THE ROLE AND REGULATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN HEART DEVELOPMENT

by

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A THESIS

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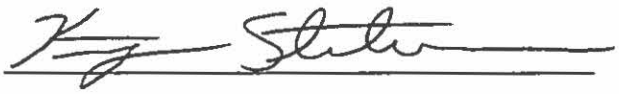
Approved: 

Kryn Stankunas

The most common birth defects are due to malformations that occur during the development of the heart. The valves, trabeculae, and surrounding vasculature are all targets for molecular mishaps that can lead to lethality and other severe consequences in later life. The development of the various structures of the heart is complex and composed of multiple steps that ultimately contribute to the formation of the organ responsible for the distribution of blood and nutrients throughout the body.

Vascular Endothelial Growth Factor (VEGF) signaling is one of the pathways involved in the formation of the various heart structures. This thesis presents the known regulation and spatiotemporal roles of this pathway in an attempt to compile scattered research done on the topic. In addition, new hypotheses are presented alongside updated potential models to explain the dynamic roles of VEGF signaling during development. Furthermore, potential experiments and areas of future research are outlined to spur new studies to further understand this signaling pathway and its interactions during heart development.

Acknowledgements

I would like to thank Drs. Kryn Stankunas and David Frank as well as PhD candidate Kate Karfilis for helping me fully examine this specific topic and consider the various perspectives and contexts related to this subject matter. I am sincerely grateful for their guidance through this strenuous but rewarding process and for their encouragement to pursue a thesis through scientific research. In addition, I would like to thank the members of the Stankunas lab for their continued support, encouragement, and advice throughout my time on campus as an undergraduate researcher. I would like to extend a special thanks to the American Heart Association and the University of Oregon Women in Graduate Sciences for helping fund my research pursuits throughout my time at the University of Oregon.

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Chapter 1: Prologue

The human body is incredibly complex and requires specific instructions in order to develop correctly. While all human beings contain the same basic components needed for development, a set of 23 chromosomes known as DNA, there is vast genetic variation seen amongst our population. More specifically, each cell has a control center—the nucleus—that contains the DNA molecules. Within those molecules, there are specific sequences, labeled as genes, which provide the cell with directions that will control its physiology. The genetic makeup is the combination of different genes that relay instructions in cells. Although the components of DNA are not visible to the naked eye, the instructions that one's genotype relays create specific traits, known as phenotypes, which can be easily identified such as eye color or a structural shape.

The role of the genes is to convert the genetic code into messages that are transported in the form of mRNA; these messages are the signals for protein formation in the cell. Each gene can be switched on or off to regulate the expression as needed throughout development and life. When a gene is turned on, a message is sent that causes the cell to behave differently than before the signal was received due to the protein interactions. When the proteins carry out specific functions in the cell, the cell's fate is ultimately determined. Through this process, specific cells are programmed to become a part of the heart, opposed to the spleen, liver, or brain. However, if there is a mutation present, changes or severe consequences can be seen in the resulting phenotype.

Gene regulation is the process by which the body forms and modifies the cardiovascular and other systems throughout life. The cardiovascular system is a

complex and highly organized vascular network that forms during embryogenesis to rid the body of waste and supply the body with blood and proper nutrients needed to survive. The *de novo* birth of blood vessels is regarded as vasculogenesis, while directions for the growth and movement of existing vessels is known as angiogenesis. While both processes can occur simultaneously, vasculogenesis must initially precede angiogenesis. The exact mechanisms of these and process are unclear; however, it is known that Vascular Endothelial Growth Factors (VEGF) interact with endothelial cells to facilitate the development of vital vessel arrangements.

Due to the implications of VEGF signaling, an abundance of research has been conducted on this protein family in order to better understand its function in relation to other signaling proteins during angiogenesis. However, much about the role of VEGF during development remains unknown and has yet to be studied. Thus far, researchers have determined that the VEGF proteins play a vital role in development by eliminating VEGF and observing the consequent phenotypes in model organisms including mice, zebra fish, and chickens. These loss-of-function studies conducted have resulted in severe defects in the cardiovascular system during embryogenesis and even embryonic lethality. Knowledge gained from these experiments provides overwhelming support for the significance of VEGF signaling to create the blood vessels that become the primitive vascular network in the organism. As the fetus grows and develops, this network is modified, remodeled, and expanded to serve the needs of the body and its vital organs. Later in life, VEGF has been shown to still serve a fundamental role in health and disease by allowing the body to recover from injuries as well as supplying tumors with needed vasculature for growth and survival.

In addition to the formation of the vascular network, VEGF has been identified as a critical molecule and signaling pathway during various phases of heart development. During proper organ formation, this protein allows for the normal and healthy development of the heart's valves, muscle, and coronary arteries. However, if this signaling protein is mutated or not correctly regulated temporally, it can lead to major morphological defects during organogenesis. Changes in gene expression that occur throughout vasculogenesis and angiogenesis during development, if well characterized, can provide insight to the cellular mechanisms that can lead to the advancement of proangiogenic and antiangiogenic therapies. These therapies will have the capability to more accurately address diseases of the cardiovascular system through both treatment and prevention.

Most cardiovascular diseases stem from congenital heart defects, which are the most prevalent form of birth defects seen in our population (Figure 2) (Bruneau, 2008). The mutations that arise during embryogenesis at the molecular level can lead to the improper formation of specific areas of the heart. While mutations may appear harmless at birth, the consequences of congenital heart defects often do not manifest until adulthood. The diseases caused by congenital defects have been categorized; however, the specific genes that are mutated remain unknown. In order to diagnose potentially life-threatening diseases of the heart, these genes must first be identified before mutations can be recognized.

The potential benefits of knowing the precise gene for each disease include genetic counseling, family planning, and early prevention for the imminent adult diseases. Identifying these genes will also lead to the ability to further regenerative

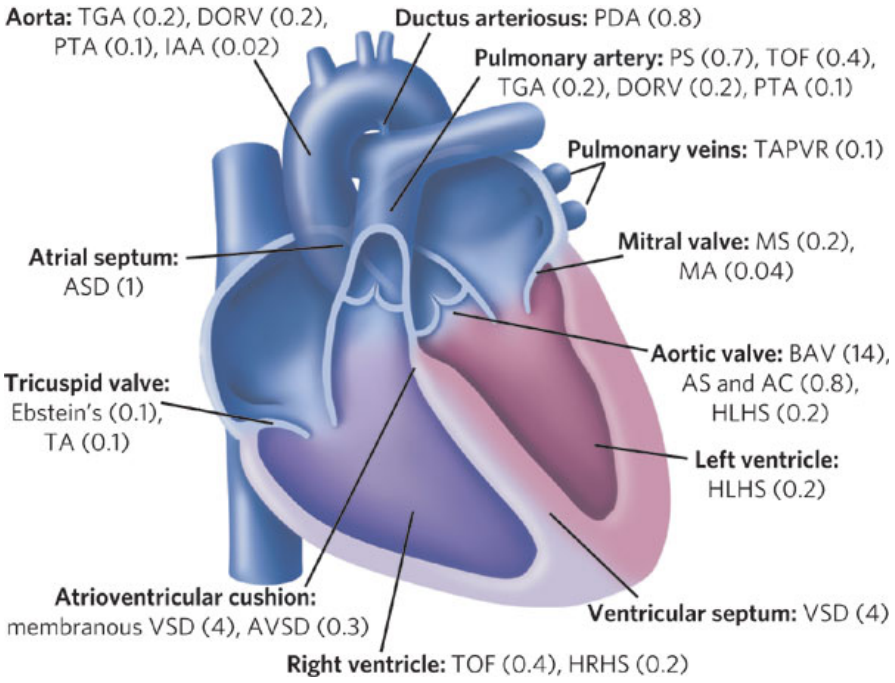

Figure 1: Congenital Heart Defects.

