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The Role of Sphingosine-1-Phosphate in Cardiac Remodeling

A dissertation submitted in partial satisfaction of the requirement of the degree

Doctor of Philosophy

in

Biology

by

Nicole Marie Gellings Lowe

Committee in charge:

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Professor Mark A. Sussman

2008

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The dissertation of Nicole Marie Gellings Lowe is approved, and it is acceptable in quality and form for publication of microfilm:

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Chair

University of California, San Diego

San Diego State University

2008

DEDICATION

To Sean
My best friend and accomplice
For celebrating every triumph and supporting me through every failure
For making me stronger and happier through your council and your companionship

To Mom and Dad
My heroes
For your gifts of financial and emotional support
For teaching me to believe that I could do anything and be anyone

EPIGRAPH

When nothing seems to help, I go look at a stonecutter hammering away at his rock perhaps a hundred times without as much as a crack showing in it. Yet at the hundred and first blow it will split in two, and I know it was not that blow that did it, but all that had gone before.

-Jacob August Riis

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LIST OF ABBREVIATIONS

α-smooth muscle actin	(α-SMA)
ATP binding cassette transporters	(ABC)
cardiac fibroblast	(CF)
cardiac myocyte	(CM)
ceramide	(CER)
dimethylsphingosine	(DMS)
extracelluar signal-regulated kinases	(ERK)
extracellular matrix	(ECM)
G-protein coupled receptor	(GPCR)
high density lipoprotein	(HDL)
interferon-γ-inducible protein	(IP-10)
interleukin-1β	(IL-1β)
interleukin-6	(IL-6)
matrix mettaloproteinases	(MMP)
mitogen-activated protein kinase	(MAPK)
monoclonal antibody	(mAb)
monocyte chemoattractant protein-1	(MCP-1)
myocardial infarction	(MI)
neutral sphingomyelinase	(nSMase)
platelet derived growth factor	(PDGF)
sphingomyelin	(SM)

sphingosine	(SPH)
sphingosine kinase	(SphK)
sphingosine-1-phosphate	(S1P)
tissue inhibitors of metaloproteinases	(TIMP)
transforming growth factor-β	(TGF-β)
tumor necrosis factor-α	(TNF-α)

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FIELD OF STUDY

Studies in Cellular and Molecular Biology
Professor Roger A. Sabbadini

ABSTRACT OF THE DISSERTATION

The Role of Sphingosine-1-phosphate in Cardiac Remodeling

by

Nicole Marie Gellings Lowe

Doctor of Philosophy in Biology

University of California, San Diego, 2008

San Diego State University, 2008

Professor Roger A. Sabbadini, Chair

Following myocardial infarction (MI), cardiac fibroblasts (CF) proliferate, undergo myofibroblast transformation and generate excess extracellular matrix (ECM). Increased ECM production leads to fibrosis resulting in diastolic dysfunction, ultimately reducing cardiac output. Sphingosine-1-phosphate (S1P), a bioactive lysophospholipid, regulates function of numerous cell types, including certain cardiac cells. The research presented in this dissertation determined the role of S1P in promoting pro-fibrotic actions of CF. Real-time PCR of adult mouse ventricular CF

and cardiac myocytes (CM) revealed that, by comparison to CM, CF exhibit higher expression of S1P receptors and sphingosine kinase-1 (SphK1), the enzyme responsible for S1P production. In agreement with this data, both cardiac tissue and isolated cells reveal large cellular pools of S1P in CF, while low levels of S1P are detected in CM. S1P enhanced CF proliferation in an ERK-dependent manner. S1P increased expression of α-smooth muscle actin (α-SMA; a myofibroblast marker) and induced collagen production. Both were Rho kinase and $S1P_2$ receptor-dependent. siRNA to $S1P_1$ and $S1P_3$ enhanced basal α-SMA and collagen production suggesting (1) that myofibroblast transformation and collagen production are inhibited by signaling through these receptors and (2) S1P stimulates basal collagen production through autocrine and paracrine signaling. The well-known pro-fibrotic mediator, TGF-β, upregulated SphK1 expression and activity and this was necessary for TGF-β-stimulated collagen production by CF. The anti-S1P monoclonal antibody (mAb) inhibited TGF-β-stimulated collagen production further suggesting that CF utilize autocrine and paracrine signaling wherein SphK produced S1P intracellularly which was secreted and activated $S1P_2$ located on the cell surface. The anti-S1P mAb, when administered 48 hr after a permanent coronary artery ligation, abolished perivascular fibrosis at two weeks as compared to saline-treated control animals. These findings demonstrate that the S1P signaling machinery is increased in CF compared to CM and that CF may serve as a source of S1P in the heart. In addition, S1P promotes pro-fibrotic function of isolated CF both on its own and in conjunction with TGF-β. Lastly, the anti-S1P antibody reduces cardiac fibrosis in mice following MI. Thus, the

anti-S1P mAb may serve as a novel therapeutic to attenuate CF function and resultant tissue fibrosis following ischemic cardiac injury.

I. Introduction

Fibrosis is defined as the formation of excessive connective tissue in an organ or tissue in response to injury. Fibrotic diseases, such as hepatic, pulmonary, and cardiac fibrosis, can affect whole organs, or they can be more systemic such as scleroderma. The thickening and stiffening that result from excessive fibrosis lead to decreased mechanical function and nutrient delivery. In the case of cardiac fibrosis, decreased nutrient delivery, limited electrical coupling and myocardial stiffening compromises the ability of the heart to act as a pump, leading eventually to heart failure.

Cardiac fibrosis can result from one of several cardiovascular events or diseases, including myocardial infarction (MI), inflammatory heart muscle disease (myocarditis), pressure overload (aortic stenosis, hypertension), idiopathic dilated

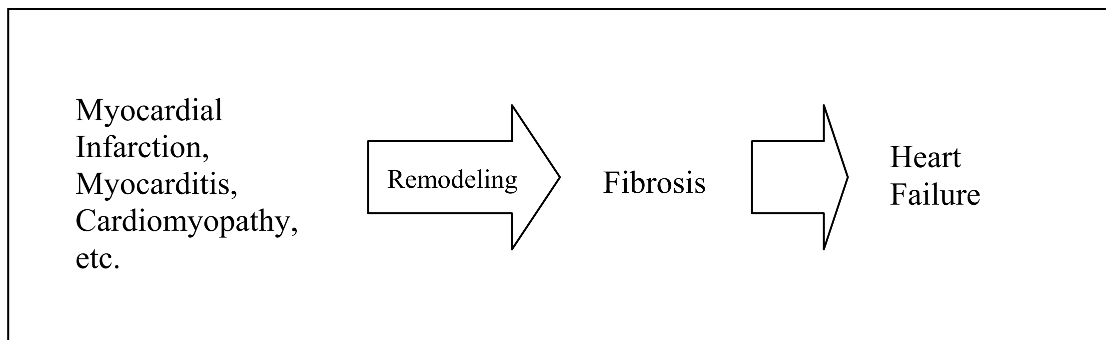


Figure 1. Myocardial damage leads to heart failure through excess fibrosis caused by remodeling.

cardiomyopathy, and volume overload (valvular regurgitation) (Cohn, Ferrari et al., 2000). The process leading from these diseases to a fibrotic heart is referred to as remodeling. Remodeling is an adaptation of the heart to exaggerated signaling and excessive mechanical stress. Although initial remodeling responses are considered adaptive, the continuation and progression of remodeling is maladaptive and causes cardiac fibrosis leading to heart failure (Cohn, Ferrari et al., 2000). Heart failure is characterized by the heart's inability to meet the metabolic demands of the body. More than 5 million people in the United States live with heart failure and almost 60,000 people of these people will lose their lives each year (2006). Although knowledge about cellular and molecular mechanisms that contribute to remodeling and heart failure expand each day, mitigating the impact of this disease requires amore comprehensive knowledge of its etiologies.

A. Fibrosis

1. The Fibroblast

Fibroblasts are dispersed in connective tissue throughout the body where they provide structural support primarily through secretion of non-rigid extracellular matrix (ECM)(Alberts, 2002). They have few actin-associated cell-cell or cell-matrix contacts(Hinz, Phan et al., 2007). Fibroblasts are mesenchymal in origin and can differentiate into multiple cell types including osteoblasts, chrondocytes, adipocytes and smooth muscle cells (Alberts, 2002). In response to injury, fibroblasts migrate into the injured area, proliferate and transform into a secretory, mature phenotype called the myofibroblast.

2. Migration and Proliferation

There are three sources of increased fibroblast in an injured tissue: increased proliferation of resident fibroblasts, conversion of epithelial cells and endothelial cells to fibroblasts, and migration of blood-borne mesenchymal stem cells, called fibrocytes (Wynn, 2008). Chemokines, cytokines and growth factors released by inflammatory cells in the injured area stimulate resident fibroblasts to proliferate. Considering cardiac fibroblasts as an example, they proliferated in response to platelet derived growth factor (PDGF)(Diez, Nestler et al., 2001), insulin-like growth factor (IGF)(van Eickels, Vetter et al., 2000), and tumor necrosis factor-α (TNF-α)(Jacobs, Staufenberger et al., 1999). The process of epithelial-to-mesenchymal transition (EMT) is a process whereby epithelial cells differentiate and become fibroblasts (Wynn, 2008). Both transformation processes have been shown to occur in response

to transforming growth factor-β (TGF-β) (Kim, Jang et al., 2007;Zeisberg, Tarnavski et al., 2007). The blood borne mesenchymal stem cells that are referred to as fibrocytes, have recently been identified as contributors to fibrotic lesions in lung(Andersson-Sjoland, de Alba et al., 2008), kidney(Sakai, Wada et al., 2008), and skin (Quan, Cowper et al., 2006). Interestingly, it has also been suggested that fibrocytes are involved in tumor invasion and may be prognosticators of tumor malignancy (Quan, Cowper et al., 2006).

3. Myofibroblast and Extracellular Matrix Production

Increased ECM production by fibroblasts responding to injury involves transformation of quiescent fibroblasts, responsible for basal ECM homeostasis, to activated myofibroblasts (Long and Brown, 2002). Myofibroblasts are a unique group of smooth muscle-like fibroblasts that have a similar appearance and function, regardless of their tissue of residence (Powell, Mifflin et al., 1999). In distinction from fibroblasts, myofibroblasts contain a well-developed actin cytoskeletal network with cell-to-cell and cell-to-matrix contacts (Hinz, 2007). In addition, myofibroblasts are characterized by the presence of α-smooth muscle actin (α-SMA), the most reliable marker of myofibroblast cells (Serini and Gabbiani, 1999). These well developed cytoskeletal networks allow myofibroblasts to play a critical role in generating passive force to close a wound(Hinz, 2007). In addition, fibroblasts are highly proliferative while myofibroblasts have well developed focal adhesion complexes and are not considered proliferative (Tomasek, Gabbiani et al., 2002). Though the stages through which fibroblasts change into myofibroblasts are complex and not fully understood (Tomasek, Gabbiani et al., 2002), intermediate cells have

been identified and termed “proto-myofibroblasts”. These contain cytoplasmic actins and generate small traction forces(Hinz, 2007).

Altered number and function of myofibroblasts are implicated in diseases associated with increased ECM deposition, primarily type I and III collagen (Schurch, Seemayer et al., 1998) and the resultant fibrosis. These diseases can effect the liver, skin, lung, kidney and heart (Mitchell, Woodcock-Mitchell et al., 1989;Hewitson, Wu et al., 1995;Long and Brown, 2002;Desmouliere, Darby et al., 2003). In some diseases, myofibroblasts undergo apoptosis, thus completing a repairative process, while in others the myofibroblasts survive, resulting in pathological fibrosis(Schurch, Seemayer et al., 1998).

There are two main stimulators of myofibroblast formation: cytokines released locally by inflammatory and resident cells and changes in the mechanical properties and composition of the ECM (Hinz, 2007). Among the cytokines that transform fibroblasts to myofibroblasts are the following: PDGF, fibroblast growth factor (FGF), granulocyte macrophage-colony-stimulating factor (GM-CSF), TGF-β, γ-interferon (γ-INF), TNF-α, interleukin-1 (IL-1) and interleukin-2 (IL-2) (Schurch, Seemayer et al., 1998). Prominent among these is TGF-β, which directly promotes α-SMA expression, ECM proteins and other cytoskeletal proteins that make up the contractile apparatus of the myofibroblast (Hinz, 2007). Interestingly, TGF-β can control both proliferation and myofibroblast transformation through modulation of signaling at the post-receptor level (Grotendorst, Rahmanie et al., 2004).

B. Cardiac remodeling and fibrosis

Several myocardial disorders can cause cardiac fibrosis that can lead to heart failure. Myocardial infarction (MI) is a particularly common cause of heart failure and a very prevalent disease. Myocardial infarction affects 8 million Americans a year (2006). It is also an effective animal model to study remodeling, cardiac fibrosis and heart failure. To this end, this introduction will focus on MI as a stimulus for cardiac fibrosis.

Myocardial infarction occurs when blood supply to a portion of the heart is compromised to the extent that cardiac tissue dies. Most MIs happen when an unstable atherosclerotic plaque forms an intracoronary thrombus, thereby occluding coronary artery blood flow. As a result, the main contractile, cellular component of the heart, the cardiac myocyte (CM), undergoes both necrotic and apoptotic death. Coronary artery occlusion leads to a lack of sufficient oxygen and nutrient supply to meet the metabolic demands of CM (Braunwald, E. Braunwald 1997;Bajzer, 2002). While necrosis and apoptosis are both determinants of the extent of injury, apoptosis is the most important determinant of subsequent remodeling and heart failure (Takemura and Fujiwara, 2004).

The process leading from MI to a fibrotic heart is referred to as remodeling. Remodeling is an adaptation of the heart to exaggerated signaling and excessive mechanical stress. Although initial remodeling responses are considered adaptive, the continuation and progression of remodeling is maladaptive and causes cardiac fibrosis leading to heart failure (Cohn, Ferrari et al., 2000). When initiated by a MI, remodeling can be divided into three phases: inflammation, reparative remodeling and

reactive remodeling. Initially, following an ischemic insult, inflammation facilitates phagocytosis of dead CMs. These are replaced by granulation tissue laid down by cardiac fibroblasts (CF), which is further replaced by a collagen-rich scar. Because cardiac function is impaired through loss of contractile tissue, the continuous stress results in maladaptive, reactive remodeling, whereby excessive fibrosis throughout the heart further impairs function and leads to heart failure. The following pages will elaborate on the three phases of remodeling.

1. Inflammation

The inflammatory phase of healing following MI is initiated by necrotic CM death. Inflammation responses are increased in reperfused myocardium, but still exist in permanently ischemic tissues (Vandervelde, van Amerongen et al., 2006). During necrotic death, CM release their intracellular contents thereby activating the innate immune response (Frangogiannis, 2006). The following events are critical to the innate immune response: complement activation, which recruits neutrophils and monocytes (Hill and Ward, 1971;Pinckard, Olson et al., 1975;Rossen, Michael et al., 1988); reactive oxygen species (ROS) generation, which induces cytokine expression(Lefer and Granger, 2000) and leukocyte chemotaxis (Patel, Zimmerman et al., 1992;Lakshminarayanan, Lewallen et al., 2001); toll like receptor activation, which stimulates Mitogen-activated Protein Kinase (MAPK) and NF-κB (Frangogiannis, 2006); and NF-κB activation which strongly upregulates cytokine, chemokine and adhesion molecule expression (Lenardo and Baltimore, 1989;Morishita, Sugimoto et al., 1997;Kupatt, Habazettl et al., 1999). Of the

responses initiated by the innate immune response, perhaps most important to development of a strong inflammatory response is the induction of chemokines and cytokines.

