ABSTRACT

Fibroblast-Endothelial Cell
Interactions in the Heart

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Angiogenesis in the heart requires cell-cell communication between fibroblasts and endothelial cells (ECs). We have demonstrated that fibroblast-EC interactions involve the cell surface molecule N-cadherin. In tube formation assays, co-culturing ECs with fibroblasts and N-cadherin blocking antibodies decreased tube formation. Moreover, our studies demonstrated that cell-cell interactions between fibroblasts and ECs result in altered gene and protein expression, specifically with the pro-angiogenic factors IL-6, MCP-1 and VEGF. These changes in expression for IL-6 and MCP-1 require direct cell-cell interactions, while VEGF regulation is through indirect interactions. We have previously shown that fibroblasts and ECs can exchange intracellular material through tight gap junctions both *in vitro* and *in vivo*. We hypothesize that small microRNAs (miRNAs) can pass freely through these tight gap junctions. Our initial studies have focused on the pro-angiogenic miRNA, let-7f, which we have shown passes between fibroblasts and ECs and aids in vascular remodeling in the heart. We will continue to characterize the exchange of intracellular materials between cells and examine their roles in the vascular remodeling process following cardiac injury.

FIBROBLAST-ENDOTHELIAL CELL INTERACTIONS IN THE HEART

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CHAPTER ONE

Review Article for the *Journal of Molecular and Cellular Cardiology*

I wrote this article to be published in the *Journal of Molecular and Cellular Cardiology*, in the January 2014 edition. It serves as an introduction and a background to my scientific Senior Thesis, and describes the cell-cell communication that takes places between the different cells and extracellular matrix in the heart. My thesis will go into further detail regarding the cardiac endothelial cells and fibroblasts, specifically the communication that takes place between them.

Abstract

Recent studies have placed an increasing amount of emphasis on the cardiovascular system and understanding how the heart and its vasculature can be regenerated following pathological stress, such as hypertension and myocardial infarction. The remodeling process involves the permanent cellular constituents of the heart including myocytes, fibroblasts, endothelial cells, pericytes, smooth muscle cells and stem cells. It also includes transient cell populations, such as immune cells (*e.g.* lymphocytes, mast cells and macrophages) and circulating stem cells. Following injury, there are dramatic shifts in the various cardiac cell populations that can affect cell-cell interactions and cardiac function. Cardiac fibroblasts are a key component in normal heart function, as well as during the remodeling process through dynamic cell-cell interactions and production of extracellular matrix (ECM). Fibroblasts interact with various cardiac cells, through mechanical, chemical (autocrine and/or paracrine) and electrophysiological means to alter cellular processes and ultimately cardiac function. Better understanding these cellular interactions and their biological consequences should provide novel therapeutic targets for the treatment of heart disease. In this review we discuss the nature of these cell-cell and cell-ECM interactions and the importance of these interactions in maintaining normal heart function, as well as their role in the cardiac remodeling process.

Introduction

The heart is composed of two main classes of cells: pacemaker and non-pacemaker cells [1]. The former group includes the Sinoatrial Node (SAN), the Atrioventricular Node (AVN), the Bundle of His and the left and right bundle branches. These cells transmit rhythm throughout the heart, causing the different chambers to contract systematically. The SAN is a modified myocyte, whose cells contract relatively weakly, and their primary purpose is to stimulate a heartbeat by depolarizing and transmitting an electrical signal [2]. On the other hand, the non-pacemaker cells include myocytes, surrounded by a collagen network that contains fibroblasts, with the myocytes making up the largest cellular volume. In addition, non-pacemaker cells also include pericytes, endothelial cells (EC) and vascular smooth muscle cells (VSMC), which are confined to the vasculature [3]. There are also transient cell types present in the heart, such as macrophages, lymphocytes and mast cells (Fig. 1). This cellular and acellular organization allows for the fibroblasts to exert mechanical force on the myocytes, by contracting the collagen network that forms the extracellular matrix (ECM).

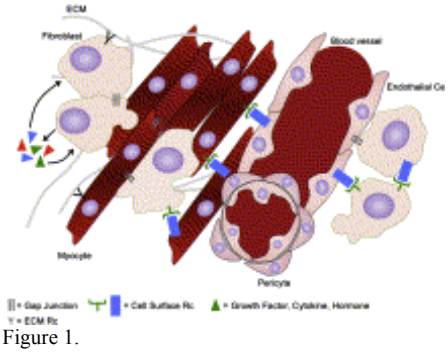


Figure 1.

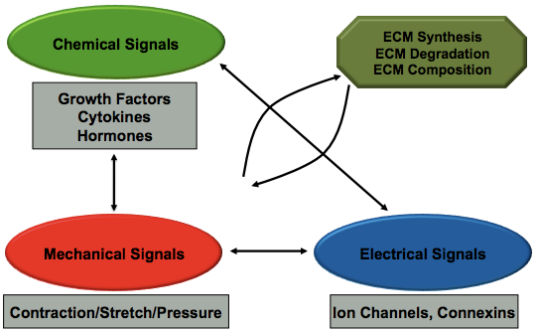


Figure 2.

Fibroblasts can also increase or decrease the rate of synthesis and degradation of the ECM. The development, maintenance and remodeling of the heart involves interactions between the different cell types, as well as interactions with the ECM. These dynamic interactions occur via chemical, electrical and mechanical signals that help to maintain cardiac structure and function (Fig. 2). Disruption of any of these signals alters the other signal types leading to changes in cardiac form and output.

Over the years, most research has tended to focus on the cardiac myocyte, but recently more attention has been drawn to the fibroblast and better understanding its role in cardiac function. While the fibroblast has long been regarded as a sentinel cell in the heart, sensing changes and waiting to act, recent data has demonstrated that the fibroblast performs a wide array of functions in both the normal and injured myocardium [4,5]. As mentioned above, it is through dynamic interactions between cells and the ECM in the heart that allow for normal cardiac form and function. These include chemical signals, such as growth factors and cytokines that can act in either an autocrine or paracrine fashion. Indeed, studies have demonstrated that interleukin-6 (IL-6), transforming growth factor-b (TGF-b) and connective tissue growth factor (CTGF) can all be secreted by fibroblasts in the heart and play a key role in cardiac function [6-8]. In addition, direct cell-cell interactions and electrophysiological interactions play important roles in cardiac physiology and pathophysiology. Gap junctions are important for cell-cell interactions between myocytes and fibroblasts through connexins (Cx40, Cx43 and Cx45) to function in electrical signaling in the heart [9-11]. It has also been shown that various cadherins, specifically N-cadherin and Cadherin-11, are important for myocyte-fibroblast and fibroblast-endothelial cell interactions [12]. In our lab, we have demonstrated that when N-cadherin blocking antibodies are added to a co-culture of ECs and fibroblasts, there is a significant increase in the number of unattached cells at specific time points [13]. Furthermore, the addition of these antibodies while performing a 3-D collagen tube formation assay decreased the tube formation. Since this protein mediates cell-cell adhesion and recognition, it can be understood that N-cadherin is necessary for the direct cell-cell communication that takes place between these cells. Our lab has also demonstrated that in co-cultures of ECs and fibroblasts, there is an increase in the amount of IL-6, a cytokine, in the media [13]. However, when N-cadherin blocking antibodies are present, there is a statistically significant decrease in IL-6 expression. This evidence demonstrates that direct cell-cell communication, mediated in part by N-cadherin, is necessary for the stimulation of this cytokine expression. Furthermore, when studying the expression of VEGF, there was no significant decrease in expression when N-cadherin antibodies were added to the cells. This result implies that VEGF expression is due to secreted factors, and not due to direct cell-cell communication [13]. Better

understanding of these dynamic interactions may provide insight into potential therapeutic targets for the treatment of the failing heart. In this review, we will discuss the communication that takes place between fibroblasts, the ECM and other cells in the heart.

Cardiac Fibroblasts

What is a fibroblast? Fibroblasts are generally defined as cells of mesenchymal origin that arise from the proepicardial organ and produce ECM [14-15]. Additionally, fibroblasts arise via bone marrow-derived cells, known as fibrocytes, in the neonatal and adult heart [16]. Fibroblasts can be identified by their flat, spindle-shaped morphology with multiple filopodia originating from the main cell body. One unique characteristic of the fibroblast is that they lack a basement membrane, and this separates it from the other permanent cell types of the heart which all contain such a membrane. Other features of the fibroblast are that it displays an extensive Golgi apparatus, as well as a relatively large endoplasmic reticulum. The obvious enlargement of these features emphasize the fibroblast's role in synthesizing and secreting protein destined for roles outside of the cell itself, such as the deposition of ECM.

One of the factors complicating the identification of fibroblasts *in vivo* has been the lack of a cell-specific marker. One marker that was thought to be a fibroblast-specific marker was fibroblast-specific protein-1 (FSP-1); however, FSP-1 is also expressed on other cell types, so its use as a fibroblast marker is debatable [17-18]. Several years back, the Goldsmith lab demonstrated that the discoidin domain receptor 2 (DDR2) is specifically expressed on fibroblasts [19]. DDR1 and DDR2 represent a newly discovered family of collagen-specific receptor tyrosine-kinases that aid in the conversion of extracellular signals to intercellular responses. Additionally, several studies have demonstrated that cadherin-11 is also localized to fibroblasts [20-21,6]. In addition to these potential fibroblast-specific markers, there are other proteins that are expressed by cardiac fibroblasts and may be used in identifying fibroblasts including vimentin and the matricellular protein belonging to the fasciclin family, periostin [22-26].

Fibroblasts play a variety of different roles in cardiac development, homeostasis and remodeling. Numerous studies have demonstrated that there is phenotypic

heterogeneity among fibroblasts, and that during times of pathological stress, the resting fibroblast can obtain an active and contractile phenotype, expressing characteristics of smooth muscle cells [27]. These traits have come to describe what is known as the myofibroblast. These cells are more mobile than the normal fibroblast, they are able to contract collagen, and are hypothesized to aid in wound closure and in maintaining the structural integrity of healing scars [27]. Fibroblasts can also be derived from cells that originate in the bone marrow, or from epithelial cells, via a process known as epithelial-mesenchymal transformation (EMT) [16]. Myofibroblasts are expressed especially when there has been tissue damage, as they participate in fibrosis and organogenesis [28]. It has been shown that myofibroblasts play an important role in reparative fibrosis in the heart following myocardial infarction [29]. Moreover, the differentiation of the fibroblast into a myofibroblast is encouraged by the secretion of growth factors such as TGF-b and cytokines such as IL-6 [16]. This chemical signaling is necessary for proper function of the heart, especially in response to pathological stress; however, unregulated myofibroblasts result in destructive tissue remodeling and fibrosis. Clearly myofibroblasts play a critical role in cardiac pathology and understanding their regulation is important for the cardiac remodeling process.

Fibroblasts also participate in mechano-electrical signaling, which can account for changes in the contractility of the heart in response to an increased or decreased cardiac load [30]. Fibroblasts respond to the contractions of the myocardium by changing their membrane potential; this phenomenon is known as mechanically induced potential (MIP), and is a feature of the mechano-electrical feedback system of the heart. When mechanical compression is experienced, fibroblasts experience an increased permeability of their membranes and allow for an influx of cations, depolarizing the cell. However, post-myocardial infarction, fibroblasts become more sensitive to stretch, causing contractile arrhythmia when the heart rate becomes depressed. [31]. Fibroblasts also play an intimate role with many cells of the heart, in addition to the extracellular matrix, as will be shortly discussed.

