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APPENDIX 1

HL-1 Cell Culture Procedures

Provided by Dr. William Claycomb

I. HL-1 Media Solutions

Complete Claycomb Medium

	50 mL	100 mL	200 mL	500 mL
FBS	5 mL	10 mL	20 mL	50 mL
Pen/Strep	0.5 mL	1 mL	2 mL	5 mL
Norepi (10mM)	0.5 mL	1 mL	2 mL	5 mL
L-Glut (200mM)	0.5 mL	1 mL	2 mL	5 mL
Claycomb	43.5 mL	87 mL	174 mL	435 mL

Wrap in aluminum foil, since the medium is extremely light sensitive.

- Supplemented media is good for two weeks;
    - At 2 weeks, can replenish the L-glutamine **once**

Norepinephrine (mw 319.3): 10 mM Stock in 30 mM Ascorbic Acid

1. Add 0.148 g ascorbic acid to 25 ml of cell culture grade distilled water.
2. Add 80 mg norepinephrine to the 25 ml of 30 mM ascorbic acid.
3. Filter-sterilize using 0.2 μm Acrodisc syringe filter
4. Aliquot in 1 ml volumes into sterile freezer vials with screw caps, and store at -20C. This is 10mM (stock) norepinephrine. Use 1 mL of stock per 100 mL medium for a 0.1 mM final concentration.

- Good for one month in -4 C freezer

Gelatin/Fibronectin

1. Prepare 200 ml of 0.02% gelatin – 0.04 g in 200 ml dH_2O.
2. Autoclave and allow to cool to room temperature.
3. Add 1 ml fibronectin solution, mix.
4. Aliquot 12 ml volumes in 15 ml conical tubes and freeze. This can be refrozen after thawing.

L-Glutamine

- Comes as 100x solution
- Aliquot 12 mL volumes into 15 mL conical tubes

Claycomb Wash Medium

Media 95 mL
FBS 5 mL

Freezing Medium

FBS 9.5 mL
DMSO 0.5 mL

- This can be stored up to a week at 4°C.

II. HL-1 Cell Culturing

A. Coating Plates

1. Coat plate with gel/fibro and incubate for at least an hour before culture
    a. 1 mL/well for 6-well or 3 mL for 100 mm/T75
2. Remove the gelatin/fibronectin and wash plate with Claycomb Wash
3. Add new media then place back in incubator until ready to add cells
    a. 1 mL for 6-well
    b. 5 mL for 100 mL
    c. 10 mL for T75

B. Thawing

1. Gelatin/fibronectin coat a plate for at least an hour in the incubator
2. Remove the gelatin/fibronectin from the flask and replace with 10 mL of supplemented Claycomb medium. Place this flask in incubator.
3. Transfer 10 mL wash medium into an empty 15 mL centrifuge tube. Incubate tube in 37°C water bath
4. Quickly thaw cells in a 37°C water bath (~2 min) and transfer into the 15 mL centrifuge tube containing the wash medium.
5. Centrifuge for 5 min at 500xg
6. Remove the tube from centrifuge and remove the wash medium by aspiration.
7. Gently resuspend the pellet in 5 mL supplemented Claycomb medium and add to the 10 mL of medium already in the plate.
8. Replace the medium with 15 mL of fresh supplemented Claycomb medium 4 hours alter (after cells have attached).

C. Culturing

1. Coat culture plates with gelatin/fibronectin for at least 1 hour at 37C. This can be done overnight if more convenient. Be sure to agitate the plates in order to spread the solution evenly.
    a. 1 ml per well for a 6-well plate
    b. 3 ml for a 100 cm plate or T75 flask
2. Remove the gelatin/fibronectin and add Complete Claycomb to the plates.
3. Thaw vial of HL-1 cells and pour into 9 ml Claycomb Wash Medium.
4. Count the cells.
5. Centrifuge the cells @200 xg for 10 min. Remove wash medium and resuspend in an appropriate volume of Complete Claycomb Medium.
    a. Final volume for each type of plate:
        i. 2 ml/well for a 6-well dish
        ii. 10 ml for a 100 mm plate
        iii. 15 ml for a T75 flask
6. Add the cells to the plates and incubate at 37C.
7. Check the cells daily and replace the media every 2 days. Double the volume if leaving the cells over the weekend.