This figure depicts the prevalence of congenital heart defects in each structure of the adult heart. The estimated incidence of each disease per 1,000 live births is given in parenthesis next to each defect (Bruneau, 2008).

medicine, which will allow for the production of artificial hearts and valves to be used for life saving surgeries. By uncovering the fundamental patterns of VEGF during heart development, the scientific community can progress in the treatment of these immense health issues.

This thesis serves to fill current gaps in the scientific literature about the spatial and temporal roles of VEGF during heart development. A comprehensive literature review contributes to the field of developmental biology by serving as the first compiled analysis of the most current knowledge surrounding the role and regulation of VEGF during heart development. Throughout this literature review are proposed mechanisms with justification for the hypothesized role of VEGF. This review then functions as the basis for the development of updated models that draw novel conclusions about the role

and regulation of VEGF during the development of the valves and ventricular trabeculae, with insight to the importance coronary arteries of the heart. To provide future direction for researchers studying this topic, proposed areas of study and potential experiments to test the hypotheses made are discussed in the chapter following the proposed models.

Chapter 2: VEGF Family and Heart Development Overview

Within the platelet-derived growth factor, VEGF contains multiple ligands (VEGFA, VEGFB, VEGFC, VEGFD, VEGFE and placental growth factor PlGF) and three tyrosine kinase receptors. The different pathways are responsible for roles in vascular development as will as the development of the lymphatic system and many organs. Together, with the addition of co-receptors such as neuropilins and integrins, the signaling cascade leading to development, growth, or remodeling is initiated and will effectively respond to changes in cellular VEGF concentration.

Prior to discussing the roles of VEGF in heart development, the interactions amongst the ligands and receptors belonging to the VEGF family must be identified. In addition, a basic understanding of how the heart develops is essential before looking in detail to the development of the individual components.

VEGF Ligands and Receptors: Key Players and Interactions

Members of the VEGF family each play distinct roles in order to accomplish the needs of a developing and growing organism. The relationships between the ligands and receptors become complex with the understanding of which ligands bind and activate the phosphorylation of specific VEGF receptors. When the tyrosine receptor is phosphorylated, it activates a subsequent pathway until degradation or internalization of the ligand occurs (Koch & Claesson-Welsh, 2012). Each pathway creates a cellular response by producing signals that create transcriptional changes; therefore, it is necessary to understand the role of each player in the VEGF family.

As depicted in Figure 2, VEGFA has the ability to bind with VEGFR1, VEGFR2, or Neuropillin-1. VEGFB has only been shown to bind to VEGFR1.

Meanwhile, VEGFC has been shown to interact with VEGFR2 for a role in angiogenesis as well as VEGFR3 for a role in lymphatic vessel development. VEGFD, can interact with either VEGFR2 or R3 for roles in lymphatic system development (Olsson, Dimberg, Kreuger, & Claesson-Welsh, 2006). Finally, VEGFE in viruses has been shown to interact with VEGFR2 (Takahashi & Shibuya, 2005).

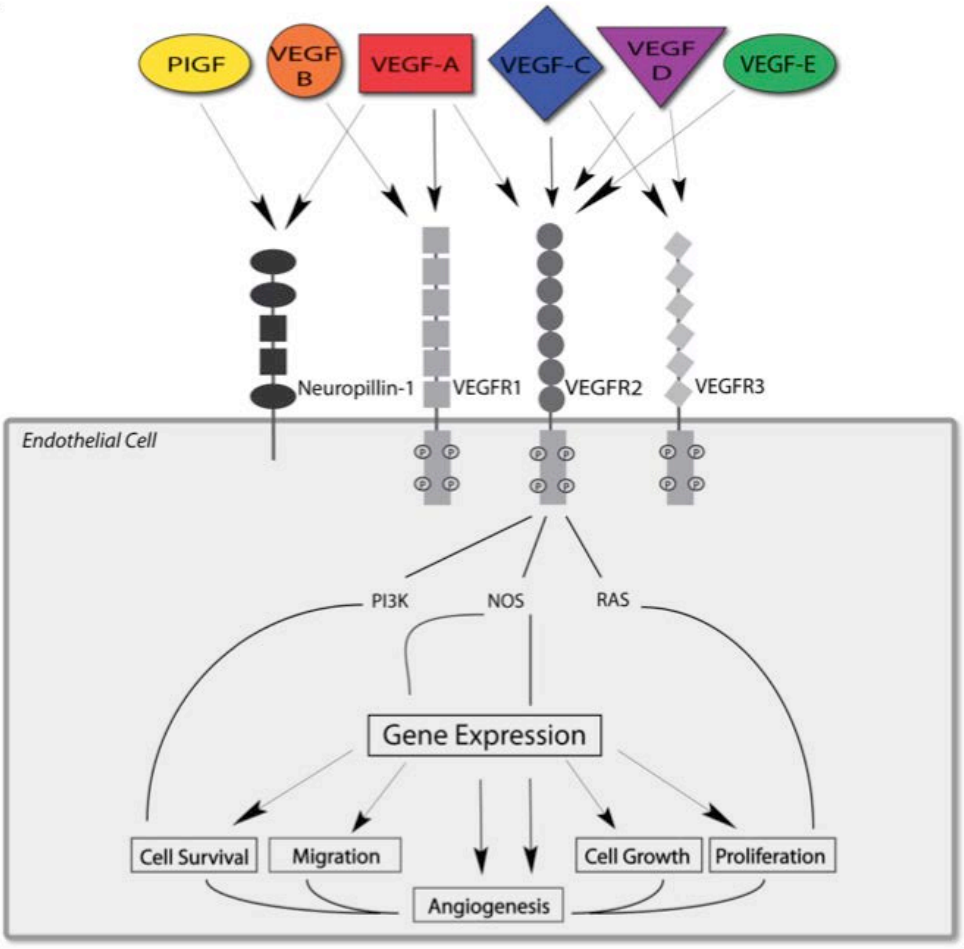

Figure 2: VEGF: Key Players and Roles.

This schematic illustrates the ligands and receptors of the VEGF family. When a ligand binds to a receptor, a resulting signal is produced that leading to gene expression (Kate Karfilis, unpublished).

In order to focus on the role of VEGF in the development of the heart, the ligands and receptors that have been shown to play critical roles outside of blood vessel

and heart development will not be discussed further. While there is the potential that these ligands and receptors do have roles in blood vessel and heart formation, until further research is performed and these potential connections are made, there is no substantial evidence to support making these claims in this thesis. As a result, VEGF ligands and receptors critical to lymphatic vessel development such as VEGFR3 and VEGFC, and the role of ligand E in viruses will not be included in the literature review or proposed models sections.

In addition to basic roles of the ligands and receptors, these pathways have the ability to inhibit each other in order to produce a greater response. In order to characterize the function of each VEGF component, studies have been performed on the various VEGF family ligands and receptors. Researchers have demonstrated the different functions of VEGF members by causing vascular defects through loss-of-function knockout studies. The results range in severity from minute defects in vessel disorganization to embryonic lethality, which provides evidence for the responsibilities of the different players in the VEGF family. For instance, while complete deletion of VEGFR1 is lethal by embryonic day 9.0 (E9.0) in mice due to disorganized vessels that lack a lumen and endothelial precursors, the deletion of the receptor's tyrosine kinase domain is not lethal. This allows researchers to conclude that VEGFR1 does not have a role as an endothelial cell signaling receptor (Fong, Rossant, Gertsenstein, & Breitman, 1995). Instead, VEGFR1 has the ability to negatively regulate VEGFR2 by appropriating the VEGFA ligand (Koch & Claesson-Welsh, 2012; Stankunas, Ma, Kuhnert, Kuo, & Chang, 2010). Lethality was also seen at E8.5 in mice due to complete deletions of VEGFR2, or the ligand VEGFA (Shalaby et al., 1995; Ferrara et al., 1996).