Cardiac Myocytes

Myocytes are the muscle cells of the heart and have a striated appearance, due to their parallel actin and myosin filaments. They contain numerous mitochondria that provide the necessary energy to allow conduction and contraction of the cell. Darker regions can be seen on the outside of the cells where the cells are connected and communicate with one another [32-34]. Known as the intercalated discs, these regions can be further subdivided into three functional zones: the adherens junctions, the desmosomes and the gap junctions. The adherens junctions have been shown to link the intercalated discs to the actin cytoskeleton. From a mechanical standpoint, this allows for the transmission of a signal between cells, in order for the contraction of the A band to be passed from cell to cell throughout the heart [33]. Electrically, these cells depolarize in response to signals from the SA Node. Calcium (Ca^{2+}) is responsible for translation of the signal into the muscular contraction that expels blood from the atria and ventricles of the heart [35]. Fearnley and colleagues determined that within the lumen of the sarcoplasmic reticulum (SR), calsequestrin is the major Ca^{2+}-binding and storage protein. When this receptor is mutated, it can lead to a pathological state of the heart in which delayed afterdepolarization becomes prevalent throughout the myocytes [35]. Hence, Ca^{2+} plays a key role in the depolarization of the heart and electrical signaling, allowing for proper functioning of the myocardium.

Myocytes have also been shown to exhibit a phenomenon known as mechano-electric feedback, in which mechanical force influences the electric potential of the myocyte membrane. In particular, if the myocardium is stretched, then stretch-activated receptors become functional, allowing an increase in the penetration of sodium across the membrane, generating new action potentials that propagate and increase the rate of contraction of the heart [30]. Gap junctions also allow for the passing of ions and possibly other intracellular material between cells. Several proteins are responsible for the continuity of the ion channels, such as Cx40, Cx43 and Cx45 [36].

Like cardiac fibroblasts, myocytes can act via chemical signaling by secreting different growth factors and cytokines. For example, myocytes can secrete tumor necrosis factor-α (TNF-α) in order to aid in the immune response [37]. It has been demonstrated that myocytes increase TNF-α expression in response to trauma or sepsis [38]. However,

in a diseased state, TNF-α has a negative inotropic effect leading to cardiodepression. Disrupting the signaling pathway that leads to increased synthesis of TNF-α has been shown to correct myocardial contraction and relaxation deficits [38]. Myocytes also express and secrete vascular endothelial growth factor (VEGF) and may play an important role in the vascular remodeling process [39]. Additionally, nitric oxide (NO), a modulator of vasodilation, as well as myocyte contraction and heart rate is activated by endothelial nitric oxide synthase (eNOS), which is also expressed by myocytes and allows for NO involvement in myocyte-endothelial cell interactions [40,41]. However, as an effect of pathological hypertrophy, there is a decrease in NO signaling that interrupts myocyte-endothelial cell communication. Such interference alters the contractility of the myocardium, leading to altered contraction and the compromise of vascular integrity [42]. Myocytes can also interact with the ECM, endothelial cells and fibroblasts, in manners that will be discussed below.

Cellular Organization in the Heart

Early descriptions of the cellular organization in the heart described the myocytes arranged in sheets termed laminae [43]. The ECM, specifically collagen, connects the laminae and affects the mechanical and chemical properties [44]. Fibroblasts, which are the main non-myocyte cell type in the heart, are spread throughout the ECM that surrounds the myocytes. On the other hand, the endothelial cells and smooth muscle cells are confined to the vasculature. The endothelial cells form the inner layer of epithelial cells in blood vessels and express matrix metalloproteinases (MMPs) in response to environmental cues. These are the same MMPs that can activate the fibroblasts, causing them to secrete collagen, as will be discussed shortly [45]. Moreover, during development it has been demonstrated that the ECM's structure is responsible for the rod-shaped phenotype of the myocytes, which communicate with the ECM through receptors that belong to the integrin family [46,47]. Myocytes interact with the integrin binding domains of the ECM differently than fibroblasts, as the latter is more of a migratory cell and makes transient attachments. The myocyte, however, attaches perpendicularly to the ECM at specific sites that are near the Z line of the sarcomere [47]. This information is key in the hypothesis that the myocyte-ECM interaction is necessary for maintaining the

structural integrity of the heart. Studies have shown that inhibition of the myocyte-ECM junctions results in abnormally shaped hearts [48]. In addition to their cell-ECM contacts, fibroblasts show heterotypic cell-cell contacts with myocytes and homotypic contacts with each other and vice versa [4,19,46]. Some of these contacts involve connexins, such as fibroblasts association with myocytes via Cx43, and with other fibroblasts through Cx45 [6]. N-cadherin and cadherin-11 are also involved in these cell-cell interactions [13]. This dynamic organization of cells and ECM forms a network for mechanical, chemical, and electrical signaling.

The Extracellular Matrix

The ECM is composed of many different proteins, including hyaluronan, the most abundant glycosaminoglycan in the heart, fibronectin, fibrillin, periostin, and collagens [49]. Hyaluronan provides a hydrated environment in which cellular motility and proliferation can occur. Fibronectin is a multi-domain protein that interacts with proteoglycans and collagens to mediate cellular function. It has several different splice sites, and each of its exons, especially EIIIA and EIIIB, are essential to normal cardiac function and growth during development. Periostin is another key extracellular protein that is able to interact with other components of the ECM, as well as with fibroblasts, playing a role in their differentiation [49]. Additionally, collagen is essential for maintaining the elasticity and integrity of the heart. There are over 28 different types of collagen, many of which are expressed in the developing valves after birth, and even in the developing adult heart [49].

Extensive research has shown that fibroblasts aid in the deposition and degradation of the ECM. This is achieved via the use of matrix metalloproteinases (MMPs), which are proteolytic enzymes that, when cleaved into active forms, degrade ECM proteins [50]. When tissue is injured under pathological conditions, there is an increased expression of MMPs, and hence increased degradation of the ECM. This promotes wound-healing and scar formation. However, excessive degradation can often occur, and is prohibited by endogenous tissue inhibitors of MMPs (TIMPs); there is a delicate balance between MMPs and TIMPs, which can help achieve angiogenesis and ideal vascular remodeling. Interestingly, certain cytokines, such as IL-6, are able to aid

in the *de novo* synthesis of TIMPs; this cytokine can also stimulate the production of growth factors like TGF- β1 and VEGF, which have angiogenic roles [51,52]. Furthermore, within the ECM, fibroblasts are able to differentiate into myofibroblasts in the diseased state [53]. Since myofibroblasts express α-smooth muscle actin, they are responsible for the contraction of filaments, exerting mechanical tension that permits wound closure. Since the fibroblasts lie within the ECM, they are able to contract the collagen, which exerts a mechanical force on the myocytes, narrowing any gaps or wound openings between the cells [16]. This mechanical function of the fibroblasts and the ECM allows for proper healing of the diseased heart. However, the enhanced activity of fibroblasts in the pathological state initially aids in healing and scar formation, but it can also eventually lead to fibrosis, or the thickening and scarring of connective tissue. This results in the reduction of capillary density, and a reduced diffusion of oxygen across the tissues [54]. Regardless, this delicate balance between aid and harm is simultaneous with changes in the ECM.

Cardiac Fibroblast-Myocyte Interactions

As a result of the specific arrangement of the myocardium, fibroblasts are able to exert mechanical force on myocytes. During the formation of the ECM, the integrins are responsible for making myocyte to collagen connections [16]. The cardiac fibroblasts themselves form netting around the myocytes, from within the ECM. Hence, they are able to contract the collagen, and thus exert a mechanical force upon the myocytes. Additionally, through Cx43 and Cx45 connections, fibroblasts and myocytes are able to communicate with one another electrically. In fact, studies from Louault and colleagues showed that fibroblast coupling can occur, suggesting that fibroblasts may form bridges that allow myocytes in different locations to communicate with one another [30]. As Kohl et al noted, myocytes that are interconnected solely by fibroblasts still experience the conduction of rhythm, demonstrating that fibroblast-based excitation is possible [56]. This hypothesis is significant in that the studying of Cx43 and Cx45 could allow for the enhancement of communication between these cells during a state of disease [56].

Both fibroblasts and myocytes have K^+ channels, which are activated by interactions between these cells. They express several different voltage-gated K^+

channels, which have also been linked to the Angiotensin II (Ang II) pathway [57]. Upregulation of Ang II has effectively increased secretion of cytokines and growth factors of myocytes, including TGF-β1 and endothelin-1 (ET-1) [58]. TGF- β1 acts as an inducer of transcription, and alters the transcription and translation of collagen, fibronectin, and other genes associated with the ECM [59]. ET-1 is a peptide growth factor known to stimulate cardiomyocyte hypertrophy [60]. The same Ang II receptors are also present on fibroblasts, as Ang II stimulates fibroblast proliferation, collagen and ECM synthesis and expression of fibroblast growth factor 2 (FGF2) [61]. This factor acts in a paracrine manner to induce myocyte hypertrophy, and it can also act as an autocrine signal, stimulating the release of pro-hypertrophic factors, in addition to eliciting cardioprotective effects against postischemic cardiac dysfunction [58]. Fibroblasts also secrete IL-33, an interleukin, which is responsible for acting on the myocytes and inhibiting the effects of pro-hypertrophic factors, in addition to fibrosis caused by pressure overload [58]. Platelet-derived growth factors (PDGFs) as well play a large role in cardiac fibrosis and angiogenesis, through their binding to protein tyrosine kinase receptors. They mainly control fibroblast proliferation and migration, as well as ECM deposition. In the diseased heart, PDGF expression can be greatly increased, leading to dilated cardiomyopathy and heart failure in transgenic mice [62]. Finally, insulin-like growth factor-1 (IGF-1) also exhibits a cardioprotective function in response to stress; its secretion by fibroblasts plays a necessary role in mediating the myocardial adaptive response to pressure overload [63]. Hence, there are many chemical signals between these cells that can affect proper cardiac function, as well as repair.

Fibroblasts and myocytes can also communicate chemically through tight cell-cell junctions. This was previously shown in our laboratory by demonstrating that myocytes and fibroblasts were able to exchange fluorescent dye between tight cell junctions in cell aggregation assays [13].

Within the past few years, the study of microRNAs (miRNAs) has begun to surface, and it has been found that they play a significant role in cell-cell communication. These small base pair sequences are usually 18-24 base pairs long, and they are noncoding RNAs that regulate mRNA translation or degradation [65]. By binding to these mRNAs that have been transcribed in the nucleus, miRNAs can block them from

being translated into functional protein (Fig. 3). miRNAs are synthesized and secreted predominantly from myocytes and fibroblasts, and their normal local concentrations in the heart provide clues to gene expression and function in the healthy or the diseased heart [66]. If each miRNA's role is better understood, then regulation of their function could be used as therapy and cardiac protection. For example, miR-21 within fibroblasts is upregulated in response to stress stimuli; when this miRNA is inhibited, fibrosis and hypertrophy induced from pressure overload are significantly decreased [66]. Especially within ECs, it has been shown that miRNAs are responsible to some extent for angiogenesis. miRNA-92a, for example, is highly expressed in tumor cells, and is upregulated by ischemia. As a negative regulator of angiogenesis, its inhibition can lead to the stimulation of blood vessel growth [65]. In the diseased heart, the addition of miRNAs to the cardiac cells could be used as a therapy to stimulate the formation of new and healthy vessels.