Chemokines are a family of small (8-14kDa) proteins with similar tertiary structures (Frangogiannis and Entman, 2005). In general, chemokines regulate leukocyte locomotion and trafficking (Gerard and Rollins, 2001). In models of MI, three chemokines are consistently upregulated: interleukin-8 (IL-8), interferon-γ-inducible protein (IP-10), and monocyte chemoattractant protein-1 (MCP-1) (Kukielka, Smith et al., 1995;Kumar, Ballantyne et al., 1997;Frangogiannis, Mendoza et al., 2001). The exact roles of IL-8 and IP-10 in myocardial infarction remain elusive. Recombinant canine IL-8 increased adhesion of neutrophils to isolated CM while IL-8 neutralization decreased post-MI necrosis without affecting neutrophil infiltration (Kukielka, Smith et al., 1995;Boyle, Kovacich et al., 1998). IP-10 is known to have a direct inhibitory effect on fibroblast migration which suggests an anit-fibrotic role (Shiraha, Glading et al., 1999). In contrast to IL-8 and IP-10, much is known about the role of MCP-1 post-MI. MCP-1 is a strong chemoattractant for monocytes, T cells and natural killer cells (Frangogiannis and Entman, 2005). In addition, MCP-1 affects fibroblasts by increasing collagen production and regulating matrix metalloproteinase synthesis (Gharaee-Kermani, Denholm et al., 1996). MCP-1 knock-out mice that underwent experimental MI by coronary ligation exhibited delayed macrophage infiltration, decreased replacement of dead CM with granulated tissue and decreases in cytokines and growth factors, such as TNF-α and TGF-β (Dewald, Zymek et al., 2005).

Expression and secretion of pro-inflammatory cytokines, especially TNF-α, interleukin-1β (IL-1β), and interleukin-6 (IL-6), are observed consistently in experimental models of MI (Herskowitz, Choi et al., 1995;Dewald, Ren et al., 2004). TNF-α is upregulated early in the infarcted myocardium and plays a complex role in both inflammation and CM apoptosis. When subjected to MI, TNF-α knock-out mice exhibit decreased chemokine expression (Maekawa, Wada et al., 2002) and TNF-α neutralizing antibodies pre-conditioned rabbit hearts subjected to MI (Belosjorow, Bolle et al., 2003). In contrast, mice lacking the receptors to TNF-α had larger infarcts than wild-type controls (Kurrelmeyer, Michael et al., 2000). Interestingly, TNF-α can activate sphingomyelinase (SMase), the first enzyme in the production of bioactive sphingolipids. SMase hydrolyzes sphingomyelin (SM) to produce ceramide (CER), which can lead to sphingosine (SPH) production and, eventually, to the generation of sphingosine-1-phosphate (S1P), a major signaling mediator that is also a major focus of this dissertation. While CER and SPH are generally recognized as pro-apoptotic molecules, S1P is considered anti-apoptotic. This may, in part, explain how TNF-α can be both protective and damaging, as its effects could depend on the ratio of CER to S1P. Less is known about the roles of IL-1β and IL-6 in inflammation in the heart; however, IL-6 may modulate adhesion of neutrophils to CM (Youker, Smith et al., 1992).

As a result of activation of the innate immune response and the production of chemokines and cytokines, inflammatory cells move into the heart. The three main classes of cells involved in this response are leukocytes, mononuclear cells and mast cells (Frangogiannis, 2006). The most prominent type of leukocyte, neutrophils, assist

in clearing of dead cells and debris both directly and through recruitment of additional cells (Frangogiannis, 2006). These cells also affect CM death. For example, neutrophil depletion in animals subjected to myocardial ischemia/reperfusion reduced infarct size (Romson, Hook et al., 1983;Litt, Jeremy et al., 1989). Following a decline in neutrophils, macrophages become the primary phagocytic cell in the infarction. Monocytes are recruited and stimulated by several growth factors including macrophage-colony stimualting factor (M-CSF) and GM-CSF to mature into macrophages (Frangogiannis, Mendoza et al., 2003). Macrophages not only phagocytose dead CM and neutrophils but also release growth factors which assist in scar formation (Frangogiannis, 2006). Depletion of macrophages increases remodeling after myocardial cryoinjury (van Amerongen, Harmsen et al., 2007). Mast cells are resident in the heart (Frangogiannis, Burns et al., 1999) and contribute to the inflammatory response by releasing several inflammatory and pro-fibrotic mediators (Gordon, Burd et al., 1990).

Throughout the inflammation process, the regulation of ECM is essential to the function of the inflammatory cells because it allows for efficient signaling and migration(Clark, R. A. Clark 1995). The ECM is composed primarily of collagen type I, but also contains fibronectin, proteoglycans, laminins, and other collagen types (Swynghedauw, 1999). The ECM around the dead CM is degraded by a series of enzymes called matrix mettaloproteinases (MMP). MMP-1 is the first to exhibit increased activity post-MI and primarily degrades collagen. This is then followed by MMP-8 (a gelatinase) and the fragments produced by MMP-1 and MMP-8 are further degraded to amino acids by MMP2, -3 and -9(Sun and Weber, 2000). IL-1β and TNF-

α increase MMP activity. TGF-β, in contrast, decreases MMP activity through activation of tissue inhibitors of metaloproteinases (TIMP) (Siwik, Chang 2000) (Verrecchia, Chu, 2001). TIMP up-regulation is important to suppress MMP activity and occurs as early as 1 week post-MI in the rat (Sun and Weber, 2000).

2. Repairative Remodeling

Arguably the most important cell type involved in both repairative and reactive remodeling is the CF. Cardiac fibroblasts are the most abundant cell type in the heart, commonly recognized to constitute two-thirds of the total cell population, although a recent study disputes this figure(Banerjee, Fuseler et al., 2007). Under normal conditions, CF are responsible for ECM deposition, creating the scaffold for CM (Eghbali, 1992). In response to growth factors, such as TGF-β, that are upregulated during inflammation, CF migrate, proliferate, and undergo a phenotypic alteration resulting in the production of excessive ECM (Virag and Murry, 2003). Sources of migrating CF remain unclear; however, possibilities include pericytes, fibrocytes (circulating fibroblasts), circulating monocytes or bone marrow-derived progenitor cells (Sun, Kiani et al., 2002). Several studies have shown that resident interstitial CF proliferate, indicating that this is likely the most abundant source of CF for remodeling (Virag and Murry, 2003;Vandervelde, van Amerongen et al., 2006).

Increased ECM production by CF involves transformation of quiescent fibroblasts, which are responsible for basal ECM homeostasis, to activated myofibroblasts, which play a key role in healing of injured myocardium (Long and Brown, 2002). Increased myofibroblasts have been observed in the infarction as early

as 4 days post-MI in the mouse (Vandervelde, van Amerongen et al., 2006). In addition, the role for myofibroblasts in the healing MI is unique, as compared to other diseases, because whereas in other tissues myofibroblasts die once the scar if fully formed, these cells are detectable in the heart years after an MI in humans (Willems, Havenith et al., 1994;Sun and Weber, 2000).

Transformation of fibroblasts to myofibroblast is stimulated by various growth factors which are primarily released by macrophages and mast cells. In fact, depletion of monocytes and macrophages delayed or inhibited scar formation in a post-MI model and thereby lead to exaggerated remodeling (Leibovich and Ross, 1976;van Amerongen, Harmsen et al., 2007). In addition, isolated macrophages stimulate fibroblast proliferation (Leibovich and Ross, 1975). Mast cells are known to release TGF-β(Pennington, Lopez et al., 1992), basic fibroblast growth factor (bFGF) (Qu, Liebler et al., 1995) and vascular endothelial growth factor (VEGF) (Boesiger, Tsai et al., 1998). In addition, mast cells accumulate post-MI in areas of proliferation and scar formation (Frangogiannis, Lindsey et al., 1998).

Myofibroblasts are initially responsible for depositing a temporary granular network consisting primarily of osteopontin (Murry, Giachelli et al., 1994) and fibronectin (Casscells, Kimura et al., 1990;Willems, Arends et al., 1996) to replace the dead and dying CM. As healing proceeds, the temporary matrix is absorbed and a collagen network is established by myofibroblasts resulting in a thin scar (Virag and Murry, 2003). The establishment of a scar in place of dead CM is essential to maintaining the structural integrity of the heart and preventing ventricular rupture (Sun and Weber, 2000).

3. Reactive fibrosis

Although initial MI healing processes are considered adaptive, the continuation and progression of myofibroblast activity leads to the maladaptive, reactive remodeling through cardiac fibrosis. Cardiac fibrosis is characterized by overproduction of ECM, predominantly collagen types I and III, into the interstitial and perivascular space (Diez, Lopez et al., 2001). Excess collagen deposition leads to myocardial stiffening, impaired cardiac relaxation and filling (diastolic dysfunction), and is associated with pathophysiological overload of the heart (MacKenna, Summerour et al., 2000). Progression of remodeling to heart failure is always associated with a poor outcome (Gaudron, Eilles et al., 1993). Therefore, slowing or reversing remodeling would potentially improve heart function and quality of life for patients.

Treatments which directly decrease cardiac fibrosis thereby treating heart failure are limited and include angiotensin-converting-enzyme (ACE) inhibitors and angiotensin II (AngII) receptor blockers. Decreased cardiac output in patients with compromised function activates the rennin-agiotensin-aldosterone system in an effort to increase cardiac output through CM hypertrophy and increased vascular flow rates (Weber, 1997;Andrew, 2002). The production of angiotensin also has the direct negative consequence of upregulating TGF-β thereby increasing fibrosis. Other, indirect treatments which can improve heart failure patients include β-blockers, digitalis, diuretics, vasodilators, blood thinners and calcium channel blockers. There is a clear need for additional treatments which target cardiac fibrosis and could lessen

the extent of reactive remodeling thereby avoiding or decreasing the severity of heart failure.

C. Sphingolipid-signaling pathway

Sphingolipids are bioactive metabolites which are primarily produced from the degradation of sphingomyelin (SM), a plasma membrane outer leaflet phospholipid (Koval and Pagano, 1989). Interestingly, the genitive *sphingo* derives from the Greek word sphinx which reflects the enigmatic nature of sphingolipids. While SM primarily serves as a structural component of cell membranes, the down-stream components of SM hydrolysis serve as cellular signaling and regulatory molecules (Hannun and Bell, 1993;Igarashi, 1997). Figure 2 shows the sphingolipid signaling cascade. Ceramide (CER), sphingosine (SPH) and sphingosine-1-phosphate (S1P) have been widely studied and have diverse effects on the cardiovascular system (Levade, Auge et al., 2001).

1. Neutral Sphingomyelinase

The neutral form of sphingomyelinase (nSMase), commonly associated with surface membranes (Das, Cook et al., 1984;Dobrowsky, 2000), is a key early component of the sphingolipid signaling pathway (Chatterjee, 1993;Liu, Obein et al., 1997). There are multiple stimuli known to activate nSMase including ROS (Bielawska, Shapiro et al., 1997;Cordis, Yochida et al., 1998;Hernandez, 2000), TNF-α (Ségui, Cuilier et al., 2001), T cell receptors (Tonnetti, Veri et al., 1999), and ionizing radiation (Haimovitz-Friedman, Kan et al., 1994). Interestingly, activation of nSMase has been shown as one of the earliest events in cardiac ischemia-reperfusion

(Bielawska, Shapiro et al., 1997;Cordis, Yochida et al., 1998;Hernandez, 2000). In addition, inhibition of nSMase is associated with slowed heart failure development in rats after chronic MI (Adamy, Mulder et al., 2007).

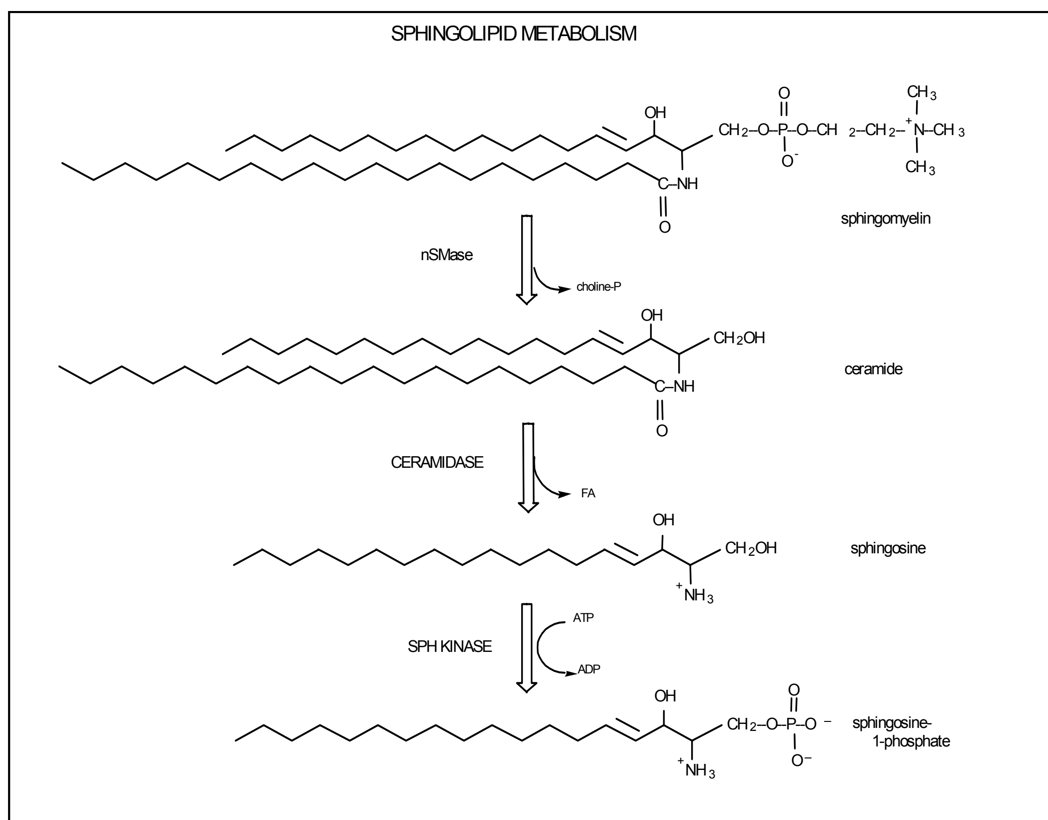


Figure 2. Sphingolipid-signaling pathway. A schematic diagramming the sphingolipid-signaling pathway and the enzymes involved.

2. Ceramide

Ceramide is the immediate downstream product of nSMase. Ceramide induces apoptosis in many different cell types, including CM (Obeid, Linardic et al., 1993;Hannun and Obein, 1995;Andrieu-Abadie, Jaffrezou et al., 1999;Kolesnick, 1999). Like nSMase activation, stimulation of apoptosis by CER is associated with cell surface membranes (Ko, Lee et al., 1999) and a mitochondrial site of action(Lin,

Chen et al., 2005). CER generation occurs in response to hypoxia/reoxygenation (H/R) in cultured CM (Bielawska, Shapiro et al., 1997;Hernandez, 2000) and during ischemia-reperfusion (I/R) in whole hearts (Bielawska, Shapiro et al., 1997;Cordis, Yochida et al., 1998;Zhang, Fryer et al., 2001). The Sabbadini laboratory has also shown that inhibition of CER production by knocking-down nSMase and/or its adaptor protein, factor associated with neutral SMase (FAN), mitigates experimental hypoxia-induced apoptosis in cultured CM (O'Brien, Gellings et al., 2003).

3. Sphingosine

Sphingosine is another potent sphingolipid second messenger, with diverse downstream signaling effects. Sphingosine is formed from CER by ceramidase. Work from the Sabbadini laboratory and others suggests that SPH may mediate the negative inotropic effects of TNF-α in the heart through direct actions on the ryanodine receptor and the L-type channel (Dettbarn, Betto et al., 1994;McDonough, Yasui et al., 1994;Krown, Yasui et al., 1995;Yasui and Palade, 1995;Oral, Dorn et al., 1997;Sugishita, Kinugawa et al., 1999;Friedrichs, Swillo et al., 2002). SPH has also been implicated as a very potent inducer of apoptosis in a wide range of cell types (Ohta, Yatomi et al., 1994), including both neonatal and adult rat CM (Krown, Page et al., 1996;Zechner, Craig et al., 1998). In addition, the Sabbadini laboratory has shown that SPH is produced and released from hypoxic CM (Cavalli AL, 2002) and that serum levels of SPH are elevated in an experimental model of heart failure (Dalla Libera, Sabbadini et al., 2001. Most importantly, SPH and S1P are elevated in clinical

trial patients with severe coronary artery disease (Deutschman, Carstens et al., 2001;Deutschman, Carstens et al., 2003).

