THE IMPACT OF THE EXTRACELLULAR MATRIX AND TYPE 1 DIABETES ON CARDIAC FIBROBLAST ACTIVATION

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LIST OF ABBREVIATIONS

ANG II; angiotensin II

α-SMA; α-Smooth muscle actin

BAPTA/AM; 1,2-bis (o-Aminophenoxy)ethane-N,N,N,N’-tetra acetic acid

Tetra(acetoxyethyl) Ester

Ca$^{2+}$; calcium

CF; cardiac fibroblast

DAG; diacylglycerol

ECM; extracellular matrix

EGF; epidermal growth factor

EGFR; epidermal growth factor receptor

IP3; inositol 1,4,5-triphosphate

MAPK; mitogen activated protein kinase

MI; myocardial infarction

PI3K; phosphatidylinositol 3-kinase

PIP2; phosphatidylinositol-4,5-bisphosphate

PKC; protein kinase C

PMA; phorbol 12-myristate 13-acetate

TGF-β; transforming growth factor-β
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CHAPTER ONE

INTRODUCTION

Impact of cardiovascular diseases

A vast spectrum of cardiovascular diseases including coronary artery disease, hypertension, stroke, and heart failure plagues more than one third of the U.S. population (Lloyd-Jones et al., 2009). According to the American Heart Association and the National Health and Nutrition Examination Survey (NHANES) study, in 2006 approximately 81.1 million people, 36.9% of the U.S. population, endured at least one form of cardiovascular disease (Lloyd-Jones et al., 2009). The same study revealed approximately 5.8 million people, 2.6% of the population, suffered from heart failure and 8.5 million, 3.6% of the population, experienced a myocardial infarction (MI) (Lloyd-Jones et al., 2009). Diabetes mellitus, obesity, and smoking are proven risk factors that contribute to the progression of these diseases. It is important to study the mechanisms of these cardiovascular diseases to develop new targets and means of intervention for its treatment and prevention.

The myocardium and the importance of cardiac fibroblasts and extracellular matrix

The myocardium is a dynamic environment composed of cells and extracellular matrix proteins to function as a pump, distributing blood throughout the body. Myocytes, the contractile cells in the heart, express numerous proteins including actin and myosin that allow these cells to contract; upon stimulation, the action potential of the contraction spreads from myocyte to myocyte enabling the heart to beat in a coordinated rhythm. Also imperative for the contraction conductance, gap junctions generate a low resistance
Figure 1. Cardiac myocyte: cytoskeleton and focal adhesions. A. The cardiac myocyte cytoskeleton is specially designed to aid in contraction through the overlapping actin and myosin of sarcomere structure. Gap junctions line the border of myocytes to facilitate intercellular communication and accelerate the contraction process. Integrin cell-matrix receptors bridge communication barrier between the myocyte and the ECM. B. A closer look at the integrin receptor reveals the structure of the focal adhesion, a subcellular region of proteins that transduce signals from the matrix to the cell and vice versa. The integrin receptor interacts with the ECM and with intracellular proteins, talin, vinculin, and paxillin. A major component to the focal adhesion is the actin cytoskeleton; therefore the focal adhesion proteins of myocytes interact with the contractile sarcomere structure to influence contraction.
Panel A

Cytoplasmic actin
Myosin
Integrin
Gap Junction
Type IV collagen
Type I collagen
Type VI collagen

Panel B

Cytoplasmic actin
Myosin
Integrin
Talin
Vinculin
Paxillin
Focal Adhesion Kinase (FAK)
Type IV collagen
Type I collagen
passageway for ions to flow and permit communication between cells, myocyte to myocyte as well as myocyte to the other cardiac cells (Fig. 1).

Cardiac fibroblasts (CFs) are the major non-muscle cell type in the heart, comprising of approximately 20% of the cardiac mass and, depending upon the species, 30-70% of the total cell number. This is a very interesting figure because for decades investigators have cited a particular study by Nag et al. (1980) stating that fibroblasts make up 70% of the myocardium, but this study was performed in rats (Nag, 1980). Recently the Baudino lab revealed that fibroblasts comprise only 27% of the total cell number in the mouse myocardium (Banerjee et al., 2007). This discrepancy leads to questions that need to be addressed in the future. Is the human myocardium more similar to that of the mouse or the rat? Does one species accurately represent another species? Fibroblast function varies greatly from that of the contractile myocyte; upon activation fibroblasts are capable of proliferation, migration, and differentiation to the hypersecretory myofibroblast phenotype. The primary function of CFs is matrix production to provide a supportive meshwork around the cells in the myocardium and for repair during an injury or disease state.

The extracellular matrix (ECM) operates as the glue of the heart and is tissue specific, therefore the myocardium boasts its own distinct ECM composition. ECM is composed of fibers, including collagen, elastic and reticular and ground substance, water, proteoglycans and glycoproteins such as laminin, perlecan, and fibronectin. Each matrix component possesses a particular, important function in creating a well-organized matrix to support the surrounding cells. Typically the proteoglycans and glycoproteins act as adhesive connectors in the matrix while fibrillar collagens, including types I and III,
provide the primary structure and other collagens such as type IV, can form networks that function more like connectors. Type VI collagen is a structurally unique protein due to its beaded filament arrangement and operates as an adhesive connector. Overall, the different components work together through precise interactions to create a supportive meshwork for organization and function.

For decades the importance of collagen types I and III has dominated the literature, although collagen type V and the non-fibrillar types IV and VI are also significant structural components in the heart, especially in the basement region around the myocytes (Eghbali et al., 1989; Kuo et al., 1997; LeBleu et al., 2007; Weber et al., 1994; Yurchenco and Schittny, 1990). Fibrillar collagens are accepted as the structural support of the matrix because they can withstand tensile and shear stresses while the non-fibrillar collagens anchor and organize these fibers together increasing matrix strength. Non-fibrillar type IV collagen constitutes a large portion of the basement membrane, a specialized region of matrix surrounding the myocyte, interfacing the cell with the outer, interstitial matrix (LeBleu et al., 2007; Yurchenco and Schittny, 1990). Proteoglycans and glycoproteins, such as laminin and fibronectin, also function in the basement membrane to create a tight meshwork fastening the ECM components together (LeBleu et al., 2007; Yurchenco and Schittny, 1990). Type VI collagen may interact with fibrillar collagens in the interstitial matrix and with type IV collagen in the basement membrane, creating a firm bridge between the matrix regions (Kuo et al., 1997). Collagen VI is a filamentous collagen functioning as a scaffold for type I collagen in the interstitial matrix (Badylak, 2002). To fully comprehend cardiac remodeling and the importance of the matrix components, we must study the changes in deposition, relative ratios and impact
Figure 2. Cardiac fibroblast: cytoskeleton and focal adhesions.  

A. The cardiac fibroblast cytoskeleton contains cytoplasmic actin that interacts with integrin receptors at focal adhesions.  

B. The focal adhesions are the major facilitator in cell-matrix and matrix-cell interactions and communication. Talin, vinculin, and paxillin associate with the cytoplasmic side of the integrin receptor and interact with cytoplasmic actin and have the potential to trigger many signaling pathways through focal adhesion kinase activation.
Panel A

- Cytoplasmic actin
- Cofilin
- Integrin
- Type VI collagen
- Type IV collagen
- Type I and III collagen

Panel B

- Cytoplasmic actin
- Integrin
- Talin
- Vinculin
- Paxillin
- Focal Adhesion Kinase (FAK)
- Type IV collagen
- Type I collagen

Signaling Cascade
of these collagens on the surrounding cells during pathological conditions such as response to myocardial infarction (MI), which will be discussed later in this chapter.

Importantly, there is continual communication between the matrix and the fibroblasts; components of the ECM and the surrounding environment interact and signal to the fibroblasts through integrins, membrane spanning receptors that also interface with the actin cytoskeleton and other intracellular proteins at focal adhesions (Fig. 2). Talin, vinculin, and paxillin are focal adhesion proteins that aid in the cell-matrix signal transduction via their interaction with the integrin receptor and the actin filaments and their ability to stimulate focal adhesion kinase (FAK) autophosphorylation. Therefore the ECM components not only function structurally in the heart, but they also regulate the activity of cardiac fibroblasts via cell-matrix signaling. Studies from our lab have demonstrated that collagen types I and III induce in vitro cardiac fibroblast proliferation whereas type VI promotes myofibroblast differentiation (Naugle et al., 2006).

The dynamic fibrous matrix is continually remodeled by the fibroblast: matrix metalloproteinases (MMPs) secreted by the fibroblasts degrade old fibers while new fibers are synthesized and released by the CFs. Cardiac matrix metalloproteinases (MMPs), the regulators of ECM degradation, are classified as collagenases (MMP-1, 8, 13), gelatinases (MMP-2, 9), and stromelysins (MMP-3, 7) (Lindsey, 2006). Collagenases initially break down the helical structure of fibrillar collagens, such as types I and III, which are further degraded by gelatinases (Tyagi, 1997). Surprisingly, fibrillar type V and non-fibrillar type IV are initially broken down by gelatinases and stromelysins and are not degraded by collagenases (Lindsey, 2006; Tyagi, 1997). MMPs- 1, 2, 3, and 9 are unable to break down type VI collagen, but rather type VI is
Figure 3. Cardiac myofibroblast: cytoskeleton and focal adhesions.  

A. The myofibroblast cytoskeleton is specifically organized to facilitate wound contraction via well-organized stress fibers containing α-smooth muscle actin.  

B. Myofibroblasts also posses focal adhesions and upon differentiation, are referred to as supermature focal adhesions from the increased vinculin and paxillin association.
Panel A

α-Smooth muscle actin
Cytoplasmic actin
Cofilin
Integrin
Type VI collagen
Type IV collagen
Type I and III collagen

See focal adhesion insert

Panel B

Actin
α-SMA
Integrin
Talin
Vinculin
Paxillin
Focal Adhesion Kinase (FAK)
Type IV collagen
Type I collagen

Collagen I and III
Collagen IV

Integrin
Signaling Cascade
degraded by serine proteases (Kielty et al., 1993). Cardiac fibroblasts regulate the dynamic myocardium through the production and breakdown of the ECM that in turn has a major impact on fibroblast activity.

**Myofibroblasts: specialized wound healing cells**

The myofibroblast is a hypersecretory cell found in all major tissues that critically mediates wound healing via wound contraction and creation of a replacement scar. Myofibroblasts secrete copious amounts of extracellular matrix at the injury site creating the necessary fibrotic scar. In addition to scar formation, the myofibroblast also prevents injury expansion and promotes wound closure through its semi-contractile property. The hallmark of the myofibroblast is stress fiber formation from increased expression of α-smooth muscle actin (α-SMA), one of the proteins that facilitate the contractile ability of the cell (Fig. 3). Differing from myocyte contraction, the myofibroblast regulates wound closure through a sustained contraction rather than a beat-like one. The force of the myofibroblast contraction is produced by the stress fibers containing α-SMA, non muscle myosin, and numerous actin binding proteins such as cofilin and through their interaction with focal adhesions (Fig. 3B) (Tomasek et al., 2002). Contraction may be stimulated through two different signaling pathways, myosin light chain kinase (MLCK) and Rho-kinase (Tomasek et al., 2002). Increased intracellular Ca\(^{2+}\) activates the MLCK pathway increasing MLC (myosin light chain) phosphorylation, which promotes rapid contraction (Chrzanowska-Wodnicka and Burridge, 1996; Katoh et al., 1998). The major pathway for myofibroblast sustained contraction is Rho kinase, which may activate MLC through either direct phosphorylation of MLC or via inactivation of myosin phosphatase.
Figure 4. Origin of the cardiac myofibroblast? There is still debate in the current literature as to the potential origin of cardiac myofibroblast following a myocardial infarction. One possible candidate is the resident fibroblast that undergoes differentiation. Local smooth muscle cells may de-differentiation to a myofibroblast-like cell following an injury. Circulating stem cells may also be attracted to the injury site and possess the potential to differentiate to myofibroblasts.
Regulation of myofibroblast contraction differs from myocytes since myofibroblasts are in a state of sustained contraction. Elevation in intracellular calcium alone does not stimulate sustained myofibroblast contraction, but Rho and Rho kinase inhibition significantly reduced myofibroblast contraction force to clearly demonstrate the importance of the Rho pathway and its role in myosin phosphatase inactivation and contraction (Parizi et al., 2000; Tomasek et al., 2002).

The differentiation process is regulated by many factors such as mechanical stretch, inflammatory cytokines, and as our lab has previously demonstrated the ECM protein, type VI collagen (Naugle et al., 2006). Following a myocardial infarction, there are multiple steps to the remodeling process that will be discussed further in a later chapter, but one process is myocyte hypertrophy. This hypertrophy can stretch the surrounding CFs, and the mechanical stress triggers alignment of the cytoskeletal filaments forming the hallmark stress fiber bundles and mature focal adhesions (Tomasek et al., 2002). There is also a potent inflammatory response that includes many inducers of differentiation, the most notable being transforming growth factor β (TGF-β). The inflammation occurs at the injury site that requires a fibrotic scar to replace the necrotic and apoptotic myocytes and to prevent further expansion of the infarcted area.

Myofibroblasts are found in all major tissue types although the specific origin of cardiac myofibroblasts following an infarction is still debated (Fig. 4) (Porter and Turner, 2009). Do resident fibroblasts differentiate in response to the inflammation and ECM changes? Do peripheral stem cells arrive at the injury site post-MI? Do smooth muscle cells de-differentiate? Interestingly, TGF β blockade post-MI attenuated myofibroblast appearance at 3 days which may indicate a necessary role for TGF β in early post-MI.
Figure 5. Cardiac cell responses following a myocardial infarction. Upon injury such as a myocardial infarction, myocytes and fibroblasts activity changes significantly. Many of the myocytes at the injury die from the ischemia and other factors post-MI. The surrounding viable myocytes will compensate through hypertrophic growth but eventually fail, leading to wall thinning and decompensation. Following a myocardial infarction, the fibroblasts become active to proliferate, migrate, differentiate to the wound healing myofibroblast, and secrete ECM. The ECM also influences fibroblast activity post-MI; collagens I and III increase proliferation and collagen VI induces differentiation.
Pathological Cardiac Remodeling: post-MI

Myocyte
- Cell Death
- Hypertrophy
- Thinning

Heart Failure

Fibroblast
- ↑ ECM production
- Proliferation
- Differentiation
- Migration

Collagen
- I
- III
- VI

???
myofibroblast differentiation (Kuwahara et al., 2002). Also, the myofibroblast origin in other tissue types is still under dispute; therefore, uncovering the mechanism of cardiac myofibroblast appearance may apply to other tissue types as well.

**Cardiac Remodeling**

In the healthy myocardium continual, gradual matrix turnover occurs. The fibroblast balances new matrix deposition and matrix degradation through ECM synthesis and MMP activation. During pathological conditions, fibroblasts become overactive; CFs proliferate to increase in number, differentiate to hypersecretory myofibroblasts, migrate to the site of injury, and these active fibroblasts secrete copious amounts of collagen to assemble the necessary fibrotic scar (Fig. 5). Typically, once the myofibroblasts have performed their duty of building the scar, they die via apoptosis and the other fibroblasts return to their basal levels. If this process does not occur, then matrix deposition may continue indefinitely and reach the areas remote from the injury, resulting in excess matrix secretion and fibrosis. Fibrosis is a detrimental condition that impedes normal heart function and leads to a stiff myocardium and impairs proper beating and contraction of the myocytes. Eventually the compromised myocardium will progress into heart failure. This is why it is necessary to study the specific mechanisms regulating fibroblast function, so that we can develop interventions that target CFs to prevent the deleterious effects from their overactivation and persistent ECM deposition.

Following an injury the left ventricular environment changes drastically. Myocardial infarction, typically from an occlusion of a coronary vessel, yields severe ischemia, lack of nutrients and oxygen, causing myocyte death (Fig. 5). Since myocytes are incapable of replication, the surrounding viable myocytes become hypertrophic and the left
ventricular wall thickens. Unfortunately the myocytes can only function in this compensatory state for a short period of time before left ventricular decompensation occurs. During this state, the left ventricular wall becomes thin and the chamber dilated, eventually progressing to dilated cardiomyopathy and heart failure.

It is well established that collagen types I and III deposition increases following an MI to help create a fibrotic replacement scar, but one unclear aspect is the change in proportions of these collagens during post-MI remodeling. Wei et al. (1999) revealed that type III collagen is deposited during early remodeling, whereas type I collagen deposition predominates during the intermediate and late remodeling stages (Wei et al., 1999). It has been proposed that collagen I accumulation is accountable for the decreased compliance and fibrotic stiffening of the left ventricle leading to overall dysfunction since type I is more tightly organized and cross-linked compared to type III (Weber, 1989).