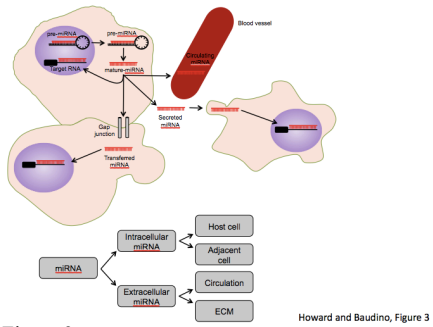


Figure 3.

Cardiac Fibroblast-Endothelial Cell Interactions

Recently studies have shown that the endothelial cell (EC) as well is involved in the regulation of the formation, function, and remodeling of the vasculature [67]. It has been known that ECs evoke vasomotor responses in smooth muscle cells via the release of paracrine factors [68]. They release substances such a NO and prostaglandins, which promote K^+ efflux. The presence of myoendothelial gap junctions allows such an electrical impulse to be transmitted from cell to cell. Also significant is the synergistic effect that ECs and fibroblasts have on changes in ECM deposition, cytokine and growth factor secretion, and gene expression [69]. In our lab, we observed that fibroblasts enhance tube formation when plated with ECs, and that this effect differs based on the tissue involved, whether it is from the heart of from the lung. In the heart, tube length and tube width were both enhanced by the presence of fibroblasts, while the results remained unaltered in the lung. When using transmission electron microscopy (TEM),

direct physical interactions between these two types of cells can be observed. The exact mode of transmission of signals is under further investigation. Furthermore, ECs and fibroblasts are able to communicate with one another, even where tight junctions do not exist, through the presence of ECM fibers that link the cells. There is possible mechanical influence here, as the fibroblasts can cause the ECM to exert a physical force on the ECs, communicating a signal in such a way [70]. The ECM can govern whether an EC exists in a state of growth, differentiation, or apoptosis.

It has also been suggested that fibroblasts interact with endothelial cells in order to stimulate angiogenesis [16]. In our lab, we seek to explore this area of research, in the hopes of finding therapeutic remedies to heart damage, ultimately desiring to stimulate angiogenesis.

Myocyte-Endothelial Cell Interactions

As previously discussed, there is a delicate balance between MMPs and TIMPs, which control the degradation and preservation of the ECM. When the expression of these two proteinases becomes unbalanced, the cell-cell communication and organization causes uncoupling between myocytes and endothelial cells. It has been demonstrated that from an early stage in development, the interactions between these cells is necessary for proper growth and development. In the heart, there is a capillary next to almost every myocyte, and ECs outnumber myocytes nearly 3:1 [71]. When there is either an overexpression or a deficit of vascular endothelial growth factor (VEGF), the result is cardiac dysfunction [72]. Studies have shown that this VEGF paracrine pathway must remain untroubled in order for myocytes to function properly. When mice were engineered with a myocyte-specific deletion of the VEGF gene, or with a cardiomyocyte-specific knockout for a secreted factor, the result was thinned ventricular walls, a decrease in contractile function, and lack of neural stimulation [73]. The method of transmission between myocytes and ECs is known to be via gap junction proteins, such as connexin 43 (Cx43), which links electromechanical processes in the myocardium with the vasculature [74]. However, it has also been shown that VEGF affects the expression of Cx43 in myocytes, suggesting a possible VEGF-dependent pathway as a mode of

communication between ECs and myocytes [72]. Further studies continue to enhance the understanding of such pathways and modes of signaling between these cell types.

Perspectives and Conclusions

Tissue interactions encompass many different components. Each cell type has its own gene expression, protein secretion, and response to extracellular signals. They also associate with one another in diverse ways, altering their behavior based on mechanical, chemical, and electrical stimuli. Much research is being performed that continues to investigate the cell receptors, the paracrine and autocrine signals, and the differences between the pathological and physiological states of the heart. By manipulating hearts and analyzing the differences in protein secretion and response to stimuli, we as the research community can gather more knowledge about how the cells interact with one another. Once our understanding is sufficient, we can gather data on how to stimulate angiogenesis and vessel growth in the diseased heart, hoping to develop new remedies that will more successfully address the issue of heart disease. What can be done for those patients whose vessels are unfit for angioplasty or bypass? It is this question that we are relentlessly seeking to answer, through the study of cell-cell interactions that take place in the myocardium.

Acknowledgements

This work was supported by American Heart Association.

CHAPTER TWO

Materials and Methods

Immunofluorescent Confocal Microscopy (Results Figure 1a/b)

1. Left ventricular sections of mouse heart cells were washed with PBS and fixed with fresh 2% paraformaldehyde in PBS, pH 7.4, for 4 h.
2. Sections were then washed with PBS containing 0.01 M glycine and 0.1% Triton X-100, and the endothelial cells were stained with phalloidin conjugated to Texas Red (T7471, Invitrogen, CA).
3. Fibroblasts were stained with antibodies against DDR2 (SC-7555, Santa Cruz, CA) In addition, antibody against collagen I (C2456, Sigma, St. Louis, MO) and antibodies against N-cadherin (SC-1502), OB cadherin (Cadherin-11, SC 6463), connexin 40 (Cx 40, SC-20466), connexin 45 (Cx 45, SC-7679), and connexin 43 (Cx 43, SC-9059) were purchased from Santa Cruz and used as previously described (Goldsmith et al., 2004; Bullard et al., 2005). The fibroblast nuclei were stained with DAPI. Sections were analyzed using a Zeiss white light laser confocal microscope. The fibroblasts and their nuclei were shown to associate with the vasculature of the heart.

Transmission Electron Microscopy (Results Figure 2a/b)

1. Wild-type adult murine hearts (12 weeks old) were isolated, cut into approximately 1–2 mm blocks, rinsed in PBS, and fixed in 4% buffered glutaraldehyde and treated with 2% aqueous osmium tetroxide for 1 h at room temperature.
2. Hearts were then rinsed, dehydrated, transferred to propylene oxide, embedded, and sectioned. Samples were examined on a JEOL 200CX TEM (JEOL, Tokyo, Japan) at 160 KV.

Cell–Cell Adhesion Assay (Results Figure 3a/b, 6a/b)

1. Adhesion between fibroblasts and endothelial cells was assayed by plating freshly isolated neonatal cardiac endothelial cells on aligned collagen as described below in the 3-D collagen tube formation assay.
2. After 48 h in culture, 5 X 10^5 neonatal cardiac fibroblasts were added to the endothelial cells. Individual dishes were plated for each of the time points, so that the cells would be subjected to minimal agitation. At various time intervals (4, 8, and 24 h), 50 µl of media were withdrawn and viable cells counted using erythrocin blue and a hemocytometer. Data were analyzed by plotting the number of unattached cells as a function of time.

3-D Collagen Tube Formation Assay (Results Figure 4a/b, 5a/b)

For this experiment, we wanted to see if communication between the fibroblasts and the endothelial cells enhanced tube formation, as compared to endothelial cells by themselves.

I. Endothelial Cells Alone
   a. We trypsinized cells and resuspended cells in 1× medium 199 (M199) for a concentration of 10^7 cells/ml. Cells were mixed with a pipette to prevent any clumps, and placed on ice until use.
   b. We prepared the gels: (when running many cultures, we prepared the gels minus Collagen I before collecting cells, and added Col I right before the addition of cells). It is important to keep all reagents on ice to prevent the collagen from polymerizing.
   c. For 1 mL of 2.68mg/ml collagen (high concentration), we combined: 525 µl 5 mg/ml type I collagen, 58.5 µl 10× M199, 3.15 µl 5 *N* NaOH, and 213 µl 1 × M199.
   d. The collagen gel solution was mixed thoroughly.
   e. We then added 200 µl of cold cell suspension to the cold collagen solution for a final concentration of 2×10^6 cells/ml. Gently pipette to mix the cells with the collagen solution without making any air bubbles (brief vortex, followed by time for it to settle from the sides of the tubes before pipetting). The cell-collagen mix was then added at 28 µl per well in 96 half-area well clear flat bottom TC-treated microplate (Corning). After every third to fourth well, we tapped the plate gently on each side to evenly spread out the cell-collagen mix within each well.
   f. The plate was placed in an incubator (37°C with 5% CO_2) for 30 min to allow collagen to polymerize and equilibrate.
   g. 100µL 10% FBS in DMEM was added to the plate, and incubated at 37°C with 5% CO_2 to allow the endothelial cells to undergo tube formation.

II. Endothelial Cells Plus Fibroblasts
   a. We repeated Steps I.a-g, using both endothelial cells and fibroblasts. 100µL of each cell type were added to the plate.

III. Staining
   a. In order to visualize tube formation, it was necessary to stain the collagen gels.
   b. We carefully placed the gel into a 48-well plate containing 500 ul of Wash Buffer (5%BSA in PBS).
   c. Gels were washed on a rocker for 10 min.
   d. Buffer was aspirated carefully, and blocking buffer (5%BSA + 10% serum in PBS) was added and incubated overnight at 4°C (on slow rocker).
   e. We aspirated and added primary antibody in 5%BSA/PBS. We then incubated overnight at 4°C on slow rocker.
   f. Gels were washed with the initial "quick washes" where buffer is immediately aspirated and new buffer put on, performing at least 4 changes of 30 min washes on rocker at RT.

g. Secondary antibody was added, and incubated 1.5h at room temp on a slow rocker.
h. It was washed well again, and PBS alone was used.
i. We then incubated with DAPI for 30 minutes, and finally washed with PBS and imaged.

Cell Aggregation Assay (Results Figure 8a/b)

1. 3 X 10^6 freshly isolated neonatal cardiac endothelial cells were cultured together with 3 X 10^6 neonatal fibroblasts in 10 ml of media in a 50-ml Erlenmeyer flask and subjected to rotational culture using an Innova 2000 platform shaker (New Brunswick, NJ) at 80 rpm in the incubator at 37°C, 5% CO_2 for 16 h.
2. Individual aggregates were isolated using a dissecting microscope and disrupted into single cell suspensions via rigorous pipetting and then counted using trypan blue and a hemocytometer. In addition, whole aggregates were fixed in fresh 2% paraformaldehyde for further immunohistochemical analyses. Further examination of cell–cell communication (via lucifer yellow dye transfer) and expression of connexins, cadherins, and DDR2, by immunofluorescence was performed as described.
3. For studies involving disruption of cell–cell contacts we used antibodies against cadherin-11 (SC-30314, Santa Cruz, CA), N-cadherin (SC-1502, Santa Cruz, CA), E-cadherin (SC-31020, Santa Cruz, CA), Cx 40 (SC-26658, Santa Cruz, CA), Cx 43 (SC-9059, Santa Cruz, CA), Cx 45 (SC-7680, Santa Cruz, CA), and antibodies raised against the cardiac fibroblast plasma membrane (1611). Cardiac fibroblasts were preincubated alone, with control IgG, with preimmune sera, or with the respective antibody for 30 min prior to coculture with endothelial cells. Cells were then cultured as described above.