4. Sphingosine-1-phosphate

Sphingosine-1-phosphate is made by sphingosine kinase (SphK). Two isoforms of SphK have been identified, SphK1 and SphK2 (Liu, 2000;Nava, Hobson et al., 2002) and both isoforms are expressed in the heart (Kohama, Olivera et al., 1998;Melendez, Carlos-Dias et al., 2000;Fukuda, Kihara et al., 2003;Jin, Goetzl et al., 2004). Interestingly, SphK1 and SphK2 have different sequences, locations and functions (Maceyka, Sankala et al., 2005). SphK1 is primarily cytoplasmic, but translocates to the plasma membrane in response to multiple growth factors where it can produce S1P which acts extracellularly to promote survival, proliferation and migration (Spiegel and Milstien, 2003;Maceyka, Sankala et al., 2005). SphK2, in contrast, is primarily found in the endoplasmic reticulum and it stimulates apoptosis independent of extracellular mechanisms (Maceyka, Sankala et al., 2005). Platelets, the main source of serum S1P, express a constitutively active SphK which converts SPH to S1P (Igarashi, Yatomi et al., 1998). As a result, S1P is found in human serum at an average concentration of 0.5µM (Yatomi, Igarashi et al., 1997).

Although much is known about S1P generation, comparatively little is known about how S1P is released. Two recent studies have identified a role for ATP binding cassette transporters (ABC) in releasing S1P. In the first study, Mitra et al. demonstrated that mast cells, previously shown to be potent sources of S1P, rely on ABCC1 for S1P release (Mitra, Oskeritzian et al., 2006). Subsequently, Sato et al.

demonstrated that rat astrocytes rely on ABCA1 for release of S1P (Sato, Malchinkhuu et al., 2007).

S1P signaling is implicated in multiple biological processes; most prominently of these are vascular maturation (Liu, 2000), lymphocyte trafficking (Brinkmann and Lynch, 2002;Sanna, Liao et al., 2004), and cancer (Xia, Gamble et al., 2000;Visentin, Vekich et al., 2006). S1P exerts its effects through stimulation of cellular responses such as proliferation, survival, migration and differentiation. S1P's diverse effects can be attributed to the fact that S1P elicits responses through intracellular and extracellular mechanisms (Cuvillier, Pirianov et al., 1996;Van Brocklyn, Lee et al., 1998). Intracellularly, S1P mobilizes calcium stores (Meyer Zu Heringdorf, 2004). Extracellularly, S1P activates a series of G protein-coupled receptors (GPCRs) known as S1P receptors (formerly known as endothelial differentiation gene (EDG) receptors). There are five known S1P receptors and each is coupled differentially to specific G proteins. Nearly all cells express at least one S1P receptor isoform. $S1P_1$, $S1P_2$, and $S1P_3$, are considered to be evenly distributed among tissues while $S1P_4$ and $S1P_5$ are more sporadically expressed (Rosen and Goetzl, 2005). $S1P_1$, $S1P_2$ and $S1P_3$ have been detected in the mouse, rat, and human heart (Nakajima, Cavalli et al., 2000;Ishii, Friedman et al., 2001;Mazurais, Robert et al., 2002).

S1P receptors couple differentially to three G proteins. Each of these, G_i, G_q, and $G_{12/13}$, activate various down-stream signaling pathways. G_i activation is associated with Ras/ERK pathway, phospholipase C, and Akt/Pi3K, all of which contribute to cellular proliferation. Through $G_{12/13}$, S1P stimulates activation of the Rho GTPase which can regulate cellular migration and cellular transformation(Ridley

and Hall, 1992). G_q is known to activate phospholipase C (PLC) (Hla, 2003). The following section will further elucidate how S1P can play a role in cardiac remodeling through these signaling mechanisms.

Interestingly, S1P's production and S1P receptor activation are linked in an autocrine and paracrine signaling paradigm (Takabe, Paugh et al., 2008). S1P produced intracellularly can be released and then elicit autocrine and paracrine effects through activation of S1P receptors on the cell surface. For example, platelet derived growth factor (PDGF) -stimulated fibroblast migration (Hobson, Rosenfeldt et al., 2001) and mast cell chemotaxis (Jolly, Bektas et al., 2004) rely on activation of SphK, production of S1P, and activation of S1P receptors. Therefore, it was necessary to both make S1P and activate extracellular S1P receptors to induce the cellular responses. This cellular signaling mechanism has been implicated in multiple diseases, include inflammation, cancer, and atherosclerosis, and could theoretically play a role in cardiac fibrosis (Takabe, Paugh et al., 2008).

C. S1P in Cardiac Remodeling

1. Inflammation

S1P exerts effects on multiple cells of the immune system including neutrophils, monocytes and macrophages, mast cells and lymphocytes. Contradictory evidence regarding S1P's role in neutrophils activity shows that, on one hand, SphK is necessary for cytokine release by neutrophils, while, on the other hand, S1P inhibits neutrophil chemotaxis and migration (Kawa, Kimura et al., 1997;Ibrahim, Pang et al.,

2004). This might be due to differences in intracellular and extracellular effects, but either way, S1P may influence neturophil activity. Effects on monocytes and macrophages have proven to be more consistent and suggest that S1P is pro-inflammatory in its effects on macrophages. S1P stimulates expression of IL-1β and TNF-α from macrophages (Lee, Liao et al., 2002). In addition, SphK activation is associated with degranulation, chemotaxis, and cytokine production from macrophages (Melendez and Ibrahim, 2004).

S1P is also intimately involved in mast cell function. For example, S1P is released when mast cells are activated to degranulate (Prieschl, Csonga et al., 1999;Melendez and Khaw, 2002;Jolly, Bektas et al., 2004;Jolly, Bektas et al., 2005). Consequently, mast cells could be (1) a rich source of S1P after a thrombus-associated ischemic insult and the inflammatory response that ensues and (2) can add to the release of S1P by activated platelets in the infarct zone.

Lymphocytes do not play a particularly robust role in post-MI inflammation; however, it is interesting to note that S1P prevents egress of lymphocytes form secondary lymphatic tissues (Rosenfeldt, Hobson et al., 2001). In addition, the drug FTY720, which is an S1P analogue, is in clinical trials as a treatment to prevent organ transplant rejection by mitigating the role of lymphocytes in tissue rejection reaction (Baumruker, Billich et al., 2007).

2. Cardiovascular effects

S1P is appreciated as having multiple effects on the cardiovascular system (Karliner, 2002). During development, S1P is necessary for blood vessel maturation.

Specifically, $S1P_1$ knock-out mice are embryonically lethal because they have excessive hemorrhage and lack neovascularization. This suggests that $S1P_1$ is the key receptor involved in blood vessel formation (Liu, Wada et al., 2000). $S1P_2$ and $S1P_3$ knock-out mice are viable (Ishii, Friedman et al., 2001;Means, Xiao et al., 2007) although $S1P_2$ knock-out mice display slightly altered vasoconstriction (Lorenz, Arend et al., 2007). In addition to blood vessel maturation, preliminary studies in zebrafish indicated that S1P may be necessary for fusion of the bilateral heart primordial (Kupperman, An et al., 2000).

S1P exerts potent effects on endothelial cells and, in fact, the first S1P receptor to be cloned, $S1P_1$, was cloned from endothelial cells (Hla and Maciag, 1990). Migration, proliferation, shape change and survival in endothelial cells are all promoted by S1P (Hla, Lee et al., 2001). These effects on vascular endothelial cells lead to S1P stimulated angiongenesis in multiple models (Hla, Lee et al., 2001;Chae, Paik et al., 2004;Visentin, Vekich et al., 2006). In addition, S1P mediates cell-cell junctions of endothelial cells and therefore might control vascular permeability (Lee, Thangada et al., 1999).

S1P has been termed by some as a “villain provoking cardiovascular disease” (Siess, Essler et al., 2000;Levade, Auge et al., 2001). In this cardiotoxic role, S1P induces arrhythmias and produces coronary vasoconstriction (Sugiyama, Yatomi et al., 2000;Liliom, Sun et al., 2001) (Sanna, Liao et al., 2004). S1P is also atherogenic and high density lipoprotein (HDL)-mediated inhibition of SphK is a possible explanation for HDL’s protective effects (Xia, Vadas et al., 1999). Lastly, as a result of its ability to activate platelets and to be produced by activated platelets (Yatomi, Ruan et al.,

1995;Yatomi, Yamamura et al., 1997), S1P may contribute to coronary ischemia through intravascular thrombus formation.

In stark contrast to the potential detrimental effects on the cardiovascular system, S1P is able to activate survival pathways in a wide variety of cell types, including CM (An, Zheng et al., 2000;Karliner, Honbo et al., 2001), and in so doing, S1P can be cardioprotective. For example, Karliner *et al*. have extensively shown that low S1P levels administered in a preconditioning paradigm can protect the heart from hypoxic injury (Karliner, Honbo et al., 2001;Karliner, 2002;Jin, Goetzl et al., 2004;Karliner, 2004). In agreement with this finding, the SphK inhibitor, dimethylsphingosine (DMS), increases hypoxia-induced cell death in Langendorf perfused mouse and rat hearts subjected to hypoxia/reoxygenation (Karliner, Honbo et al., 2001;Jin, Goetzl et al., 2004). Subsequent studies revealed that a mutation in SphK increases I/R-induced infarct size and the number of apoptotic cells(Jin, Zhang et al., 2007). In addition, it was shown that signaling through $S1P_1$ is responsible for protection of adult CM from hypoxia-induced death (Zhang, Honbo et al., 2007).

Additional evidence for the cardioprotective role of S1P comes from Theilmer et al. who demonstrated *in vivo* that S1P, as a component of HDL, injected 30 min. before MI decreases infarct size through activation of $S1P_3$ (Theilmeier, Schmidt et al., 2006). Further, studies from Means *et al.* showed that double knock out mice lacking both $S1P_2$ and $S1P_3$, but not either alone, have decreased infarct sizes after coronary ligation (Means, Xiao et al., 2007). Taken together, there is compelling evidence the S1P is cardioprotective particularly as an early player in mitigating cell death due to acute I/R injury.

It is not know what roles S1P has, if any, during post-MI remodeling. It is possible that S1P is cardioprotective during the initial ischemia but then becomes maladaptive during later-stage remodeling. Although CM death occurs in the first few days after an infarction, promotion of fibrosis occurs for an extended period of time. As the following pages will illustrate, S1P may contribute to cardiac fibrosis through its effects on CF and its cross-talk with other pro-fibrotic mediators.

3. Roles in cardiac fibrosis

During acute ischemic events, the heart takes advantage of the ability of S1P to protect CM against cell death; however, if S1P promotes CF function, the effect of S1P on remodeling may not be beneficial to long-term heart function. It was recently demonstrated that mouse CF express $S1P_{1-3}$; however, this study did not investigate fibrogenic effects of S1P on CF (Landeen, Aroonsakool et al., 2007). On the other hand, S1P is fibrogenic to several non-cardiac fibroblast lineages. NIH 3T3 fibroblasts and mouse embryonic fibroblasts proliferate in response to S1P (Wang, Nobes et al., 1997;Olivera, Kohama et al., 1999;Hobson, Rosenfeldt et al., 2001). In addition, S1P promotes differentiation of human lung fibroblasts to the pro-fibrotic, myofibroblast phenotype (Urata, Nishimura et al., 2005). Studies utilizing the S1P analogue, FTY720, showed that it is capable of transforming human foreskin derived fibroblasts into myofibroblast through $S1P_3$ activation (Keller, Rivera Gil et al., 2007). In addition, $S1P_2$ knock out mice display reduced quantity of hepatic myofibroblasts after liver injury as compared to wild type animals (Serriere-Lanneau, Teixeira-Clerc et al., 2007). Further, mice subjected to skin damaging radiation and treated with a

SphK activator (K6PC-5) have increased collagen and fibroblast numbers (Park, Youm et al., 2008).

Interestingly, a comparison of SphK activity in CF and CM revealed that CF have approximately 14 fold higher kinase activity levels, suggesting that CF could be a potent source of S1P in the heart(Kacimi, Vessey et al., 2007). However, it is clear that more work needs to be done in understanding not only the source of S1P in the heart during the remodeling period, but also the effects of that S1P on CF function and remodeling.

There are several signaling pathways stimulated by S1P which could contribute to CF activation. An activated CF demonstrates increased proliferation, myofibroblast transformation, and collagen production. S1P could potentially increase all of these responses through the diversity of S1P coupling at the post-receptor level. $S1P_1$ couples exclusively to G_i (Hla, 2004) while both $S1P_2$ and $S1P_3$ can signal through three different G proteins, G_i, G_q, and $G_{12/13}$ (Sanchez and Hla, 2004). Through G_i, S1P activates pro-proliferative signaling pathways, including the MAPK extracelluar signal-regulated kinases (ERK) (Sugden and Clerk, 1997;Kluk and Hla, 2002). Upon activation, ERK is phosphorylated and translocates to the nucleus where it activates multiple transcription factors(Gonzalez, Seth et al., 1993). G_i also activates the MAPKs c-Jun N-terminal kinase (JNK) and p38 kinases, but these can serve as both pro- and anti-proliferative (New and Wong, 2007). G_q primarily binds to and activates PLC-β which results in increased intracellular Ca^{2+}. This increase is associated with activation of multiple signaling enzymes and, although insufficient on their own to induce proliferation, contribute to its up-regulation most likely through

activation of ERK (Radeff-Huang, Seasholtz et al., 2004). $G_{12/13}$ exclusively activates the small GTPase Rho, which is necessary for myofibroblast formation and function (Sah, Seasholtz et al., 2000). Interestingly, constitutively active Rho upregulates α-SMA promoter activity and Rho is necessary for stress fiber and focal adhesion formation (Sugden and Clerk, 1997;Anderson, DiCesare et al., 2004). Rho kinase knock-out mice exhibit dramatic decreases in perivascular fibrosis (Rikitake, Oyama et al., 2005) and the Rho kinase inhibitor (Y-27632) decreases interstitial fibrosis in mice post-MI (Hattori, Shimokawa et al., 2004). siRNA directed against $S1P_2$ and $S1P_3$ reduces TGF-β-stimulated α-SMA expression in lung fibroblasts (Kono, Nishiuma et al., 2007). These data suggests that in some fibroblast types both $S1P_2$ and $S1P_3$ are responsible for myofibroblast transformation. Again, this has yet to be demonstrated in CF and is the topic of this dissertation.

4. Synergy with TGF-β

S1P may facilitate the action of other well-known pro-fibrotic factors, such as TGF-β. TGF-β is one of the most widely studied and recognized contributors to cardiac fibrosis (Desmouliere, Geinoz et al., 1993). TGF-β plays a particularly important role in both reparative and maladaptive remodeling and it's expression is increased in all areas of the heart post-MI (Hao, Ju et al., 1999;Deten, Holzl et al., 2001). TGF-β increases both production and secretion of collagens (Lijnen and Petrov, 2000). It is directly linked to myofibroblast transformation (Okada, Takemura et al., 2005). *In vivo* gene transfer of TGF-β induces myocardial fibrosis (Lijnen and

Petrov, 2000). In addition, a soluble TGF-β receptor, which blocks TGF-β signaling, attenuates remodeling post-MI (Okada, Takemura et al., 2005).