Non-fibrillar collagens not only operate structurally in the normal myocardium but they are also key mediators of fibroblast activity during pathological remodeling. Our lab determined that type VI collagen accumulates in the infarcted region of the rat heart 7 days post-MI and remains elevated for up to 20 weeks, and we established that type VI collagen-induced myofibroblast differentiation in vitro (Bryant et al., 2009; Naugle et al., 2006). Furthermore, we determined that during the later stages of remodeling, at 16-20 weeks post-infarction, both type VI collagen and myofibroblast content were elevated in the infarcted areas (Naugle et al., 2006). Other labs have revealed that myofibroblasts may persist for up to 17 years post-infarction in human myocardial scars (Willems et al., 1994). We postulate that the appearance of myofibroblasts in the very late stages of
Figure 6. Epidermal growth factor-stimulated MAPK activation. Epidermal growth factor (EGF) binds to its respective receptor, EGFR, to induce dimerization. The dimerization triggers autophosphorylation recruits SOS which exchanges GTP for GDP to activate Ras. Activated Ras recruits Raf, the first kinase of the MAPK cascade. Raf phosphorylates and activates MEK which in turn activates ERK, propagating the MAPK phosphorylation cascade.
Translocation to nucleus to activate transcription
remodeling may be due to the increased type VI collagen, which in turn may induce myofibroblast differentiation via cell-matrix interactions.

Type IV collagen is another non-fibrillar collagen that appears to play a key role in post-MI remodeling; type IV collagen increases expression in the peripheral zone at day 3 post-infarction and reaches its maximal expression in this zone between 7 and 11 days (Yamanishi et al., 1998). Surprisingly, in the outer infarct zone type IV collagen does not accumulate until day 4 and in the inner portion of the infarct region at day 10. The increased expression of type IV in the inner portion of the infarct zone is minimal compared to the outer, peripheral zones which may be more detrimental to cardiac function (Yamanishi et al., 1998). This pattern of early peripheral deposition that slowly progresses inward and lastly appears in the infarct scar is unique to type IV. Watanabe et al. (1998) revealed that type IV collagen appeared in the myocyte basement membrane and in the areas of replacement fibrosis of hypertrophic cardiomyopathy patients (Watanabe et al., 1998).

**Signaling and Regulation of Cardiac Fibroblast Activation**

Upon stimulation by hormones and cytokines, active fibroblasts may proliferate, migrate, or differentiate. Classic inducers of CF proliferation include fibroblast growth factor (FGF) and epidermal growth factor (EGF). These potent growth factors bind their respective tyrosine kinase receptors and trigger intrinsic kinase activity. The activated receptors signal to SOS through GRB2, activating Ras and the classical MAPK pathway of Raf, MEK and ERK 1/2. ERK 1/2 signals to transcription factors such as Elk-1 and c-Myc to induce proliferation (Fig. 6).
Figure 7. Angiotensin II triggers calcium release and PKC activation in cardiac fibroblasts. Angiotensin II (ANG II) activates the classical Gq pathway via the AT1R. The Gq α subunit signals to phospholipase C β (PLC β) to cleave phosphatidylinositol-4,5-bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 triggers intracellular calcium release from the endoplasmic reticulum (ER). The intracellular calcium and DAG have to potential to activate classical PKC isoforms. DAG alone may activate novel PKC isoforms. Atypical PKC isoforms are activated independently of intracellular calcium and DAG.
Another potent inducer of CF proliferation is angiotensin II (ANG II). Typically ANG II is a key regulator of blood pressure by inducing contraction of the vascular smooth muscle cells that encircle blood vessels. ANG II has other cardiovascular effects including stimulation of CF proliferation, ECM production and myofibroblast differentiation (Booz et al., 1994; Brilla et al., 1995; Naugle et al., 2006; Olson et al., 2005; Swaney et al., 2005). ANG II binds to either the AT1 or AT2 cell membrane receptors, although each boasts a distinctive downstream effect. The AT1 receptor is the predominant subtype in VSMCs and CFs and modulates the classical blood pressure effects as well as the ANG II-induced CF proliferation and ECM production (Booz et al., 1994; Brilla et al., 1997; Brilla et al., 1995; Olson et al., 2005). The specific signaling intermediates of ANG II-induced ERK 1/2 activation were yet to be elucidated. The AT2 receptor is important during fetal development and has an antagonist effect against the AT1 receptor functioning in cardioprotection (Kim and Iwao, 2000).

Activation of the AT1 receptor signals to the classical Gq signaling pathway which triggers release of calcium from intracellular stores and production of diacylglycerol (Orr et al.) (Fig. 7). Protein kinase C (PKC), a key mediator in a multitude of signaling cascades, may be activated by calcium and DAG or a phorbol ester such as phorbol 12-myristate 13-acetate (PMA) (Larsson, 2006). There are three major classifications of PKC isoforms: classical, novel, and atypical. Classical PKCs, PKCα, PKCγ and PKCβ, require both calcium and a phorbol ester or DAG for activation. Novel PKCs, PKCδ, PKCe, PKCη and PKCθ require a phorbol ester or DAG for activation but do not contain a calcium binding site. Atypical PKCs, PKCζ and PKCλ, do not require neither calcium nor a phorbol ester for activation.
Figure 8. Transforming growth factor-β induces myofibroblast differentiation. Transforming growth factor-β (TGF-β) binds its receptor inducing dimerization. The activated receptor recruits and phosphorylates SMAD2/3, which interacts with SMAD4 and this SMAD complex translocates to the nucleus and functions as a transcription factor. The activated receptor also activates the RhoA cascade to induce stress fiber formation.
TGFβ → SMAD2/3 → ROCK → LIMK → Cofilin → Stress Fibers

SMAD2/3 → SMAD4 → Translocation to nucleus to activate transcription

RhoA
ANG II stimulates CF proliferation through the AT1 receptor and activation of the mitogenic activated protein kinase (MAPK) cascade, with ERK 1/2 acting as the main effector. In other cardiac cell types, ANG II induces ERK 1/2 phosphorylation through multiple mechanisms; in vascular smooth muscle cells (VSMCs) this occurs via Ca\(^{2+}\) and Src-dependent EGFR transactivation (Bokemeyer et al., 2000; Eguchi et al., 1998; Voisin et al., 2002). In neonatal CFs, ANG II activates ERK 1/2 through the intracellular Ca\(^{2+}\) and classical PKC activation, and in MCF-7, breast cancer cells, phosphotidylinositide 3-kinase, PI3-K, and PKC\(\zeta\) contribute to ANG II-induced ERK 1/2 phosphorylation (Booz et al., 1994; Liao et al., 1997; Muscella et al., 2003; Nakanishi et al., 1993). The specific pathway for ANG II-induced ERK 1/2 phosphorylation has yet to be elucidated in adult cardiac fibroblasts and is the subject of the studies described in chapter 4.

Akt, also known as protein kinase B, regulates a diverse array of cell activities including protein synthesis, cell survival, glucose metabolism, and proliferation. Akt manipulates cell proliferation through suppression of cyclin-dependent kinase inhibitors, including p21 (Zhou et al., 2001). Akt activates MDM2, a key repressor of p53, a tumor suppressor that is also a classic regulator of the cell cycle (Zhou et al., 2001). Inhibition of p21 and p53 allows for cell cycle progression and proliferation.

Myofibroblast differentiation is regulated by factors including mechanical stress, collagen VI, and the most commonly studied TGF-\(\beta\). The classical TGF-\(\beta\) differentiation pathway begins with receptor activation, which phosphorylates and activates SMAD2/3 (Fig. 8). SMAD2/3 binds to a coSMAD, SMAD4, and the SMAD complex translocates to the nucleus to function as a transcription factor. SMAD 3 is required for maximal collagen I expression but not for \(\alpha\)-SMA expression in myofibroblasts (Schnabl et al.,
Figure 9. Cardiac fibroblast migration. CF migration is a precisely orchestrated process with specifically regulated activities at both the trailing and leading edge of the cell. At the trailing edge, focal adhesions are broken down and the cell detaches from the matrix. At the leading edge, focal adhesions are re-assembled, actin polymerization occurs, and the cell attaches to the matrix.
Migration

- focal adhesion disassembly
- cell detachment

- lamellipod formation
- focal adhesion assembly
- actin polymerization
- cell attachment
A distinct feature of the myofibroblast that is regulated by TGFβ is the formation of supermature focal adhesion, containing vinculin, talin, paxillin, and FAK. Activation of the TGF-β receptor may also signal to RhoA to induce stress fiber formation through the ROCK/LIMK pathway. Mechanical stress stimulates cytoskeletal rearrangement and the formation of the myofibroblast stress fibers (Tomasek et al., 2002). Interestingly, the direct pathway for type VI collagen-induced differentiation has yet to be elucidated.

Fibroblast migration requires multiple steps for proper motion; the major stages are cytoskeletal reorganization, lamellipod extension, cell adhesion at the leading edge, contraction of the cell body pulling it forward, and release of the trailing edge (Fig. 9) (Lauffenburger and Horwitz, 1996). The cytoskeletal reorganization is an important aspect to consider when studying fibroblasts and myofibroblasts, since the hallmark of the myofibroblast is an increase in the cytoskeletal protein α-SMA. The increased cytoskeletal filaments, along with more mature focal adhesions, may hinder myofibroblast migration compared to normal fibroblasts.

Cell migration can be regulated by multiple factors but focal adhesion kinase (FAK) is the best described mediator (Schlaepfer et al., 2004). FAK regulates cell-to-matrix and matrix-to-cell signaling through the intracellular focal adhesion that is comprised of integrin receptors and multiple linker proteins including talin, paxillin, and vinculin. These focal adhesion linker proteins connect the integrin receptor that is interacting with extracellular matrix components, to the actin cytoskeleton. Activation of the focal adhesion triggers autophosphorylation of FAK, in turn, activating a multitude of signaling pathways such as Akt for survival and ERK 1/2 for proliferation. FAK may
also stimulate cell adhesion causing the formation of focal adhesions that are required for migration (Richardson and Parsons, 1996). An important aspect of signaling from the focal adhesion is that it is bi-directional; ECM components may signal to the cell in an outside-in signaling fashion and other agonists can signal to the integrin by activating their respective receptors triggering a cascade with the final target of the integrin which is referred to as inside-out signaling. The Rho family is another important regulator of the migration. Typically Rho/Rac regulates actin remodeling and cytoskeletal reorganization, which are necessary steps to facilitate cell migration.

**Significance of diabetes and hyperglycemia on the myocardium**

The American Diabetes Associated reported that in 2007 approximately 23.6 million Americans suffered from Diabetes Mellitus, a disease characterized by hyperglycemia. The three major forms of diabetes are type 1, type 2 and gestational, all of which arise due to complications from insulin, the key signaling component of glucose metabolism. After a carbohydrate-rich meal, glucose enters the beta cells of the pancreas through GLUT1/2 receptors, providing the substrate for glycolysis and the Krebs cycle to produce ATP. Insulin release is triggered by ATP that initiates ATP-K⁺ channel closure and depolarization of the cell membrane. Depolarization causes the opening of the L-type Ca²⁺ channels and Ca²⁺ flows into the cell that signals to the secretory vessels containing the stored insulin to be released and completes the first stage of insulin release. The second stage of insulin release is a slower, glucose-independent process that involves the production of new insulin. The major target cells for insulin are adipocytes since they are the main energy storage site in the body and muscle cells due to their high energy
requirement to produce movement, but insulin also signals to many other cell types including cardiac fibroblasts. Once insulin binds its receptor, insulin receptor substrate (IRS) is recruited to the receptor, activated, and signals to numerous mediators, the most important of which is PI3-K. PI3-K phosphorylates Akt which activates GLUT4 translocation, allowing glucose to enter the cell, as well as glycolysis to facilitate the conversion of glucose to pyruvate and free energy release. Akt also triggers multiple energy storage pathways such as fatty acid synthesis and glycogen synthesis.

Type 1 diabetes, formally known as juvenile diabetes, arises when the pancreas does not produce or secrete insulin. Type 2 diabetes arises from either insufficient insulin production, but not complete ablation of insulin release as occurs in type 1, or more commonly from the inability of target cells to recognize insulin, also known as insulin insensitivity. Gestational diabetes may arise in women during pregnancy and cause a higher risk of developing type 2 diabetes following parturition.

The progression of diabetes affects multiple organs producing complications including kidney disease, retinopathy, stroke, and deficits in wound healing. In the vasculature, complications such as hyperlipidemia, atherosclerosis, and hypertension often arise. Many of these complications occur since the pathways downstream of insulin signaling regulate fatty acid synthesis and metabolism. Angiogenesis is also altered by diabetes: diabetic patients may suffer from both abnormal and insufficient peripheral angiogenesis and coronary angiogenesis (Abaci et al., 1999; Martin et al., 2003).

Type 1 diabetic patients are ten times more likely to develop cardiovascular disease compared to the general population (Deckert et al., 1978; Dorman et al., 1984; Orchard et al., 2006). Cohen-Solal et al. (2008) reported that 30% of patients with heart failure were also
diabetic (Cohen-Solal et al., 2008). Post-MI, diabetic patients are at a higher risk to develop heart failure (Mak et al., 1997). Boyer et al. (2004) disclosed that up to 75% of diabetic patients display diastolic dysfunction (Boyer et al., 2004). Diabetic patients are prone to developing diffuse cardiac fibrosis, specifically a drastic increase in fibrillar type III collagen (Shimizu et al., 1993). The specific cause of this collagen deposition has yet to be determined, therefore, we will investigate the potential influence of high glucose as a possible source of fibroblast and myofibroblast activation. Hyperglycemia alters proliferation and migration in numerous cell types; Neumann et al. (2002) revealed that high glucose stimulated myofibroblast proliferation (Neumann et al., 2002). Interestingly, neonatal fibroblasts plated on type I collagen migrate slower and proliferate less when cultured in high glucose media yet our lab previously determined that type I collagen stimulates adult CF proliferation (Naugle et al., 2006; Zhang et al., 2007). Hyperglycemic conditions in vitro increase cardiac fibroblast collagen production (Muona et al., 1993; Tang et al., 2007). We will investigate how type 1 diabetes and hyperglycemia alters fibroblast function both in vivo and in vitro in chapters 5 and 6.
Specific Aims and Hypotheses

The overall goals of the studies outlined in this dissertation are to determine the impact of the ECM and diabetes on cardiac fibroblast function, and our hypothesis was that both the ECM and hyperglycemia will activate CFs in distinct ways. First we focus on the influence of ECM components on fibroblast function. Second, we concentrate on the specific signaling of ANG II-induced ERK 1/2 activation and fibroblast proliferation. In the last two chapters we apply both the influence of the matrix and specific signaling to a diabetic disease state and the overall effect on fibroblast activation and function through utilization of a well established type 1 streptozotocin diabetic model. Given that cardiac fibrosis is a common diabetic complication, we set out to determine how hyperglycemia affects fibroblast function that would account for the increased matrix deposition.

1.) To determine the influence of collagen types I, III, VI on fibroblast and myofibroblast migration and the specific integrin receptor that mediates collagen VI myofibroblast differentiation.

   We hypothesize that collagen types I and III will promote migration, whereas type VI will slow migration and induce differentiation through the α3 integrin.

2.) To determine the specific PKC isoform mediating Angiotensin II-induced ERK 1/2 phosphorylation.

   We hypothesize that a classical PKC isoform, PKCα or PKCβ, will mediate ANG II-induced ERK 1/2 activation.
3. a.) To determine whether hyperglycemia alters cardiac fibroblast proliferation, migration, and myofibroblast differentiation.

We hypothesize that hyperglycemia will promote fibroblast proliferation and inhibit migration and myofibroblast differentiation.

b.) To determine whether changes in ECM composition can alter the function of cardiac fibroblasts in hyperglycemic conditions.

We hypothesize that the addition of collagen types I and III will increase hyperglycemia-induced fibroblast proliferation and counteract the high glucose reduced migration.

4.) To determine the influence of type 1 diabetes in vivo on fibroblast activation (proliferation and differentiation).

We hypothesize that type 1 diabetes will increase fibroblast proliferation and myofibroblast differentiation in the diabetic rat myocardium.
CHAPTER TWO

General methods (common to all chapters)

Isolation of cardiac fibroblasts

Cardiac fibroblasts were isolated as previously described (Bryant et al., 2009; Naugle et al., 2006; Olson et al., 2008). Left ventricles were excised from adult male Sprague Dawley rats and rinsed with cold PBS. Following a brief rinse with isolation buffer, individual ventricles were minced and digested for 90 minutes in isolation buffer containing collagenase type 2 (100 U/mL) and trypsin (0.6 mg/mL). The fibroblasts were collected by centrifugation, resuspended in DMEM containing 10% FBS and plated on tissue culture dishes. After 3-4 days, the confluent cultures were passaged by trypsin treatment and split 1:3. Passage 2 cells were used for all fibroblast experiments and passage 4 or 5 for all myofibroblast experiments.