Engineering of a Plasmid, which is to be used for the transfection of Mouse with Trans-Aortic Constriction, with induced pressure-overload hypertrophy of the myocardium. (Results Figure 9a/b, 10a/b)

I. Transformation of E. Coli with Let 7F-microRNA and pAAV-IRES-GFP.
   a. We have a stock of Let-7F 1 and Let-7F 2 plasmids, which we wish to transform into E. Coli in order to increase the amount of plasmid present.
   b. We took 2µl of each Let7F and added it to five separate *E. Coli* containing tubes. These were put on ice for 20 min, then placed at 42°C for 60 sec, then back on ice for 2 min, and then put in the bacteriological shaker at 37°C, at 250 rpm, for one h.
   c. The same protocol was performed with our pAAV-IRES-GFP plasmid.

II. Bacterial Replication
   a. 100 µl from each of the tubes was plated onto ampicillin containing LB agar petri dishes, and these were incubated at 37°C overnight.

b. There was growth on the plates the next morning, which means that in each *E. Coli* colony, the bacteria had taken up the Let 7F1, Let 7F2, or pAAV plasmid, and was able to express the ampicillin resistance gene.
c. We took a micropipet tip and picked a colony from each plate. These were then added to three separate culture tubes, each with 5 mL LB broth and 50 µg/mL ampicillin.
d. These were then put in a bacterial shaker at 37°C and shaken at 250 rpm for 8 h.
e. In order to further increase the plasmid yield, we poured each tube's contents into a separate Erlenmeyer flask and added 50 mL LB broth and 50 µg/mL ampicillin to each one. These were put in the shaker at 37°C, 250 rpm, overnight.

III. Isolation of the Plasmids
a. Plasmid isolation was accomplished using the QIAGEN Plasmid Plus Midi Kit.
b. DNA Isolation
1. Harvest bacterial culture by centrifuging at 6000 x g for 15 min at 4˚C. Aspirate supernatant.
2. Completely resuspend the pelleted bacteria in 2 mL Buffer P1 (after adding 2 µl LyseBlue reagent).
3. Add 2 mL Buffer P2, gently mixing by inverting until the lysate appears viscous and the cell suspension turns blue. Incubate at room temperature for 3 min.
4. Place the QIAfilter Cartridge into a new and suitable tube (50 mL conical tube).
5. Add 2 mL Buffer S3 to the lysate, and mix by inverting 4-6 times. Mix the solution until it is completely colorless.
6. Transfer the lysate to the QIAfilter Cartridge and incubate at room temperature for 10 min.
7. During incubation, place QIAGEN Plasmid Plus spin columns into the QIAvac 24 plus. Insert Tube Extenders into each column.
8. Gently insert the plunger into the QIAfilter Cartridge and filter the cell lysate into the tube.
9. Add 2 mL Buffer BB to the cleared lysate, and mix by inverting 4-6 times.
10. Transfer lysate to a QIAGEN Plasmid Plus spin column on the QIAvac24 Plus.
11. Apply the vacuum until the liquid has been drawn through all columns.
12. To wash the DNA, add 0.7 mL Buffer ETR and apply vacuum until the liquid has been drawn through all columns.
13. To further wash the DNA, add 0.7 mL Buffer PE and apply vacuum until the liquid has been drawn through all columns.
14. To completely remove the residual wash buffer, centrifuge the column at 10,000 x g for 1 min in a tabletop microcentrifuge.
15. Place the QIAGEN Plasmid Plus spin column into a clean 1.5 mL microcentrifuge tube. To elute the DNA, add 200 µl Buffer EB or water to the center of the QIAGEN Plasmid Plus spin column, let it stand for 1-2 min, and centrifuge for 1 min.
16. I then measured the concentrations of each of the types of eluted DNA:

a. Let-7F1: 709.0 ng/µl
b. Let-7F2: 659.8 ng/µl
c. pAAV-IRES-GFP: 1295.7 ng/µl

IV. Confirm Presence of Let7F1/2 and pAAV plasmids.

a. In order to ensure that the 5.3kb Let7F plasmid was successfully cloned, as well as the 6kp pAAV plasmid, we ran a gel via gel electrophoresis.
b. I added ladders, as well as 500 ng of each DNA type, to 2 µl loading buffer, and ran a 1.2% agarose gel for approximately 1 h at 110V. The results of the test indicate that our plasmids are in fact present, as a band is present at the proper corresponding line on the ladder.

V. Digest Let-7F 1/2

a. Now that we have pure isolated DNA, we must digest the plasmids to obtain the insert to place into the expression plasmid. For the Let-7F 1/2, when we digest with the restriction endonuclease XhoI, it will cut out the 300 bp fragment that serves as our Let-7F microRNA precursor. When we digest the pAAV plasmid, it will open it up, allowing us to later ligate the Let-7F 1/2 300 bp fragment into the pAAV vector.
b. The following digestion mixes were concocted:
   i. Let-7F1:
      1. 2.0 µl H_2O
      2. 1.5 µl XhoI
      3. 0.2 µl BSA
      4. 2.0 µl 10X Buffer D
      5. 14.3 µl DNA (10 µg)
   ii. Let 7F2:
      1. 1.1 µl H_2O
      2. 1.5 µl XhoI
      3. 0.2 µl BSA
      4. 2.0 µl 10X Buffer D
      5. 15.2 µl DNA (10 µg)
c. These were digested at 37˚C for 90 min.
d. A 1.2% agarose gel was then run at 110V for 1 h, with 2 µl loading buffer and 8 µl digested DNA. In order to increase the precision of all future gels, I made the agarose without ethidium bromide (EtBr), and then I soaked the gel in EtBr for 15 min once it had run. There was a 300 bp fragment for Let-7F1 and Let-7F2. This was then cut out of the gel and will shortly be isolated.

VI. Digest the pAAV-IRES-GFP

a. For the pAAV plasmid, the digestion mix was concocted:
      1. 8.6 µl H_2O
      2. 1.5 µl XhoI
      3. 0.2 µl BSA
      4. 2.0 µl 10X Buffer D
      5. 7.7 µl DNA (10 µg)

ii. These reagents were added to a microcentrifuge tube and incubated at 37°C for 90 min.
iii. Then we had to precipitate this DNA:
    1. Precipitate DNA by adding 0.1 volume of 3 M sodium acetate (2 µL), pH 5.2, and two volumes of 100% ethanol (44 µl). Put in -20°C for 35 min.
    2. Centrifuge at 12,000 x g for 10 min at 4°C.
    3. Remove supernatant and add 200 µl 70% ethanol and centrifuge at 12,000 x g for 5 min at 4°C.
    4. Remove supernatant and air-dry pellet.
    5. Add 20 µl H_2O to the pellet and nonodrop. The concentration of the pAAV-IRES-GFP is 0.65µg/µL.
iv. Then, we dephosphorylated the 5' ends of the pAAV-IRES-GFP plasmid, in order ensure that the ends do not ligate back together.
    1. To the microcentrifuge tube, add:
    2. 18.5 µl pAAV-IRES-GFP DNA
    3. 1 µl CIAP
    4. 5 µl 10x Buffer
    5. 25.5 µl H_2O
v. Incubate 15 min at 37°C, and then 15 min at 56°C.
vi. Add another 1 µl CIAP and then repeat the incubations.
vii. Then, to stop the reaction, add 2 µl 0.5 M EDTA, and heat at 65°C for 20 min.
viii. A 1.2% agarose gel was then run at 110V for 1 h, with 2 µl loading buffer and 8 µl DNA. A 6 kb fragment was visualized and excised form the gel. It will be isolated and purified from the agarose gel along with the Let-7F1/2.

VII. Isolate the DNA from the agarose gel

a. Gel solubilization
    i. We added 200 µl NT Buffer to each 100 mg of agarose gel slice. At this point in time, we have three slices of DNA: Let-7F 1, Let-7F 2, and pAAV-IRES-GFP. The weight and amount of NT buffer added to each PrepEase Clean-Up Column is as follows:
        1. Let-7F1: 200 mg, 400 µl NT Buffer
        2. Let-7F2: 200 mg, 400 µl NT Buffer
        3. pAAV-IRES-GFP: 400 mg, 800 µl NT Buffer
    ii. We incubated the samples at 50°C for 10 min, until each gel was completely dissolved, while vortexing.
b. Bind DNA sample to column
    i. We placed the three Clean-Up Columns into three 2 mL Collecting Tubes, and loaded the samples directly to the center of each column.
    ii. We centrifuged for 1 min at 11,000 x g.
    iii. The flow-through was discarded and the clean-up columns were placed back into the collecting tubes.
c. Wash Column
    i. We added 600 µl NT3 Buffer to each clean-up column.

ii. These were then centrifuged 1 minute at 11,000 x g.
iii. Flow through was discarded.

d. Dry Column
   i. We centrifuged 2 min at 11,000 x g to remove excess NT3 Buffer.

e. Elute DNA
   i. The clean-up columns were transferred to a clean microcentrifuge tube.
   ii. 20 µl of RT NE Buffer was added.
   iii. The samples were incubated at RT for 1 min to increase the yield of eluted DNA.
   iv. We centrifuged for 1 min at 11,000 x g. The flow-through was then collected.

f. Measure concentration
   i. Using the ThermoFisher nanodrop, we measured the concentration of each sample of DNA. They are as follows:
      1. Let-7F1: 22.8 ng/µl
      2. Let-7F2: 23.9 ng/µl
      3. pAAV-IRES-GFP: 166.2 ng/µl

VIII. Ligate the Plasmid Vector pAAV-IRES-GFP and each Let-7F1/2 insert.

a. We will be using a 1:6 molar ration of vector : insert. I first diluted the DNA until it was 40 ng/µl. The rest of the pAAV-IRES-GFP DNA (166.2 ng/µl) was put in the freezer, with the rest of the Let-7F1/2 DNA.

b. Equation: (ng of vector x kb size of insert / kb size of vector) x molar ratio of insert / vector = ng of insert.
   Equation: (50 ng of vector x 300 bp insert) / 6.0 kb vector x 6/1 = 15 ng of insert.

c. Set up the following reaction using the proper vector : insert ratio:
   i. Let-7F1 Insert and pAAV-IRES-GFP Vector: (tube 1)
      1. 1.25 µl Vector DNA
      2. 0.65 µl Insert DNA
      3. 0.5 µl T4 DNA Ligase (10 u)
      4. 1.0 µl Ligase 10x Buffer
      5. 6.6 µl Nuclease – Free Water
   ii. Let-7F2 Insert and pAAV-IRES-GFP Vector: (tube 2)
      1. 1.25 µl Vector DNA
      2. 0.65 µl Insert DNA
      3. 0.5 µl T4 DNA Ligase (10 u)
      4. 1.0 µl Ligase 10x Buffer
      5. 6.6 µl Nuclease – Free Water
   iii. Control and pAAV-IRES-GFP Vector: (tube 3)
      1. 1.25 µl Vector DNA
      2. 0.5 µl T4 DNA Ligase (10 u)
      3. 1.0 µl Ligase 10x Buffer
      4. 7.25 µl Nuclease – Free Water

d. This should then be incubated. Usually for blunt ends: 22°C for 4 h. These tubes (1-3) were then refrigerated overnight. At this point, we hope that our 300 bp

fragment of Let-7F1/2 is present within the pAAV vector. We will now increase our plasmid amount by cloning E. Coli.