Original work done by Trojanowska and colleagues established a cross-talk between S1P and TGF-β in fibroblasts (Sato, Markiewicz et al., 2003). Using YSR2, the yeast equivalent to SphK, fused to a luciferase reporter, they found that TGF-β stimulates its activity. In the same study S1P induced activity of a collagen promoter (COL1A2) and overexpression of YSR2 increased endogenous type I collagen gene expression(Sato, Markiewicz et al., 2003). Additional evidence comes from dermal fibroblasts whereby, TGF-β upregulates SphK1 expression and activity leading to increased expression of TIMP-1, a protein that inhibits ECM degradation (Yamanaka, Shegogue et al., 2004). Increased expression of TIMP-1 is linked to interstitial fibrosis and diastolic dysfunction in heart failure patients (Heymans, Lupu et al., 2005). Conversely, S1P stimulates expression and release of TGF-β (Norata, Callegari et al., 2005). Lastly, using a model of mouse lung fibrosis where the drug bleomycin is used to induce fibrosis, α-SMA expression coordinates with increased SPHK1 expression (Kono, Nishiuma et al., 2007). In the same study WI-38 cells, a human fetal lung fibroblast line, responded to TGF-β treatment with increased SphK1 and α-SMA expression, which was inhibited by DMS or another commercially available SphK inhibitor (Kono, Nishiuma et al., 2007).

Signaling pathways associated with S1P and TGF-β cross-talk appear to depend on cell type. In a non-fibroblast study utilizing a murine embryonic Langerhans cell line (XS52), S1P-induced migration depended on Smad3 activation (Radeke, von Wenckstern et al., 2005). Smad activation is the classical pathway for

TGF-β-induced signaling (Radeke, von Wenckstern et al., 2005). In contrast, in esophageal cancer cells TGF-β–stimulated ERK 1/2 activation is Smad3 dependent while S1P activates ERK independent of Smad3 (Miller, Alvarez et al., 2008). Furthermore, TGF-β increases S1P levels and this increase is necessary for ERK 1/2 activation and subsequent invasion and migration (Miller, Alvarez et al., 2008).

D. Summary

Cardiac remodeling after MI is initially beneficial but progresses to a maladaptive process with time. Cardiac fibroblasts are the main cell responsible for cardiac remodeling. They respond to injury by proliferating, transforming to myofibroblasts and increasing production of ECM. Excessive ECM production leads to cardiac fibrosis, diastolic and eventually systolic dysfunction.

S1P is a bioactive lipid which can increase proliferation, survival and transformation of multiple cell types. In addition, S1P may be important to mediating TGF-β-stimulated effects in fibroblasts. This dissertation focuses on the roles that S1P plays in influencing CF activation both on its own and in conjunction with TGF-β. Further, *in vivo* studies were undertaken to evaluate the effect of S1P post-MI using an anti-S1P mAb. Although S1P has been implicated in multiple fibrotic diseases, this is the first time that its role in post-MI remodeling has been explored. Considering its potentially important role in myocardial healing it is important to elucidate the potential for anti-S1P treatments in heart failure. Therefore the thesis statement of this dissertation is that S1P activates cardiac fibroblasts, both on its own and in

conjunction with TGF-β, and these effects result in exaggerated post-MI remodeling *in vivo*.

II. Methods

A. Cardiac cell culture

1. Isolation and dissociation of cardiac fibroblasts

Cardiac fibroblasts (CF) were prepared from the ventricles of 5-6wk male C57BL/6 mice. Ventricles were excised and placed in Hanks Balanced Salt Solution (HBSS)(Mediatech, Manassas, VA) supplemented with 100µM HEPES (Invitrogen, Carlsbad, CA). Excised heart ventricles were minced and digested in 0.03 mg/ml Liberase Blendzyme 4 (Roche, Indianapolis, IN) and 0.014% Trypsin (Mediatech) at 37 °C for 10 minutes. Digestion solution was aspirated off and fresh digestion solution was added for an additional 75 minutes. Isolated cells were pelleted (1000 rpm for 5 min), washed three times and re-suspended in growth media (DMEM/10% FCS/ 200U/ml penicillin/ 200ug/ml streptomycin 0.5µg/ml amphotericin B) (Mediatech) and incubated at 37°C with 90% air/10% CO_2. Cells were allowed to adhere to T75-cm^2 flasks for 1-2 hrs after which attached cells were supplemented with fresh media. All procedures involving animal use were approved by the San Diego State University Institutional Animal Care and Use Committee (IACUC).

2. Plating and culture of cardiac fibroblasts

All cells were allowed to grow for 4 days which is equivalent to approximately passage 2. Cells were trypsinized, resuspended in growth media and plated at appropriate densities for future experiments.

3. Isolation, plating and culture of adult mouse cardiac myocytes

Adult mouse cardiac myocytes (CM) were isolated and cultured as previously described (Horikawa, Patel et al., 2008). Briefly, hearts were excised and digested via retrograde perfusion with a collagenase II (Worthington Biochemicals) solution. After pelleting CM away from CF, RNA was isolated for RT-PCR or quantitative real-time PCR. For immunofluorescence, cells were plated on culture plates pre-coated with laminin (Sigma).

B. siRNA Transfection

For Collagen synthesis or immunoblot analysis 3 x 10^4 cells were plated on 12-well plates, for Rho assays 1 x 10^5 cells were plated on 6-well plates, and for BrdU incorporation 5 x 10^3 cells were plated on 96-well plates in growth media and allowed to adhere overnight. Media was then changed to 0.4ml, 0.8ml or 0.08ml DMEM (0% FCS) without antibiotics, respectively. Lipid complexes of siRNA and Lipofectamine RNAiMax (Invitrogen) were prepared using yielding 1 μM siRNA. The following siRNA constructs were used: s74219 for SphK1, 61102 for $S1P_1$, 164753 for $S1P_2$, 66573 for $S1P_3$ (Ambion, Austin, TX). After 4 hr of transfection 2%BSA/DMEM without antibiotics was added to yield a final concentration of 1% BSA and cells were incubated for an additional 44 hr before performing the appropriate assays.

C. RT-PCR and Real Time PCR

Total RNA was column extracted from adult mouse cardiac fibroblasts (CF) or cardiac myocytes (CM) according to manufacturer’s protocol (RNeasy, Qiagen, Valencia, CA). cDNAs were amplified by reverse transcription with random

hexamers as primers according to manufacturer's protocol (Superscript III, Invitrogen). PCR was run on cDNA samples to amplify ~500 base pair segments of each gene using the following sequence specific primers: $S1P_1$, 5'-aactttgcgagtgagctggt-3' and 5'-agagactgagctgcggagag-3'; $S1P_2$, 5'-aaaaccaaccactggctgtc-3' and 5'-ttgctgagtggaacttgctg-3'; $S1P_3$, 5'-aagcctagcgggagagaaac-3' and ttcccggagagtgtcatttc-3'; $S1P_4$, 5'-ggctactggcagctatcctg-3' and ggcccagacattagaaccaa-3'; $S1P_5$, 5'-tatggctgcagcagaaattg-3' and 5'-ttcctctgtagccagccact-3'; SphK1, 5'-agaagggcaagcatatggaa-3' and 5'-agggctctactggggatgtt-3'; SphK2, 5'-tggcctgctcactgtagatg-3' and 5'-ggggaggaacattagtgcaa-3'; and GAPDH, 5'-aactttggcattgtggaagg-3' and 5'-ccctgttgctgtagccgtat-3'. For real-time PCR, Taqman labeled primers for $S1P_{1,}$ $S1P_2$, $S1P_{3,}$ $S1P_4$, SphK1, SphK2 and GAPDH (Mm00514644_m1, Mm01177794_m1, Mm00515669_m1, Mm00474763_m1, Mm00448841_g1, Mm00445021_m1, and 4352339E, respectively) and Collagen Iα1 (Invitrogen, MLUX3302453) and real-time PCR master mix from Applied Biosystems (Foster City, CA) were used for amplification of cDNA using iCycler from Bio-Rad (Hercules, CA). Standard curves for each gene were created using above listed primers on mouse reference total RNA standard from Stratagene (La Jolla, CA). Total cDNA representing each gene was back calculated using standard curve and normalized to GAPDH signal for that particular cDNA sample.

D. Cell Proliferation Assays

1. [^{3}H]-Thymidine Incorporation

CF were plated at on 24-well plates at 3x 10^5 cells per well. Cells were serum-starved for 48 hr in DMEM (0%FCS) followed by 48 hr treatment with media alone (control) or with treatments of interest in the presence of 0.5 μCi [^{3}H]-thymidine (Perkin Elmer Life Sciences, Waltham, MA). Cells were washed once with cold PBS, twice with cold 10% tricholoracetic acid (TCA) and lysed in 1M NaOH. Lysates were added to 5ml scintillation fluid and radioactivity was counted.

2. BrdU Incorporation

5 x 10^3 cells per well were plated in 96 well plates. Cells were allowed to adhere overnight in growth media followed by 48 hr serum starvation in DMEM (0%FCS). CF were pre-incubated with U0126 (200nM) (Sigma, St. Louis, MO) for 30 min for the inhibition of ERK or 24 hr with pertussis toxin (PTX) at 100ng/ml (Sigma, St. Louis, MO) for the inhibition of G_i. Appropriate treatments were then applied (media alone for control) for 48hr in the presence of BrdU according to manufacturer's directions. Cells were then fixed and BrdU incorporation was quantified with anti-BrdU antibody staining according to manufacturer's protocol (Calbiochem, Gibbstown, NJ).

E. Cell Viability (WST-1 ELISA).

5 x 10^3 cells per well were plated in 96 well plates. Cells were allowed to adhere overnight in growth media followed by 48 hr serum-starvation in DMEM

(0%FCS). Appropriate treatments were then applied (media alone for control) for 48 hr. Wells were incubated an additional 3 hr in the presence of WST solution and detected for formazan product according to manufacturer's protocol (Millipore, Temecula, CA).

F. Collagen Synthesis ([^{3}H]-proline Incorporation).

CF were plated overnight on 12 well plates at 3 x 10^5 cells per well followed by 48 hr of serum starvation in DMEM (0%FCS). Cells were then treated with media alone (control) or with indicated treatments in the presence of 1 μCi [^{3}H]-proline (Perkin Elmer Life Sciences). Cells treated with Y-27632 (Sigma, St. Louis, MO) were pre-treated for 30 min. Cells were removed from dishes with trypsin and precipitated in 20% trichloroacetic acid (TCA) overnight at 4 °C. After centrifugation, cell pellets were washed three times with 1 ml of 5% TCA then dissolved in 0.2M NaOH and the solutions were titrated to neutral pH with 0.2M HCl. Collagenase II (Worthington Biochemical, Lakewood, NJ) (100 μl, 2mg/ml) in Tris-$CaCl_2$-*N*-ethylmaleimide buffer (Appendix C) was added to each sample and incubated at 37 °C for 1 hr. Samples were then precipitated with 10% TCA for 1 hr at 4 °C and centrifuged at 18 x *g* for 10 min. Radioactivity of the supernatant was quantified using liquid scintillation counting.

G. Rho Activation

CF were plated overnight on 6-well plates at 1 x 10^6 cells per well followed by 48 hr of serum starvation in DMEM (0% FCS). Cells were then treated with media alone (control) or with 1 μM S1P for indicated times. Rho activation was assessed

using G-LISA RhoA Activation Assay Biochem Kit from Cytoskeleton (Denver, CO) using the manufacturer's recommended protocol.

H. Immunoblot analyses

1. Protein isolation

CF were cultured in 12-well plates at a density of 7 x 10^4 cells per well. Wells were lysed in 100µl of lysis buffer (Appendix C) and the resulting lysates were sonicated for 10 seconds. 10µl of cell lysate was diluted into 90 µl of lysis buffer and protein concentration was determined using BCA reagent (Pierce, Rockford, IL).

2. Protein fractionation and Western blotting

Protein isolated from CF was suspended in Laemmli buffer (Invitrogen) at approximately 1:2. For α-Smooth Muscle Actin (α-SMA) detection, 1µg of protein was loaded per lane while, generally speaking, 5-10µg of total protein was loaded per lane for all other proteins detected. Protein was then boiled for 5 minutes and was fractionated by SDS-PAGE, using pre-cast Tris-Glycine gels (Invitrogen). Proteins were then electroblotted onto PVDF membranes (GE Healthcare, Piscataway, NJ) in transfer buffer for one hour at 60 volts, blots were rinsed in Tris buffered saline with Tween-20 (TBST, Cellgro) and either stored at 4 °C or immunostained immediately.

3. Immunostaining

Blocking of immunoblots was performed in either 5%BSA in TBST or 1.5% non-fat dry milk in TBST for one hour at room temperature. Blots were then incubated at 4 °C with agitation over night with appropriate concentration of primary

antibody (see Appendix B) diluted in 5%BSA/TBST or 1.5% non-fat dry milk/TBST. Blots were then washed with agitation three times for 10 minutes each in TBST. Secondary antibody incubations were performed with the appropriate HRP-conjugated antibodies (see Appendix B) with agitation for 1-2 hr at room temperature. Blots were rinsed again three times as above. Detection was performed using SuperSignal West Pico (Pierce) or Lumigen TMA-6 (GE Healthcare).

I. Immunofluoresence

CF cultured on 4-well chamber slides (Becton Dickinson; Franklin Lakes, NJ) were serum-starved in DMEM (0%FCS) for 48 hr followed by stimulation for the indicated times with media alone (control) or with treatments of interest. CM were plated, allowed to adhere for 30 min and fixed. For S1P, cells were fixed in 5% neutral buffered formalin, permeabilized with 0.1% Triton X 100 for 10 min., blocked with 1% BSA in PBS for 20 min. and stained in the same blocking solution with anti-S1P mAb at 1:50 overnight at 4 °C. Phalloidin was used to label actin (Invitrogen) during secondary incubation at 1:40. For all other stains, cells were washed once with PBS, fixed for 5 min in ice-cold methanol, blocked in 10% goat serum in PBS for 1 hr at room temperature. Slides were incubated with primary antibodies diluted in 10% goat serum in PBS for 1 hr at room temperature, washed 3 times for 5 min each in PBS and then detected with secondary antibodies (1:500 for goat anti-rabbit FITC and goat anti-mouse FITC) diluted in PBS supplemented with 10% goat serum for an additional 1 hr. Cells were mounted using Vectashield mounting media from Vector

Laboratories (Burlingame, CA). Confocal microscopic imaging was done as previously described (O'Brien, Gellings et al., 2003).

J. Sphingosine Kinase Assays

Sphingosine kinase (SphK) assays were performed as a modified version of Olivera et al. (Olivera, Barlow et al., 2000). 1.7 x 10^5 cells were plated on 6-well plates in growth media and allowed to adhere overnight followed by 48 hr of serum starvation in DMEM (0%FCS). Cells were then treated media alone (control) or with appropriate treatments in duplicate wells. Duplicate wells were lysed in a total of 300 µl sphingosine kinase (SphK) assay buffer (Appendix C) on ice. Further cell lysis was performed through 3 freeze/thaw cycles using dry ice and a room temperature water bath. Protein concentrations were determined as described in immunoblot analysis section. 15 µg of protein in 180 µl was incubated with 10 µl 1mM sphingosine complexed to BSA and 10 µl 20mM ATP in 200mM $MgCl_2$ for 30 min in a 37 °C water bath. Reactions were terminated by placing samples on ice and adding 20 µl 1M HCl. Lipid extraction was performed by adding 0.8ml of chloroform/methanol/HCl (100:200:1, v/v) followed by vigorous vortexing and further addition of 240 µl of chloroform and 240 µl of 2M KCl. The reactions were then vortexed and centrifuged 10 min at 1000g to separate the organic and aqueous phases. The organic phase was separated and dried down in a Speed Vac followed by resuspension in 400 µl of delipidized human serum (DHS). S1P levels were quantified as described in S1P ELISA.