In vitro wound healing assay

Cardiac fibroblasts and myofibroblasts were plated onto collagens type I, III, VI and tissue culture plastic. Cells were grown to 90-100% confluency, and then scratched with a yellow 200 μL pipet tip. Wounds were washed with 10% PBS and fresh 2% FBS DMEM media was added. Time-lapsed images were taken at 0, 6, 12, 24 and 48 hours.
Western blot analysis

Left ventricles were isolated from adult Sprague-Dawley rats and tissue samples placed on ice in lysis buffer (62.5mM Tris•HCl, 2 mM EDTA, 2.3% SDS, 10% glycerol (pH6.8), and protease inhibitors (1mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, and 5 μg/ml aprotinin) for 15 minutes then homogenized with a polytron for 10 seconds, this was repeated three times with a 10 minute incubation on ice between each homogenization. Samples were centrifuged at 4,500 g for 10 minutes and the supernatant was collected and stored at -20°C. Protein levels were quantified with the BCA method (Pierce) and equal amounts of protein were mixed with 2X sample buffer (100mM Tris base, 20% glycerol, 2% SDS, and 0.01% bromophenol blue) and boiled for 5 minutes. The proteins were separated by SDS-PAGE and transferred to a PVDF membrane by electrolysis. The membranes were blocked in 0.1% Tween-20-TBS containing either BSA or milk for one hour at room temperature then incubated overnight in primary antibody and washed three times in 0.1% Tween-20-TBS following antibody incubation. The membranes were incubated in secondary antibody for one hour at room temperature then washed five times in 0.1% Tween-20-TBS. The protein signals were detected using ECL supersignal (Pierce) and the band intensity was quantified by densitometric scanning using a Kodak 1D Digital Science Imaging System.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4.0 statistical software. Statistical significance (p<0.05) between groups was determined by one-way ANOVA followed by paired t-tests or tukey’s post hoc test.
CHAPTER THREE

The influence of matrix components on cardiac fibroblast activation: focus on proliferation, migration, and differentiation

INTRODUCTION

The adult myocardium is comprised of a network of fibrillar (I, III, and V) and non-fibrillar (IV and VI) collagens, with collagen types I and III accounting for approximately 90% of the ventricular collagen (Borg et al., 1982; Eghbali et al., 1989; Weber et al., 1994). Classically, the ECM has been viewed merely as a structural meshwork encapsulating the cells of the myocardium, however, our lab and others have elucidated the potential of these matrix components to alter cell proliferation and differentiation. Elliott et al. (2005) determined that type I collagen in its native fibrillar form reduces smooth muscle cell proliferation (Elliott et al., 2005). Type I collagen also reduced H9C2 myoblast proliferation while type IV enhanced proliferation (Macfelda et al., 2007). Collagen type V repressed both proliferation and attachment of VSMCs (Sakata et al., 1992). Our lab revealed that collagen types I and III potently stimulate cardiac fibroblast proliferation whereas type VI-induced myofibroblast differentiation in vitro (Naugle et al., 2006). We next intended to determine the influence of the collagen substrates on fibroblast migration.
Collagen and other components of the ECM interact and signal to the fibroblasts through integrins, membrane spanning receptors that also interact with the actin cytoskeleton and other intracellular proteins at focal adhesions. Integrin receptors are a large family of heterodimeric receptors composed of α and β subunits. To date, 18 α subunits and 8 β subunits have been identified; in the heart, myocytes express the α1, α3, α5, α6, α7, α9, and α10 subunits whereas fibroblasts express the α1, α2, α3, α5, α9, α10, and αV subunits, which mainly dimerize with the β1 subunit although few studies have reported β3 and β5 interaction (Ross and Borg, 2001). We previously determined that collagen VI promotes myofibroblast differentiation and revealed that type VI is elevated at 7 days post-MI and remains elevated at 20 weeks, both correlated to increased myofibroblast content (Bryant et al., 2009; Naugle et al., 2006). The next aim was to determine the specific integrin that facilitates type VI collagen-induced differentiation. Collagen VI has been shown to interact with α3β1 in corneal fibroblasts, therefore we selected α3β1 as one potential target for the cardiac myofibroblast collagen VI mediated differentiation (Doane et al., 1998; Wayner and Carter, 1987).

MATERIALS AND METHODS

Materials

DMEM, fungizone, penicillin/streptomycin, and FBS were all purchased from Invitrogen/GIBCO (Carlsbad, CA). Collagenase type 2 and trypsin were acquired from Worthington Biochemical Corporation (Lakewood, NJ). The anti-α-SMA, collagen I substrate, and collagen III substrate were purchased from Sigma-Aldrich (St. Louis, MO).
Native collagen type VI was isolated according to Doane et al. (1992). Anti-GAPDH antibody was obtained from Cell Signaling Technology (Boston, MA). The anti-α3 integrin antibody and function blocking antibody were both acquired from Chemicon/Millipore (Billerica, MA). The BrdU proliferation assay was purchased from Roche Applied Science (Indianapolis, IN). Vectashield Mounting Medium was purchased from Vector Laboratories (Burlingame, CA). All other reagents and chemicals were obtained from VWR (West Chester, PA).

**BrdU proliferation assay**

BrdU labeling and detection was executed following the instructions provided by Roche Applied Science. In brief, *in vitro* wound healing assays were performed on glass dot-well slides with the addition of BrdU into the media added immediately following wound-induction. After 9 hours, cells were fixed with an ethanol/glycine fixative at -20°C for 30 minutes. The slides were washed three times in PBS/tween followed by 30 minute incubation with anti-BrdU working solution. The slides were covered with Anti-mouse-Ig-AP solution for 30 minutes and then washed. Color-substrate solution was added to the cells for 30 minute incubation. The slides were mounted with Vectashield mounting medium containing DAPI for nuclear visualization.

**α3 integrin blockade of collagen VI mediated myofibroblast differentiation**

Cardiac fibroblasts were plated onto collagens type I, III, VI and tissue culture plastic. Two hours post-plating, the media was aspirated and fresh serum-free media was added with and without the α3 function blocking antibody. Whole cell lysates were
Figure 10. Collagen-induced wound healing, migration and proliferation of cardiac fibroblasts.  

A. Passage 2 fibroblasts were plated onto the specific collagen substrates and wounds were photographed at 0, 12, and 24 hours. At the 12 H time point the wounds were near closure, approximately 90% confluent, on both types I and III collagen. By 24 H, the wounds were fully closed on tissue culture plastic, type I and type III, this was not the case with type VI collagen.  

B. Since both migration and proliferation occur during wound healing, BrdU (green) incorporation was measured to determine proliferation by counting the number of cells with BrdU incorporated into the nuclei and dividing by the total number of cells in the wound indicated by DAPI (blue) nuclear stain. At 9 hours approximately 5-15% of the wound closure on each substrate occurred through proliferation. Therefore, collagens type I and III mainly increase the rate of migration of CFs rather than stimulating proliferation during wound healing.
A.

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<th>Tissue Culture</th>
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<th>12 Hour</th>
<th>24 Hour</th>
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<td>Type VI</td>
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B.

Brdu

Type:

Type I

Type III

Type VI
collected after 48 hours in lysis buffer (62.5 mM Tris-HCl, 2 mM EDTA, 2.3% SDS, 10% glycerol, pH 6.8, protease inhibitor cocktail containing pepstatin A, E-64, bestatin, leupeptin, aprotinin, and phosphatase inhibitor cocktail 1 containing cantharidin, bromotetramisole, and microcystin LR). Cell lysates were sonicated 3 times for 5 seconds. Total protein content was determined by the BCA method and equal amounts of protein were run on SDS PAGE and subjected to Western blot analysis for the \( \alpha \)-SMA and GAPDH.

RESULTS

**Collagen types I and III enhance cardiac fibroblast migration; collagen VI slows fibroblast migration**

Our lab previously revealed that collagen types I and III stimulate CF proliferation and collagen VI promotes myofibroblast differentiation, we next set out to determine how the collagen environment influences fibroblast migration (Naugle et al., 2006). Utilizing the *in vitro* wound healing assay, we determined wound closure of CFs plated on control tissue culture plastic was approximately 10-20% 6 hours after wound induction and confluency of CFs on type I and III was 40-50% and 75-85%. By 12 hours the wounds were completely closed on type III (95-100% confluent) and 80-90% confluent on collagen type I, but the CFs on control plastic lagged behind at 40-50% confluency (Fig. 10A). At 24 hours the wounds were fully closed on all three substrates (Fig. 10A). To confirm the wound closure represented the migration of fibroblasts as opposed to proliferation, BrdU assays were performed with the *in vitro* wound healing
Figure 11. Reduced migration of myofibroblasts. Passage 4 CFs/myofibroblasts were plated onto specific collagen substrates. Wounds were photographed at 0, 12, and 24 hours. Unlike P2 CFs, full wound healing did not occur. Note and compare the wounds on collagens type I and III at 12 H for both P2 (fig. 10) and P4. P2 were approximately 90% confluent and P4 were only 40% confluent. These data demonstrate that myofibroblasts (P4) migrate at a slower rate than cardiac fibroblasts (P2).
Tissue Culture Control

Type I

Type III

Type VI

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<td>Type VI</td>
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assay. At 9 hours post-wound induction, 13% of the cells in the wound on control plastic stained positively for BrdU, and 17% and 8.5% were BrdU positive on types I and III, respectively (Fig. 10B).

Utilizing the *in vitro* wound healing scratch assay we determined type VI collagen reduced the rate of cardiac fibroblast migration. Wounds were 5-15% confluent 6 hours post-injury. At 12 hours after injury, the confluency of the wounds was 10-20% on type VI collagen and 40-50% on tissue culture plastic (Fig. 10A). 24 hours post-injury, the wounds were fully closed on plastic but the wounds on type VI collagen did not fully close until 48 hours after wound induction (Fig. 10A).

**Myofibroblasts have reduced migratory ability**

Migration occurs through the turnover of focal adhesions and the cytoskeleton; actin is broken down at the trailing edge and is re-assembled at the leading edge of a migrating cell. The hallmark of the myofibroblast phenotype is the appearance of both supermature focal adhesions and stress fibers, large aggregate bundles containing $\alpha$-SMA fibers. Since the myofibroblasts have increased cytoskeletal filaments such as $\alpha$-SMA and well-defined supermature focal adhesions, we hypothesized that cardiac myofibroblasts will migrate slower than undifferentiated fibroblasts. Our lab has previously established that fibroblasts spontaneously differentiate in culture by passage 4 (Swaney et al., 2005). Passage 4 myofibroblasts were plated onto specific collagen substrates and tissue culture plastic. Wounds were observed at 0, 12, and 24 hours and unlike P2 CFs, full wound healing at 24 hours did not occur on any substrate (Fig. 11).
Figure 12. \( \alpha_3 \) integrin receptor blockade inhibits type VI collagen induced \( \alpha \)-SMA expression.  

A. Cardiac fibroblasts were plated overnight on tissue culture plates pre-coated with 20 µg/ml of purified collagen I, III, or VI and cross-linked to the matrix with DTSSP. The cells were dissolved away and the remaining focal adhesion proteins were subjected to SDS-PAGE. Immunoblots for \( \alpha_3 \) integrin demonstrate the interaction with the collagen substrates.  

B. CFs were plated on the indicated substrates in the presence and absence of the \( \alpha_3 \) function blocking antibody. After 48 hours cell lysates were collected and subjected to SDS-PAGE and Western blot analysis for \( \alpha \)-SMA and GAPDH for loading controls. The image is representative of 5 separate experiments.  

C. Representative napthol blue loading control blot (non-specific protein stain).
A. α3 integrin
118 kD

TC plastic  Collagen I  Collagen III  Collagen VI

B. α3 FB Ab

α-SMA

GAPDH

36 kD

TC Plastic  Collagen I  Collagen III  Collagen VI

C. Loading control: napthol blue

% Control

Collagen VI  VI + FB Ab
Note and compare the wounds on collagen type III at 12 H for both P2 and P4: P2 were 95% confluent and P4 were only 40% confluent.

**Type VI collagen interacts with the α3 integrin to induce myofibroblast differentiation**

We previously revealed that the α3 integrin subunit is elevated at 3 days post-MI in both the infarcted and non-infarcted regions and type VI collagen is elevated at 7 days post-MI, correlating to an increase in myofibroblast expression (Bryant et al., 2009). We also determined that collagen types III and VI interact with the α3 integrin in cardiac fibroblasts (Bryant et al., 2009). We next wanted to determine whether the interaction between collagen VI and the α3 integrin receptor contributes to the type VI collagen-induced myofibroblast differentiation. We blocked the interaction of the collagen substrates and the α3 integrin receptor by utilizing a function blocking antibody. The α3 function blocking antibody reduced type VI collagen-induced α-SMA expression by 26.2 ± 13% over control, but had little effect on α-SMA expression on naive plates and type I and III collagen (Fig. 12).

**DISCUSSION**

The cardiac ECM provides structural integrity for the myocytes and fibroblasts and regulates cell activity by interacting with integrin receptors on the cells surface. In this study the aim was to determine the influence of the ECM on fibroblast and
myofibroblast migration and differentiation. We observed that the fibrillar collagen types I and III highly promoted CF and myofibroblast migration. Given our data, we postulate a role for type III collagen in fibroblast and myofibroblast recruitment to the injury site, since collagen III most potently stimulated fibroblast migration in our wound healing assays. Interestingly, collagen III protein expression is the first significantly elevated collagen in the infarct site at 3 days post-MI (Cleutjens et al., 1995b; van den Borne et al.). Collagen I deposition increases after collagen III and is significantly less than the initial collagen III deposition following an MI (Cleutjens et al., 1995b; van den Borne et al.). Many investigators deem the increased type I collagen post-MI as the important structural component to the fibrotic scar since it provides the most tensile strength, but the function of type III collagen is less understood.

Interestingly, the sequence of events in skin remodeling suggests a role for collagen III in early remodeling; during the proliferative phase initially collagen type III is synthesized and later during the remodeling phase collagen I is deposited. This expression pattern may indicate that collagen III fibroblast recruitment may have more global importance (Clark, 1985). The specific mechanism of CF regulated migration has yet to be determined. Carragher et al. (1999) revealed that collagen I promoted focal adhesion breakdown and cell detachment, therefore one potential mechanism is through accelerated focal adhesion turnover (Carragher et al., 1999). Collagen type I also enhances the migration of other cell types, 3T3 fibroblasts, human dermal fibroblasts and keratinocytes (Gaudet et al., 2003; Li et al., 2004; O'Toole, 2001). Blockade of the $\alpha_2\beta_1$ integrin receptor inhibits the collagen type I-induced keratinocyte migration (Kim et al.,
Collagen types I and III promoted airway SMC migration through Src activation, although migration was greater on collagen type III than both control and collagen type I (Parameswaran et al., 2004). Blockade of the $\alpha_5$, $\alpha_v$, $\beta_1$ integrin subunits prevented the SMC collagen I-induced migration through cell adhesion inhibition (Parameswaran et al., 2004). Surprisingly collagen I impaired MDCK cell migration through loss of the lamellipodia and focal adhesion breakdown via activation of $G\alpha_{12}$ signaling and the Rho pathway (Kong et al., 2009). We postulate that the discrepancy between our data and other studies is due to cell type specificity and integrin interaction. Integrin receptors are the key mediators between the matrix and the cell; each cell type expresses a unique integrin profile that can change during pathological conditions. Cardiac fibroblasts express $\alpha_1$, $\alpha_2$, $\alpha_3$, $\alpha_5$, $\alpha_9$, and $\alpha_{10}$, and $\alpha_v$ whereas MDCK epithelial cells express $\alpha_2$, $\alpha_3$, and $\alpha_6$ (Ross and Borg, 2001; Schoenenberger et al., 1994). Integrin receptors exhibit substrate specificity: the four major $\alpha$ subunits that interact with fibrillar collagen are $\alpha_1$, $\alpha_2$, $\alpha_{10}$, and $\alpha_{11}$, while $\beta_1$ is the classical $\beta$ subunit in the collagen-integrin interaction (Hynes, 2002; Vogel, 2001). The $\alpha_3$, $\alpha_6$, and $\alpha_7$ subunits are the laminin receptors that typically interact with either the $\beta_1$ or $\beta_4$ subunit. It is important to note that we have shown the interaction of $\alpha_3$ and collagen VI, and given that $\alpha_3$ is a laminin binding integrin, this interaction must be occurring at the glycoprotein globular heads of type VI (Hynes, 2002).