IX. Transform the *E. Coli*

a. Then, we took the three tubes and added 2 µl of each to three separate E. Coli tubes, transformed them (Step I), and then plated them (Step II.a).

X. Pick Colonies and Grow the *E. Coli*

a. From the Let-7F1/2 plates, we picked 10 colonies from each and incubated them overnight at 37˚C, 250 rpm, in LB broth and ampicillin.
b. The plates correspond to the following tubes (only including tubes with growth):
   i. Plate 1: culture tubes 1-12
   ii. Plate 2: culture tubes 13-17
c. We had growth in the culture tubes the next morning. We then isolated the plasmids using the QIAGEN kit (see Step III.b), resuspending the plasmids in 25 µl AE elution buffer.

XI. Verify Presence of Let-7F

a. In order to verify the presence of the 300 bp fragment, we redigested the plasmids with XhoI, using the following mixture:
   i. 2 µl DNA (from each of the 17 tubes)
   ii. 1 µl 10x buffer
   iii. 0.2 µl BSA
   iv. 0.4 µl XhoI
   v. 6.4 µl H_2O
b. This digested for 90 min at 37°C. Then a gel was run, and tubes 4, 6, and 9 had a visible Let-7F1 300 bp fragment, and tubes 14 and 16 had a visible Let-7F2 300bp fragment.

XII. Verify Correct Orientation of the Let-7F1/2 Fragment Using BamHI.

a. There is a possibility that, because both sticky ends of the vector would align with any other XhoI cut site, the fragment may have inserted in the improper orientation. To verify this, we will digest the plasmid with insert with BamHI. Because the BamHI site on the vector is very close to the BamHI site on the insert (if inserted the proper way), then after we cut with BamHI, we should not notice any other bands on the gel, because the excised fragment is so small.
b. We digested the pAAV-Let7F1/2 plasmids with BamHI using the following mixture:
   i. 2 µl DNA
   ii. 1 µl 10x buffer
   iii. 0.2 µl BSA
   iv. 0.4 µl BamHI
   v. 6.4 µl H_2O
c. This digested, and then we ran a gel. There was no 300 bp fragment visible, which leads us to believe that our insert was in the proper orientation.

XIII. Verify Correct Orientation of the Let-7F1/2 Fragment Using NheI and ClaI.
   a. To fully verify that our insert is in the proper orientation, we decided to perform a double digest with NheI and ClaI. This will cut the insert back out, so we should see the reappearance of the 300 bp fragment.
   b. We used the following mixture:
      i. 2 µl DNA
      ii. 1 µl Multi-core buffer (100% with both Res)
      iii. 0.2 µl BSA
      iv. 0.2 µl NheI
      v. 0.2 µl ClaI
      vi. 6.4 µl H_2O
   c. We allowed this to digest, and then we ran a gel, and there was the reappearance of the 300 bp fragment (Figure 13a). This confirms that each Let-7F insert is contained within the pAAV-IRES-GFP vector in the proper orientation for transcription and translation to take place.

XIV. Glycerol Stocks
   a. We then made glycerol stocks of tube #4, 6, 14, and 16. To do this, I autoclaved a mixture of 80% glycerol and 20% H_20, and then in a tube I mixed 500 µl bacterial culture with 500 µl 80% glycerol.

XV. Isolate pure plasmid DNA for transfections
   a. Then I incubated 0.5 mL of the bacterial culture from tubes 6 (Let-7F1) and 14 (Let-7F2) and incubated them in 50 mL LB broth and ampicillin overnight. I then isolated this DNA using the QIAGEN kit (see Step III.b). The concentrations are as follows:
      i. Let-7F1: 3892 ng/µl
      ii. Let-7F2: 2475 ng/µl
   b. These were stored in my box at 4°C.

Transfection

This is how a transfection is performed. This is the next step in my experiment. I had the opportunity to practice a transfection, and have included the materials and methods below. For this experiment, we transfected endothelial cells (NREC, P.31, 7.25.13), in antibiotic-free media, with Let-7F1/2 DNA.

1. We diluted the DNA to 1 µg/µl. This was done using DPBS.
2. We ensured that the cells were plated in 6-35mm wells with Penn/Strep-free media.
3. In a microcentrifuge tube, we aliquoted 2 µl DNA and 2 µl OptiMEM media.
4. In one other tube, we put 10.0 µl Lipofectamine and 15.0 µl OptiMEM media.
5. The contents of the original aliquot in step 3 were added to the tube in step 4.
6. This was mixed gently and incubated for 30 min at RT.
7. We then added 800 µl OptiMEM media and overlayed this mixture onto rinsed (DPBS) cells.

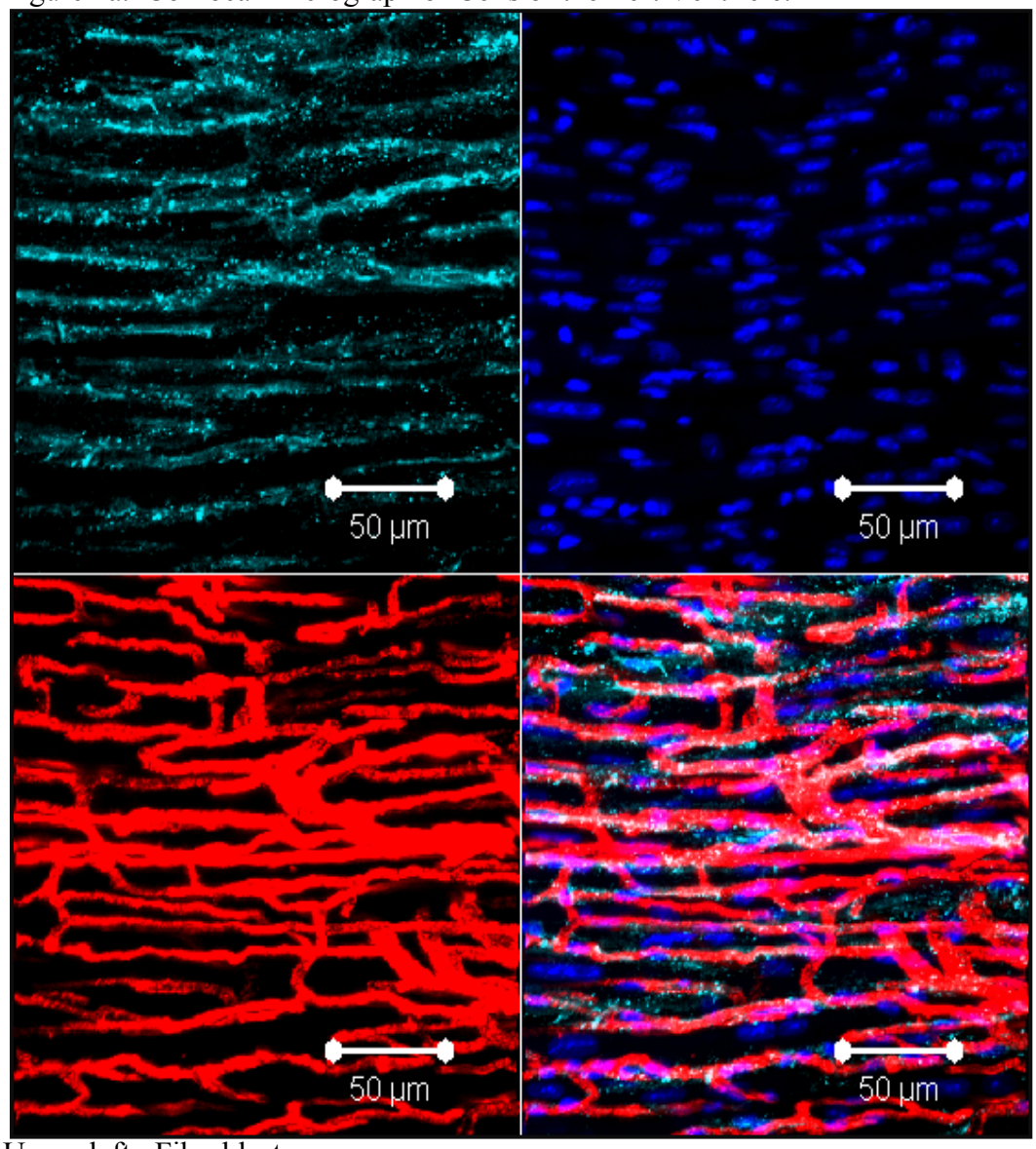
8. We let it incubate for 5-6 h and then added 800 µl antibiotic-free media containing 40% fetal bovine serum (FBS).
9. The cells were incubated overnight.
10. The next day, we aspirated the media and then we added 1 mL antibiotic-free media containing 20% FBS. This again incubated overnight.
11. The next morning, the cells were analyzed by flow cytometry to determine which cells were successfully transfected.

When I continue my project, I will be infecting sham and trans-aortic constriction (TAC) mice with my AAV-Let-7f. TAC mice are a mouse model of pressure overload hypertrophy.

CHAPTER THREE

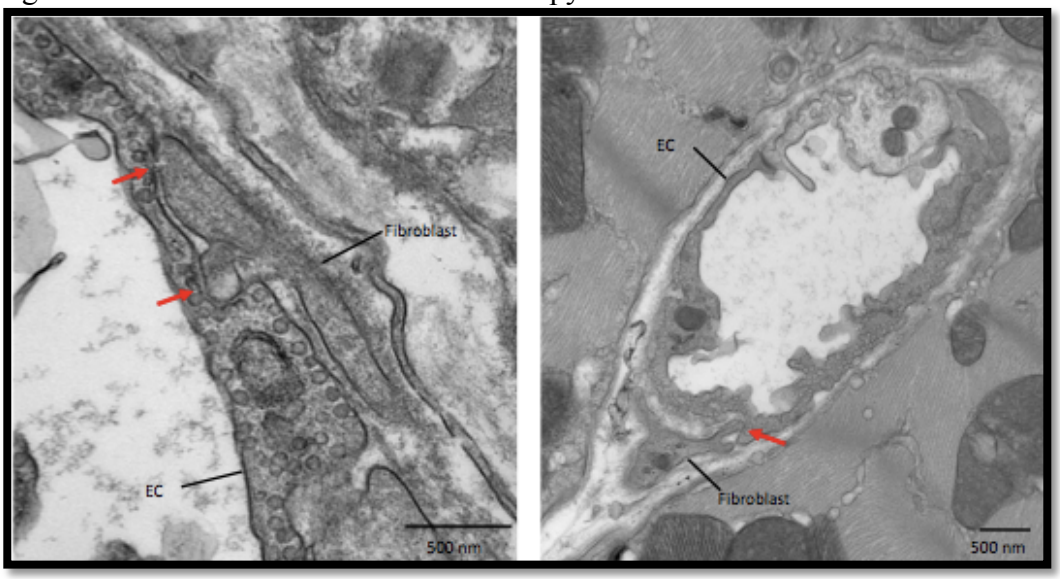
Figures and Results

Figure 4a. Confocal Micrograph of Cells of the Left Ventricle.