K. S1P ELISA

S1P levels in were determined by competition ELISA. S1P coating material was diluted to 0.1 uM in carbonate buffer (Appendix C). Plates were coated with 100ul/well of this coating solution and incubated at 37°C for 1 hour. The plates were then washed 4X with PBS (Appendix C) and blocked with 150 ul/well PBS + 1% BSA + 0.1% tween-20 for 1 hour at room temperature. S1P standards were prepared by diluting 100uM stock S1P into DHS at the following concentrations: 2 uM, 1 uM, 0.5 uM, 0.1 uM, 0.01 uM, and 0.0 uM. Samples were diluted appropriately so that the ODs fall within the linear range of the standard curve (0.5 uM-0.1 uM). The primary antibody (biotinylated anti-S1P mAb) was diluted to 0.8 ug/ml in PBS +0.1 % tween-20 and combined with the samples or standards at a 1:3 ratio of antibody to sample on a non-binding plate (recommended 100 ul Ab and 300 ul samples/standards, enough to run in triplicate). The plates were washed 4X with PBS and then incubated for 1 hour at room temperature with 100 ul/well of the primary antibody combined with the samples/standards. Next the plates were washed 4X with PBS and then incubated for 1 hour at room temperature with 100 ul/well of HRP-conjugated streptavidin antibody diluted 1:60,000 in PBS + 1% BSA + 0.1% tween-20. Again the plates were washed 4X with PBS and developed using 100 ul/well TMB substrate at 4°C. After 8 minutes, the reaction was stopped with 1M H_2SO_4, 100ul/well. The OD was measured at 450 nm. Data is analyzed using Graphpad Prism software. The standard ODs were graphed using a four parameter equation and this is used to calculate the S1P in the samples. Excel software was used to calculate the uM S1P in each sample by correcting the values for the dilution factor.

L. Myocardial Infarction

Mice were housed in an air-conditioned room with a 12 hr light-dark cycle and given standard chow with free access to water. Ligation of the proximal left anterior artery was performed according to previously established methods with the following modifications (Bayat, Swaney et al., 2002). Briefly, anesthetic induction was accomplished using isoflurane (4% in 100% O_2). Mice were then intubated using a 20 gauge angiocatheter and mechanically ventilated with 1.5% isoflurane in 100% O_2 to maintain anesthesia during surgery. Animals then were placed in the supine position and the chest cavity was opened at the third intercostal space to visualize the left side of the heart. The left coronary artery was ligated approximately 2 mm below the edge of the left atrial appendage by placing 6-0 silk suture (tapered needle) around the artery. LV blanching indicated successful occlusion of the vessel. The chest was then closed by suturing together adjacent ribs (6-0 vicryl), and the skin was closed as a separate layer. Antibody or vehicle only treatments were administered in a double-blinded manner. Anti-S1P mAb was administered to animals at 25mg/kg via intraperitoneal (IP) injections. Animals were treated at 48 hours after MI and again every three days until the study was terminated at 2 weeks. *In vivo* efficacy, lack of toxicity, and basic pharmacokinetic properties have previously been demonstrated for the anti-S1P mAb (Visentin, Vekich et al., 2006).

M. Echocardiography

Echocardiography was performed in lightly anesthetized mice using a Sequoia C256 echocardiography system (Siemens, Munich, Germany) and a 15L8 MHz linear

transducer. Inhaled isoflurane (1% in 100% O_2) was administered by a facial mask and M-mode echocardiography of the mid-ventricle was recorded at the level of papillary muscles. LV end-diastolic and end-systolic diameter (LVIDd and LVIDs, respectively), end-diastolic LV septal wall thickness, and LV posterior wall thickness were measured from these images. LV fractional shortening (FS) was calculated as FS (%) = [(LVIDd-LVIDs)/LVIDd] x 100.

N. Histology

Paraffin sections (5 μm) were obtained from heart specimens and stained using Weigert's Iron Hematoxylin Set and Masson's Trichrome Kit (Sigma). Infarct size was calculated using ImageJ (National Institutes of Health) whereby the area of collagen staining was outlined and expressed as a percentage of the total left ventricle. Four separate sections from base to apex of the heart were averaged. Perivascular fibrosis was also quantified using Image J in a blinded manner from at least 4 sections from each heart containing up to 5 vessels per section. Perivascular fibrosis was calculated as the ratio or fibrosis area surrounding the vessel to the total vessel area. Results were obtained from 6 hearts in each of the vehicle control and anti-S1P antibody treated groups and 3 hearts in the sham group. Vessels between 100 and 500 microns were used for analysis. Interstitial fibrosis was evaluated using Adobe Photoshop and Image J. At least 8, 40X photos of masson's trichrome stained sections were taken of at least 5 sections from each heart. All blue or red areas on each section were selected in Adobe photoshop, copied, converted to a grey scale image and each image was saved. In Image J total pixels for each image (red or blue) were quantified.

Interstitial fibrosis was expressed as the ratio of the blue pixels over the total pixels (red + blue).

O. Statistical Analysis

One- and Two-way analysis of variance (ANOVA) and graphical representations were performed using PRISM 4.0 from GraphPad (San Diego, CA). Statistical significance was set at $P < 0.05$

III. Results

A. S1P effects on cardiac fibroblasts, *in vitro*

S1P is a bioactive lipid which increases proliferation, survival and transformation of multiple cell types. In addition, S1P may be important to mediating TGF-β-stimulated effects in fibroblasts. Therefore the thesis statement of this dissertation is that S1P activates cardiac fibroblasts, both on its own and in conjunction with TGF-β.

1. PCR analysis of cardiac fibroblasts and cardiac myocytes.

The expression profiles of the S1P receptors and SphK isoforms vary among tissues (Melendez, Carlos-Dias et al., 2000;Sanchez and Hla, 2004;Rosen and Goetzl, 2005). In order to appreciate the putative role of S1P signaling in the heart, the profile of $S1P_{1-5}$ receptors and SphK1 and SphK2 in the two principal cell types of the heart, CF and CM was assessed. Previous studies have demonstrated expression of $S1P_{1,}$ $S1P_2$ and $S1P_3$ in CF(Landeen, Aroonsakool et al., 2007). As shown in Figure 3A, both CF and CM express $S1P_1$ and $S1P_3$, as well as SphK1 and SphK2. $S1P_2$ was detectable in CF but not in CM. $S1P_4$ and $S1P_5$ were not detected in either cell type. In order to further appreciate the relative abundance of each receptor and kinase to signaling in CM and CF, their relative mRNA expression levels were quantified by quantitative real-time PCR (QPCR). As is evident from Figure 3B, $S1P_{1,}$ was detected at very low levels in CF and CM. In contrast to $S1P_{1,}$ $S1P_2$ and $S1P_3$ were expressed

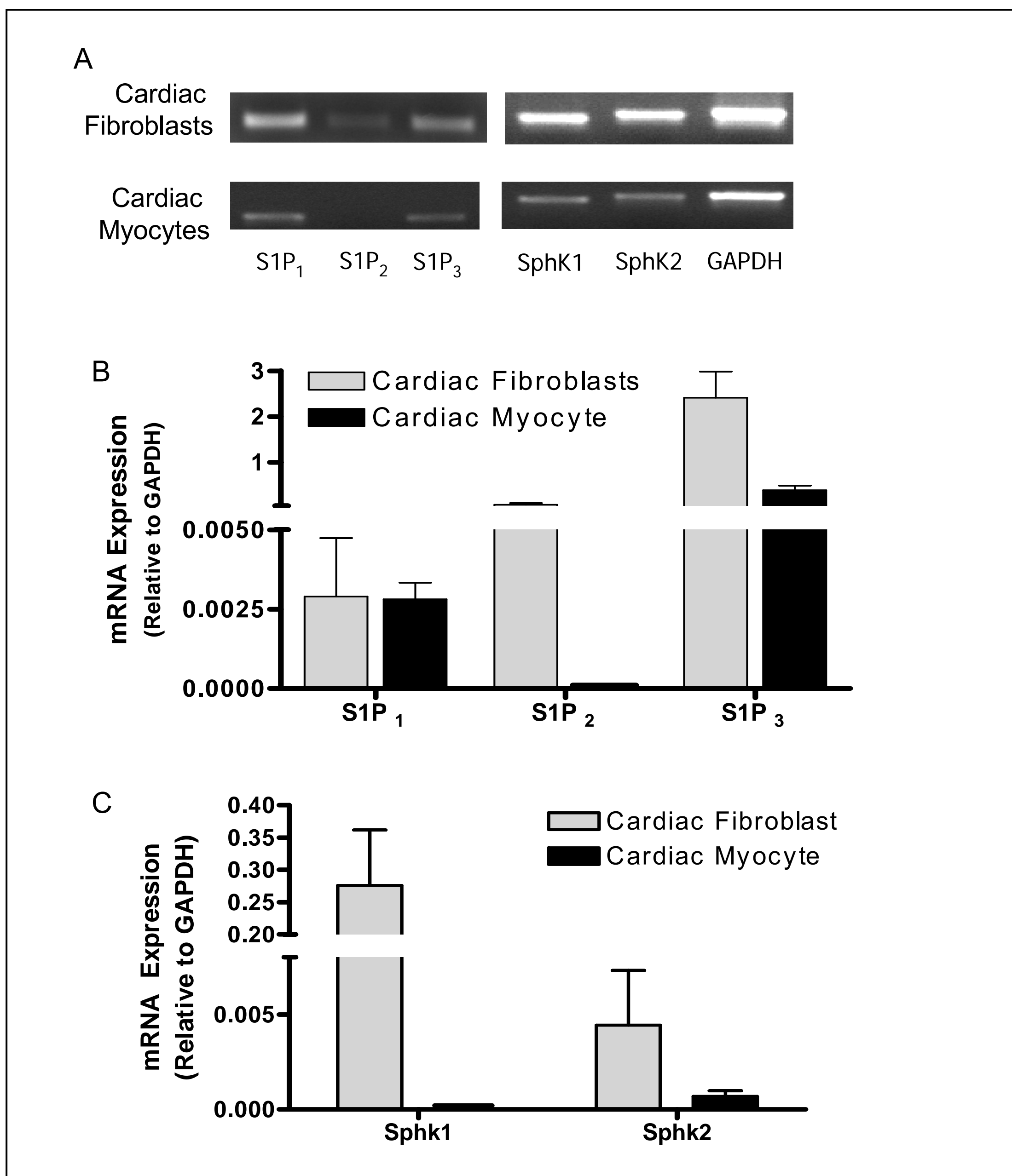


Figure 3. Mouse cardiac fibroblasts express a more robust S1P signaling system than cardiac myocytes. Reverse Transcriptase (RT-PCR), real-time PCR, and immunoblot analysis were performed on cardiac fibroblasts at passage 2 and cardiac myocytes using gene specific primers and fluorescently labeled gene specific primers. (A) Representative bands from RT-PCR products. (B) Quantitative PCR values are expressed relative to GAPDH and normalized for the amount of cDNA. $S1P_2$ and SphK1 were not detectable in cardiac myocytes using QPCR.

at relatively high levels. Consistent with RTPCR, $S1P_2$ was detectable in only CF and not in CM. $S1P_3$ was detectable in both cell types, with CF exhibiting a 38-fold higher level of expression when compared to CM. This suggests that CF possess key receptors involved in S1P signaling. In comparing CF and CM with respect to kinase expression, a similar pattern emerged. Figure 3C demonstrates that CF express very high levels of SphK1 and that CM do not express detectable levels of SphK1. In CF, SphK1 expression was 44-fold higher than SphK2. While CM displayed no detectable SphK1, negligible but detectable levels of SphK2 were observed (Figure 3C).

2. S1P localization in cardiac tissue and isolated cells.

Using the anti-S1P mAb as a reagent for immunohistochemistry (IHC), the presence and localization of S1P in mouse cardiac tissue and in isolated CF and CM was investigated. Co-staining of murine cardiac tissue sections for S1P and caveolin-3 (Cav-3; myocyte marker) revealed prominent, granular S1P staining which localized primarily in the region surrounding CM (Figure 4A, top panels), as indicated by Cav-3 which localizes in a punctate manner along the sarcolemmal membrane of CM. As shown in the higher magnification (100x) images, S1P appears to localize primarily in the zone between adjacent CM, in a region where CF are known to reside (Figure 4A, bottom panels). Although some overlap in staining was observed between S1P and Cav-3, this occurred primarily along the periphery of CF, in the border zone between CF and CM.

To confirm the S1P content of CF in the myocardium, sections were co-stained for S1P and S100A4/FSP1, a fibroblast-specific marker. As shown in Figure 4B,

prominent S1P staining was associated with CF (top panels) and appeared to exist primarily within the cytosolic space of CF (bottom panels). Based on these findings, additional studies to examine S1P staining in isolated CF and CM were conducted (Figure 5). Consistent with the tissue staining, CF contained a large, apparently

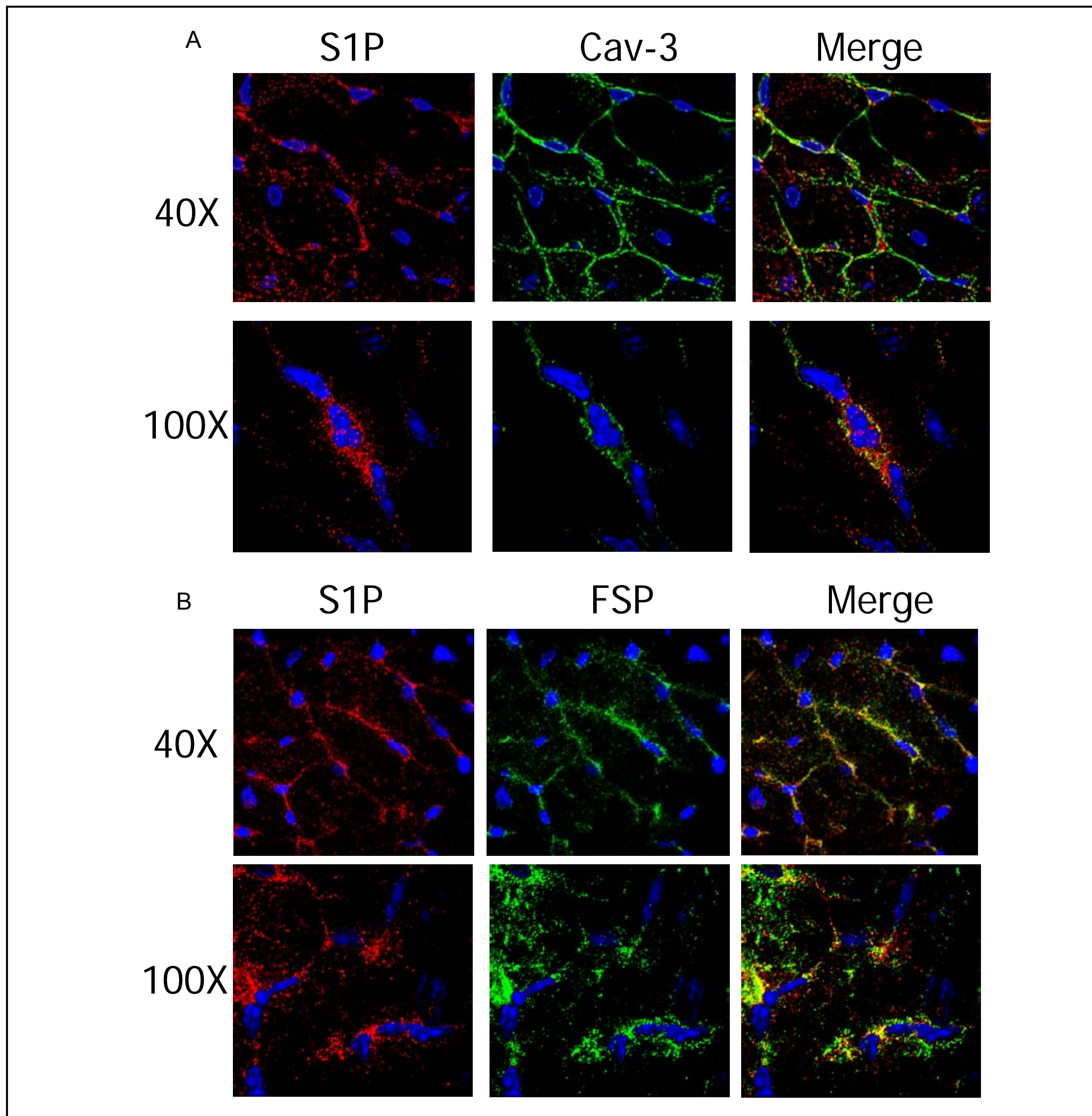


Figure 4. S1P localizes toCF in tissue sections. Frozen mouse cardiac tissue sections were stained using the anti-S1P mAb (red) and (A) Cav-3, a membrane cardiac myocyte marker (indicated by white arrows) or (B) FSP, an intracellular fibroblast marker (green). Nuclei are labeled with Dapi and appear blue in all images.

intracellular pool of S1P (Figure 5, top panels). In contrast to CF, CM did not display appreciable intracellular S1P by IHC, a finding that is consistent with the lack of appreciable SphK1 and SphK2 expression detected in CM by QPCR (Figure 3C).