Unlike the fibrillar structure of collagen types I and III, type VI is assembled in a unique end to end fashion forming a beaded filament structure with large globular glycoprotein ends flanking the short alpha helices containing center region (Bashey et al., 1992).
Figure 13. Summary model of the role of specific collagen substrates in cardiac in vitro wound healing. Following an injury, cardiac fibroblasts secrete copious amounts of ECM. The ECM influences fibroblast activity; collagen types I and III promote proliferation and migration, whereas collagen VI induces myofibroblast differentiation.
Wound Healing

Collagen

Type I
- Migration
- Proliferation

Type III
- Migration
- Proliferation

Type VI
- Differentiation
1992; Pfaff et al., 1993). Our lab has recently focused on the importance of type VI in the heart demonstrating that collagen type VI is a potent inducer of cardiac myofibroblast differentiation in vitro and collagen VI slightly stimulated fibroblast proliferation but did not reach statistical significance (Naugle et al., 2006). We next focused on the influence of collagen VI on fibroblast migration and as predicted type VI highly reduced CF migration. At 12 hours, the fibroblasts plated on collagen VI lagged behind the cells plated on plastic by approximately 30%. Since we also determined that myofibroblasts migrate slower compared to early passage fibroblasts, we initially hypothesized that the type VI collagen-induced migratory lag was from collagen VI stimulating myofibroblast differentiation. We next explored the impact of collagen types I, III, and VI on myofibroblast migration and determined that types I and III promoted myofibroblast migration but to a lesser extent than that of normal fibroblasts. Surprisingly, myofibroblasts migrated much slower on collagen VI compared to control and the other collagens, indicating that the type VI collagen-induced fibroblast migration lag is not due to myofibroblast differentiation as we initially hypothesized. Another potential explanation is that the adhesive nature of the beaded filament structure slows migration. Again, integrin receptors mediate migration and the specific integrin receptors interacting with collagen VI may be anti-migratory in nature. Our lab has thoroughly explored the influence of collagen types I, III, and VI on fibroblast activation in vitro, these data are summarized in Figure 13.

Collagen VI has previously been perceived as a minor ECM component in the myocardium, although recently a few studies have suggested an important role for type
VI collagen in cardiac pathologies. Type VI collagen is elevated in hypertensive and diabetic rat models (Spiro and Crowley, 1993), as well as in human hypertrophic cardiomyopathy (Kitamura et al., 2001). Our lab revealed that collagen type VI is a potent inducer of cardiac myofibroblast differentiation *in vitro* and is elevated at both 7 and 14 days as well as 20 weeks post-MI (Naugle et al., 2006). Integrin receptors are important transducers of ECM signaling in both myocytes and fibroblasts. Type VI collagen can potentially interact with the $\alpha_1$, $\alpha_2$, $\alpha_3$, and $\alpha_v$ integrin receptors (Vogel, 2001). Cardiac fibroblasts have been reported to possess the multiple integrin receptors including the $\alpha_3$ subunit that has been demonstrated to interact with collagen VI (Doane et al., 1998; Thibault et al., 2001; Wayner and Carter, 1987), and our cross-linking data also reveal that it interacts with types III and VI collagen. Our *in vitro* function blocking data indicate that the binding of type VI collagen and the $\alpha_3$ integrin receptor contributes to the myofibroblast differentiation and that blockade of this interaction attenuates the differentiation process. *In vivo*, we reported that this receptor is significantly elevated 3 days following MI and returns to control levels by day 14, but is elevated again at 16 weeks post-MI along with increased collagen VI and myofibroblast content (Bryant et al., 2009; Naugle et al., 2006). During the early phase of post-MI remodeling, expression of inflammatory mediators, such as TGF $\beta$, is increased and function as the likely candidates responsible for inducing myofibroblast appearance (Petrov et al., 2002; Sun et al., 2000). Sun et al. (200) revealed that TGF-$\beta$ expression declines 14 days post-MI, therefore is not likely to play a significant role in the late remodeling phase (Sun et al., 2000). An interesting biphasic pattern to myofibroblasts appearance has emerged from our data and importantly the $\alpha_3$ integrin also displays a biphasic pattern preceding the myofibroblast
appearance, therefore, we hypothesize that the $\alpha_3$ integrin is mediating the collagen VI-induced myofibroblast appearance during the late phase of remodeling. Cell-matrix interactions are a critical aspect of tissue remodeling and repair that require further studies to understand the basic mechanisms that regulate normal and pathological cardiac remodeling.
CHAPTER FOUR

*Angiotensin II-induced ERK1/2 activation is mediated by intracellular calcium and PKCδ*

INTRODUCTION

Cardiac fibrosis is characterized by an increase in the structural collagens (I and III) and may develop from overactive fibroblasts and aberrant remodeling, eventually leading to reduced contractility which ultimately contributes to heart failure (Brilla, 2000; Weber and Brilla, 1991). Cardiac fibroblasts, key regulators of cardiac remodeling, comprise approximately 20% of the myocardial mass (Dostal et al., 1992). During normal remodeling new matrix is laid down by the active fibroblasts and old matrix is degraded by MMPs, but if remodeling is not regulated properly there will be an accumulation of matrix leading to fibrosis. In addition to secreting ECM components, activated cardiac fibroblasts proliferate, migrate, and differentiate to the hypersecretory myofibroblast, a cell type that is critical to the remodeling and wound healing of damaged tissue (Campbell et al., 1995; Petrov et al., 2002; Sun et al., 2000). The classical stimuli that activate cardiac fibroblasts include angiotensin II and TGF-β; angiotensin II induces proliferation and TGF-β induces myofibroblast differentiation (Dostal et al., 1996; Petrov et al., 2002; Schorb et al., 1993). ANG II has been shown to be a potent activator of CFs, and in cultured CFs, activation of the ANG II type 1 receptor (AT₁R) stimulates both cellular proliferation and de novo collagen
synthesis (Dostal et al., 1996; Schorb et al., 1993; Swaney et al., 2005). Our lab and others have demonstrated the mitogenic properties of ANG II on cardiac fibroblasts. ANG II-induced proliferation of CFs has been shown to be dependent upon activation of the extracellular signal-regulated kinase (ERK) 1/2 (Olson et al., 2005; Schorb et al., 1993).

Increased circulating angiotensin II (ANG II) commonly occurs during multiple cardiovascular diseases such as hypertension. ANG II activates cardiac fibroblasts to stimulate both proliferation and de novo collagen synthesis in vitro through activation of the ANG II type 1 receptor (AT_1R) (Brilla et al., 1997; Dostal et al., 1996; Schorb et al., 1993; Schorb et al., 1995; Swaney et al., 2005). Our lab and others have established the mitogenic properties of ANG II on fibroblasts and ANG II-induced proliferation of CFs has been shown to be dependent upon activation of the extracellular signal-regulated kinase (ERK) 1/2 cascade (Olson et al., 2005; Schorb et al., 1993; Schorb et al., 1995; Stockand and Meszaros, 2003), however, the signaling intermediates leading to ERK1/2 activation were not yet elucidated.

ANG II stimulates the release of intracellular calcium from the endoplasmic reticular and triggers the production of diacylglycerol (Orr et al.), together they stimulate various isoforms of protein kinase C (PKC). Calcium is a major mediator in multiple signaling pathways including the mitogenic ERK 1/2. One potential mechanism by which GPCRs, such as the AT_1R, induce ERK 1/2 activation is by transactivation of the epidermal growth factor receptor (EGFR). In vascular smooth muscle cells (VSMCs), AT_1R-induced ERK 1/2 activation occurs via Ca^{2+} and Src-dependent transactivation of
the EGFR (Bokemeyer et al., 2000; Eguchi et al., 1998; Voisin et al., 2002). In neonatal CFs, phorbol ester-sensitive PKCs and intracellular Ca\(^{2+}\) have been shown to mediate MAPK signaling in response to ANG II (Booz et al., 1994). The EGFR also mediates β₂-adrenergic receptor (β₂-AR)-induced ERK 1/2 activation in adult CFs (Kim et al., 2002; Prenzel et al., 1999). An alternative Ca\(^{2+}\)-independent mechanism by which the AT₁R-induces ERK 1/2 activation is through phosphatidylinositide 3-kinase (PI3-K) and subsequent activation of atypical PKCζ (Liao et al., 1997; Muscella et al., 2003; Nakanishi et al., 1993). In VSMCs and in MCF-7 cells, PKCζ is critical in mediating ANG II-induced ERK 1/2 activation and cellular proliferation, since direct inhibition of PKCζ resulted in a loss of ANG II-induced ERK 1/2 phosphorylation (Liao et al., 1997; Muscella et al., 2003). Additionally, PKCζ inhibition reduced both basal and transforming growth factor-β-induced [\(^{3}\)H]-thymidine incorporation in neonatal CFs (Braun and Mochly-Rosen, 2003). Thus, the mechanism of ERK 1/2 activation by ANG II potentially involves multiple signaling mechanisms including EGFR transactivation, PKCs and intracellular Ca\(^{2+}\).

The various isoforms of PKC are common mediators for multiple cellular regulatory activities including proliferation and migration. We next set out to determine whether inhibition of specific PKC isoforms altered fibroblast migration. We particularly focused on PKCδ since inhibiting PKCδ reduced the migration of many cell types including MCF-7 cells, NR6 cells, astrocytes, and human T lymphocytes (Fanning et al., 2005; Iwabu et al., 2004; Kruger and Reddy, 2003; Renault-Mihara et al., 2006). PKCα inhibition suppressed human T lymphocyte migration through impediment of actin
polarization but PKC α inhibition did not affect MCF-7 cell migration (Fanning et al., 2005; Iwabu et al., 2004; Kruger and Reddy, 2003). The PKC isoforms may be involved in different processes of migration including cell attachment to ECM and motility through the actin cytoskeleton (Renault-Mihara et al., 2006). The aim was to determine the specific PKC isoform regulating CF migration.

**MATERIALS AND METHODS**

**Materials**

Dulbecco’s Modified Eagle’s Medium (DMEM), penicillin/streptomycin, fungizone and fetal bovine serum (FBS), Lipofectamine RNAiMax were all purchased from Invitrogen/GIBCO (Grand Island, NY). [3H]-Thymidine was from ICN Biomedicals (Irvine, CA). Anti-phospho-tyrosine, anti-phospho-PKCζ, anti-PKCα, and anti-EGFR antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-phospho-ERK 1/2, anti-ERK1/2, and anti-PKCδ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). AG 1478 and anti-PKCζ antibodies were from Sigma-Aldrich (St. Louis, MO). Fura-2/AM, BAPTA/AM, LY 294002, PP2, rottlerin, hispidin, Gö6976 and the myristoylated PKCζ pseudosubstrate were from Calbiochem (La Jolla, CA). PKCδ siRNA was from Upstate/Millipore (Lake Placid, NY).

**[3H]-Thymidine incorporation assay**

Proliferation assays were performed as previously described (Olson et al., 2005). CFs were plated on 12-well tissue culture plates and grown to 40-50% confluency. Cells
were treated in triplicate for a period of 48 hrs, with [³H]-thymidine (2 µCi/ml) being added to the media during the final 4 hrs. The media containing the label was removed and the cells were washed twice with PBS and incubated in cold 5% TCA for 30 min. Cells were washed and solubilized in 1 ml 0.5 mol/L sodium hydroxide for 30 min at room temperature and the amount of incorporated label was determined by liquid scintillation counting.

**Pretreatment of CFs using pharmacological inhibitors and PKCδ siRNA**

CFs were pretreated with the specific PKC isoform inhibitors, 10 µmol/L rottlerin for PKCδ, 10 µmol/L hispidin for PKCβ, and 1 µmol/L Gö6976 for PKCα, in either the absence and presence of BAPTA for 30 min prior to 5 min stimulation by 100 nmol/L ANG II. CFs were incubated with Lipofectamine RNAiMax, 1.25 µg/ml PKCδ siRNA, and serum/antibiotic free DMEM or DMEM alone for 24 hrs, followed by pretreatment for 30 min with BAPTA and BAPTA with rottlerin prior to 5 min stimulation by 100 nmol/L ANG II. Equal protein amounts (10 µg) were loaded on SDS-PAGE gels and subjected to Western analysis as described in chapter 2.

**Immunoprecipitation of the EGFR**

CFs were treated as indicated, washed with ice-cold PBS and then scraped on ice in lysis buffer containing (in mmol/L) 20 Tris, 150 NaCl, 1 EDTA, 1% Triton X-100, 1 Na₃VO₄, and Protease Inhibitor Cocktail, pH 7.5. Cell lysates were sonicated 3 times for 5 sec, centrifuged at 10,000 x g for 10 min at 4°C and supernatants collected. Equal amounts of protein (500 µg) were immunoprecipitated with either the anti-EGFR or anti-
phospho-tyrosine antibodies overnight at 4°C. Protein A-agarose beads were then added to the lysates and rotated for an additional 2 hrs at 4°C. Antibody complexes were centrifuged and dissociated by incubating with 3X sample buffer (187.5 Tris-HCl, 6% SDS, 30% glycerol, 150 mmol/L DTT, 0.03% bromophenol blue, pH 6.8) and boiling 5 min. Equal protein amounts were loaded on SDS-PAGE gels and subjected to Western analysis as described in chapter 2.

**Measurement of intracellular calcium**

Cardiac fibroblasts were loaded with 1 μmol/L Fura-2/AM at 37 °C for 30 min in HEPES buffered saline (HBS; in mmol/L: 130 NaCl, 5 KCl, 10 glucose, 1 MgCl₂, 1.0 CaCl₂, 25 HEPES, pH 7.4), and groups of 5–8 cells were monitored using an inverted Olympus IX-70 microscope. Spectrofluorometric measurements were collected using the Delta Scan System spectofluorometer (Photon Technology, Ontario, Canada), where the field was alternately excited at 380 and 340 nm and the emission ratio was collected at 511 nm.

**Plasma membrane translocation of PKC**

CFs were treated, washed with ice-cold PBS and collected on ice in Buffer A (20 mmol/L Tris, 250 mmol/L sucrose, 1 mmol/L EDTA, protease inhibitor cocktail, and phosphatase inhibitor cocktail). Cells were sonicated 3 x 5 sec followed by centrifugation at 1000 x g for 10 min at 4°C. Cell lysates were then spun at 100,000 x g for 1 hr at 4°C and the supernatants containing the cytosolic fraction were saved while the pellets
Figure 14. Chelation of intracellular Ca\(^{2+}\) does not block ANG II-induced ERK 1/2 activation.

A. Cardiac fibroblasts were loaded with Fura-2/AM (1 µmol/L) and pretreated for 30 min with the calcium chelator BAPTA/AM. As anticipated, BAPTA/AM reduced the ANG II-induced intracellular Ca\(^{2+}\) elevation in a concentration-dependent manner with full blockade at 30 µmol/L. Triton X-100 was added following treatment with ANG II for normalization of the ANG II response. The fluorescence ratios are representative data of 3 similar experiments. B. Representative Western blots demonstrating that pretreatment for 30 min with BAPTA/AM had no effect on ANG II-induced ERK 1/2 phosphorylation after 5 min.
Figure 15. Downregulation of PKC with PMA does not block ANG II-induced ERK 1/2 activation.  

A. Representative Western blot demonstrating PMA-induced activation of a classic isozyme PKCα. Cytosolic and membrane fractions were separated as described in Materials and Methods and subjected to SDS-PAGE. Cytosolic PKCα was reduced following treatment with 100 nmol/L PMA for 15 min, whereas membrane associated PKCα was increased over basal. 

B. Representative Western demonstrating the rapid activation of ERK 1/2 in CFs in response to PMA (5 min). 

C. Representative Western blot displaying the effects of 18 hr treatment with PMA (100 nmol/L) on PKCα expression. 

D. Representative Western blot demonstrating that downregulation of PKC with PMA treatment (18 hr) had no effect on ANG II-induced ERK 1/2 phosphorylation (5 min).
containing the membranes were resuspended in Buffer B (Buffer A containing 0.8% Triton-X 100) and kept on ice for 45 min. Cytosolic and membrane fractions were assessed by Western analysis as previously described.

RESULTS

**Mechanism of angiotensin II-induced ERK 1/2 activation is controlled by parallel calcium and phorbol ester-sensitive PKC signaling pathways**

Release of calcium from intracellular stores contributes to PKC activation and potentially to ERK 1/2 phosphorylation. Incubating CFs with BAPTA/AM is an effective means in which to buffer intracellular Ca\(^{2+}\) and prevent downstream signaling (Fig. 14A). Sequestering calcium with 30 μmol/L BAPTA/AM inhibited intracellular Ca\(^{2+}\) elevation by ANG II, but did not have a significant effect on ANG II-induced ERK 1/2 activation (Fig. 14B). We attempted to utilize BAPTA/AM in longer-term proliferation assays in this study, however, overnight treatments of this chelator proved to be cytotoxic to the cells, hence a minor limitation of the study.

Short-term exposure to PMA stimulated translocation of phorbol ester-sensitive classic and novel PKC to the plasma membrane (Fig. 15A) and induced rapid activation of ERK 1/2 (Fig. 15B). When CFs are exposed to PMA for longer time periods, phorbol ester/DAG-sensitive PKCs are down regulated. Figure 15C demonstrates that CFs normally express PKC\(\alpha\), but after treatment with 100 nmol/L PMA for 18 hr, there is no apparent PKC\(\alpha\) expression. The downregulation of phorbol ester/DAG-sensitive PKCs
Figure 16. Inhibition of ANG II-induced ERK 1/2 activation by concurrent blockade of Ca$^{2+}$ and PKC. A. Representative Western blot demonstrating that downregulation of PKC with 18 hr 100 nmol/L PMA and concomitant chelation of intracellular Ca$^{2+}$ with 30 μmol/L BAPTA/AM reduces ANG II-induced ERK 1/2 phosphorylation. B: Summary graph of all experiments measuring ANG II-induced ERK 1/2 activation in the presence of BAPTA/AM and/or PMA. All blots are representative of at least 3 separate experiments in cells cultured from 3 separate animals. Data are expressed as mean ± SEM. *p < 0.05, statistically significant vs. Basal, †p < 0.05, statistically significant vs. ANG II. Significant differences between conditions were determined by one-way ANOVA and Tukey’s multiple comparison test.
Figure 17. Inhibition of PKCδ and intracellular Ca2+ attenuates ERK1/2 activation.