Upper left: Fibroblasts.
Upper right: Nuclei.
Lower left: Vasculature.
Lower right: Overlay.

Figure 5a. Transmission electron microscopy of fibroblasts and endothelial cells.



Red arrows: electron dense regions of cell communication.

Figure 6a. 3-D cell-adhesion assay between fibroblasts and endothelial cells, with and without N-cadherin blocking-antibody.

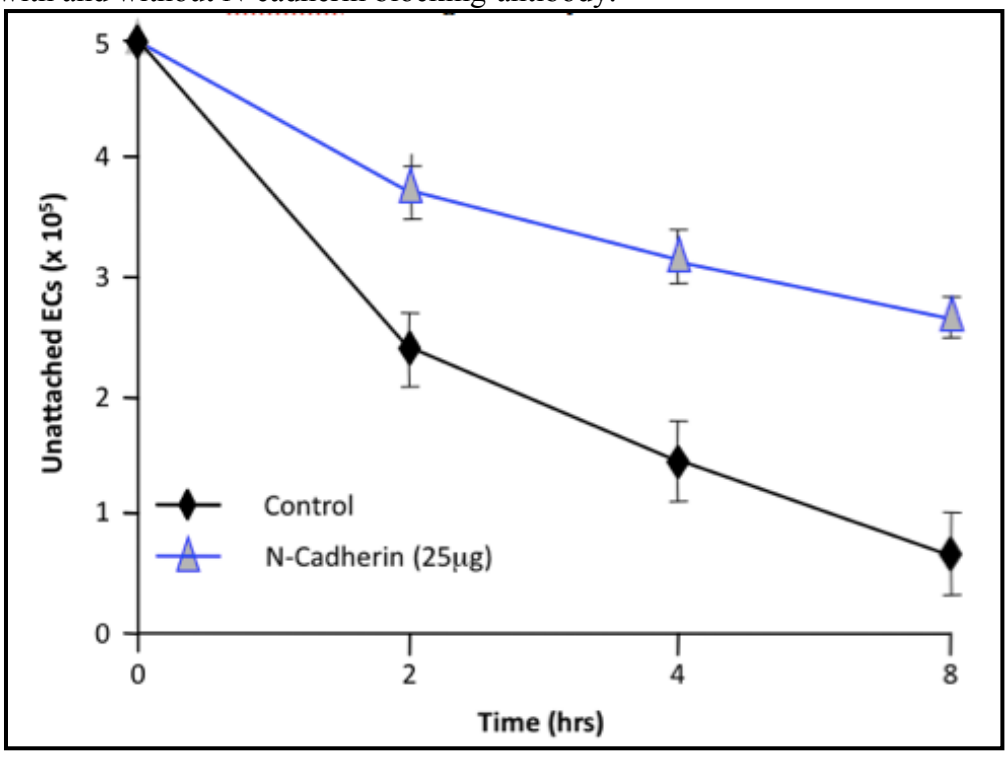
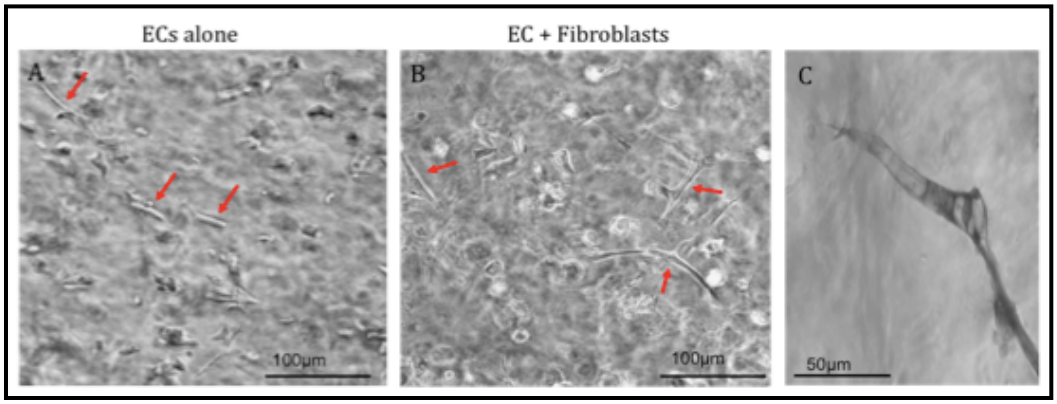


Figure 7a. Endothelial cell and fibroblast cell-cell interactions and tube formation.



Red arrows: tube formation.

Figure 8a. Endothelial cell and fibroblast cell-cell interactions and tube formation with N-cadherin blocking antibody.

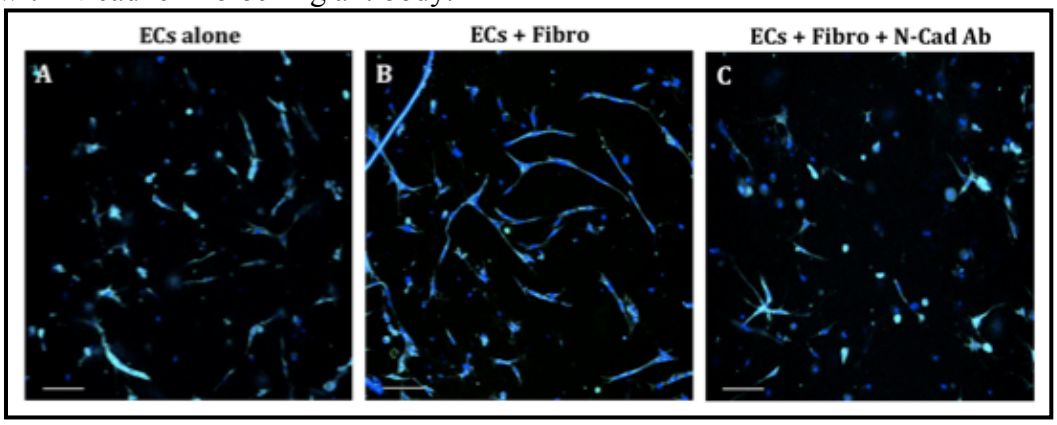


Figure 9a. Single and co-cultures of endothelial cells and fibroblasts with N-cadherin blocking antibody.

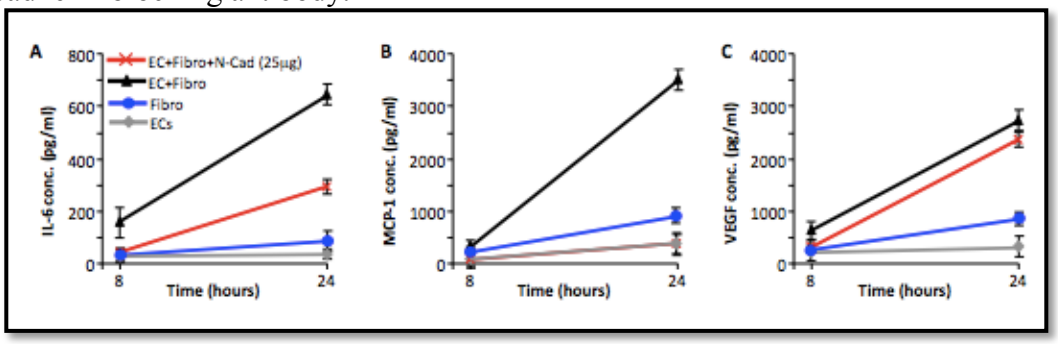


Figure 10a. (A) siRNA knockdown of dicer in cardiac fibroblasts. (B) Dicer protein levels with dicer expression knocked down. n=4

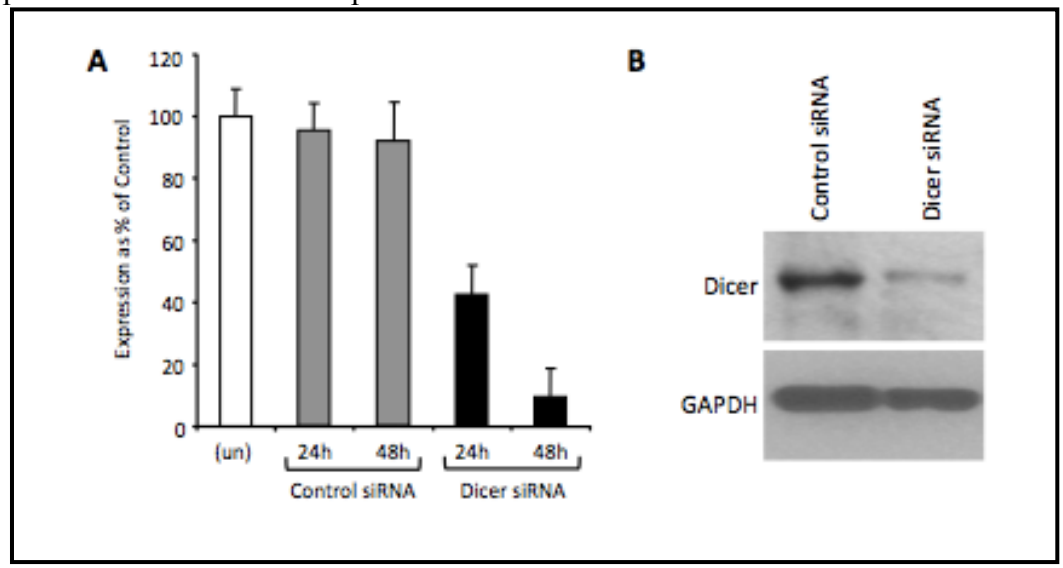


Figure 11a. 3-D cell aggregation assays with single or cocultures of endothelial cells and fibroblasts, with dicer knockdown.

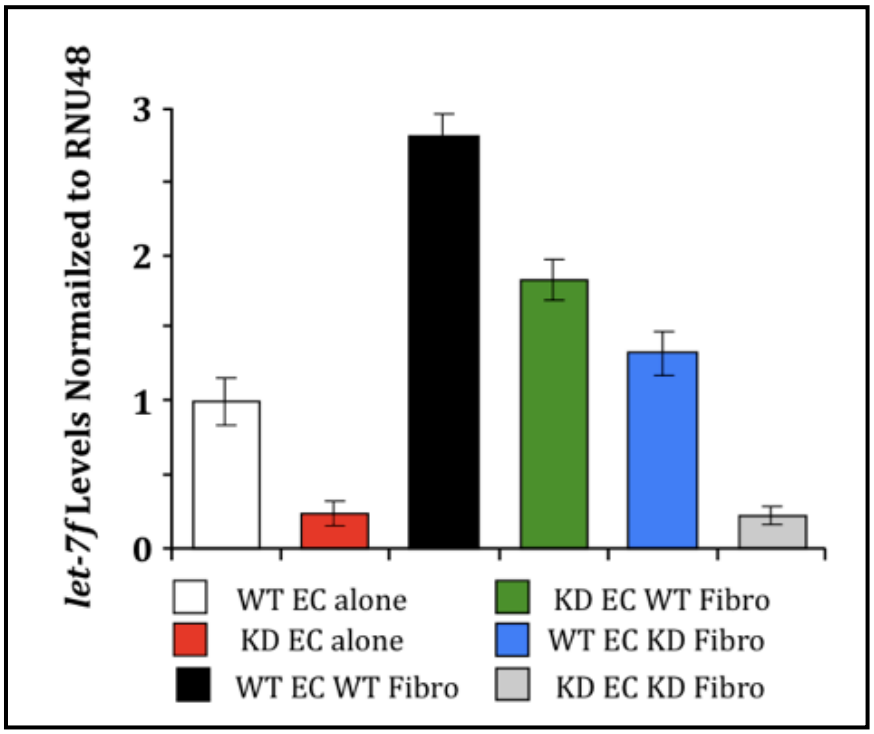


Figure 12a. The pAAV-IRES-GFP vector, which was engineered with a Let-7f precursor within the MCS (Multiple Cloning Sites).