This implies that vascular development is caused by the interaction of VEGFA with VEGFR2. Meanwhile, abnormal increases in ligands have also been shown to cause malformations in the vascular system (Miquerol, Langille, & Nagy, 2000). From the combination of these studies it is clear that both the timing of expression and the amount of each ligand is vital to creating the proper signals that form the vascular network and the developing heart.

Heart Development Overview

The heart develops through a series of intricate steps during embryogenesis (Figure 3). The multistep process of heart formation begins with cardiomyocyte differentiation in the two heart fields at E7.0 in mice. The heart then becomes a linear tube that is composed of two layers, the outer myocardium and inner endocardium, which are separated by cardiac jelly made up of extracellular matrix. At E8.5, the primitive heart tube then loops to form distinct atria and ventricles the following day. Later on, the valves, trabeculae, and coronary arteries are formed in the developing heart prior to prenatal completion. In humans, this process occurs by day 22 (Rosenthal & Harvey, 1999), which implies the urgency for identifying how mutations arise in order to provide education on how to prevent congenital defects in the human population.

In order to further define the spatial and temporal roles of the VEGF family, it is necessary to understand the details of when and how the different structures of the heart develop. This information provides critical clues that show the potential roles and regulation of VEGF in the development of the heart, and how the development of different structures is related during embryogenesis.

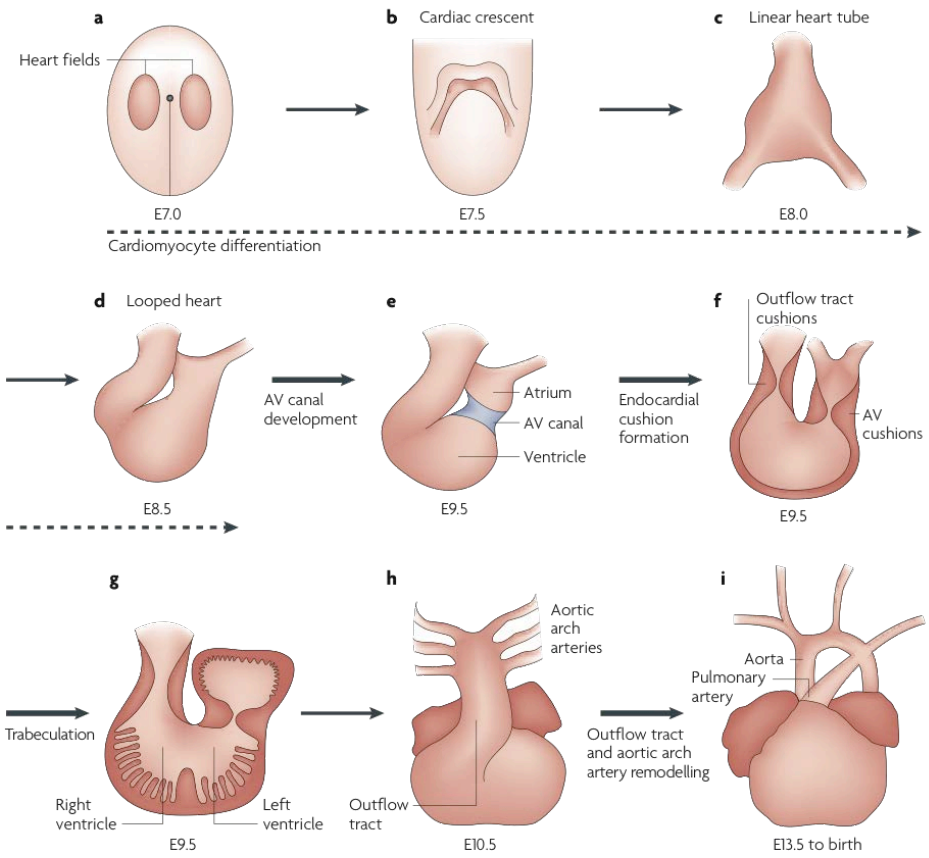

Figure 3: The embryonic development of the heart.

This flowchart illustrates the timing and order of heart development during organogenesis in mice (High & Epstein, 2008).

Chapter 3: Heart Valves

Heart valves regulate the flow of blood throughout the four chambers of the developed heart. One pair of valves, known as the atrioventricular valves, separates the atrial chambers from the muscular ventricles. The other pair of valves regulates the flow between the ventricles and the pulmonary vessels and aorta. These outflow tract valves are commonly referred to as the semilunar pulmonic and aortic respectively (Figure 4).

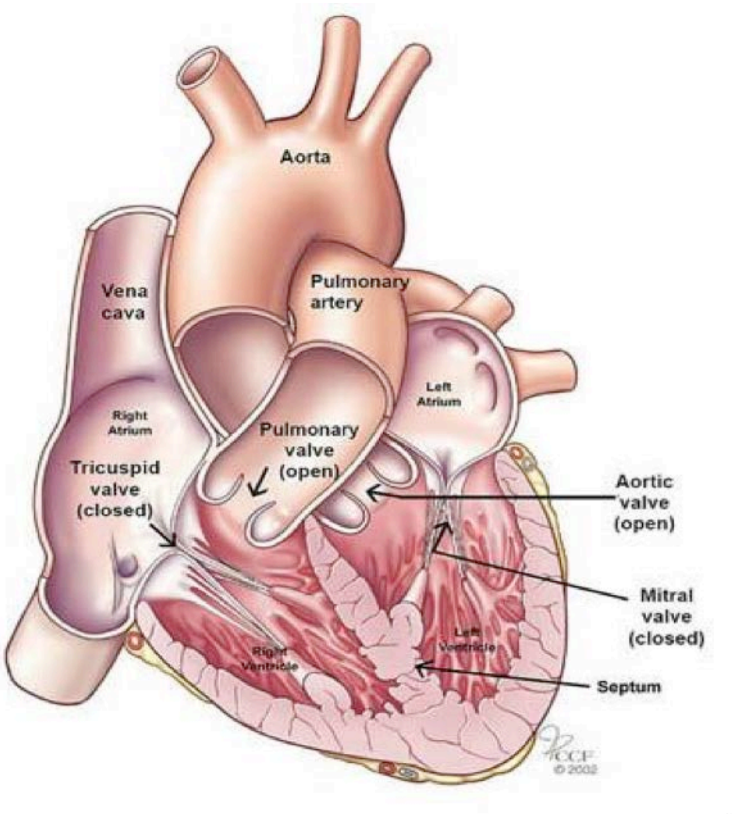

Figure 4: An illustration of the valves in an adult heart.

This illustration shows an adult heart section to make the atria, ventricles, and valves visible. In this image, the atrioventricular valves are referred to as the tricuspid and mitral valves (http://www.cardiachealth.org)

Both sets of valves contain cusps that work to form a seal to prevent the back flow of blood during each contraction of the heart. The valves are designed to withstand large amounts of pressure to regulate blood flow; therefore, malformations in design

lead to severe consequences. Hoffman and colleagues determined that defects in the valves make up 20-30% of congenital defects seen in the cardiovascular system, with many of the valve defects linked to other syndromes including Down syndrome (Hoffman & Kaplan, 2002). When a heart valve is not properly functioning, blood can regurgitate back into the previously occupied chamber instead of moving forward to be oxygenated or distributed throughout the rest of the body. By gaining a complete understanding of the contribution of endothelial-lineage cells to developing valves, we can identify when embryonic heart malformations first originate. The identification of which signaling molecules are present during the time of valve formation and remodeling can lead to the knowledge of which genes, when mutated, cause congenital heart defects. This knowledge supports the development of therapeutics to prevent defective valves from progressing to a state of disease that requires a valve replacement surgery.