A. Cardiac fibroblasts were pre-treated for 30 min with the following isoform-specific PKC inhibitors: 10 μmol/L rottlerin for PKCδ; 10 μmol/L hispidin for PKCβ; 1 μmol/L Gö6976 for PKCα prior to 5 min stimulation by 100 nmol/L ANG II. Cell lysates were collected and subjected to SDS-PAGE and Western blot analysis as described above. The representative image was taken from one of three Western blot experiments performed in cells isolated from three separate animals, and demonstrates that pharmacological inhibition of PKCδ, but not PKCα or β, attenuates ANG II-induced ERK1/2 activation.

B. CFs were incubated with PKCδ siRNA for 24 hrs. and then pretreated for 30 min with BAPTA alone or BAPTA with rottlerin prior to 5 min stimulation by 100 nmol/L ANG II. Cell lysates were collected and subjected to SDS-PAGE and Western blot analysis as described above. The representative Western blot validates the pharmacological data displaying that ANG II activates ERK 1/2 via PKCδ.

C. Western blot confirmation of PKCδ siRNA efficiency demonstrating that PKCδ protein levels decreased and PKCα protein levels were unaffected upon PKCδ siRNA treatment.
by chronic PMA treatment did not have a significant effect on ANG II-induced activation of ERK 1/2 (Fig. 15D). However, when PKC was downregulated and intracellular Ca\(^{2+}\) was chelated simultaneously, ANG II-induced ERK 1/2 phosphorylation was significantly inhibited (Fig. 16), indicating that ANG II activates ERK1/2 through parallel signaling of PMA-sensitive PKCs (phorbol ester-sensitive isoforms) and intracellular Ca\(^{2+}\).

**Pharmacological and siRNA-mediated inhibition of PKC\(\delta\) attenuate ANG II-induced ERK1/2 phosphorylation**

Since ANG II activates ERK 1/2 through both intracellular Ca\(^{2+}\) and phorbol ester-sensitive PKC pathways in isolated adult CFs, our next goal was to determine the specific PKC isoform involved in this pathway. Pretreatment of CFs with rottlerin, a PKC\(\delta\) inhibitor, did not inhibit ANG II-mediated ERK 1/2 activation, but the combination of rottlerin and BAPTA blocked this activation (Fig. 17A). Additionally, ANG II-induced ERK 1/2 activation was not attenuated in cells pretreated with BAPTA and either Gö6976 (PKC\(\alpha\)) or hispidin (PKC\(\beta\)), indicating that ANG II signals through PKC\(\delta\) and not through \(\alpha\) nor \(\beta\). PKC \(\delta\) silencing by siRNA partially attenuated the ANG II-induced ERK 1/2 phosphorylation, whereas concurrent treatment with the siRNA and BAPTA led to a complete inhibition (Fig. 17B). PKC\(\delta\) siRNA treatment reduced PKC\(\delta\) protein levels and had no effect on PKC\(\alpha\) protein levels (Fig. 17C). Thus, pharmacological and molecular evidence demonstrates that ANG II utilizes PKC\(\delta\) as a key signaling intermediate to activate ERK1/2 in adult CFs.
Figure 18. Angiotensin II-induced cardiac fibroblast proliferation and ERK 1/2 activation is EGFR-independent. A. \[^{3}H\]-Thymidine incorporation was used to measure ANG II- (100 nmol/L) or 10 nmol/L EGF-induced proliferation of serum-starved CFs over 48 hrs. Each independent experiment was performed in triplicate. Data were pooled and expressed as percent of Basal ± SEM. *p < 0.05, statistically significant vs. Basal, †p < 0.05, statistically significant vs. EGF. B. Pretreatment of CFs with 1 μmol/L AG 1478 for 30 min was effective in blocking the activation of ERK 1/2 induced by stimulation with 10 nmol/L EGF for 5 min. However, AG pretreatment had no significant effect on 100 nmol/L ANG II-induced ERK 1/2 activation. Western blot is representative of 3 separate experiments (n=3). Data were pooled and expressed as percent of the ANG II response ± SEM. *p < 0.05, statistically significant vs. Basal, †p < 0.05, statistically significant vs. EGF. C. Stimulation with 10 nmol/L EGF for 5 min induced EGFR phosphorylation as determined by immunoprecipitation. There was no significant tyrosine phosphorylation induced by 100 nmol/L ANG II after 5 min. D-F. Pretreatment of cultured cardiac fibroblasts with the PP2 (src inhibitor), LY294002 (PI3K inhibitor), or ζ-PS (PKC zeta inhibitor) did not alter ERK 1/2 activation by ANG II.
**Angiotensin II induces cardiac fibroblast proliferation and ERK 1/2 activation in an EGFR-independent manner**

EGFR transactivation is a common mechanism for ERK activation and proliferation by ANG II and a number of other GPCR ligands in several cell types. Stimulation of cardiac fibroblasts with ANG II resulted in a 44.6 ± 2.6% increase in [3H]-thymidine incorporation over basal (Fig. 18A). In CFs pretreated with AG 1478 (EGFR kinase inhibitor) 30 min prior to ANG II stimulation, the measured increase in [3H]-thymidine incorporation was 30.2 ± 6.2% over basal and was not significantly different from ANG II alone. However, EGF-induced proliferation (75.3 ± 19.6% over basal) was completely inhibited by pretreatment with AG 1478, as expected.

Since ERK 1/2 is a common mediator of both ANG II- and EGF-induced CF proliferation, we determined whether ERK 1/2 phosphorylation was affected by AG 1478 pretreatment. Figure 18B demonstrates that AG 1478 blocked the EGF-induced increase in phosphorylated ERK 1/2 (107.2% reduction), but had no effect on phosphorylation induced by ANG II (21.2% reduction, not significant vs. ANG II alone). This finding suggests that ANG II activates ERK 1/2 through a pathway that does not involve transactivation of the EGFR. To confirm this hypothesis, immunoprecipitation of phospho-tyrosine containing proteins in cell lysates was performed followed by Western blotting for the EGFR. We determined that EGFR phosphorylation was not induced by ANG II, since densitometric analysis indicated that the levels of tyrosine phosphorylation were not significantly different from basal (Fig. 18C). Src tyrosine kinase is a common intermediate of ANG II-induced mitogenesis and ERK 1/2 activation through
Model for ANG II-induced ERK 1/2 activation in adult rat cardiac fibroblasts. ANG II induces ERK 1/2 activation through independent parallel pathways originating at Ca\(^{2+}\) and PKCδ. Inhibition of one pathway does not block ANG II-induced ERK 1/2 phosphorylation, whereas simultaneous chelation of intracellular Ca\(^{2+}\) and downregulation of phorbol ester-sensitive PKCs and more specifically, PKCδ, suppresses ANG II-induced ERK1/2 signaling. The EGFR, c-Src, PI3-K and the atypical PKCζ do not play a role in ANG II-induced ERK 1/2 activation in adult rat cardiac fibroblasts.
Figure 20. Blockade of PKC δ and α slows cardiac fibroblast migration. CFs were plated onto specific collagen substrates. Wounds were observed at 0, 12, and 24 hours. A. 10 µM rottlerin, a PKC δ inhibitor, was added to the media after the wound. The wounds on all substrates did not fully heal at 24 H, indicating that PKC δ promotes migration. B. 1 µM Go6976, a PKC α inhibitor, was added after wound induction. At 12 H it is demonstrated that the CFs migrated slower since the wounds were sub-confluent on all substrates. At 24 H, the wounds on collagens type I, III, and VI were near closure but the wounds were still open on tissue culture plastic. These data demonstrate that both PKC δ and α affect the migration of CFs.
### A. Rottlerin

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### B. Gö6976

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transactivation of the EGFR (Bokemeyer et al., 2000). PI3-kinase has been identified as a potential upstream mediator of the ANG II pathway as well in various cell types. Inhibition of c-src signaling with PP2 or PI3-K inhibition by LY29004 had no effect on ANG II-induced ERK 1/2 activation (Fig. 18D,E). Lastly, since PKCζ has previously been shown to be activated by ANG II in vascular smooth muscle and MCF-7 cells (Liao et al., 1997; Muscella et al., 2003), we treated adult CFs with the myristoylated inhibitory PKCζ pseudosubstrate (Fig. 18F) and found that ANG II-induced ERK 1/2 activation was unaffected by this inhibitor, contrary to the signaling in these other cells.

The multiple signaling pathways examined in this study and the proposed mechanisms of ANG II-induced ERK1/2 activation in adult cardiac fibroblasts are summarized in Figure 19.

**PKC α and δ contribute to fibroblast migration**

We determined PKC δ mediates ANG II-induced ERK activation and CF proliferation. We next sought to determine if certain PKC isoforms were key regulators of CF migration through pharmacological inhibition. Fibroblasts were treated with rottlerin for PKCδ, hispidin for PKCβ, and Gö6976 for PKCα. Inhibition of PKCβ did not alter fibroblast migration compared to no treatment (data not shown). Blockade of PKCα and PKCδ both severely delayed fibroblast migration. Typically untreated wounds fully close by 24 hours but inhibiting PKCα and PKCδ prevented full wound closure with wounds only reaching 20-30% confluency at 24 hours (Fig. 20).
DISCUSSION

It is well established that ANG II-induced proliferation of CFs is dependent on ERK 1/2 phosphorylation, since it has been shown that selective inhibition of this pathway will attenuate this process (Stock and Meszaros, 2003). However, several of the intermediates between the AT1R and ERK 1/2 in CFs remain to be elucidated. The current study, therefore, was designed to identify the signaling mediators of angiotensin II-induced proliferation and ERK 1/2 activation specifically in adult rat cardiac fibroblasts.

Intracellular calcium and PKC are common intermediates between ERK 1/2 and Gq-coupled receptors. Classic and novel PKCs are activated by diacylglycerol and phorbol esters and can directly phosphorylate Raf-1 to stimulate MAPK activation (Chiloeches et al., 1999). Ca$^{2+}$ has several intracellular targets, including multiple cytosolic tyrosine kinases like the Src family and the focal adhesion kinase/PYK2 family, which have been implicated in ERK 1/2 activation (Dikic et al., 1996). We report here that concurrent cytosolic Ca$^{2+}$ chelation and PKC downregulation were necessary to fully attenuate ERK 1/2 activation in our cells. This result is similar to one report which focused on ANG II and PDGF effects on MAPK signaling in neonatal cardiac fibroblasts (Booz et al., 1994); we have extended this to include the identification of the PKCδ isoform as a specific mediator of ERK1/2 signaling in adult CFs. Our current findings indicate two separate and parallel pathways involved in ERK 1/2 activation; one through PKCδ (and/or phorbol ester-sensitive PKCs) and another which is mediated by intracellular Ca$^{2+}$. 
In addition to signaling through PKC, ERK 1/2 phosphorylation resulting from G protein coupled receptor stimulation has been attributed to multiple alternative intracellular signaling pathways. ANG II has been shown to induce ERK 1/2 activation by both Ca\(^{2+}\)/c-Src-dependent and -independent EGFR transactivation (Bokemeyer et al., 2000; Saito and Berk, 2001; Wang et al., 2000). Our findings indicate that in adult CFs, neither the EGFR nor c-Src is involved in signal transduction between the ANG II receptor and ERK 1/2, which distinguishes adult CFs from both neonatal CFs and VSMCs, which utilize EGFR transactivation.

Another important mediator of ANG II-induced mitogenesis is PI3-K, which has been found to mediate ANG II-induced growth and proliferation by activation of ERK 1/2 (El Mabrouk et al., 2001) or through ERK 1/2-independent pathways (Chiu et al., 2005; Dugourd et al., 2003). PI3-K-induced ERK 1/2 activation and proliferation is commonly found in cell types where ANG II induces EGFR transactivation (Bokemeyer et al., 2000; Godeny and Sayeski, 2006). Through pharmacological inhibition of PI3-K with LY 294002, we demonstrate that ERK 1/2 activation by ANG II is not dependent on this pathway in adult CFs. An alternative pathway from PI3-K to ERK 1/2 involves activation of protein kinase C\(\zeta\), which is also a proposed mediator of ANG II-induced signaling. ERK 1/2 activation and cellular proliferation caused by agonist stimulation is dependent on PKC\(\zeta\) in VSMCs, MCF-7 cells and neonatal rat cardiac fibroblasts (Braun and Mochly-Rosen, 2003; Liao et al., 1997; Muscella et al., 2003). In mouse embryonic fibroblasts expressing the AT\(_1\) receptor, ANG II-induced ERK1/2 activation and cell proliferation has been shown to be mediated by PKC\(\zeta\) and c-src (Godeny and Sayeski, 2006). Collectively, our data suggest that PI3-K and PKC\(\zeta\) are not signaling
intermediates of ANG II-induced mitogenesis in adult CFs, which points out another major difference in the signaling pathways utilized in adult CFs versus those in neonatal CFs, VSMCs and other cell types. Moreover, this result is consistent with our previous finding that ANG II did not stimulate Akt phosphorylation since both Akt activation and PKCζ translocation require PI3-K (Olson et al., 2005).

We next set out to determine whether inhibition of specific PKC isoforms altered fibroblast migration. We focused on PKCδ since blockade of PKCδ delayed the migration of MCF-7 cells, NR6 cells, astrocytes, and human T lymphocytes (Fanning et al., 2005; Iwabu et al., 2004; Kruger and Reddy, 2003; Renault-Mihara et al., 2006). We also investigated PKCα since inhibition suppressed human T lymphocyte migration but PKCα inhibition did not affect MCF-7 cell migration (Fanning et al., 2005; Iwabu et al., 2004; Kruger and Reddy, 2003). We determined that blockade of PKCα and PKCδ both severely delayed fibroblast migration, but PKCβ inhibition had no effect indicating an isoform specific regulation of migration.

Our findings demonstrate that ANG II-induced ERK 1/2 activation in adult cardiac fibroblasts occurs through parallel Ca^{2+}-dependent and PKC-dependent mechanisms, neither of which require the activity of the EGFR or c-Src. Selective inhibition of PI3-K and PKCζ also had no effect on ERK 1/2 activation. These findings indicate that there are significant differences between the signaling pathways mediating ANG II-induced ERK 1/2 activation in adult CFs as compared to VSMCs and neonatal CFs. In conclusion, both calcium and PKCδ independently mediate the activation of ERK 1/2 by ANG II through parallel signaling pathways to control proliferation of adult cardiac fibroblasts.
CHAPTER FIVE

The influence of hyperglycemia on fibroblast proliferation, migration and differentiation

INTRODUCTION

In my previous chapters I investigated the role of the ECM and ANG II on cardiac fibroblast activation, and in the next two chapters I will apply this knowledge to a disease level and determine the impact of hyperglycemia and type 1 diabetes on fibroblast activation function.

Type 1 diabetic patients are ten times more likely to develop cardiovascular disease compared to the general population (Deckert et al., 1978; Dorman et al., 1984; Orchard et al., 2006). After a myocardial infarction, diabetic patients are at a higher risk to develop heart failure and prone to developing diffuse cardiac fibrosis, specifically a drastic increase in fibrillar collagen (types I and III) (Aragno et al., 2008; Kelly et al., 2007; Loganathan et al., 2006; Mak et al., 1997; Shimizu et al., 1993; Tsutsui et al., 2007; Van Linthout et al., 2007). The specific cause of this collagen deposition has yet to be established, therefore, we investigated the influence of high glucose on cardiac fibroblast and myofibroblast activation to determine the possible source of fibrosis.

Hyperglycemia alters proliferation and migration in other cell types; it promotes smooth muscle cell (SMC) proliferation in vitro (Natarajan et al., 1992; Watson et al., 2001; Yasunari et al., 1995). Interestingly, Peiro et al. (2001) and Zheng et al. (2007) reported
contradictory results that high glucose inhibited SMC proliferation (Peiro et al., 2001; Zheng et al., 2007). Varma et al. (2005) reported that hyperglycemic concentrations of 20 mM and 40 mM glucose significantly decreased human umbilical vein endothelial cell (HUVEC) proliferation compared to 5 mM glucose media (Varma et al., 2005). High glucose stimulates myofibroblast proliferation in vitro and increased fibroblast collagen production (Asbun et al., 2005; Muona et al., 1993; Neumann et al., 2002; Tang et al., 2007). We investigated the role of hyperglycemia on adult rat CF proliferation and the potential signaling pathways that mediate it.