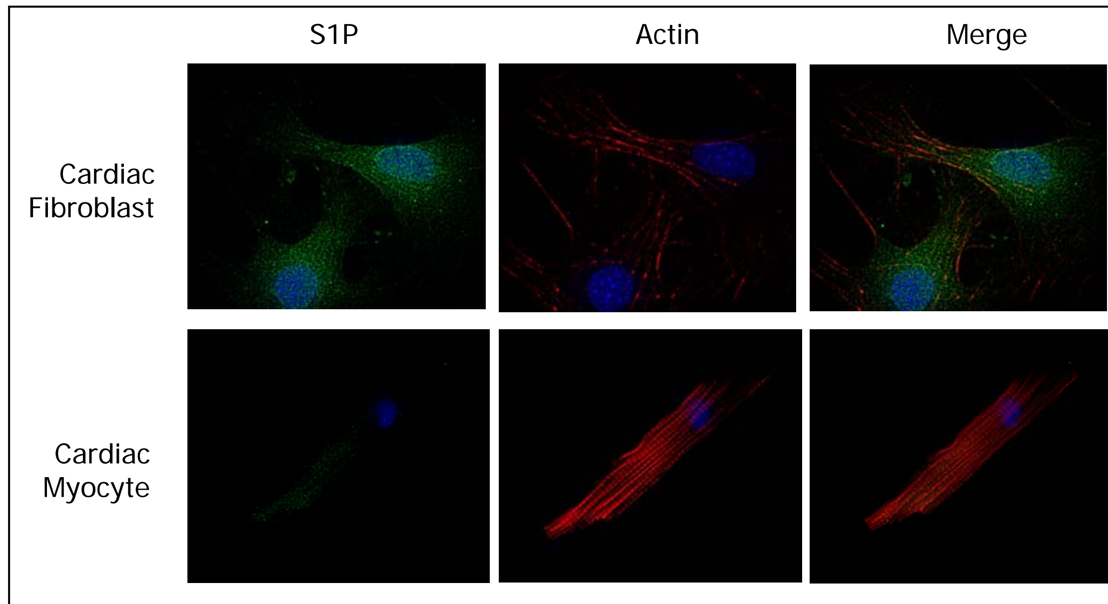


Figure 5. S1P localizes to CF in isolated cells. Isolated cells were fixed within 30 min or 24hr of isolation (CM and CF, respectively) and stained with the anti-S1P mAb (green) and α-actin (red). Nuclei are labeled with Dapi and appear blue in all images.

Combined, these findings demonstrate that the majority of S1P in cardiac tissue is associated with CF.

3. S1P increases proliferation of primary cardiac fibroblasts.

S1P promotes proliferation of numerous cell types including non-cardiac fibroblasts (Wang, Nobes et al., 1997;Olivera, Kohama et al., 1999;Hobson, Rosenfeldt et al., 2001); therefore, S1P was tested for its effects on the proliferation of

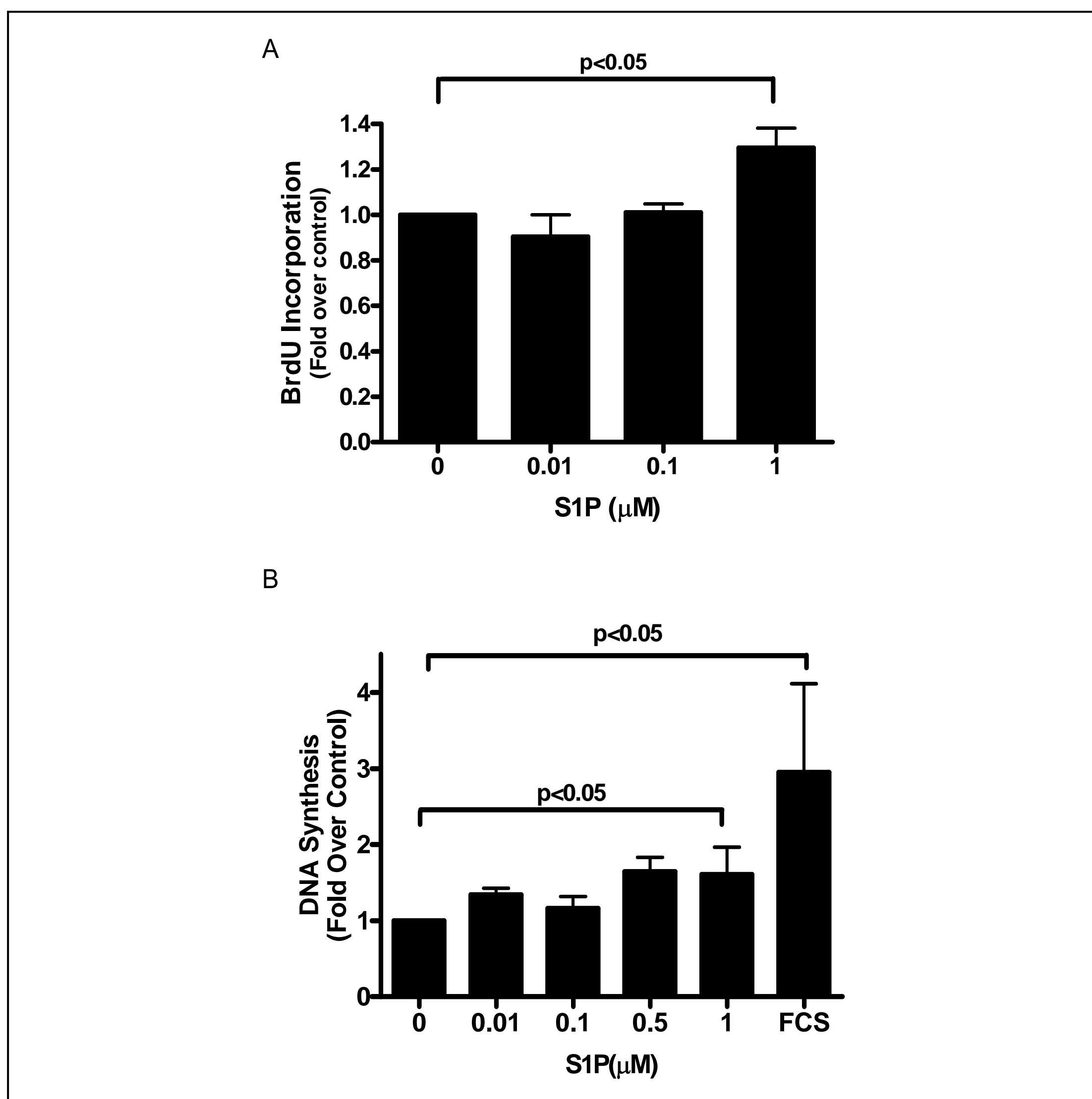


Figure 6. S1P increases DNA synthesis in CF. DNA synthesis was measured using BrdU incorporation (A) or [3H]-thymidine incorporation (B) in CF that were serum starved for 48 hr and then stimulated for 48 hr with serum-free media alone (control) or 0.01, 0.1, 0.5 or 1 μM S1P or 2.5% FCS as a positive control. Data are represented as fold change over control. Values represent mean ± SEM of at least 3 independent experiments. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

CF. Using a BrdU incorporation assay, S1P increased CF proliferation by 1.3-fold at 1 μM concentration (Figure 6A). In confirmation, S1P increased proliferation measured by increased [^{3}H]-thymidine incorporation into DNA. Significance was achieved at 0.5 μM S1P and 2.5% fetal calf serum (FCS), a positive control (Figure

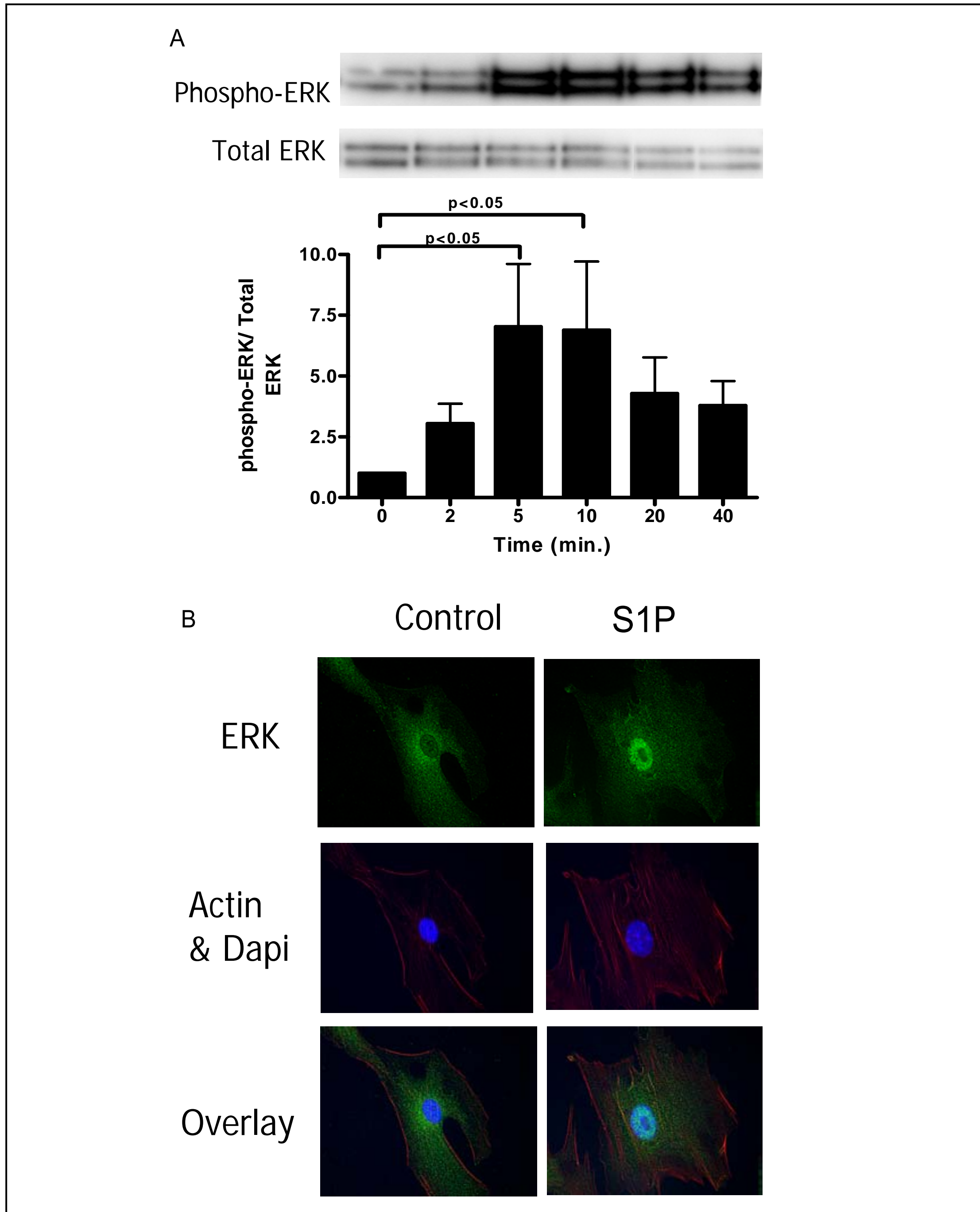


Figure 7. S1P increases ERK phosphorylation in CF. ERK Phosphorylation and nuclear translocation was measured in CF that were serum starved for 48 hr and then stimulated for (A) 0 (control), 2, 5, 10, 20, or 40 min with 1 μM S1P; and (B) 3 hr with serum-free media (control) or 1 μM S1P and stained for ERK (green), α-actin (red) and dapi (blue). Data are represented as fold change over control and phospho-Erk data are expressed relative to total Erk. Values represent mean ± SEM of at least 3 independent experiments. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

6B). The MAP kinase ERK is well-recognized for its role in S1P-induced cell proliferation (Carpio, Stephan et al., 1999;Kim, Lee et al., 2006). Accordingly, effects

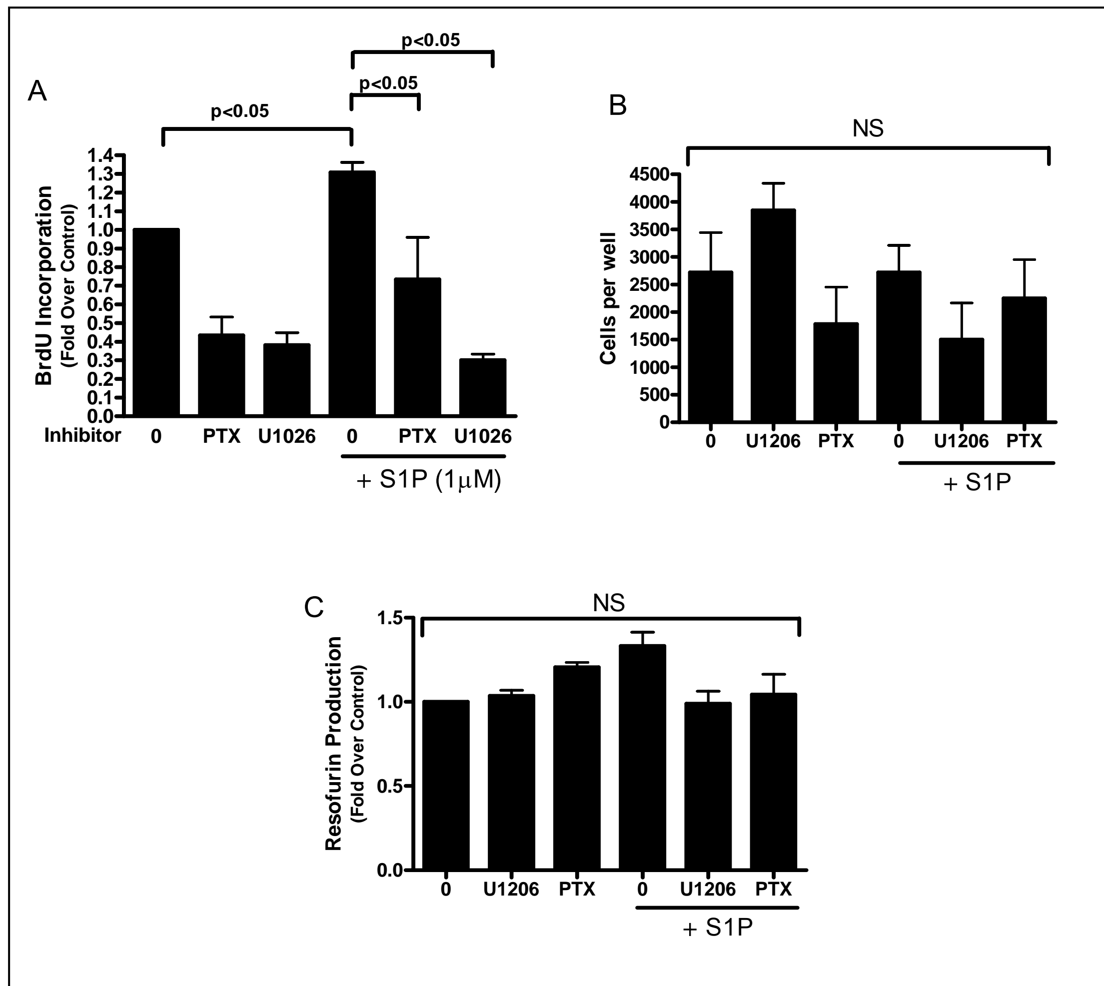


Figure 8. S1P increases DNA synthesis in an Erk-dependent manner in CF. DNA synthesis (BrdU incorporation), cells per well and cellular viability (resorufin production) were measured in CF that were serum starved for 48 hr and then stimulated for 48 hr with serum-free media alone (control) 1 µM S1P with or without U0126 (Erk inhibitor) or pertussis toxin (PTX, G_i inhibitor). Data are represented as fold change over control. Values represent mean ± SEM of at least 3 independent experiments. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

of S1P on ERK phosphorylation were examined. Treatment of CF with S1P (1 µM) caused a time-dependent increase in phospho-ERK that was maximal at 5 and 10 min (Figure 7A). Consistent with the literature reports, phospho-ERK translocated to the

nucleus of CF (Figure 7B), an event which is linked to increased ERK activation and regulation of transcription (Gonzalez, Seth et al., 1993).