Heart Valve Development

Heart valve development is a complex, multi-step process. A thorough understanding of the cellular and molecular mechanisms driving these steps is critical for identifying the causes of congenital valve defects. During development, endocardial cushions form at specific locations in the early heart tube at E9.5 (Figure 5). These cushions form before endocardial cells undergo delamination and differentiation in a process known as epithelial-mesenchymal transformation (EMT). In mice, EMT takes place between embryonic day 9.5 and embryonic day 10.5, and it is a vital process to understand since the heart valves are derived from the cardiac cushions. As

delamination occurs, cells invade the cardiac jelly causing the cushions to swell with a population of mesenchymal cells.

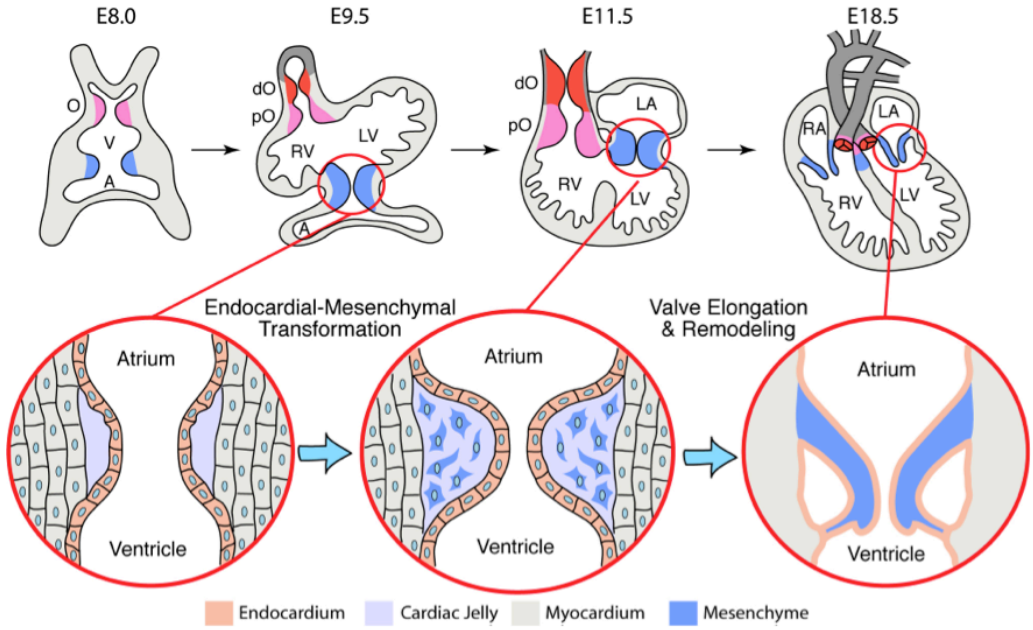

Figure 5: The development of atrioventricular valves.

This schematic depicts the developmental timeline of the atrioventricular valves starting at embryonic day 8.0 in mice and ending after the elongation and remodeling of the valve leaflets (Stankunas).

While the atrioventricular valve mesenchyme is derived from EMT alone, the mesenchyme of the outflow tract valves found at the ventriculo-arterial junctions originates from EMT and additional sources. In order to correctly form the aortic and pulmonic valves, neural crest cells that originate in the neural crest migrate to populate the outflow tract of the developing heart. The cardiac cushions are then able to remodel extensively from E11.5 through postnatal stages to form the thin, leaflet semilunar valves of the adult heart.

The Role and Regulation of VEGF in Valve Development

In order for valves to develop properly, many genes must coordinate temporally

and specifically throughout embryogenesis to send the correct developmental signals. It has been previously determined through explant and knockout studies that the signals originate in the myocardium, the endocardium uniquely responds to the differentiation signals in a temporal manner, and mutations in genes can cause malformed phenotypes in model organisms (Bernanke & Markwald, 1982; Runyan & Markwald, 1983; Schroeder, Jackson, Lee, & Camenisch, 2003). In regards to VEGF, it has been determined that while VEGFB does not play a large role in EMT (Carmeliet et al., 2001), the interactions between VEGFA and the receptors VEGFR1 and VEGFR2 are critical to valve development. VEGFA has been found to be present in the myocardium, resulting in the activation of expression of VEGF receptors in the endocardium (Dor et al., 2001). In addition, a VEGFR2 knockout study caused lethality as earlier as E8.5 in mice (Shalaby et al., 1995). In both studies, expression of VEGF ligands and receptors occurred immediately prior to the onset of EMT, indicating that VEGF is an EMT inhibitor in the AVC.

VEGF expression can be induced a variety of ways including growth factors, mutagens, Nitric Oxide, and tumor promoters[1]. Additional factors that have the ability to induce the expression of VEGF throughout the body and potentially impact the EMT process are hypoxia and glucose levels. Experiments on VEGFA were performed to determine the impact of hypoxia on the stimulation of vessel development. Initially, researchers determined that hypoxic conditions create an increase in the binding of HIF-1 to DNA, which leads to increased transcription of the VEGF gene (Jelkmann, 2001). A further study resulted in the observation that VEGFA was the ligand stimulated by

[1] Nitric oxide signaling is less likely to stimulate VEGF expression in heart valve progenitor cells. In zebrafish studies, induced NO signaling results in malformations of the nascent atrioventricular valves prior to EMT (Li, Jia, & Zhao, 2014).

hypoxia-inducible factor (HIF) (Germain, Monnot, Muller, & Eichmann, 2010). This research illuminates how hypoxia in the extracellular matrix during development may be leading to the formation of new vasculature and structures of the heart. A brief, developmentally programmed period of hypoxia during cardiac development could signal the need for growth, leading to increased transcription and production of VEGF mRNA. Further support for this hypothesis comes from a study where reduced oxygen levels *in vitro* have been shown to induce EMT in AVC explants (Dor, Klewer, McDonald, Keshet, & Camenisch, 2003). The tissue specificity of VEGF expression prior to EMT could be caused by interleukin-1, which is activated by the increased binding of HIF-1 to DNA as a result of hypoxia (Jelkmann, 2001). While this specific interleukin has been shown to be present during developmental processes as well as in diseased states of the adult heart, it has not been studied in the developing heart and could be an area of future study.

Although hypoxia is possible during development, it has been shown that severe or prolonged hypoxia can cause congenital heart defects as a result of inhibited EMT (Dor et al., 2001; Lueder, Kim, Buroker, Bangalore, & Ogata, 1995). Additional studies in high altitude show that hypoxia can result in a variety of congenital valve defects due to severe increases of VEGF expression that subsequently prevent EMT (Miao, Zuberbuhler, & Zuberbuhler, 1988). The severe consequences of high concentrations of VEGF suggest there are other signals that are tightly regulating the expression of VEGF, specifically during EMT at E9.5-10.5. Additionally, if hypoxia is a signal for activating transcription of VEGF during gestation, then normal levels of oxygen must be restored soon after the initiation of expression to prevent overexpression. The

induced hypoxia may be kept spatiotemporally under control by the gene *Cited2*, which acts as a negative feedback inhibitor of HIF-1 in hypoxic conditions. *Cited2*, when deleted has been linked to many congenital heart defects similar to those caused by extreme hypoxia (Xu et al., 2014).

Meanwhile, increased levels of glucose at E9.5 decreases EMT in the atrioventricular valves due to decreased levels of VEGFA in the myocardium (Enciso et al., 2003). While high levels of VEGFA in the endocardium prevent EMT, this study indicates that absence of the ligand in the myocardium also causes inhibition of the formation of the endocardial cushions. The effect of under-expressing VEGF further indicates the need for strict temporal and spatial regulation of this signaling molecule in order for proper development of the heart valves to occur. Reactive oxygen species are additional metabolic regulators that have been shown to stimulate the VEGF family. While studies showed that the presence of reactive oxygen species occurred as a result of tumors or tissue degradation, without evidence suggesting their presence during developmental processes (Fay et al., 2006; Ushio-Fukai & Nakamura, 2008), as a result of maternal diabetes, ROS are increased and glutathione levels are decreased in the developing fetus. This condition has been shown to ultimately lead to increased transcription of VEGFA in fetal hearts (Moazzen et al., 2014), which resultantly inhibits EMT. In addition, these studies illuminate the need for regulated maternal glucose levels during gestation that could be influenced by diabetes.