The mitogen activated protein kinase (MAPK) pathway, specifically ERK 1/2, plays a key role in agonist-induced cell proliferation. High glucose conditions increased ERK 1 phosphorylation 2.6 fold and ERK 2 activation 1.8 fold in human aortic endothelial cells (Popov and Simionescu, 2006). Venkatachalam et al. (2009) reported that a 1 hour high glucose treatment induced ERK 1/2 activation in mouse CFs (Venkatachalam et al., 2008). Hyperglycemic conditions increased ERK 1/2 activity in other cell types including mononuclear phagocytes, HepG2 cells, and SMCs (Engvall et al., ; Panchatcharam et al., ; Takata et al., 2009). Our lab has recently determined that angiotensin II (ANG II) activates ERK 1/2 through parallel calcium and PKCδ pathways (Olson et al., 2008). In this study we investigated the influence of high glucose on ERK 1/2 activation alone and in the presence of ANG II.

Panchatcharam et al. (2010) revealed that hyperglycemic conditions increased SMC proliferation and migration through promotion of filopodia formation (Panchatcharam et al.). High glucose impaired mesangial cell and human umbilical endothelial cell migration whereas it stimulated pericyte migration (Li et al., 2009; Pfister et al., 2008; Schaeffer et al.).
High glucose promotes retinal endothelial cell migration but does not affect proliferation (Huang and Sheibani, 2008).

It is well established that myofibroblasts are key mediators of wound healing which is impaired in diabetic patients, but few labs have investigated the impact of hyperglycemia on myofibroblast differentiation. Previous studies from other tissues have revealed an increased myofibroblast population in the kidneys of diabetic rats and increased circulating myofibroblast progenitor cells in type 1 diabetic patients (Ina et al., 2002; Nguyen et al., 2006). Interestingly, myofibroblasts have a delayed appearance in skin wounds of diabetic mice (Darby et al., 1997). The next aim was to use an in vitro system to determine the role of hyperglycemia on myofibroblast differentiation.

Given the severe cardiovascular complications including fibrosis that diabetic patients suffer, the aim of this was study to determine the impact of hyperglycemia and collagen types I and III on cardiac fibroblast migration, differentiation, and proliferation and the potential signaling pathways that are involved in these processes.

MATERIALS AND METHODS

Materials

Dulbecco’s Modified Eagle’s Medium (DMEM), penicillin/streptomycin, fungizone and fetal bovine serum (FBS) were all purchased from Invitrogen/GIBCO (Grand Island, NY). Anti-phospho-Akt and total Akt antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-phospho-ERK 1/2 and anti-ERK 1/2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). α-SMA
antibody, angiotensin II and epidermal growth factor were acquired from Sigma-Aldrich (St. Louis, MO). The non-radioactive cell proliferation assay was purchased from Promega (Madison, WI).

**Isolation of Cardiac Fibroblasts**

Cardiac fibroblasts were isolated from adult, male, Sprague-Dawley rats as described by our previous studies and in chapter 2 (Naugle et al., 2006; Olson et al., 2005; Swaney et al., 2005). Upon initial passing, cells were divided into three groups: low glucose, long-term high glucose, short-term high glucose. Low glucose cells were cultured in 5 mmol/l DMEM, which is comparable to the normal physiological level of blood glucose. Long-term high glucose cells were cultured in high glucose, 25 mmol/l, DMEM after initial passing till the time of experimentation at passage 2. Short-term high glucose cells were cultured in low glucose DMEM until time of experimental stimulus when the cells were treated with high glucose DMEM. Passage 2 cells were used for all cardiac fibroblast experiments and passage 5 cells for myofibroblasts, and cells were switched to serum-free DMEM, with the appropriate glucose concentration, 24 hours prior to experimentation.

**Assessment of Hyperglycemia-Induced Myofibroblast Differentiation**

Cardiac fibroblasts were serum-starved for 24 hours, followed by 24 hour incubation in high glucose, and whole cell lysates were collected. Cell lysates were also collected from fibroblasts treated with low glucose or long-term high glucose at each passage up to passage 5 myofibroblasts and Western blot analysis was performed to assess changes in α-SMA.
ERK Phosphorylation Assessment Following High Glucose Treatment

CFs from each group were plated onto 60 mm dishes and protein lysates were collected at 0 min, 5 min, 20 min, 60 min, 4 hrs, and 24 hrs following the addition of the high glucose stimulus for the short-term group, the other groups were treated with their original glucose levels. Protein lysates were subjected to Western blot analysis for phosphorylated and total ERK and Akt. Cells were also pretreated with 20 nm ANG II and 20 nm EGF.

Measurement Cardiac Fibroblast Proliferation

Passage 2 fibroblasts from each group were plated onto a 96 well plate on tissue culture plastic and precoated type I collagen and type III collagen wells. At this step, high glucose media was added to the short-term high glucose level cells. Proliferation was measured using an MTT assay kit, an indicator of proliferation from metabolic activity, and Edu incorporation, and indicator of proliferation from DNA replication. The promega MTT kit was used to measure proliferation because it is an efficient technique to provide quantitative proliferation data. Since MTT kits measure mitochondrial activity and there is some debate in the literature as to how accurately this represents cell proliferation, the Edu Click-it 96 well plate kit from Invitrogen was also utilized which measures proliferation through DNA incorporation.

RESULTS

Hyperglycemia accelerates in vitro spontaneous myofibroblast differentiation

Our goal was to establish the direct impact of short-term and long-term hyperglycemia. The hallmark of the myofibroblast phenotype is increased α-SMA stress
Figure 21. Hyperglycemia accelerates α-SMA expression. Cardiac fibroblasts were cultured in either low glucose (5 mM) or high glucose (25 mM) for the designated time frame and cell lysates were collected and subjected to Western blot analysis. A-B. Representative Western blot and summary graph for α-SMA expression following a 24 hour high glucose incubation and for α-SMA expression at each cell culture passage. Each Western blot was normalized to bands from the napthol stain of the same membrane. Each blot and summary graph are mean fold change ± SEM and representative duplicate wells from 2 separate animals (n=2).
A.

\[ \alpha\text{-SMA} \]

\[ \text{LG} \quad \text{HG 24 hr} \]

B.

\[ \alpha\text{-SMA Expression} \]

(Fold change over P2 LG)

\[ \alpha\text{-SMA} \]

P2 LG, P2 HG, P3 LG, P3 HG, P4 LG, P4 HG, P5 LG, P5 HG
Figure 22. High glucose stimulates cardiac fibroblast migration. A-B. Hyperglycemia promotes fibroblast migration and the additive effects of hyperglycemia and collagen types I and III on cardiac fibroblast migration. Data are representative of triplicate wells from 4 separate animals (n=4).
A.

0H LG  
0H LG Collagen I  
0H LG Collagen III

24H LG  
24H LG Collagen I  
24H LG Collagen III

B.

0H HG  
0H HG Collagen I  
0H HG Collagen III

24H HG  
24H HG Collagen I  
24H HG Collagen III
Figure 23. Hyperglycemia stimulates cardiac fibroblast proliferation. 

A. Proliferation was measured via a colorimetric 96-well assay on fibroblasts in low glucose (LG), high glucose (HG), collagen type I and collagen type III. High glucose significantly induced fibroblast proliferation. Data are representative of 5 separate wells from 3 separate animals (n=5). 

B. Proliferation was assessed using the click-it EdU assay. Collagen types I and III significantly stimulated proliferation. *p<0.05 statistically significant vs. LG, #p<0.05 statistically significant vs. HG. Significant differences between conditions were determined by one-way ANOVA followed by paired t-tests.
fibers, and we determined that CFs cultured in high glucose for 24 hours expressed a 1.28±0.10 fold increase in α-SMA expression (Fig. 21A). During the cell culture process, cardiac fibroblasts spontaneously differentiate to myofibroblasts and α-SMA expression increases at each passage and hyperglycemia accelerates the increased α-SMA expression at passage 4 and 5 (Fig. 21B).

**Collagen types I and III potently promote cardiac fibroblast migration**

Since our lab has previously revealed that collagen types I and III stimulate CF proliferation and collagen VI promotes myofibroblast differentiation (Naugle et al., 2006), the next aim was to determine how the collagen environment influences fibroblast migration in the hyperglycemic environment. Utilizing the *in vitro* wound healing assay, we determined the hyperglycemic wounds were 10% more confluent compared to low glucose-treated (Fig. 22A). Collagen types I and III stimulated fibroblast migration in both normo- and hyperglycemic conditions and again the confluency was 10% higher in the hyperglycemic wounds compared to normoglycemic wounds with the same collagen substrate (Fig. 22B).

**Hyperglycemia induces proliferation**

Proliferation was assessed via the colorimetric nonradioactive 96-well (MTT) assay and revealed high glucose significantly increased proliferation 1.43±0.11 fold (p<0.05) (Fig. 23A). Collagen types I and III promoted the proliferation of fibroblasts to the relative control of low glucose, though this did not reach statistical significance (1.11±0.15 and 1.17±0.17 fold, respectively) and high glucose (1.17±0.07 and 1.28±0.32,
Figure 24. High glucose significantly attenuates basal ERK 1/2 phosphorylation. Cardiac fibroblasts were cultured in either low glucose (LG) or high glucose (HG, HGLT = high glucose long-term) for the designated time and cell lysates were collected and subjected to Western blot analysis for phospho-ERK and total ERK. Basal ERK phosphorylation was decreased in fibroblasts treated with high glucose for 20 minutes (0.13±0.06 fold, *p<0.05), 60 minute (0.08±0.42 fold, #p<0.001), and 4 hour (0.20±0.16 *fold, p<0.05). Data are representative of duplicate wells from 4 separate animals (n=4). Significant differences between conditions were determined by one-way ANOVA followed by paired t-tests.
Figure 25. High glucose inhibits ANG II-induced ERK 1/2 activation. CFs were treated with high glucose for the specified time frame then stimulated with ANG II or EGF for 5 minutes. Cell lysates were collected and subjected to Western blot analysis for phospho-ERK and total ERK. A. Representative Western blot and summary graph depicting that high glucose pretreatment attenuates ANG II-induced phosphorylation (*p<0.05). B. High glucose does not prevent EGF-induced ERK 1/2 activation. Data are representative of duplicate from 4 separate animals (n=4). Significant differences between conditions were determined by one-way ANOVA followed by paired t-tests.
A. & B. Bar graphs showing p-ERK/total ERK (fold change) with LG, LG + ANG II, HG 20 min + ANG II, LG + EGF, and HG 20 min + EGF conditions. 

A. 
- LG 
- LG + ANG II 
- HG 20 min + ANG II

B. 
- LG 
- LG + EGF 
- HG 20 min + EGF
respectively) (Fig. 23A). Surprisingly, the click-it EdU proliferation assay revealed no significant hyperglycemia-induced proliferation (0.93±0.07 fold) but collagen I and III significantly stimulated CF proliferation compared to tissue culture plastic control (1.79±0.65 fold and 2.15±1.10 fold, p<0.05) (Fig. 23B).

**Short-term high glucose incubations attenuate basal ERK 1/2 phosphorylation**

Our next goal was to determine which signaling pathway was activated by hyperglycemia. Initially we focused on the classical MAPK pathway because ERK 1/2 is a common mediator for high glucose stimulated proliferation in other cell types (Popov and Simionescu, 2006; Venkatachalam et al., 2008). Surprisingly, 20 minute (0.13±0.06 fold, p<0.05), 60 minute (0.08±0.42 fold, p<0.001), and 4 hour (0.20±0.16 fold, p<0.05) high glucose incubations significantly decreased basal ERK phosphorylation (Fig. 24). By 24 hours, phosphorylated ERK returned to basal levels (Fig. 24).

**High glucose attenuates ANG II-induced ERK 1/2 phosphorylation**

We have previously reported that ANG II is a potent activator of ERK 1/2 through concurrent calcium and PKCδ pathways (Olson et al., 2008). As expected, ANG II stimulated ERK 1/2 phosphorylation (p<0.05) (Fig. 25A). Surprisingly, high glucose significantly reduced the ANG II-induced ERK phosphorylation (0.36±0.19 fold over low glucose with ANG II p<0.05) (Fig. 25A). Hyperglycemic conditions did not inhibit EGF-induced ERK 1/2 phosphorylation (Fig. 25B).
Figure 26. Akt is activated by short term hyperglycemia. Cells were cultured in the indicated glucose concentrations for the time frame specified. The representative Western blot and summary graph reveal that 5 minute and 20 minute glucose incubations stimulate Akt phosphorylation. Data are representative of duplicate wells from 4 separate animals (n=4).
Short-term high glucose treatment stimulates Akt phosphorylation

Previous studies in other cell types, including endothelial cells and mouse embryonic stem cells, revealed Akt activation mediated cell proliferation (Kim et al., 2006; Varma et al., 2005). We determined that 5 minute (1.86±0.19 fold) and 20 minute (1.661±1.000) high glucose incubations increased Akt phosphorylation, but did not reach statistical significance (Fig. 26).

DISCUSSION

The current study unveils novel data demonstrating the effects of hyperglycemia on cardiac fibroblast proliferation, migration, differentiation and that collagen types I and III promote proliferation and migration. Our overall goal was to determine the impact of ECM components and hyperglycemia on CF activation and to determine the signaling cascades activated by short-term high glucose stimulation. Previously, studies have centered on the influence of hyperglycemia on the cardiac myocyte, with few studies focusing on the fibroblast. Fibroblasts and myofibroblasts are the major matrix producing cells in the heart and are consequently responsible for aberrant remodeling and fibrosis. Diabetic patients are predisposed to cardiovascular complications including fibrosis, therefore it is important to study the impact of hyperglycemia on fibroblast activation to determine specific targets that may potentially combat the progression of the fibrosis (Aragno et al., 2008; Kelly et al., 2007; Loganathan et al., 2006; Tsutsui et al., 2007; Van Linthout et al., 2007).
We first set out to determine the impact of hyperglycemia on myofibroblast differentiation via measurement of α-SMA expression and our data collectively suggest hyperglycemia promotes myofibroblast differentiation. Similar to a study by Zhang et al. (2007) that revealed 48 hour hyperglycemia promoted α-SMA expression in neonatal CFs, we determined that 24 hour high glucose incubation increased α-SMA production (Zhang et al., 2007). We also observed that continually culturing fibroblasts in high glucose up to passage 5 accelerated α-SMA expression compared to fibroblasts cultured in low glucose at the same passage. Yuen et al. have recently reported that modified collagen treated with methylglycoxal, a glucose metabolite, promoted myofibroblast differentiation (Yuen et al., 2010).

We also observed that adult cardiac fibroblasts stimulated with high glucose migrated faster than fibroblasts in low glucose. Collagen types I and III potently promoted CF migration, and addition of the collagen substrates had an equal additive effect on the hyperglycemia-induced increased confluency at 24 hours post wound infliction. Interestingly, neonatal fibroblasts plated on type I collagen and cultured in hyperglycemic media migrate slower than in normoglycemic media (Zhang et al., 2007). These data are representative of different migration assays; Zhang et al. utilized neonatal fibroblasts aggregates in a three-dimensional collagen gel and monitored the cell spreading whereas we utilized a wound healing scratch assay in a confluent monolayer of adult CFs on the collagen substrates.

High glucose has been found to stimulate cardiac fibroblast and myofibroblast proliferation in vitro and we determined that high glucose significantly increased proliferation 1.43±0.12 fold (p<0.05) and collagen types I and III had an additive effect
to the hyperglycemia-induced proliferation. The MAPK cascade is a common pathway for high glucose stimulated proliferation in other cell types such as aortic endothelial cells (Popov and Simionescu, 2006; Venkatachalam et al., 2008). Tang et al. (2007) reported that 1 and 2 hour high glucose exposures increased ERK 1/2 activity in cardiac fibroblasts (Tang et al., 2007), but surprisingly we observed a significant decrease in ERK 1/2 phosphorylation at 20 minutes, 1 hour, and 4 hours. The differing results may be from variations in the isolation or culturing processes and importantly our data are from adult Sprague-Dawley rat CFs, whereas Tang et al. utilized CFs from 1- to 3-day-old Wistar rats. Popov et al. (2009) recently reported similar findings to our short-term high glucose ERK attenuation and they also observed increased Akt phosphorylation in human aortic smooth muscle cells (Popov et al., 2009). Venkatachalam et al. (2009) reported that a 1 hour high glucose treatment induced ERK activation in P0 mouse CFs, which they propose is downstream of Akt activation (Venkatachalam et al., 2008). We have previously established that ANG II is a potent activator of ERK 1/2 through concurrent calcium and PKCδ pathways (Olson et al., 2008). Interestingly, short-term high glucose pretreatment attenuated the ANG II-induced ERK 1/2 phosphorylation but did not affect EGF-stimulated ERK activation.

Our decreased ERK 1/2 phosphorylation data were quite surprising, therefore we next explored Akt activation as a potential pathway activated by hyperglycemia. Short-term hyperglycemic incubations significantly increased Akt phosphorylation through PI3-K activation in mouse cardiac fibroblasts (Venkatachalam et al., 2008). Hyperglycemia also activates Akt phosphorylation in mouse embryonic stem cells and mesangial cells (Kim et al., 2006; Sheu et al., 2004). We revealed that 5 minute (1.86±0.18 fold) and 20 minute
(1.66±1.00) high glucose incubations increased Akt phosphorylation, but did not reach statistical significance.