D. Subculturing

- For a 1:3 split

1. Remove media and rinse 1x with PBS.
    a. 1 ml/well for a 6-well dish
    b. 5 ml for a 100 mm plate or T75 flask
2. Add **0.05% trypsin/EDTA** to the plate and incubate for 1 min at 37C.
    a. 1 ml/well for a 6-well dish
    b. 2 ml for a 100 mm plate
    c. 3 ml for a T75 flask
3. Replace the trypsin/EDTA with fresh and incubate for 2 min at 37C.
4. Check to see if cells are dislodged. If not, rap plates on bench until completely floating; may take 5-10 min.
5. Add Claycomb Wash to plate (volume equal to double the trypsin volume)
6. Transfer the cells to a conical tube and centrifuge at 500g for 5 min.
7. Cells are now ready for passaging or freezing.
   Passaging: resuspend cells in Complete Claycomb and follow the culturing procedure.
   Freezing: suspend the cells in ice cold freezing medium (1×10^6 cells/ml). Transfer to cryovials (1 ml/vial), put in thawed freezing container, and put in ultracold freezer.

E. Freezing

One T75 flask/100 mm into one cryovial

1. Briefly rinse the plate with HL-1 culture with 5 mL of PBS warmed to 37°C. Remove via aspiration.
2. Transfer 3 mL of 0.05% trypsin/EDTA into the plate.
3. Incubate the flask at 37°C for 1 minute
4. Remove the trypsin/EDTA from the flask and replace with 3 mL of fresh 0.05% trypsin/EDTA. Incubate for 2 min.
5. Check to see if cells have dislodged. If not, rap plates on bench until completely floating; may take 5-10 min.
6. Add 8 mL of Claycomb wash medium to the flask and transfer 6 mL into a 15 mL centrifuge tube.
7. Rinse the empty plate with 8 mL wash medium and add the cells already in the 15 mL tube; 14 mL total volume
8. Centrifuge tube for 5 minutes at 500xg
9. Remove wash medium by aspiration.
10. Gently resuspend cells into a cryovial. Place the cryovial containing the cells into a Nalgene freezing jar containing room temp isopropanol. Freeze at -80°C; transfer to liquid nitrogen the next day
11. Immediately place the freezing jar in a -80°C freezer and freeze the cells at a rate of -1°C/minute.
12. 6 to 12 hours later, transfer the vial to a liquid nitrogen drawer.

APPENDIX 2

C166 Cell Culture Procedures

Provided by ATCC

I. C166 MEDIA SOLUTIONS

Supplemented DMEM

DMEM	450mL
FBS	50 mL
Pen/Strep	5mL

Freezing Medium

Media	9.5 mL
DMSO	0.5 mL

II. C166 CELL CULTURE PROTOCOL

A. Subculturing

1. Remove and discard the culture medium.
2. Briefly rinse the cell layer with Dulbecco's Phosphate Buffered Saline (PBS).
   NOTE: This step removed all traces of serum that contains trypsin inhibitors.
3. Remove the PBS from the plate.
4. Add 2.0 mL of Trypsin- EDTA Solution to the plate.
5. Incubate cells in 37°C, 50% CO_2 for 3-6 minutes; do not exceed 10 minutes
6. Once the cells are loose, add 8 mL of medium to the plate. Pipet cells and medium up and down to mix.
7. Prepare the correct number of plates (label with initials, date, cell type, passage #) and add 9 mL of complete medium to 100 mm plate (**For 6-wells**: 1 mL per well)
8. Add 1 mL of cells to each plate (**For 6-wells**: 0.5 mL per well)
9. Incubate plates in a 37°C, 5% CO_2 incubator.

B. Freezing

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with Dulbecco's Phosphate Buffered Saline (PBS).
3. Remove the PBS from the plate.
4. Add 2.0 mL of Trypsin- EDTA Solution to the plate.
5. Incubate plates in a 37 °C, 5% CO_2 incubator for 3-6 minutes.

   *Note: After 3 minutes, check to see if the cells have let go of the plate (look at the plate under the microscope). If the cells are still attached place back in the incubator for 2-3 more minutes. DO NOT EXCEED 10 MINUTES.
6. Once the cells are loose, add 8 mL of medium to the plate. Pipet cells and medium up and down to mix.
7. Add the entire content of the plate to a 15 mL centrifuge tube.
8. Centrifuge the tubes at 3000 rpm for 5 minutes.
9. Remove the medium from the tube, only leaving the pellet inside.
10. Add 1.5 mL of medium + DMSO to each tube. Pipet up and down to remove the pellet.
11. Remove all of the liquid from the tube and add it to a 2 mL tube.
12. Place all tube into the -80°C freezer overnight before placing them in the liquid nitrogen the next day.