Further studies show, that at this time, the process of VEGFA binding to VEGFR2 in the endocardium regulates the expression of the Notch ligands and receptors required to activate the Notch signaling pathway. NOTCH signaling initiates

EMT and serves to temporally control the expression of VEGF through a downstream target gene known as *Hey2* (Niessen & Karsan, 2008). These studies show that although VEGF is required early on in valve development, it is ultimately an inhibitor of EMT. In addition to *Hey2*, NFAT signaling, which is expressed in the myocardium at the initiation of EMT may be redundantly working to repress the transcription of VEGFA to ensure EMT occurs (C. P. Chang et al., 2004). Regulation of VEGFA by these mechanisms would allow the signaling pathway to be restricted to pockets of endocardial cells (Miquerol, Gertsenstein, Harpal, Rossant, & Nagy, 1999). The evidence also conincides with studies showing immediate downregulation of the signaling pathway in transforming endothelial cells (Dor et al., 2001). These cells that continue to maintain the VEGF signaling pathway may be the activators of downstream siginaling intermediates such as PDK1. PDK1 is located in endocardial cells and promotes EMT in the AVC by activating Akt and Snail. This process leads to cell survival (Feng et al., 2010) despite repression of the VEGF signaling pathway during EMT.

Although VEGF signaling is inhibited during EMT by the Notch signaling pathway, VEGFR1 expression is found within the cushion endocardium. This receptor helps facilitate the process of EMT by sequestering VEGF proteins that would otherwise bind to VEGFR2 and inhibit the transformative process (Koch & Claesson-Welsh, 2012; Stankunas et al., 2010). Without this regulation, EMT would not occur correctly in the cardiac cushions, further leading to the improper formation of the atrioventricular valves. Alternatively, the VEGF signaling pathway is shown to play an important role in the formation of the outflow tract at E9.5. The inhibition of VEGFA

during this time causes cellular arrest of the population undergoing EMT. Stankunas and colleagues labeled this transitional stage as endomesenchymal since the cells were found to be display characteristics of both endocardial and mesenchymal states. This unique role of VEGF in the the OFT could be necessary due to the presence of neural creset cells, which are not present in the AVC (Stankunas et al., 2010). In the outflow tract, Notch signaling has yet to be confirmed; however, it is likely present and working in a similar manner as in the AVC. It is a critical observation that the two valves sets may require slightly different regulatory signals or roles of VEGF, providing further evidence for the importance of VEGF spatiotemporal regulation.

While VEGF plays a significant role in valve development, many of the downstream targets of this pathway are still unknown. To date, the transcription factor NFAT has received the most attention for being a likely downstream signaling pathway. NFATc1 signaling has been observed in the development of valve endothelial tissue as well as in postnatal valve cells for rebuilding endothelial cells, potentially as a result of VEGF expression (Armstrong & Bischoff, 2004). In a series of studies, Combs and Yutzey found that VEGF treatment of adult human pulmonary valve endothelial cells (HPVECs) increased the propagation of cells within the valves that are known to express nuclear NFATc1 (Combs & Yutzey, 2009a, 2009b). Knockout studies of NFATc1 resulted in lethality by E13.5 in mice due to defects in the development of the cardiac cushions (de la Pompa et al., 1998; Ranger et al., 1998). This knowledge combined with the results showing that NFATc1 is required at E11.5 (C. P. Chang et al., 2004), provides a lot of evidence that the presence of VEGF creates the translocation of NFATc1 required for successful valve elongation. These findings have

led to the model of NFATc1 acting as the downstream transcriptional effector of VEGF that causes the determination of the fate of cells that make up the cardiac cushions (Figure 6). However, *in vivo* experiments indicate that VEGF is not an upstream regulator of NFATc1 (Stankunas et al., 2010) and VEGFRi experiments confirm these findings, leaving the downstream targets of VEGF in valve elongation unknown.

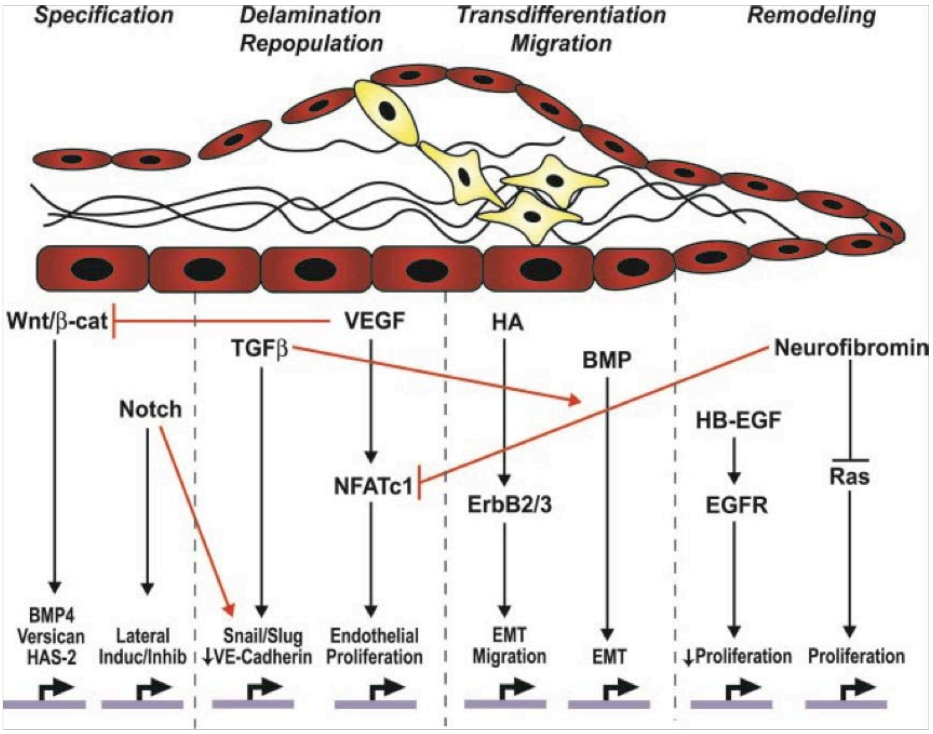

Figure 6: 2004 potential model for heart valve development.

This model illustrates a current depiction of how heart valve development occurs (Armstrong & Bischoff, 2004).

Following completion of EMT, the valve cushions must undergo remodeling in order to form the functioning valves as seen in a healthy adult heart. VEGFR2 is shown to be present in the endocardium of developing heart valves at E10.5-12.5, leading to the inhibition of EMT and ending cell proliferation. Expression of VEGF signaling at this time may be directing the transformations seen during elongation. Research

confirms the importance of the inhibition of negative regulators of VEGF by experimenting with the deletion of microRNA 126 (Stankunas et al., 2010). The absence of miR-126 at this time causes malformations in the atrioventricular valves (Stankunas et al., 2010). This data suggests the role VEGF at this time point is to direct morphological changes to produce valve leaflets after EMT (Stankunas et al., 2010). In addition, VEGF signaling immediately following EMT suggests a spatiotemporal role in the cushion endocardium to prevent further transition of endocardial cells by reinforcing the endothelial phenotype as a result of increased VEGFA concentration (Dor et al., 2001). As the valves continue to undergo remodeling, VEGF overexpression studies show lethal effects at E12.5-14.5 in mice. The increased amount of VEGF molecules resulted in delayed septation of the outflow tract as well as increased cell proliferation into the cardiac cushions (Miquerol et al., 2000). This indicates that VEGF expression is carefully regulated by another signaling pathway by E12.5. The presence of some VEGFA could potentially allow for the increase in number of endocardial cells as the heart continues to develop.