In this study we demonstrate collagen types I and III promote CF migration and hyperglycemia stimulates differentiation, migration, proliferation, and Akt phosphorylation but attenuates ERK activation. We postulate the high glucose-induced fibroblast proliferation is mediated via Akt activation. Overall our data reveal hyperglycemia and the ECM potently activate cardiac fibroblasts and that this fibroblast activation may be responsible for the detrimental fibrosis that afflicts diabetic patients.
CHAPTER SIX

*Impact of type 1 diabetes on cardiac fibroblast activation: enhanced cell cycle progression and reduced myofibroblast content in the diabetic myocardium*

**INTRODUCTION**

Diabetic patients suffer from numerous cardiovascular complications including atherosclerosis, hypertension, and myocardial fibrosis. Wound healing is impaired in diabetic patients, although the underlying causes are poorly understood. Cardiac function is also compromised during the progression of diabetes; patients with diabetes are predisposed to myocardial infarction, heart failure, and arrhythmias (Adeghate, 2004; Yuan et al., 1999). Diabetic patients are less likely to survive a myocardial infarction, perhaps due to deficits in their wound healing capabilities. Cardiac fibrosis is often associated with these cardiovascular pathologies and recent studies have reported that streptozotocin-induced type 1 diabetic animals develop cardiac fibrosis and left ventricular dysfunction (Aragno et al., 2008; Kelly et al., 2007; Loganathan et al., 2006; Tsutsui et al., 2007; Van Linthout et al., 2007).

Cardiac fibrosis is characterized by an increase in fibrillar collagen and may develop from overactive fibroblasts and aberrant remodeling, eventually leading to compromised cardiac performance (Brilla, 2000; Weber and Brilla, 1991). Cardiac fibroblasts, key regulators of cardiac remodeling, comprise approximately 20% of the myocardial mass (Dostal et al., 1992). In addition to secreting ECM components,
activated cardiac fibroblasts proliferate, migrate, and differentiate to the hypersecretory myofibroblast, a cell type that is critical to the remodeling and wound healing of damaged tissue (Campbell et al., 1995; Petrov et al., 2002; Sun et al., 2000). High glucose has been shown to stimulate cardiac fibroblast and myofibroblast proliferation in vitro (Asbun et al., 2005; Neumann et al., 2002). Previous studies from other tissues have demonstrated an increase in the myofibroblast population in the kidneys of diabetic rats (Ina et al., 2002). Nguyen et al. measured increased myofibroblast progenitor cells in the blood of type 1 diabetic patients (Nguyen et al., 2006). Interestingly, myofibroblasts have a delayed appearance in skin wounds of diabetic mice (Darby et al., 1997). The appearance of myofibroblasts in the diabetic myocardium has not been described to date.

Cardiac fibroblast activation is highly regulated by a myriad of signaling pathways. Proliferation is controlled by cell cycle regulators such as p53 and p21 (Ryan et al., 1993). Alterations in p53 expression have been shown in many diabetic models; p53 is increased in the kidney of type 1 diabetic rats and in myocytes and dermal fibroblasts isolated from type 1 diabetic animals cultured in hyperglycemic culture (Tikoo et al., 2007). In vitro hyperglycemia decreased p21 expression in mesangial cells but increased p21 expression in circulating progenitor cells (Danesh et al., 2002). The varying results indicate cell type-specific responses to high glucose.

Given the several cardiovascular complications that arise in diabetic patients, and their reduced wound healing capabilities, the aim of this study was to determine a link between diabetes and cardiac fibroblast activation with a particular focus on proliferation and differentiation to the myofibroblast phenotype and whether mediators of the cell cycle are altered in the diabetic heart.
MATERIALS AND METHODS

Materials

The streptozotocin solid and anti-α-SMA were purchased from Sigma-Aldrich (St. Louis, MO). Blood glucose monitoring equipment was from One Touch II (Milpitas, CA). Anti-p53, anti-c-jun, anti-phospho-Akt, and anti-Akt antibodies were obtained from Cell Signaling Technology (Boston, MA). Anti-p21 was purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-desmin was obtained from BD Pharmingen (Franklin Lakes, NJ) and anti-pecam from Fitzgerald Industries (Concord, MA). Trizol, DMEM, fungizone, penicillin/streptomycin, and FBS were all purchased from Invitrogen/GIBCO (Carlsbad, CA). Collagenase type 2 and trypsin were acquired from Worthington Biochemical Corporation (Lakewood, NJ).

Induction of experimental type 1 diabetes and tissue preparation

Six week old adult male Sprague-Dawley rats received i.p. injections of streptozotocin (55mg/Kg) to induce diabetes. Age matched controls were injected with vehicle only (0.1N sodium citrate). The development of diabetes was assessed by weekly monitoring of body weight and blood glucose levels with a OneTouch Ultra meter via foot pad prick to draw blood. Six weeks after STZ administration animals were sacrificed and the left ventricles were isolated and divided into three sections, one for
tissue lysates, the second for histological cryosectioning, and the third for mRNA isolation via the Trizol method. A second group of animals were utilized exclusively for fibroblast isolations.

**Isolation of cardiac fibroblasts**

Cardiac fibroblasts were isolated as previously described in chapter 2 from both control and diabetic animals (Naugle et al., 2006).

**Assessment of fibroblast proliferation**

Proliferation of the cardiac fibroblasts was assessed using the Celltiter 96 Non-Radioactive Cell Proliferation Assay from Promega. Cells were grown on a 96-well plate for 24 hours followed by 4 hour incubation in DMEM containing either 0% or 10% FBS. A colorimetric dye solution was added to each well for 4 hour incubation then a solubilization/stop solution was added for 1 hour before absorbance was read on a 96-well plate reader.

**Microarray Analysis**

Total RNA was extraction from the heart using Trizol. Total RNA was quantified and 2 μg of total RNA was utilized to make biotin-labeled cRNA following the SuperArray Truelabeling-AMP 2.0 protocol. We utilized the signal transduction Oligo GEArray HybTube microarray from SuperArray Bioscience according to the protocol provided. Briefly, the array membranes were pre-hybridized for 2 hours at 60°C, and then hybridized with 2 μg of biotin-labeled cRNA overnight at 60°C overnight with gentle
Table 1. Effects of type 1 diabetes on blood glucose and body weight

<table>
<thead>
<tr>
<th>Group</th>
<th>Duration of diabetes</th>
<th>Final blood glucose (mg/dL)</th>
<th>Body weight (g)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0 Days</td>
<td>97.5</td>
<td>288.0</td>
<td>379.4</td>
</tr>
<tr>
<td>Diabetic</td>
<td>42 Days</td>
<td>550.8</td>
<td>293.1</td>
<td>248.0</td>
</tr>
</tbody>
</table>
Figure 27. Proliferation is enhanced in isolated diabetic fibroblasts. Diabetes was induced by streptozotocin injection compared to vehicle-treated control. At six week post-STZ administration, cardiac fibroblasts were isolated from both groups and cultured. Panel A depicts proliferation of cells in serum-free media whereas panel B displays fibroblasts stimulated with serum. Data are presented as mean ± SEM from 4 separate samples (*indicates statistical significance versus control + serum, *p<0.05; n=4).
A.

B.

Proliferation (% Control)

Control  Diabetic

Proliferation (% Control)

Control + serum  Diabetic + serum

*
agitation. The array membranes were washed in an SDS solution with medium agitation and visualized on X-ray film via chemiluminescence. The images were quantitatively analyzed using GEArray Expression Analysis Suite software.

RESULTS

**Blood glucose and body weight monitoring**

Overall, the diabetic rats had higher blood glucose, as expected, and did not maintain body weight as compared to the control animals (Table 1). One week after the streptozotocin injection the diabetic rats displayed high levels of blood glucose averaging 564 mg/dL. Blood glucose monitoring occurred weekly and the diabetic rats had a final average reading of 551 mg/dL (Table 1).

**Enhanced proliferation of cardiac fibroblasts isolated from type 1 diabetic rats**

Proliferation of isolated control and diabetic cardiac fibroblasts was assessed by a non-radioactive proliferation assay. Proliferation of diabetic fibroblasts increased by 15.6% versus control fibroblasts in serum-free DMEM (Fig. 27A) and upon serum stimulation, the diabetic fibroblasts exhibited an even higher increase in proliferation of 45.0% compared to control fibroblasts stimulated with serum (Fig. 27B).

**Decreased signaling gene expression in the diabetic heart**

We utilized microarray analysis to identify signaling genes that were differentially expressed in diabetic and control rat hearts. Surprisingly, no genes were upregulated in
Figure 28. Cell cycle regulators have decreased gene expression in the diabetic myocardium. Six weeks after diabetes was induced, the animals were sacrificed and RNA was extracted from the left ventricles of animals from both groups. Microarray analysis was performed to detect changes in signal transduction genes. Panel A is the fold change of downregulated genes in the diabetic heart versus control (control values are set to 1.0). Panel B is a bar graph of the statistically significant genes with decreased expression in the diabetic heart. Data are presented as mean fold change ± SEM of 4 animals per group (*indicates statistical significance versus control, *p<0.05; n=4).
### A.

<table>
<thead>
<tr>
<th>Description</th>
<th>STZ Fold Change [Vs. Control]</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21</td>
<td>$0.14 \pm 0.17^*$</td>
</tr>
<tr>
<td>p53</td>
<td>$0.54 \pm 0.09^*$</td>
</tr>
<tr>
<td>Jun</td>
<td>$0.19 \pm 0.12^*$</td>
</tr>
<tr>
<td>Bmp4</td>
<td>$0.37 \pm 0.24$</td>
</tr>
<tr>
<td>Hspca</td>
<td>$0.49 \pm 0.30$</td>
</tr>
<tr>
<td>Ll4ra</td>
<td>$0.33 \pm 0.23$</td>
</tr>
<tr>
<td>Nfkbia</td>
<td>$0.42 \pm 0.22$</td>
</tr>
<tr>
<td>Rbp1</td>
<td>$0.10 \pm 0.14$</td>
</tr>
<tr>
<td>Rbp2</td>
<td>$0.45 \pm 0.24$</td>
</tr>
<tr>
<td>Tmepai</td>
<td>$0.61 \pm 0.10$</td>
</tr>
<tr>
<td>Wisp2</td>
<td>$0.10 \pm 0.11$</td>
</tr>
</tbody>
</table>

* $p<0.05$ [control set to 1.0]
Figure 29. p53 and jun protein levels are reduced in the diabetic myocardium. Whole heart lysates were obtained from 6-week diabetic and control animals. The proteins were separated by SDS-PAGE and subjected to Western blot analysis. Panel A is a representative Western blot and summary graph for p53. Panel B contains a representative Western blot and summary graph for jun. Panel C displays a representative Western blot and summary graph p21. Panel D is a representative naphthol blue blot; each Western blot was normalized to bands from the naphthol stain of the same membrane. Each blot and summary graph are mean fold change ± SEM from n=4 per group (* indicates significance compared to control, p<0.05).
Figure 30. Akt activity is increased in the diabetic heart. Whole tissue lysates from control and diabetic hearts were separated on SDS-PAGE and Western blotted for phosphorylated Akt and total Akt. Displayed are a representative Western blot and summary graph of mean fold change ± SEM from 4 animals per group.
the diabetic group compared to the control group. Eleven genes were downregulated in the STZ group, three were statistically significant: jun decreased 5.26 fold (0.19 fold change), p21 decreased 7.14 fold (0.14 fold change), and p53 decreased 1.85 fold (0.54 fold change) (p<0.05, Fig. 28).

**Inhibition of cell cycle proteins: p53, jun, and p21**

Since we detected a decrease in mRNA expression of p53, we next sought to validate the changes at the protein level utilizing Western blot analysis. p53 protein expression significantly decreased by 1.80±0.03 fold in the diabetic hearts (p<0.05) (Fig. 29A). To confirm that the changes in Jun gene expression were translated to the protein level, we performed Western blot analysis on protein samples from each animal. We determined a slight, non-significant 1.24±0.08 fold decrease in Jun expression in the diabetic group versus control (Fig. 29B). Since p21 mRNA levels were significantly lower in the diabetic hearts versus control hearts, we measured p21 protein expression in these hearts. Surprisingly, we did not detect any statistically significant changes in p21 protein expression between groups (Fig. 29C).

**Increased Akt activity in the diabetic heart**

Previous studies have revealed that Akt is an upstream mediator of p53 and p21, therefore, the aim was to determine whether changes in activated Akt could be detected in the diabetic hearts (Zhou et al., 2001). Left ventricular protein lysates were collected from diabetic and control rat hearts. Western blot analysis revealed a 1.52±0.60 fold increase of activated Akt in the diabetic heart versus control (Fig. 30).
Figure 31. Myofibroblast content is significantly decreased in diabetic hearts. Whole tissue lysates were subjected to SDS-PAGE and Western blot analysis. Panel A is a representative Western blot for α-SMA and summary graph below of mean fold change ± SEM including at least n=4 per group. Panel B displays a representative desmin Western blot and summary graph of mean fold change ± SEM for at least n=4 per condition. Panel C is a Pecam/cd31 representative Western blot and summary graph of mean fold change ± SEM including at least n=4 per group (*indicates statistical significance versus control, *p<0.05).
Control Diabetic

A. 

\[ \alpha\text{-SMA} \]

\[ \text{Control} \] \quad \text{Diabetic} 

\[ \text{\( \alpha \)-SMA expression (fold change)} \]

B. 

\[ \text{Desmin} \]

\[ \text{Control} \] \quad \text{Diabetic} 

\[ \text{desmin expression (fold change)} \]

C. 

\[ \text{Pecam} \]

\[ \text{Control} \] \quad \text{Diabetic} 

\[ \text{pecam expression (fold change)} \]
Figure 32. Isolated diabetic fibroblast populations contain significantly less myofibroblasts, less p53, and increased Akt activity. Cardiac fibroblasts were isolated from the 6-week diabetic and control groups. Cell lysates were separated by SDS-Page and Western blot analysis for α-SMA. Displayed is a representative Western blot and summary graph of mean fold change ± SEM from n=6 (#indicates statistical significance versus control, #p<0.01). Panel A is a representative Western blot for α-SMA and summary graph. Panel B is a representative Western blot and summary graph for p53 and panel C is a representative Western blot and summary graph for p-Akt/Akt.
A. Control Diabetic

α-SMA

[Bar graph showing α-SMA expression change between Control and Diabetic groups]

B. Control Diabetic

p53 expression (fold change)

[Bar graph showing p53 expression levels between Control and Diabetic groups]

C. Control Diabetic

p-Akt

[Bar graph showing p-Akt expression levels between Control and Diabetic groups]
Decreased myofibroblast content in the diabetic myocardium

Whole tissue lysates were obtained from the left ventricle of diabetic, and control rats. Myofibroblast content was assessed by measuring α-smooth muscle actin, α-SMA, levels and to our surprise the diabetic myocardium contained 4.37±0.16 fold less myofibroblasts (p<0.05) (Fig. 31A). The diabetic hearts also produced 1.29±0.25 fold more of the muscle intermediate filament desmin and 1.59±0.25 fold more of the endothelial cell marker pcam (Fig. 31B, C). To confirm the α-SMA decrease represented a change in myofibroblast content, we isolated fibroblasts from both control and diabetic hearts and observed a 4.78±0.01 fold reduction of α-SMA in the diabetic fibroblasts versus control fibroblasts (p<0.01) (Fig. 32A).

DISCUSSION

The current study presents novel data demonstrating enhanced proliferation of diabetic fibroblasts, decreased expression of cell cycle regulators and decreased appearance of myofibroblasts in the diabetic heart. Our overall goal was to uncover altered signaling in the diabetic myocardium and how this disease state affects the resident fibroblasts. Historically the research has been focused on the influence of diabetes on the cardiac myocyte, with few studies revolving around the fibroblast. Diabetic patients suffer from insufficient wound healing; therefore it is important to study how the disease influences the key mediators of wound healing, the fibroblasts and myofibroblasts. In our study we present decreased mRNA expression of cell cycle
mediators, p53, p21, and jun, along with decreased p53 and jun at the protein level. Our study also reveals increased diabetic fibroblast proliferation and decreased myofibroblast content in the diabetic myocardium.

The relationship between p21 and p53 and their regulation of the cell cycle has been well studied and documented; p53 is a tumor suppressor gene that signals to p21 to inhibit CDK/cyclin activation and prevent cell cycle progression (Xiong et al., 1993). Our data support cell cycle progression in the diabetic myocardium based on the observation of decreased p21 and p53. We also observed that fibroblasts isolated from the diabetic myocardium are more proliferative compared to fibroblasts from control animals. Upstream of p53, Akt phosphorylates Mdm2 leading to transcriptional repression and proteasome-mediated degradation of p53 (Zhou et al., 2001). Our data reveal an increase in Akt activation suggesting that it may mediate the p53 decreased mRNA expression and its protein degradation, enhancing cell cycle progression in the diabetic heart.