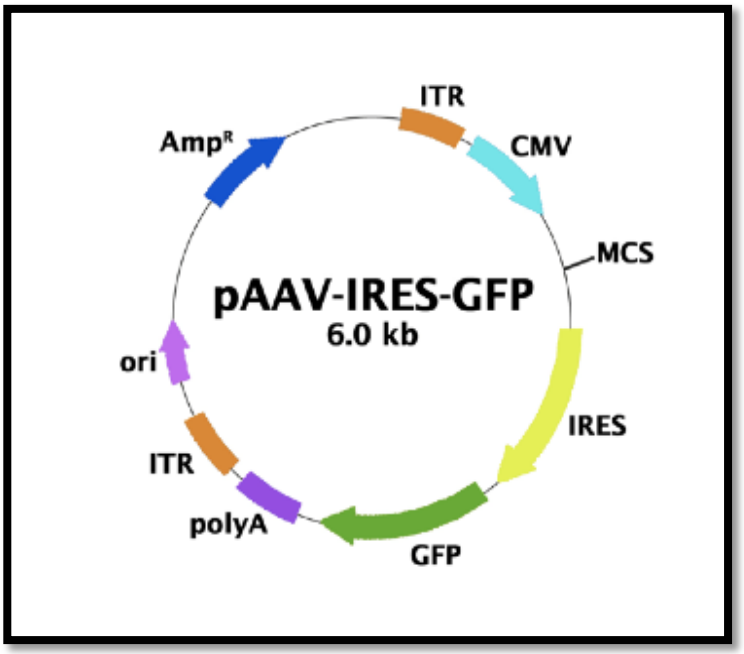
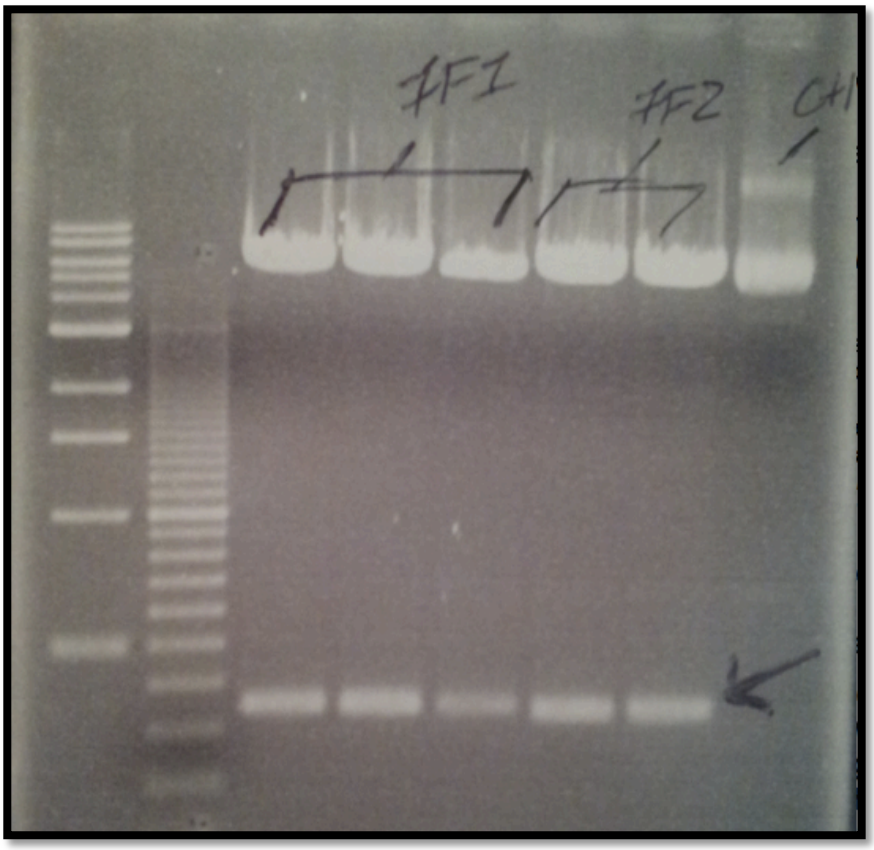


Figure 13a. A gel electrophoresis image that shows the presence of a 300bp Let-7F fragment cut out of a 6kp pAAV-IRES-GFP vector. This image confirms the success of the cloning experiment.



CHAPTER FOUR

Discussion and Conclusions

Discussion

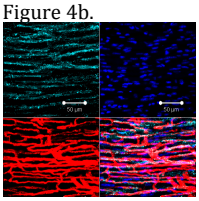
The main cell types in the heart include myocytes, which are the muscle cells that allow the heart to function as a pump, the fibroblasts, which lay down the extracellular matrix (ECM) that serves as a scaffold, and the endothelial cells, which compose the vasculature of the heart. The heart is also composed of immune system cells, which are transient and secrete important factors necessary for a proper immune response. Our laboratory focused its studies on understanding the communication that takes place between these cell types, and the influence of the secreted factors. We have moved from studying myocyte-fibroblast interactions to fibroblast-endothelial interactions with each other, as well as with the ECM.

These cells are able to interact in several different ways. Chemical signaling, which conveys a message via secretions, such as growth factors, cytokine, and hormones, which can act in autocrine or paracrine fashion. Mechanical signaling is another pathway for communication, caused by changes in stretch, pressure or muscle contraction. Finally, electrical signals are transferred directly from one cell to another through the opening and closing of ion channels, as well as through gap junctions. The dynamic interactions between these different signaling pathways allows for proper form and function of the heart. They also play a role in ECM synthesis, degradation, and composition, which is necessary for the heart to continue working properly. Any

alteration in one signaling pathway leads to changes in the others, ultimately compromising normal cardiac function.

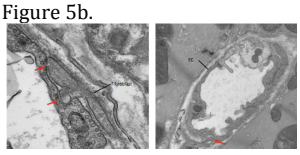
Our interest in studying fibroblast and endothelial cell (EC) interactions was originally sparked by the following data (Figure 4b). This confocal micrograph of the left ventricle of the mouse heart shows tight association of fibroblasts and endothelial cells. In the upper left panel, the teal staining is representative of fibroblasts. The right upper panel shows cell nuclei (stained with DAPI), and the lower left panel highlights the vasculature as visualized following perfusion with fluorescent microspheres. The lower right panel shows the overlay of the fibroblasts and the vasculature. As one can see, the fibroblasts are intimately associated with the vessels of the heart. This led us to believe that they may have a significant role in the formation and maintenance of the vessels. Since it is our ultimate goal to better understand how the vasculature is formed so that we can stimulate new blood vessel growth, we decided to further study the interactions that take place between the fibroblasts and the ECs.

Figure 4b.

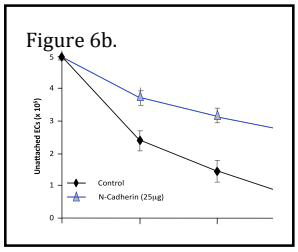


To examine these interactions more closely, we used transmission electron microscopy (TEM). In the left panel of Figure 5b, an EC and fibroblast are situated adjacent to each other in the left ventricle of the heart. The red arrows indicate electron dense regions, which indicate tight interactions directly between the two cells. In the right panel, a fibroblast and EC show electron dense regions between them, indicating that these cells communicate intimately with each other.

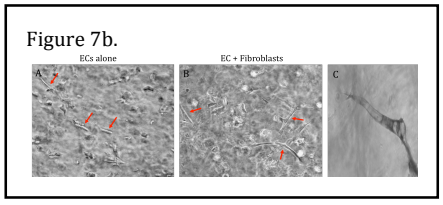
Figure 5b.



While we were able to observe this phenomenon *in vivo*, the next step was to confirm these interactions *in vitro*. To do this, we used our 3-D cell adhesion assay. For this experiment, we cultured cardiac fibroblasts and ECs with either a control antibody or a blocking antibody against N-cadherin. N-cadherin is necessary for proper cell adhesion, forming adherens junctions between cells. At different time points in our assay, the media was collected and unattached cells were counted (Figure 6b). In the control, we observed nice cell-cell interactions at the eight hour time point of the experiment. However, after addition of N-cadherin blocking antibodies, there is a significant increase in the number of unattached cells. This confirmed our hypothesis regarding fibroblast-EC interactions observed *in vivo*. Having established that these cells can directly interact, the next question is: what is the importance of these interactions?

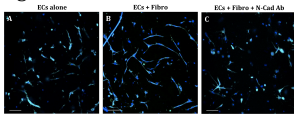


Using a 3-D collagen tube formation assay adapted from George Davis's group at the University of Missouri, we mimicked the cell's normal *in vivo* activity *in vitro*, for type I collagen acts as the ECM of the heart. In Figure 7b, one can see how ECs alone display modest tube formation. However, when cocultured with fibroblasts, there is a dramatic increase in the number of tubes being formed in this assay. As visualized in panel C at higher magnification, there is a lumen for the tubes being formed in the assay. Having demonstrated the importance of these interactions, we next asked whether direct cell-cell interactions are required for this enhanced tube formation that we observed.



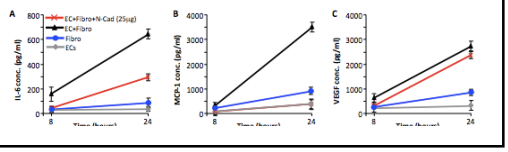
Again, using the 3-D collagen tube assay, we examined the effect of N-cadherin blocking antibodies on tube formation. The N-cadherin antibody should disrupt any direct communication between the endothelial cell and fibroblasts. In Figure 8b, once again we see enhanced tube formation when the ECs and fibroblasts are cocultured in the collagen gels; however, when N-cadherin blocking antibodies were added, enhanced tube formation is lost. These data indicate that direct cell-cell interactions are required for proper tube formation to take place. This leads to the question, what are the cells saying to each other?

Figure 8b.