Chapter 4: Trabeculae of the Heart

In addition to the formation of heart valves, it is critical to understand the process of trabeculation within the ventricles of the heart to fully understand the role of VEGF in heart development. Trabeculae are the muscular strands that work to create contractions of the heart to pump blood through the chambers and the rest of the body (Figure 7). Trabeculation, although consistent in mammal organisms, creates an individually unique multilayer spiral system of muscular fibers that contain the necessary structures for ventricular contraction (Sedmera & McQuinn, 2008). Although the trabeculae are not identical, proper cell signaling is necessary for correct trabeculation to occur. If the steps required for trabeculation do not occur, severe heart problems can be seen in adulthood due to improper or inadequate contraction (Sedmera & McQuinn, 2008). Furthermore, effects related to malformed trabeculae have been associated with congenital clinical conditions including malseptation, defective papillary muscle formation, and conduction system defects (Grego-Bessa et al., 2007).

These congenital defects can create severe consequences in postnatal adult life. For instance, if the muscles of the heart do not form properly, the organ must work overtime in order to deliver the necessary amount of blood throughout the body. When the ventricles are forced to work overtime, the muscle thickens which decreases the amount of available space in the ventricular chamber. This process then becomes a cycle since the heart must compensate for the decreased blood volume, eventually leading to a diseased heart in need for transplantation. This diseased state is caused when the compacting step of trabeculation does not occur. In humans, this congenital defect in the myocardium is known as non-compaction cardiomyopathy.

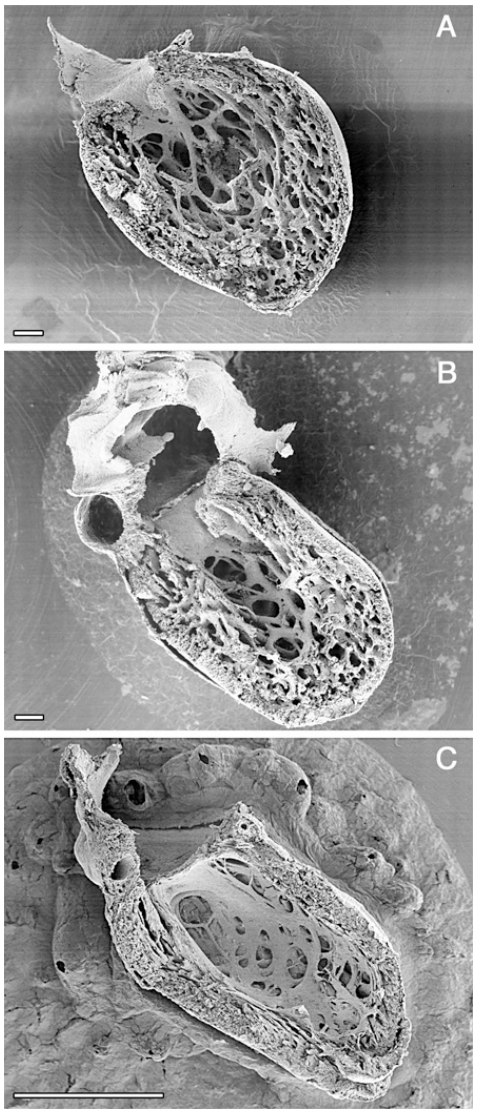

Figure 7: Trabeculation in the human left ventricle.

This series of images gives a clear visual of the dynamic process of trabeculation during development in humans from weeks 6-12 in gestation (Sedmera & McQuinn, 2008).

The Process of Trabeculation in the Heart

The formation of trabeculae is dynamic during development, beginning shortly prior to E9.5 in the murine heart (Figure 8). The formation and compaction of the trabeculae is essential for ventricular chamber morphogenesis to then occur. Similar to the formation of heart valves, the formation of trabeculae into muscular layers of cardiomyocytes relies on cell to cell signaling between the endocardium and

myocardium. Clonal clusters of myocardial cells grow inwards creating invaginations of the endocardial cells. The cells in the trabeculaes proliferate into the lumen from the myocardium at E10.0 in mice and contain gap junctions that will later be used for contractile communication (Sedmera & McQuinn, 2008). Once the trabeculae are formed, remodeling and compaction from E12.5-18.5 occurs in order to create the specific structures including the papillary muscles, interventricular septum, and conduction system occurs (Grego-Bessa et al., 2007).

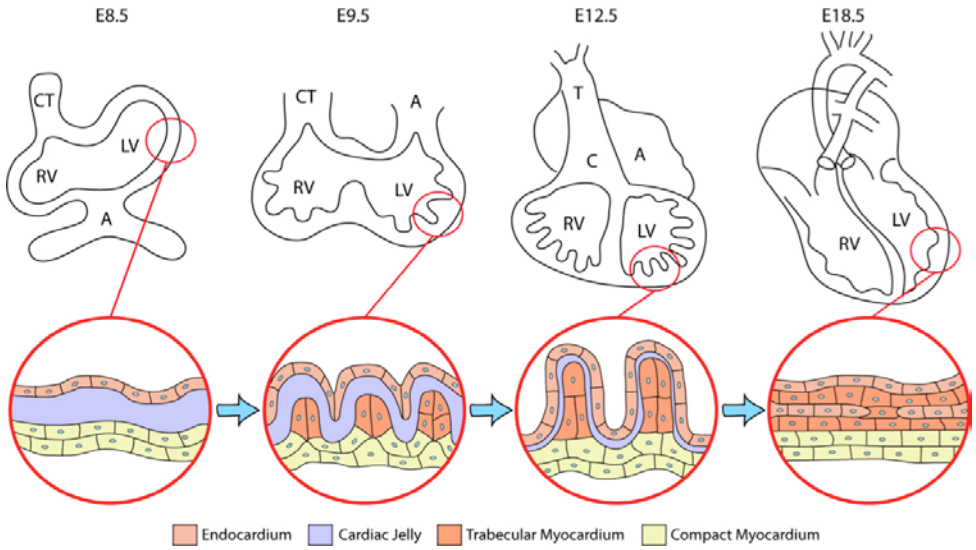

Figure 8: The formation of trabeculae in the ventricles.

This schematic illustrates the dynamic process of trabeculation during development in a mouse model (Stankunas, 2014 unpublished).

During compaction between E13.0 and E14.0, the myocardium thickens as the trabeculae flatten against the compact myocardium. The left ventricular wall is thicker than the right due to its need to pump blood through the aortic semilunar valve to rest of the organism. If the process of compaction does not occur, the heart will not have the necessary strength to be an efficient pumping mechanism.

In order for trabecular compaction to occur, the tissue must be vascularized by the coronary vessels (Sedmera & McQuinn, 2008). These vessels provide circulation and nutrients necessary for the muscles in the heart to function properly in order to distribute blood throughout the body. These vessels form from endocardial cells located in the ventricles, and are regulated by VEGFA in collaboration with VEGFR2 found within the endocardium (Zhang and Zhou, 2013). Defects in development of the coronary vessels can lead to the improper formation of the heart muscles due to lack of circulation as the ventricles thicken (Tomanek, 2005). As a result, one must consider the interconnected role of these two heart structures during developmental studies.

The Role and Regulation of VEGF in Trabeculation

Fewer studies have looked into the role of VEGF in the process of the trabeculae formation. It has been shown that altered temporal expression of VEGF results in defects including impaired ventricular trabeculae formation (Dor et al., 2001). From this research, it is clear that VEGF signaling between the endocardium and myocardium is critical for successful ventricular development. However, the spatiotemporal roles and how VEGF signaling is controlled in this developmental phase remain largely unknown.

To initiate trabeculation, it appears that VEGF expression in the ventricles is induced by the same hypoxic signal discussed in valve formation. *Cited2* also appears to be a key regulator of VEGF induced hypoxia in the development of the ventricular muscle and vasculature. Specific deletion of the gene in the developing heart resulted in ventricular wall thinning, which could be due to decreased trabeculae formation (Xu et al., 2014). *Cited2* has also been shown to play an important role in ventricular septation and coronary vasculature development, ultimately leading to proper development of the

cardiac muscle (MacDonald et al., 2013). As a result, VEGF expression is needed to properly form the endothelium, eventually leading to trabeculation.