The current study examines cardiac fibroblast activation in response to diabetes and demonstrates decreases in p53 and p21, whereas recent studies have reported an increase in these cell cycle regulators in diabetic myocytes (Fiordaliso et al., 2001). Increased cardiac fibroblast proliferation may explain the decrease in p21 and p53 expression, since p53 and p21 must degraded for the cell cycle to progress. Other labs have shown that high glucose stimulates fibroblast proliferation and we observed increased proliferation of fibroblasts isolated from diabetic versus control hearts (Asbun et al., 2005). Also the diversity of the models may explain our results since we observed
a decrease of p53 in whole heart tissue that we postulate is from fibroblast activation whereas others observed an increase in isolated myocytes that lead to apoptosis (Fiordaliso et al., 2001; Jazayeri et al., 2008).

We have also determined that myofibroblast differentiation is reduced in the diabetic myocardium within the first six weeks of induction. Since other studies reported increased myofibroblasts in the kidneys and increased circulating myofibroblast progenitor cells in diabetic patients, we originally hypothesized that diabetic hearts would contain increased myofibroblast content which would account for the development of cardiac fibrosis (Ina et al., 2002). To our surprise the diabetic myocardium contained significantly less α-SMA, indicating fewer myofibroblast in the diabetic heart. To confirm the decrease in α-SMA was from the fibroblast population and not VSMCs, we isolated fibroblasts from control and diabetic hearts and discovered decreased α-SMA in the diabetic fibroblast lysates indicating fewer resident myofibroblasts from the diabetic hearts. This reduction of myofibroblasts may contribute to the insufficient wound healing ability of diabetic hearts and begin to explain why diabetic patients are less likely to survive a myocardial infarction (Yuan et al., 1999).

In our study we demonstrate enhanced diabetic cardiac fibroblast proliferation, decreased cell cycle mediators, along with a reduction of myofibroblasts in the diabetic myocardium. We postulate that the increased proliferation may be explained by the decreased expression of the cell cycle inhibitors, p53 and p21. Although the cardiac fibroblasts are active and proliferating in the diabetic myocardium, there is a decrease in
differentiation to the myofibroblast phenotype. Overall our data reveal that diabetes significantly alters cardiac fibroblast activity and may provide a better understanding of the mechanisms that lead to impaired wound healing and cardiac fibrosis in the diabetic myocardium.
CHAPTER SEVEN

OVERALL DISCUSSION AND CONCLUSIONS

Cardiac fibrosis develops in response to several cardiovascular diseases and significantly compromises cardiac performance. There are current therapies designed to alleviate symptoms of many of these cardiovascular diseases, although few therapies target the progression of cardiac fibrosis. CFs are the major ECM producers, therefore understanding their activity following an injury such as an MI is important in combating the progression of cardiac fibrosis.

Our previous findings and the results from our migration assays in chapter three reveal that the ECM may be a new potential target for therapies. Designing a graft-like mesh to be implanted post-MI with fibroblasts and a specific collagen composition to target the fibroblast activities during early remodeling (1-2 days after the MI) may reduce adverse remodeling. Following a myocardial infarction, myofibroblasts function in wound contraction by grasping the wound edges and preventing infarct expansion, but myofibroblasts do not appear in the wound until day 7. If we could implant a graft at 1 or 2 days post MI with high collagen VI content, we could potentially accelerate myofibroblast differentiation to prevent infarct expansion to reduce the damage. We have demonstrated that collagen III highly promoted fibroblast and myofibroblast migration, consequently implanting a graft at 1 or 2 days post-MI could recruit more fibroblasts to the injury area to create and remodel the replacement scar. Further experimentation would be required to determine the most effective and beneficial
collagen ratio necessary to target correct fibroblast activity necessary for early remodeling. We hypothesize that activating fibroblasts and myofibroblasts earlier after an injury may prevent adverse remodeling because this will reduce the overall injury and myocyte death, therefore preventing infarct expansion and diffuse fibrosis.

The collagen expression profile following a myocardial infarction is quite interesting; we consider that it not only represents the formation of the replacement scar but the pattern also reflects the precise fibroblast activity at the time. After a myocardial infarction, collagen III expression was increased in the infarcted region at day 2 whereas the collagen I was not increased in the infarcted area until day 4, and both collagen I and III expression were increased in the non-infarcted region but only after day 4 (Cleutjens et al., 1995a). We have revealed that collagen VI expression was significantly elevated day 7 and 14 as well as week 20 post-MI (Bryant et al., 2009; Naugle et al., 2006). The expression profile for collagen III is rather intriguing since we determined that type III potently promoted migration and shortly after an MI fibroblasts and myofibroblasts must migrate to the infarct site. I postulate that collagen III is imperative for fibroblast and myofibroblast recruitment early at the injury site because collagen III was the first collagen elevated at the infarct site and it significantly enhanced migration. Also, it is crucial to note that at the infarct site there was a delay in collagen I expression compared to type III but in the non-infarcted region both collagen I and III expression increased within the same time frame after day 4. This is important to note because collagen expression in the non-infarcted or remote regions classically represents the detrimental fibrosis that impairs myocyte function. Fibroblast recruitment is not beneficial to the non-infarcted region, and it is interesting that no difference in the expression profile
between collagen I and III occurs in this region reflecting only a structural and non-fibroblast-activating function for the collagen expression. Squires et al. (2005) determined that fibroblasts isolated from the infarcted region 7 days post-MI were significantly more proliferative compared to fibroblasts from sham controls and the non-infarcted region and, surprisingly, fibroblasts from the infarcted area migrated slower than fibroblasts from sham controls (Squires et al., 2005). The decreased migration rate of fibroblasts isolated from an infarcted heart indicates an imperative need for the presence of haptotactic and chemotactic compounds in the infarcted region to aid in recruitment. It is important to note that collagen I also promoted migration, but to less of an extent as collagen III based on our in vitro assays. The differential collagen expression post-MI is quite interesting; during the early stages type III is abundantly produced and during later stages the pattern changes and type I dominates. The changes in the collagen expression pattern greatly indicate an early importance for type III which we speculate is structurally and also functionally necessary to attract fibroblasts to the wound and initiate remodeling.

Diabetic patients are two to four times more likely to develop coronary artery disease and at a two-fold higher chance of mortality following an MI (Backlund et al., 2004; Jacoby and Nesto, 1992; Kannel and McGee, 1979; Woodfield et al., 1996). At 12 weeks post-infarction, it was demonstrated in a previous report that the number of apoptotic cells was significantly higher in the border zone and non-infarcted area of type 1 diabetic rats (Backlund et al., 2004). Marfella et al. (2003) revealed that diabetic patients have significantly larger infarct segment lengths and significantly lower ejection fractions compared to a normoglycemic group (Marfella et al., 2003). It is well
established that myofibroblasts are critical mediators of wound healing: myofibroblasts contract to generate wound closure and secrete copious amounts of ECM to create the infarct scar to replace the apoptotic and necrotic myocytes. The highly organized α-SMA-containing stress fibers interact with supermature focal adhesions in the myofibroblast to generate a sustained contraction around the edge of the infarction to prevent infarct expansion. We determined that the diabetic, non-injured myocardium contained significantly lower myofibroblast content which may explain the increased infarct segment length and cell death in diabetic patients since there are potentially fewer myofibroblasts present to aid in wound closure and prevent infarct expansion. A future avenue to pursue is to induce myocardial infarction in type 1 diabetic rats to determine whether sufficient numbers of myofibroblasts are present in the infarcted area, since there is decreased myofibroblast content in the non-injured myocardium. The diabetic myocardium may not support myofibroblast differentiation and thus, could reduce survival in response to an MI.

We reported that type 1 diabetic rats had decreased myofibroblast content, but since type 1 diabetes is a complex disease the next aim was to determine the direct impact of hyperglycemia on myofibroblast differentiation (Shamhart et al., 2009). Similar to a study by Zhang et al. (2007) that revealed 48 hour hyperglycemia promoted α-SMA expression in neonatal CFs, we determined that 24 hour high glucose incubation increased α-SMA production although neither reached statistically significance (Zhang et al., 2007). We also found that continually culturing fibroblasts in high glucose up to passage 5 accelerated α-SMA expression compared to fibroblasts cultured in low glucose at the same passage. These data suggest a hyperglycemia-independent role for the decreased myofibroblast
content in the type 1 diabetic myocardium. Another important point to consider is the disease time frame because our data are only representative of animals that were diabetic for six weeks. If we investigate an early time point, such as one week, perhaps we would encounter a drastic increase in the myofibroblast population sparked from the hyperglycemia. Typically, myofibroblasts have a short lifespan of 2-4 weeks, then undergo apoptosis, therefore at our six week time point we could have potentially missed the hyperglycemia-induced myofibroblast population (Virag and Murry, 2003).

Recent studies have reported that streptozotocin-induced type 1 diabetic animals developed cardiac fibrosis and left ventricular dysfunction (Aragno et al., 2008; Kelly et al., 2007; Loganathan et al., 2006; Tsutsui et al., 2007; Van Linthout et al., 2007). We determined that the type 1 diabetic myocardium had significantly lower myofibroblast content, so we next explored the role of proliferation to account for the increased matrix deposition in the diabetic heart. We determined that cardiac fibroblasts isolated directly from the type 1 diabetic myocardium were more proliferative than fibroblasts from the healthy, control myocardium (Shamhart et al., 2009). Again the next aim was to verify whether this was a direct hyperglycemic effect and determined that high glucose significantly increased CF proliferation. Given our differentiation and proliferation data, we postulate that the reported cardiac fibrosis in the type 1 diabetic heart is from a highly proliferative fibroblast population rather than from an increase in the myofibroblast population.

We must note that our data only represent a type 1 diabetic model, and a future avenue is to explore myofibroblast content and function in the type 2 diabetic myocardium. Hyperglycemia is a major consequence of both type 1 and type 2 diabetes,
but type 1 arises from insufficient insulin production whereas type 2 occurs when the cells do not respond to the circulating insulin. The two forms impact cardiac myocyte morphology quite differently: myocyte diameter is decreased in type 1 diabetes but increased in type 2 diabetes (Asghar et al., 2009; Barouch et al., 2003; Fredersdorf et al., 2004; Nemoto et al., 2006). Also there is discrepancy in cardiac mass between the types and studies have revealed a decrease in cardiac mass in type 1 diabetes with increased fibrosis, whereas overall cardiac mass is increased in type 2 diabetic patients and is also accompanied by fibrosis (Asghar et al., 2009). I hypothesize that since we revealed that the type 1 decreased myofibroblast content was hyperglycemia-independent, the type 2 diabetic myocardium may not share this same characteristic. Further studies are required to determine the mechanism of the reduced cardiac myofibroblast population and to verify whether this is specific to type 1 diabetes.

Angiotensin II-induced proliferation of cardiac fibroblasts (CFs) is a major contributing factor to the pathogenesis of cardiac fibrosis. ANG II stimulates CF proliferation through the AT1R via activation of the MAPK/ERK 1/2 pathway. A major effect of ANG II stimulation in fibroblasts is the release of calcium from intracellular stores and the production of DAG which activates classical and novel PKC isoforms. However, calcium has additional cytosolic targets that have also been implicated in ERK 1/2 signaling. In vascular smooth muscle cells (VSMCs), AT1R-induced ERK 1/2 activation occurs via Ca$^{2+}$ and Src-dependent transactivation of the EGFR and the EGFR mediates β2-adrenergic receptor (β2-AR)-induced ERK 1/2 activation in adult CFs, therefore we initially investigated the role of EGFR transactivation (Bokemeyer et al., 2000; Eguchi et al., 1998; Kim et al., 2002; Prenzel et al., 1999; Voisin et al., 2002). We
determined that inhibiting EGFR kinase did not prevent ANG II-induced CF proliferation and ERK 1/2 activation, and as expected EGFR kinase inhibition did attenuate EGF-induced proliferation and ERK 1/2 phosphorylation.

An alternative Ca\(^{2+}\)-independent mechanism by which the AT\(_1\)R-induces ERK 1/2 activation is through phosphatidylinositol 3-kinase (PI3-K) and subsequent activation of atypical PKC\(\zeta\) (Liao et al., 1997; Muscella et al., 2003; Nakanishi et al., 1993). Blockade of PKC\(\zeta\) attenuated ANG II-induced ERK 1/2 activation in VSMCS and MCF-7 cells, indicating PKC\(\zeta\) (Liao et al., 1997; Muscella et al., 2003). Additionally, PKC\(\zeta\) inhibition reduced both basal and transforming growth factor-\(\beta\)-induced \([^{3}H]\)-thymidine incorporation in neonatal CFs (Braun and Mochly-Rosen, 2003). We investigated the role of PI3-K and PKC\(\zeta\) in ANG II-stimulated ERK 1/2 phosphorylation in adult rat CFs and determined that PI3-K inhibition by LY29004 and treatment of adult CFs with the myristoylated inhibitory PKC\(\zeta\) pseudosubstrate had no effect on ANG II-induced ERK 1/2 activation.

Calcium release from intracellular stores contributes to PKC activation and potentially to ERK 1/2 phosphorylation in adult rat CFs since it plays a role in neonatal CF ERK 1/2 activation (Booz et al., 1994). Surprisingly, we determined that calcium chelation with BAPTA/AM attenuated intracellular calcium elevation by ANG II, but did not have a significant effect on ANG II-induced ERK 1/2 phosphorylation. We also established that downregulation of PKCs through long-term PMA treatment did not have a significant effect on ANG II-induced phosphorylation of ERK 1/2. However, simultaneous PKC down regulation and calcium chelation significantly attenuated ERK 1/2 phosphorylation by ANG II indicating a parallel signaling of PMA-sensitive PKCs.
(phorbol ester-sensitive isoforms) and intracellular Ca\textsuperscript{2+}. To our surprise, ANG II-induced ERK 1/2 activation was not attenuated in cells pretreated with BAPTA and either Gö6976 (PKC\(\alpha\)) or hispidin (PKC\(\beta\)), but phosphorylation was blocked with BAPTA and rottlerin, a PKC\(\delta\) inhibitor. To further validate the pharmacological inhibition data, concurrent treatment with PKC\(\delta\) siRNA and BAPTA led to a complete inhibition of ANG II-induced ERK 1/2 activation and the PKC\(\delta\) siRNA treatment reduced PKC\(\delta\) protein levels, yet had no effect on PKC\(\alpha\) protein levels. We determined that intracellular calcium and PKC\(\delta\) were the key mediators of ANG II-induced ERK 1/2 phosphorylation through parallel, independent pathways. Our results were quite surprising since they demonstrate ANG II activates ERK 1/2 in a pathway unique to adult rat CFs. PKC\(\delta\) is a novel PKC isoform which does not require calcium for activation, indicating that the required calcium in ANG II-induced ERK activation must be used to activate a separate mediator in this pathway.

In the hyperglycemic setting we revealed that ANG II-induced ERK phosphorylation was blocked by short-term exposure to hyperglycemia. While investigating the potential pathway for high glucose stimulated CF proliferation, we determined that short-term hyperglycemia significantly attenuated basal ERK phosphorylation. We next explored whether this attenuation blocked other agonist-induced ERK activation and interestingly short-term hyperglycemia significantly blocked ANG II ERK phosphorylation but had no effect on EGF-induced ERK activation. These data are quite intriguing since we previously reported that intracellular calcium is required for ANG II induced ERK 1/2 activation whereas EGF activates ERK in a calcium independent manner (Olson et al., 2005). Calcium is utilized in the later stages
of GLUT4 vesicle translocation to the plasma membrane (Rose and Richter, 2005; Witczak et al., 2007; Wright et al., 2004). The calcium allocated to GLUT movement may explain the decreased ERK phosphorylation following short-term hyperglycemia. Another future avenue would be to explore the effect of short-term hyperglycemia on other MAPK family members, since MAPK p38 also facilitates GLUT 4 translocation (Sweeney et al., 1999).

**OVERALL CONCLUSIONS**

The overall aims of the studies outlined in this dissertation were to determine the impact of the extracellular matrix, angiotensin II, and diabetes on cardiac fibroblast activation. Our lab had previously reported the collagen types I and III potently stimulate CF proliferation and collagen VI induces myofibroblast differentiation and in these studies we revealed that collagens I and III promote CF migration whereas collagen VI slows migration (Naugle et al., 2006). We also established that collagen VI interacts with and induces myofibroblast differentiation through the α3 integrin subunit. Next we investigated the mechanism of ANG II induced ERK 1/2 activation and determined that this was mediated via concurrent PKCδ and intracellular calcium pathways.

During the progression of diabetes, patients develop many cardiovascular complications including fibrosis; therefore we next investigated the impact of diabetes and hyperglycemia on cardiac fibroblasts. We next examined the direct effects of hyperglycemia on fibroblast activity and determined that high glucose increases proliferation and accelerates myofibroblast differentiation. We revealed that CFs are more proliferative from a type 1
diabetic heart and that, surprisingly, the diabetic heart contains fewer myofibroblasts.

Understanding how the ECM, hormones such as ANG II, and diseases such as diabetes influence fibroblast activity are important to combat the progression of cardiac fibrosis that arises from overactive fibroblasts and myofibroblasts.
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