We next examined these cultures for the expression of various cytokines and growth factors that can promote angiogenesis. We wanted to see how their expression changed between single cultures and cocultures of cells when direct cell-cell signaling was compromised. Specifically, we examined Interleukin-6 (IL-6), Monocyte Chemotactic Protein-1 (MCP-1), and Vascular Endothelial Growth Factor (VEGF) expression. For these studies, shown in Figure 9b, we used either single or cocultures of ECs and fibroblasts in the absence or presence of N-cadherin blocking antibody. At different time points, the media was collected and expression of the various secreted proteins was measured by Multiplex analyses. In panel A, one can see that the single cultures of cardiac ECs or fibroblasts yielded minimal IL-6 expression, whereas cocultures of ECs and fibroblasts greatly increased the concentration of IL-6 in the media. However, when N-cadherin blocking antibody was added, there was a significant

Figure 9b.

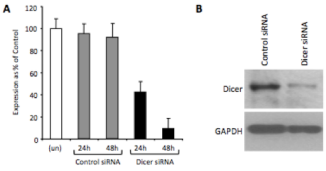


decrease in IL-6 expression. These data indicate that direct cell-cell interactions are necessary for IL-6 to be properly expressed. Similar results are obsereved with MCP-1. Minimal MCP-1 expression is present in single cultures of ECs or fibroblasts, but the cocultures showed a marked increase in MCP-1 expression. As with IL-6, N-cadherin blocking antibodies compromised MCP-1 expression. However, with VEGF, although there is in increase in expression with cocultures of ECs and fibroblasts, there is no decrease observed when cell-cell interactions are disrupted. These data indicate that direct cell-cell interactions are not required for VEGF to be properly expressed, but that indirect cell communication is necessary for proper VEGF expression.

While these studies focused on secreted factors that can act in an autocrine or paracrine fashion, we desired to know to what extent cells were passing information directly between each other via tight gap junctions. Gap junctions will allow for the passage of material that is smaller than 1000 Daltons. This limited us to examining the cells for the exchange of small signaling proteins and microRNAs (miRNA). My summer research project in particular focused on studying the miRNA let-7f in particular, which is known to have a pro-angiogenic role.

miRNAs are short, non-coding strands of RNA that affect gene expression by binding to mRNA in the cell and preventing it from being translated into protein. Let-7f is considered a pro-angiogenic factor in that it binds to and inhibits an anti-angiogenic factor from being translated. In order for immature miRNA to be processed and presented as mature miRNA, a protein called dicer is required. If we were to knock down dicer expression, then we should in theory see no mature miRNAs present in those cells with dicer knocked down. These two images in Figure 10b demonstrate that we are able

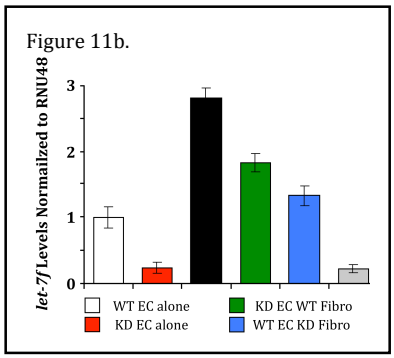
Figure 10b.



to knockdown dicer expression in cardiac cells. When using small interfering RNA (siRNA) specifically targeted against dicer, we are able to decrease the amount of dicer present in cells by about 90% at the transcript level. At the protein level, panel B reveals that there was again a dramatic decrease in dicer expression in cells treated with dicer-specific siRNA. Our knockdown of dicer is important in the next experiments that we performed, which involved studying miRNA exchange between ECs and fibroblasts.

Using a 3-D cell aggregation assay, we analyzed the exchange of miRNA between cardiac ECs and fibroblasts. As seen in Figure 11b, wild-type ECs (WT EC) cultured alone showed a certain level of mature let-7f expression (white bar) as measured by real time PCR. When we knocked down dicer in ECs (KD EC), there was a significant decrease in the amount of mature let-7f expressed, as was expected. Next, we cocultured ECs with fibroblasts, using different combinations of WT and KD ECs and fibroblasts and then we isolated the ECs and measured their mature let-7f levels. When coculturing WT ECs and WT fibroblasts, there was a three-fold increase in the amount of mature let-7f in the ECs (which were first isolated and then analyzed for protein expression, black column). There are several possible explanations for this increase in let-7f expression in the ECs. Either direct cell-cell interactions between ECs and fibroblasts resulted in increased expression at the transcriptional level in the ECs, or let-7f is being directly transferred between the two cell types through tight gap junctions. To discover which

Figure 11b.

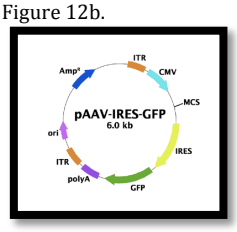


mechanism is occuring, KD ECs were cultured with WT fibroblasts. This group (green bar) sill shows a marked difference in let-7f expression than WT ECs alone (white bar). This indicates that the increase from is via direct cell-cell transfer of let-7f. If those ECs have dicer knocked down, then they cannot process let-7f into mature miRNA, no matter what factors are present. The increase in let-7f observed must have been directly transferred from the fibroblasts to the ECs through tight gap junctions. Next, WT ECs were cultured with KD fibroblasts. Here, the fibroblasts were unable to directly transfer let-7f, because they cannot process it. However, an increase is still in let-7f expression is still observed over the EC alone cultures. This can be explained by other factors being transferred directly from the fibroblasts to the ECs, or activation of a signaling cascade that transcriptionally increases let-7f expression in ECs. This experiment confirms that this miRNA is being transferred via gap junctions from the fibroblasts to the ECs. Finally, as is expected, when dicer is knocked down in both ECs and fibroblasts, there is almost no expression of mature let-7f in the ECs.

Now that we know that this pro-angiogenic factor is being transferred directly between these cell types, we sought to understand if increased expression of let-7f in these cell types leads to increased angiogenesis in a mouse model of pressure overload hypertrophy (Trans-Aortic Constriction; TAC). By in essence stimulating hypertension in a mouse, we can test whether or not let-7f will help in blood vessel regrowth and remodeling *in vivo*.

In order to test this, I had to engineer a plasmid with a let-7f precursor that could be used in mice and successfully increase expression of let-7f in the cells of the heart. To do this,

Figure 12b.



I began with an Adeno-associate virus plasmid (pAAV-IRES-GFP)(Figure 12b), and cloned the let-7f insert into it using the restriction endonuclease XhoI.

The following is the cloning strategy that I used in order to engineer and isolate a plasmid that could be used to generate AAV-let-7f.

Let-7f Cloning and Transfection Strategy

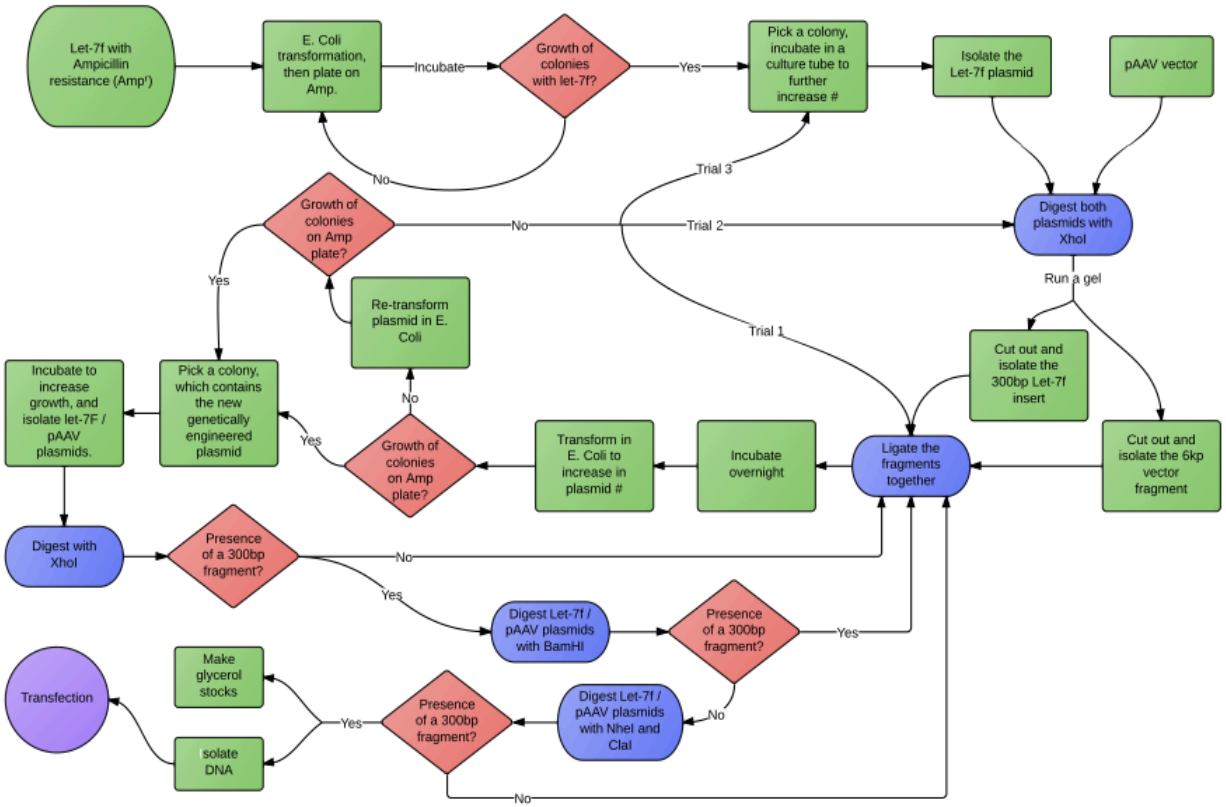
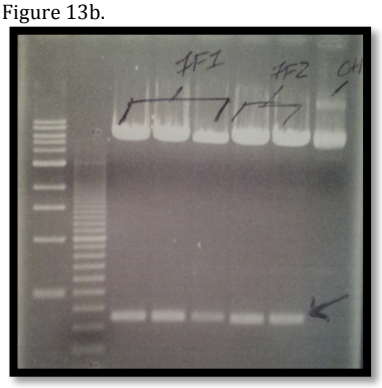


Figure 13b.



Lanes:
1: 1kp ladder
2: 100bp ladder
3-5: pAAV-IRES-GFP with Let-7f1 insert
6-7: pAAV-IRES-GFP with Let-7f2 insert
8: Control pAAV-IRES-GFP vector

This gel electrophoresis image confirms at the last step in the process that our 300 bp let-7f fragment was successfully cloned into the pAAV-IRES-GFP 6 kb vector (Figure 13ba). Glycerol stocks were made, and the next step is to generate virus and infect mice.

Conclusion

Our goal was to study the molecules that have an effect on angiogenesis in the heart; through various co-culture systems including, cell adhesion and aggregation assays, 3-D collagen tube formation assays, and numerous other tests, we have been able to investigate this question. We have demonstrated that cell-cell interactions are required for enhanced tube formation and that this occurs through multiple signaling pathways. The study of miRNAs such as let-7f confirms that these two cell types (fibroblasts and ECs) are able to exchange intracellular material (less than 1000 Da) through tight gap junctions. We are continuing to study the effects that over and under expression of let-7f has on these cells, with the hopes of better understanding the angiogenic process, and stimulating new blood vessel growth in the pathological heart.

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