The role of VEGF signaling in trabeculation is further illuminated by studies identifying Notch signaling as a director for trabeculation. These studies indicate that VEGF signaling through the binding of VEGFA to VEGFR2 inhibits trabeculation and that Notch signaling in the endocardium initiates the proliferation of cardiomyocytes in the trabeculae (Grego-Bessa et al., 2007; High & Epstein, 2008). The presence of Notch-1 in the endocardium of ventricles at E9.5 suggests the signal that activates Notch-1 is from the myocardium. This is the location where VEGFA is abundant prior to this point in time (Dor et al., 2001). As in valve formation, the presence of VEGFA subsequently causes changes in the activity of VEGF receptors in the endocardium, and in order for trabeculation to occur, Notch signaling must block VEGF signaling through VEGFR2.

Signaling to Delta receptors activates the Notch signaling pathway resulting in expression of Notch-1. This is further solidified by the presence of *Hey2* in the myocardium, which has been shown to inhibit VEGF signaling so proliferation can occur. When *Hey2* is inhibited, malformed ventricles and impaired contractile ability was seen suggesting a similar spatiotemporal regulation of VEGF as seen in valve development (Niessen & Karsan, 2008). These studies further confirm the presence of VEGF signaling prior to proliferation. The presence of NFAT signaling and VEGFR1 may once again be respectively working to regulate the expression of the VEGF proteins and to sequester VEGFA molecules.

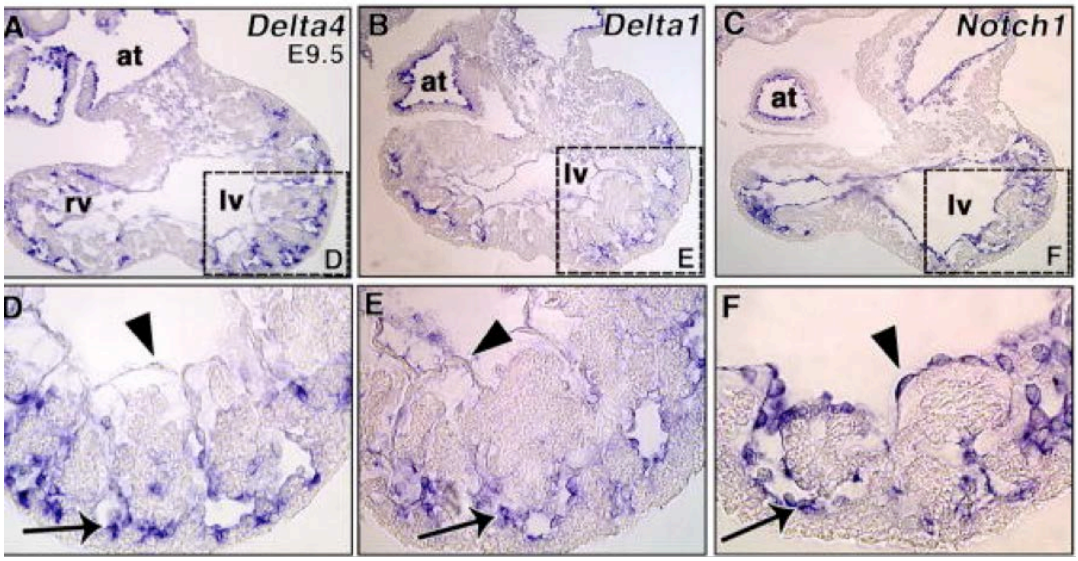

Figure 9: Expression of Notch1, Delta4 and Delta1 in the ventricles at E9.5 may be indicative to prior VEGF expression within the myocardium.

This series of images shows the presence of DLL4/1 and Notch-1 in the ventricles of a mouse heart at E9.5 (Grego-Bessa et al., 2007)

A few downstream signaling effectors of VEGF that are vital for ventricular development have been identified and include PDK1 and Akt1. These targets are expressed in the endocardium as previously reviewed in heart valve development (Feng et al., 2010). When deleted, abnormal cardiomyocyte proliferation occurs in the developing heart (Z. Chang et al., 2010). These defects could be the result of poor development of the endothelium. Additional downstream signaling effectors as well as target genes must be further classified to understand the complete role of VEGF in the process of trabeculation.

Finally, the expression of angiopoietin-1 (Ang-1) has been shown to regulate trabeculation through cardiomyocyte signaling to its endocardial receptor Tie2 at E9.5 in the ventricles. Knockout studies involving Ang-1 and Tie2 prevented trabecular formation as a result of an under-vascularized endothelium (Suri et al., 1996). Ang-1 is

known to stabilize endothelial monolayers while VEGF increases proliferation and permeability via VEGFR2 (Brindle, Saharinen, & Alitalo, 2006; Gamble et al., 2000; Satchell, Anderson, & Mathieson, 2004). As a result, VEGF may also be present during this time to complement Ang-1 expression, since they have been shown to have coordinated roles during growth, development, and disease.

Chapter 5: Proposed Models

Proposed Model for VEGF in Valve Development

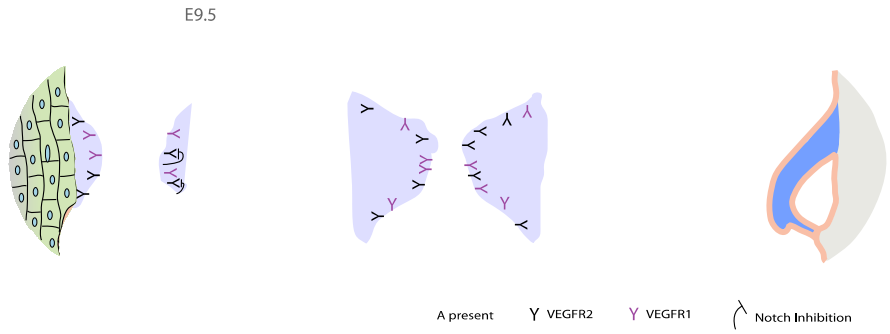

Figure 10: Spatiotemporal Roles and Regulation of VEGF in Valve Development

This flowchart aims to combine current research on VEGF in heart valve development by showing where and when VEGF is active during this phase in development (Modified Stankunas Figure).

This model shows the activation of VEGF signaling initiated by developmentally programmed hypoxia. VEGFA expression can be seen in the myocardium, and VEGF receptors are present in the endocardium. Binding between VEGA and VEGFR2 initiates the Notch signaling pathway by activating expression of Notch ligands. Since the binding of VEGFA to VEGFR2 inhibits EMT, the model shows Notch signaling in the endocardium inhibiting the pathway via inhibition of VEGFR2 expression. During EMT, VEGFA is transcriptionally controlled in the myocardium by NFAT signaling and remaining VEGFA ligands bind unproductively to VEGFR1. After EMT, microRNA-126 inhibits negative regulators of VEGF signaling, which allows for VEGFA to activate signaling through VEGFR2. This interaction prevents further EMT from occurring and directs the process of elongation and remodeling in heart valves.