V. C166 TRANSFECTION

1. Plate 0.5 mL cells in 1 mL on inserts the day before
    a. Use Claycomb Media for C166's in addition to HL-1's
2. For each transfection sample, prepare oligomer-Lipofectamine 2000 complexes as follows:
    a. 15 μL siRNA + 750 μL Opti-MEM
        i. **TWO** FoxO1 siRNA tubes/6-well plate
            1. **6** tubes/experiment
        ii. **ONE** Control siRNA tubes/6-well plate
            1. **3** tubes/experiment
    b. 10 μL Lipofectamine + 750 μL Opti-MEM
        i. **9** tubes/experiment
        ii. Incubate at room tempertaure for 5 minutes
    c. Mix two solutions together
        i. Incubate at room temperature for 20 minutes
3. Remove medium from wells using the vacuum.
4. Rinse each well with PBS.
5. Add the oligomer-Lipofectamine complexes to each well containing cells and medium.
6. Mix gently by rocking back and forth.
7. Incubate cells at 37°C, 5% CO_2 incubator for 48 hours *Note: Change media after 4-6 hours

APPENDIX 3

Western Blot Protocol

Provided by Dr. Laura Glasscock

Pouring Gel

1. Pour separating (running) gel (10%, denaturing):
   - Prop cassette up with clips on a paper towel
   - In a beaker, mix:
     3.12 ml 40% acrylamide 3.12 ml 4x running buffer
     6.25 ml dH20
   - Immediately before you pour the gel, add:
     41.26 ml 10% APS (catalyst)
     6.25 ml TEMED (polymerizes acrylamide)
   - Using a pipette, slowly pour gel in-between cassettes to spacer mark.
   - Overlay gel with approx. 800 ml butanol
   - Let gel polymerize.
   - Dispose of butanol; use a piece of filter paper to remove residual butanol.

Stacking gel:

- In a beaker, mix:
  0.624 ml acrylamide
  1.55 ml 4x stacking buffer
  4 ml dH20
  37.5 ml 10% APS
  12.5 ml TEMED
- Pipette onto top of running gel until you reach the top of the shorter plate.
- Place spacer in gel and let polymerize (approx. 15')

Sample preparation:

1. Add sample buffer to your sample.
   -Final Volume: 20 mL: 10 mL sample + 10mL 2x Sample Buffer

Running the gel:

1. Remove small clips from gel sides and remove tape.
2. Remove the spacer.
3. Using 1x running buffer (Laemlli), fill the space behind the gel to the top and the bottom chamber about 1 inch.
4. Using a syringe, remove all bubbles.
5. Load samples into each well.
6. Connect lid and electrodes to the power source. Run under constant current.
   - Set current to 15 mA for approx. 15 min.
   - Turn current up to 30-35 mA
   - Volts should be about 100.
8. When the dye front reaches the bottom, turn the power supply off and disconnect the power supply from the gel.
9. Dispose of running buffer and open cassette to remove gel

Western Blotting

Prepare blot for transfer: about 30' before gel is through:

1. Cut nitrocellulose and submerge in MeOH in a small dish for about 60 sec.
2. Pour in Towbin buffer to completely submerge, plus extra, components.
   - Let this equilibrate for approx. 20' before assembling with gel.

   1/2 x Towbin buffer for everything.
3. Remove gel from cassettes under buffer with your finger. Assemble cassette UNDER BUFFER as follows: 2 sponges, 2 pieces. filter paper, nitrocellulose (line up marks, orientation), gel ("gel side back"), 2 pieces. filter paper, 2 sponges
5. Load "sandwich" into transfer cassette then load into transfer chamber (gel side back).
7. Fill cassette with 1/2 x Towbin buffer to top of sponges (bellow screws). Fill transfer chamber about 2 inches.

Transfer:

1. Transfer at 25 V, constant voltage for 1-2 hours 1 hour
   - Current should be about 250-500
   - Check every 20' or so to see if over heating.
2. Turn power source off, disconnect, disassemble sandwich.
3. Use nitrocellulose in WB probing/detection steps.

Probing:

1. Place one nitrocellulose blot in each pipette tip lid or other small container.
2. Block with 3% milk in 1x TBS buffer, 30', rocking.
3. Rinse with TBS.
4. Add primary antibody:
   - Dilution: 1:1000
   - Make in 1% milk/TBS
   - Rock 3 hours-overnight
5. Rinse with TBS.

   Wash 3x, 5' each, with TBS while rocking.
6. Add secondary antibody:
   - Anti-rabbit HRP-conjugated (SCBT) to enzyme for substrate
   - Dilution: 1:10000
   - Rock 1-3 hours or overnight
7. Rinse with TBS.

   Wash 2x, 5' each, with TBS while rocking.

   Wash 1x with distilled water. Leave it in this until you do the ECL.
8. Detection: ECL

ECL detection:

1. Remove wash solution but leave blot in lid.
2. Prop one end of the lid up and add approx. 4 ml each ECL reagent to the bottom of the lid (do not touch blot yet). Mix 2 reagents in lid gently.
3. Un-prop lid and cover blot. Let sit for 1 minute.
4. Remove blot with tweezers, shake gently over paper towel, place on saran wrap and cover (no bubbles, wrinkles, etc.)
5. Place blot on imaging surface of Bio-Rad Gel-Doc EZ machine and image blot.