In order to demonstrate the relationship between ERK phosphorylation, translocation and proliferation, whether S1P-induced CF proliferation was ERK-dependent was examined. As Figure 8A shows, S1P-stimulated CF proliferation was abolished by the ERK inhibitor, U0126. In addition, S1P-stimulated proliferation was completely abrogated by the G_i inhibitor, PTX, suggesting that S1P effects on CF proliferation are due to G_i-mediated ERK activation. Because the inhibitors alone appear to decrease BrdU incorporation, effects of these inhibitors on cellular viability were evaluated by counting cells per well (Figure 8B) and using the MTT assay for dehydrogenase activity (Figure 8C). In both cases, the inhibitors alone did not affect cellular viability.

4. S1P-stimulated proliferation and ERK activation is not dependent on any one S1P receptor alone.

S1P-stimulated proliferation has been linked to G_i activation and $S1P_1$ and $S1P_3$ both activate G_i(Heo, Kim et al., 2008;Yoon, Hong et al., 2008). In order to determine which S1P receptor is responsible for proliferation in CF, siRNA to each receptor was employed to knock-down the receptors. As seen in Figure 9, when evaluated with specific QPCR, the RNA levels for $S1P_1$, $S1P_2$, $S1P_3$ and SphK1 were knocked down by siRNA to 26%, 11%, 2% and 33% of scramble control, respectively. When $S1P_1$, $S1P_2$ or $S1P_3$ siRNA was employed for knock-down and S1P-stimulated proliferation

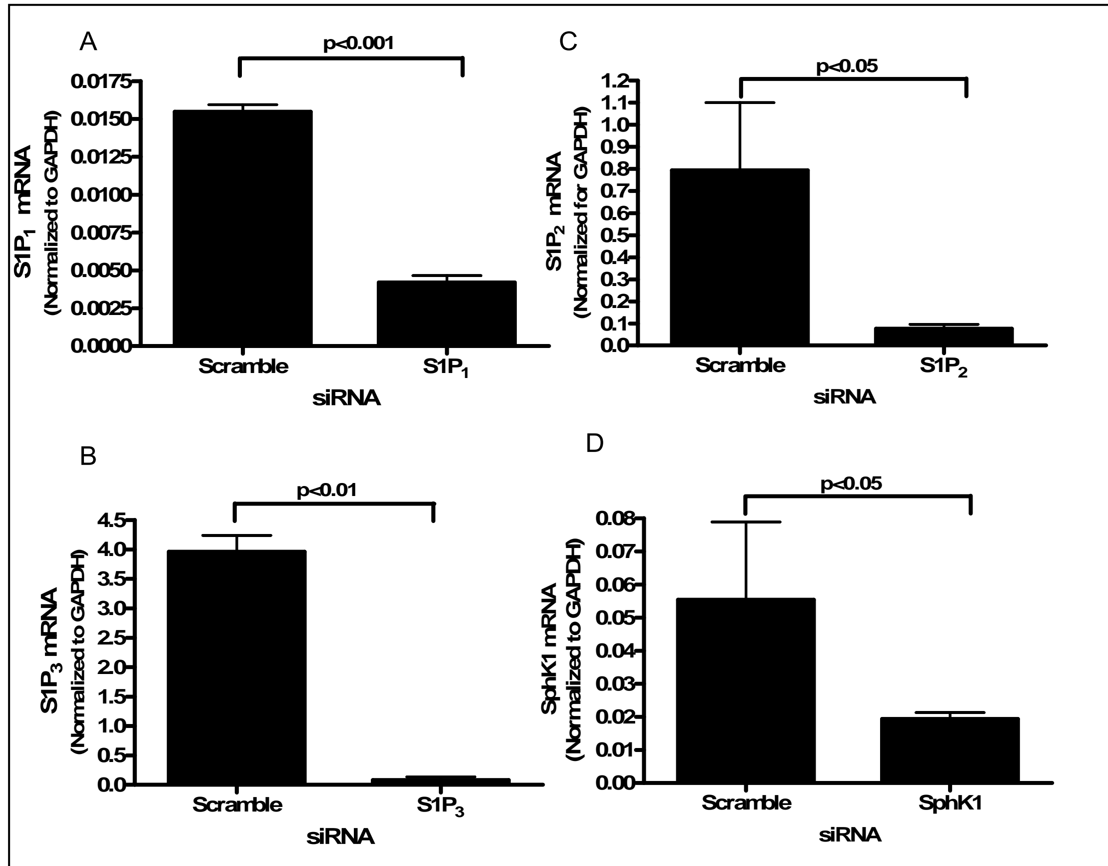


Figure 9. **siRNAs to S1P receptors of SphK1 significantly decrease mRNA.** CF were transfected with siRNA for 24hr, RNA was harvested, and gene specific QPCR analysis was used to evaluate mRNA levels. Values represent mean ± SEM of at least 3 independent experiments. Statistical analyses were performed using student's t-test.

The inability of any one siRNA to knock-down proliferation suggested that more than one S1P receptor can facilitate S1P-stimulate proliferation. Therefore, loss of one receptor could be compensated for through expression of another. To test this hypothesis, the anti-S1P mAb was added to cell treatments to immunologically absorb extracellular S1P and thereby inhibit signaling through all receptors. Figure 10B proliferation when compared to antibody treatment alone. There was an increase in

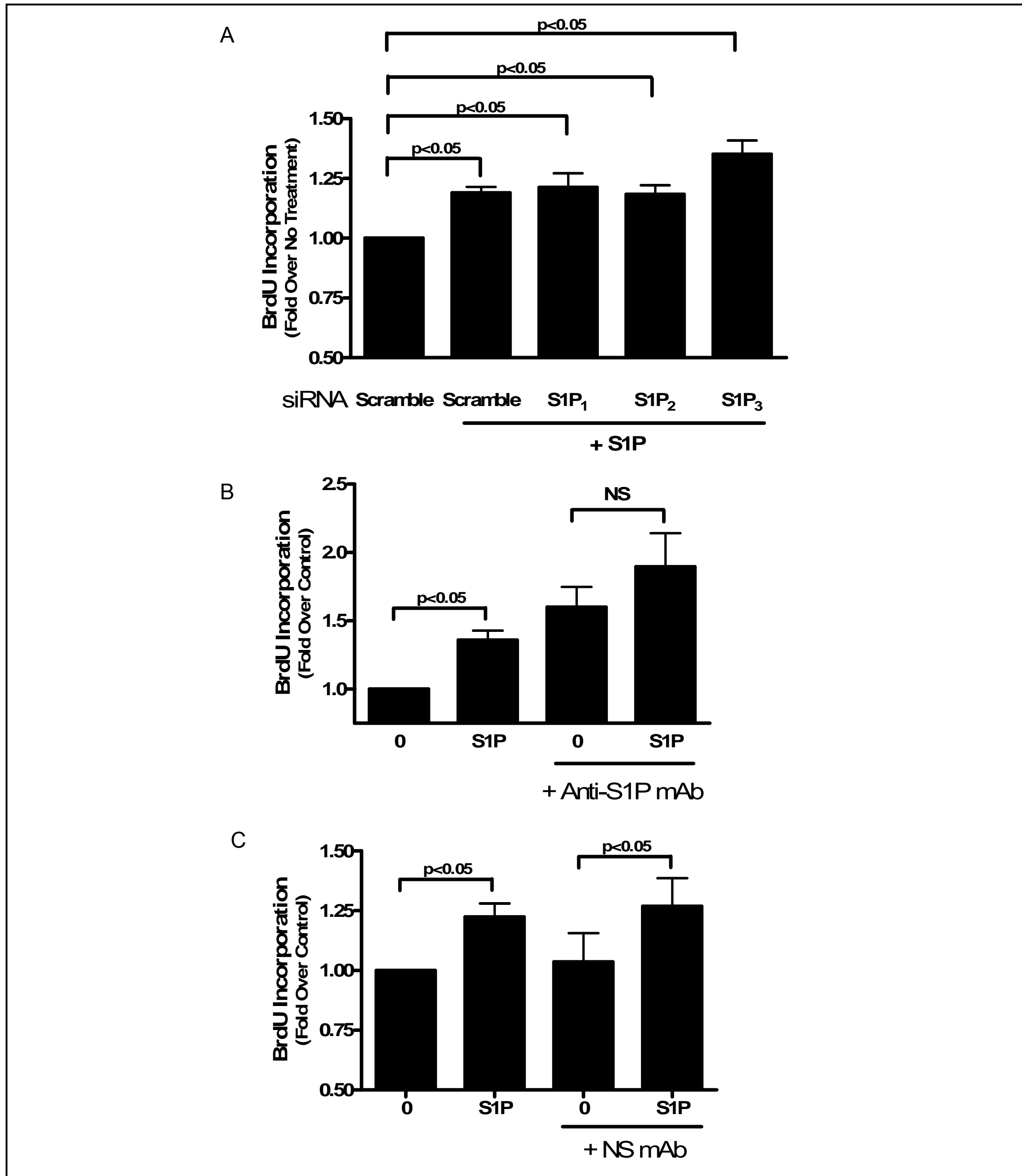


Figure 10. S1P-increased DNA synthesis is inhibited with anti-S1P mAb. DNA synthesis was measured using BrdU incorporation in CF that were (A) transfected with siRNA in serum free media for 48 hr and then stimulated for 48 hr with serum-free media alone (control) or 1 μM S1P; (B and C) serum starved for 48hr and then treated for 48hr with serum-free media alone (control) or 1 μM S1P with or without anti-S1P mAb or NS Ab. Data are represented as fold change over control. Values represent mean ± SEM of at least 3 independent experiments. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

the background level of BrdU incorporation with the addition of anti-S1P mAb and it was independent of the presence of S1P. Addition of a non-specific isotype matched antibody (NS Ab) had no effect on S1P-stimulated proliferation (Figure 10C).

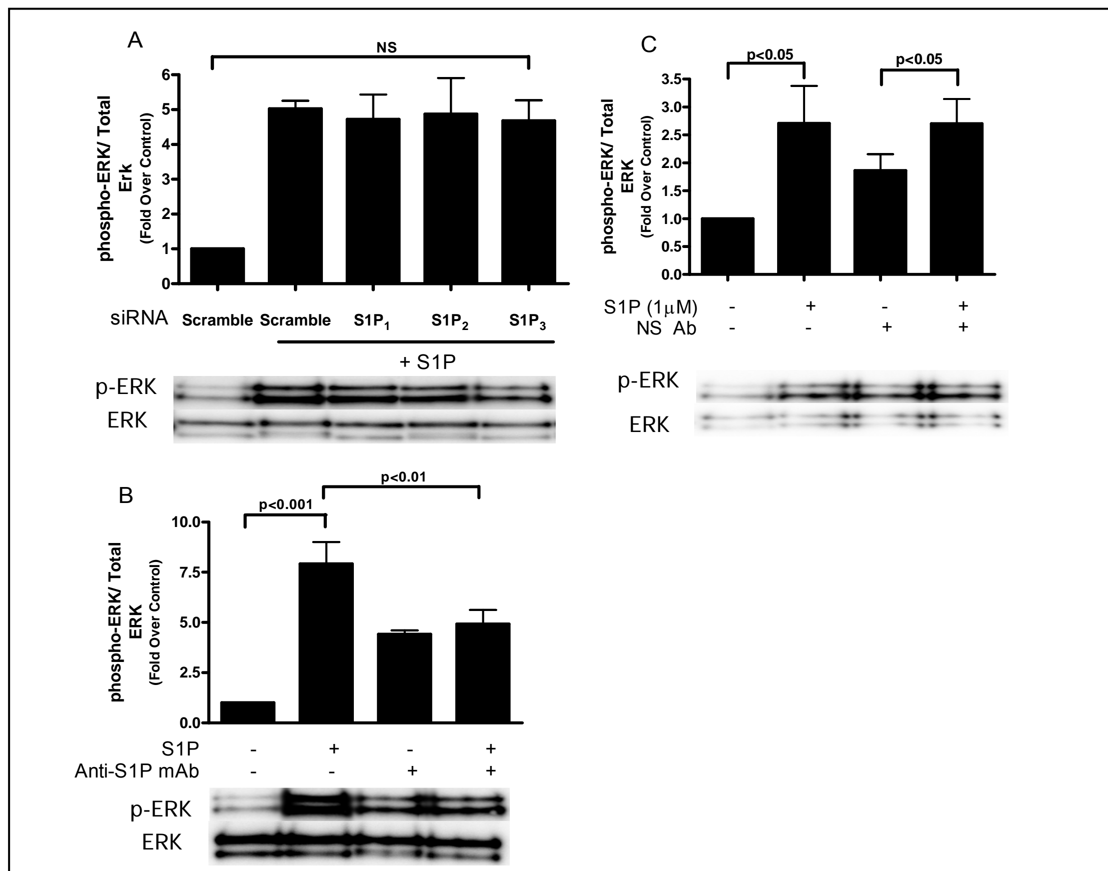


Figure 11. S1P increases DNA synthesis in an Erk-dependent manner in CF. ERK phosphorylation was measured in CF that were transfected with scramble (control), $S1P_1$, $S1P_2$, or $S1P_3$ siRNA for 48 hr in DMEM/ 1%BSA and then stimulated for 10 min. with (A) serum-free media alone (control) or 1 μM S1P (B) serum-free media alone (control) or 1 μM S1P with or without anti-S1P mAb (150 μg/ml) and (C) serum-free media alone (control) or 1 μM S1P with or without NS Ab (150 μg/ml). Data are represented as fold change over control and phosphor-ERK over total ERK. Values represent mean ± SEM of at least 3 independent experiments. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

Consistent with the lack of effects of siRNA on proliferation, siRNA to $S1P_1$, $S1P_2$ or $S1P_3$ did not inhibit S1P-stimulated ERK phosphorylation (Figure 11A). As seen in Figure 11B, the anti-S1P mAb, on the other hand, was able to decrease S1P stimulated ERK phosphorylation by approximately 50%. As with proliferation, the NS Ab did not inhibit S1P-stimulated ERK phosphorylation (Figure 11C), thus indicating that immunoabsorbtion of S1P, and not just the addition of an antibody, is necessary to inhibit S1P-stimulated ERK phosphorylation.

5. S1P-stimulated myofibroblast transformation is Rho dependent.

Over time, fibroblasts transform to myofibroblasts spontaneously when plated on a rigid surface, such as a plastic cell culture dish, and at low density (Masur, Dewal et al., 1996). To evaluate the myofibroblast nature of the CF used in these studies, lysates of cells were made at various times after initial plating: 0, 2, 4 and 6 days. These time points correspond approximately to 0, 1, 2 and 3 doublings of cell number. Figure 12A shows that α-smooth muscle actin (α-SMA), the myofibroblast marker, was not detectable until day 4 and 6. This indicates that by day 4 after initial plating, the CF had begun transforming to the myofibroblast phenotype; however, it was desirable to use cells at this stage because before that time there were not a sufficient number of cells to run the desired experiments. To determine if it was possible to further differentiate these cells, day 4 cells were serum starved for 48hr and then treated for 48hr with increasing concentrations of TGF-β, a well-known and very potent activator myofibroblast transformation. Even though these cells had already displayed characteristics of myofibroblasts, TGF-β was able to further transform them proliferation when compared to antibody treatment alone. There was an increase in

evaluating myofibroblast transformation and all future studies were done with day 4 CF.