Proposed Model for VEGF in Trabeculation

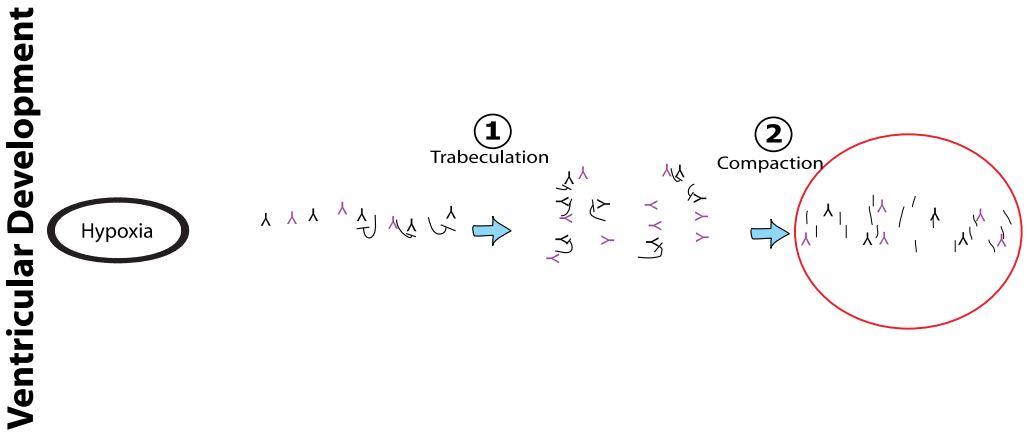

Figure 11: Spatiotemporal Role and Regulation of VEGF during Trabeculation

This flowchart aims to combine current research on VEGF during trabeculation by showing where and when VEGF is active during this phase in development (Modified Stankunas Figure).

This model shows the activation of VEGF signaling initiated by developmentally programmed hypoxia. This hypoxic moment is the same as the one activating VEGF signaling for valve formation. Binding between VEGA and VEGFR2 initiates the Notch signaling pathway. Since the binding of VEGFA to VEGFR2 inhibits trabeculation and the proliferation of cells, the model shows Notch signaling in the endocardium inhibiting the pathway via VEGFR2. During trabeculation, VEGFA is transcriptionally controlled in the myocardium by NFAT signaling and remaining VEGFA ligands bind to VEGFR1. Research at this time has not identified a role for VEGF signaling during compaction of the trabeculae.

Chapter 5: Future Directions

In order to test the hypotheses in the literature reviews and proposed models, a variety of experiments can be performed to further understand the spatiotemporal role of VEGF in heart development. Both plausible and idealistic experiments are included here to highlight the benefits of understanding of VEGF in heart development.

To expand the current knowledge on the role and regulation of VEGF in valve development, researchers can first confirm how VEGF signaling is initiated. In order to confirm the hypothesis that hypoxia is inducing VEGF signaling in the developing heart, oxygen levels would need to be measured in vivo prior to EMT and during EMT. To confirm this hypothesis, a decrease in oxygen levels would be seen immediately prior to when VEGFA is expressed in the myocardium. In addition, normalized oxygen levels should be restored shortly after this time point to prevent lethality.

Further studies should be done in valves to confirm the presence of the Notch signaling pathway in the OFT of developing valves. Although it is likely that the same inhibition process of VEGFR2 in the AVC is occurring in the OFT, there have not been studies directly confirming this hypothesis. Activators or inhibitors could be used to see if quantitative data matches the proposed model. In addition, the presence of Notch receptors in staining experiments would help solidify the process and explain why only VEGFR1 is seen in the OFT during EMT.

It is also important to remember that the different sections of the heart work together both directly and indirectly through signaling pathways. A crucial next step to further understand the spatiotemporal role of VEGF in valve development would be to identify downstream target genes and their role in furthering EMT, valve elongation and

remodeling, and potential roles in trabeculation as a result of proper endothelial formation. In the future, researchers could identify other molecular targets of VEGF signaling during embryonic angiogenesis using a combination of transcriptome profiling technology and chemical genetics to determine the direct target genes of VEGF signaling. RNA sequencing would provide further characterization of vessel development by identifying molecular targets of VEGF signaling. In order to gain these insights, TU-tagging could be employed to distinguish the temporal transcription of endothelial specific genes within *in vivo*. This innovative approach could identify the actively transcribed genes that regulate vascular endothelial growth factor (VEGF)-directed angiogenesis, which would help identify target genes and profile the effects of inhibited VEGF receptors on vessel formation and stabilization within the endocardial architecture. Integrating the knowledge gained from these various research pursuits could lead to interesting conclusions about how heart valves develop.

In initial experiments performed by members of the Stankunas lab, the small molecule inhibitor, Cabozantinib (Cabo), produced the phenotype of improperly formed vessel networks in yolk sacs. Cabozantinib inhibits VEGFR2 by blocking the receptor's ability to bind to other ligands. The preliminary data shows clear vasculature defects when the VEGFR2 receptor is inhibited. By examining the effects of VEGFR2 small molecule inhibitors, the angiogenic regulatory mechanism can be defined by observing the downstream effects on target genes. With further characterization of VEGFR inhibitors, there is potential for suppressed coronary vessel development as well as decreased formation of the endocardial matrix could be observed in the embryos treated with Cabozantinib. In addition to this technique, valve and trabeculae phenotypes could

be Cabo treated embryos could be compared to phenotypes of knock out studies of potential downstream genes to narrow down potential target genes. If similar malformations are seen in both experiments, the genes could be good candidates to screen for during RNA sequencing.

Transcription factors have also been identified to play a coordinated role leading to gene expression. In a 2009 study, the ETS family of transcription factors was linked to angiogenesis through a variety of target genes and signaling pathways (Randi, Sperone, Dryden, & Birdsey, 2009). The study identified 19 members of the ETS family that have a role during development; however, only some of these factors were specific to VEGF (Dejana, Taddei, & Randi, 2007; Hollenhorst, Jones, & Graves, 2004). As a result, looking for occurrences of these specific Foxs and Ets transcription factors may provide clues to when VEGF is stimulated in the heart. Staining with antibodies to the transcription factors previously identified in this study during developmental time points including EMT, valve remodeling, or trabeculation could provide insight to their role in relation to VEGF.

Once target genes are identified, the endothelium of the developing heart could also be analyzed using a newly developed multicolor flow cytometry technique. This method allows for endothelial cells of the heart to be isolated and analyzed *ex vivo* (Pratumvinit, Reesukumal, Janebodin, Ieronimakis, & Reyes, 2013), which could result in novel information about VEGF and downstream genes once identified. For example, using this method, researchers could confirm and measure protein expression of VEGF and downstream target genes using western blots in different cell populations and at

different time points. These experiments would increase understanding of expression levels at specific time points during heart development.

On a different note, it is important to remember the connection between the proper development of the coronary arteries and trabeculation. For example, *Cited2* has a critical role in regulating hypoxia in order to form the coronary arteries and specific deletion studies of *Cited2* showed abnormal angiogenesis of the ventricular septal coronary vessels (Xu et al., 2014). While the deletion may be causing separate, direct effects on both the arteries and ventricles, perhaps the effect on trabeculation is a result of improper ventricle vascularization. This possibility could be studied by looking at the location of the coronary arteries throughout trabeculation.

Additionally, deletion of downstream VEGF effector Atk-1 results in reduced coronary vessels (Vandoorne et al., 2013), indicating the continued importance of VEGF signaling in coronary artery development. Further research into the spatiotemporal regulation of VEGF during coronary vessel development could lead to a better understanding of how the coronary vasculature helps the ventricles grow and gain contractile strength. Interesting conclusions could be made *ex vivo*, to determine which factors from the coronary arteries are essential to allow for trabeculation to continue. Adding key metabolic factors such as calcium as well as identifying the members of the VEGF family that exist on coronary vasculature could provide insight into how VEGF is regulating trabeculation from the epicardium. To further confirm the hypothesis that proper vessel formation leads to successful trabeculation, researchers can quantify the number of vessels and trabeculae. In addition, looking at the perfusion of vessels could provide insight to the role of oxygen during this developmental process.

Chapter 5: Concluding Remarks

While this thesis leaves many questions unanswered and presents more to be considered, it has contributed to the scientific community by compiling what is currently known about VEGF in heart valve development and trabeculation. The extensive review has resulted in new hypotheses that result in updated models to explain the role and regulation of VEGF in heart development. Through extensive, continued research the targets and modifiers of VEGF will be determined which will lead to novel therapies, prevention, and education efforts to combat the increasing prevalence of congenital heart defects in our population.

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