To determine the effects of S1P on transformation of CF into collagen-producing myofibroblasts, α-SMA expression in CF in response to S1P was evaluated. As Figure 13 demonstrates, S1P stimulated α-SMA expression in a dose-dependent

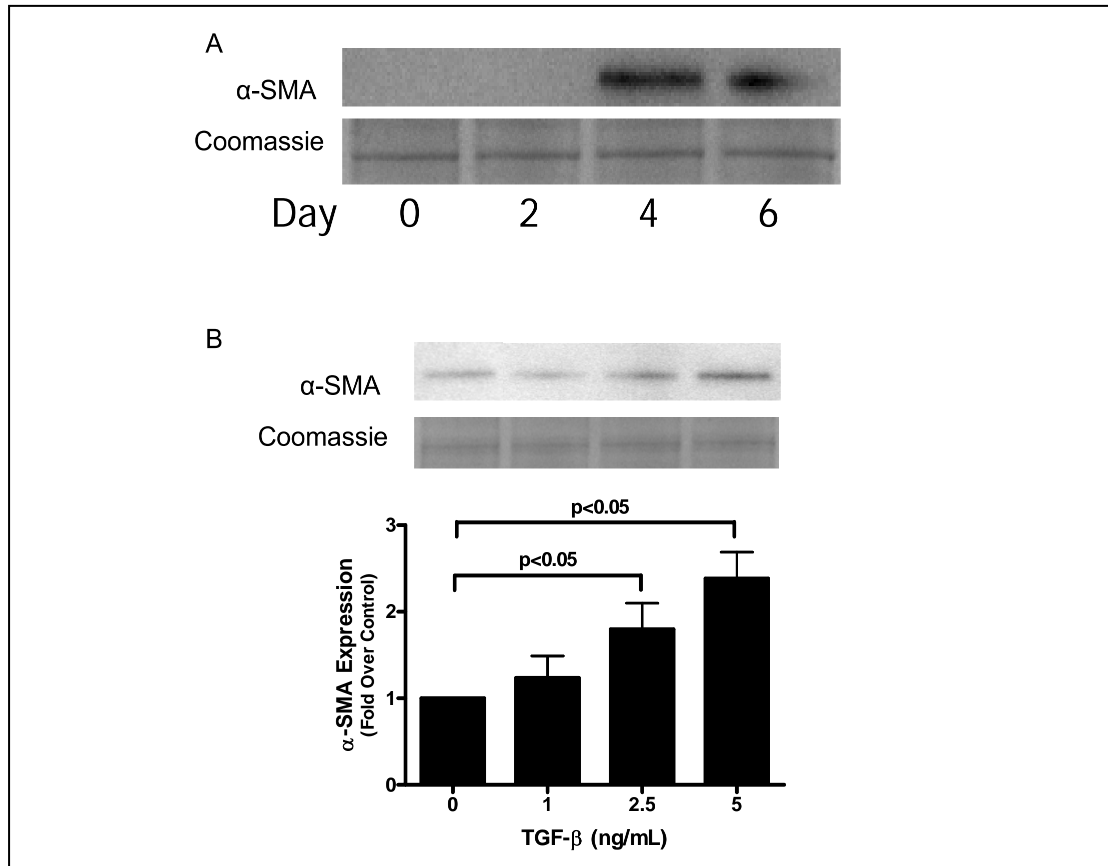


Figure 12. Day 4 CF can be further transformed to myofibroblasts. α-SMA expression was measured using immunoblot analysis in CF that were(A) freshly isolated (0) or cultured for 2, 4 or 6 days (B) cultured for 4 days, serum starved for 48hr and then stimulated for 48 hr with serum-free media alone or 1, 2.5 or 5 ng/ml TGF-β. Data are represented as fold change over control. Values represent mean ± SEM of at least 3 independent experiments. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

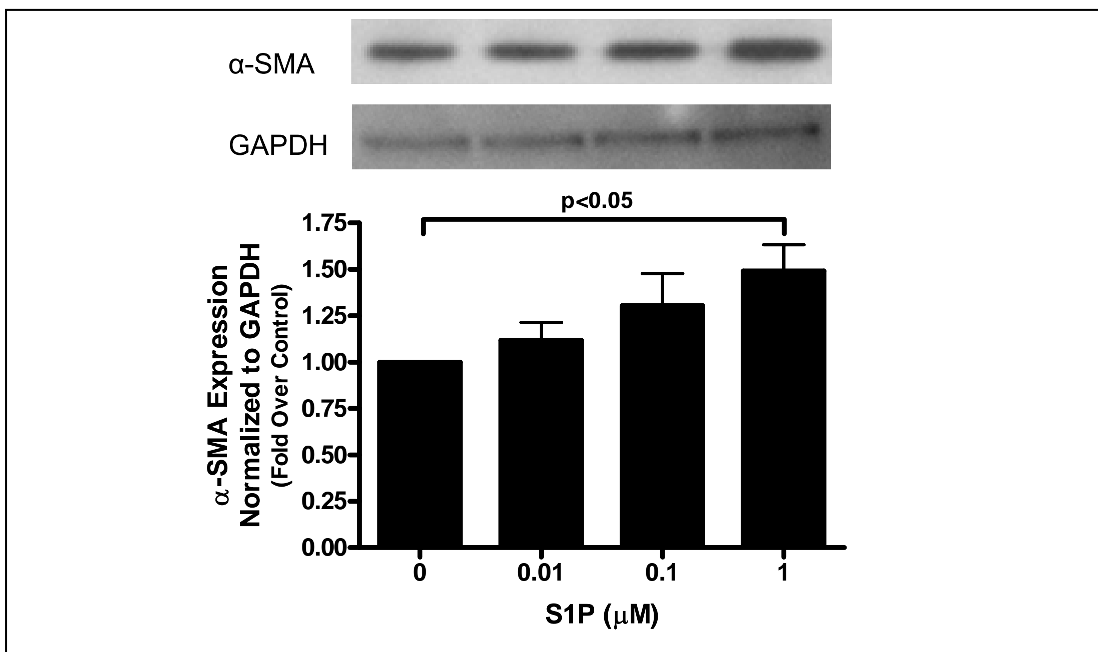


Figure 13. S1P stimulates myofibroblast transformation in CF. α-SMA expression was measured using immunoblot analysis in CF that were serum starved for 48 hr and then stimulated for 48 hr with serum-free media alone (control) or 0.1 or 1 μM S1P. Data are represented as fold change over control. Values represent mean ± SEM of at least 4 independent experiments. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

expression in 4 day old fibroblasts was significant but not as potent as TGF-β (compare to Figure 12B).

Activation of the low molecular weight G protein, Rho, is required for myofibroblast transformation (Anderson, DiCesare et al., 2004) and S1P has been shown in other cell types, to activate Rho (Sugimoto, Takuwa et al., 2003). As seen is Figure 14A, S1P (1 μM) increased Rho activation by about 3 fold with a maximal effect observed at 10 min. To examine the role of Rho in S1P-induced myofibroblast transformation, cells were pretreated for 30 minutes with the Rho kinase inhibitor, Y-27632, followed by 48 hours of treatment with 1 μM S1P.

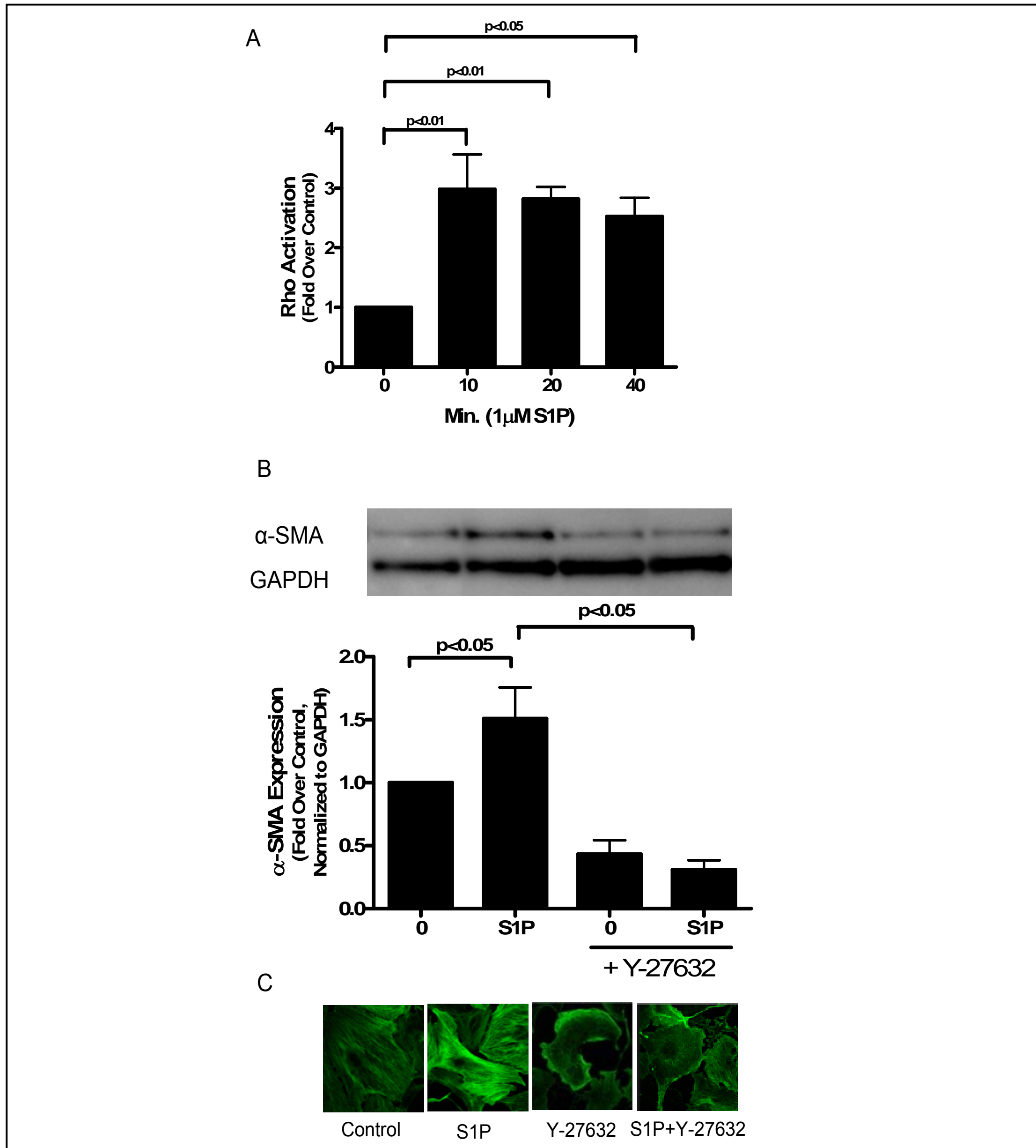


Figure 14. S1P-stimulated myofibroblast transformation is Rho-dependent. (A) Rho activation was measured using Rho ELISA on cells that were serum starved for 48 hr and then stimulated with 1 μM S1P for 10, 20 or 40 min. (B)α-SMA expression was measured using immunoblot analysis in CF that were serum starved for 48 hr and then stimulated for 48 hr with serum-free media alone or 0.5 μM S1P with or without Y-27632 (Rho Kinase inhibitor). (C) α-SMA expression was measured using immunofluoresence in CF that were serum starved for 48 hr and then stimulated for 24 hr with serum-free media alone or 1 μM S1P with or without Y-27632 (Rho Kinase inhibitor). Data are represented as fold change over control. Values represent mean ± SEM of at least 4 independent experiments. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

Addition of the Rho kinase inhibitor decreased S1P-induced α-SMA expression (Figure 14B). Importantly, the Rho kinase inhibitor also inhibited the S1P-stimulated incorporation of cytosolic α- SMA into organized microfilaments as detected by IHC (Figure 14C).

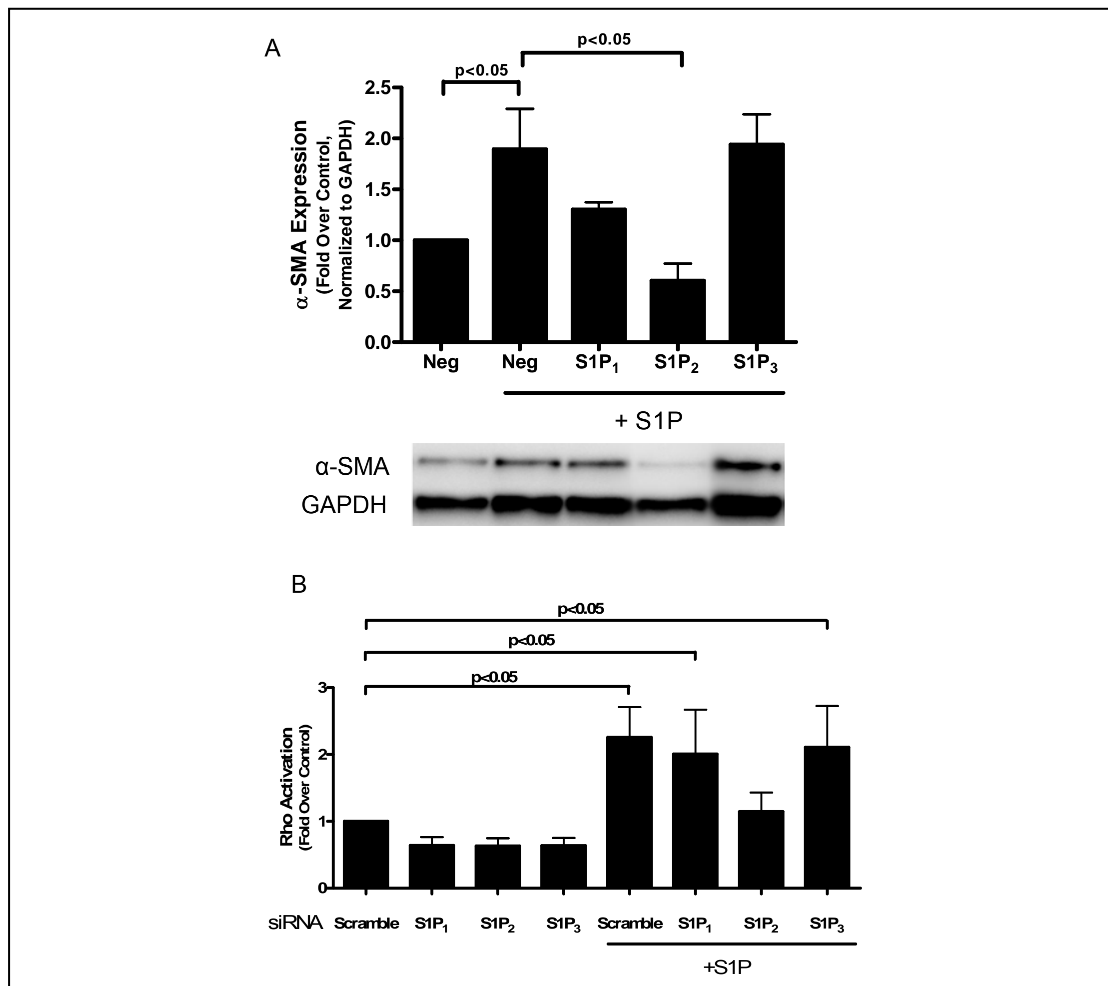


Figure 15. α-SMA expression and Rho activation are $S1P_2$ dependent. CF were transfected with scramble (control), $S1P_1$, $S1P_2$, or $S1P_3$ siRNA for 48 hr in DMEM/ 1%BSA and then (A) stimulated for 48hr with serum-free media alone (control) or 1μM S1P and α-SMA expression was measured using immunoblot analysis and (B) stimulated for 10 min with serum-free media alone (control) or 1 μM S1P and Rho activation was measured using Rho ELISA. Data are represented as fold change over control and phosphor-ERK over total ERK. Values represent mean ± SEM of at least 3 independent experiments. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

6. S1P-stimulated myofibroblast transformation and Rho activation is $S1P_2$ dependent.

To determine which receptor is responsible for S1P-stimulated α-SMA expression and Rho activation, siRNA was employed to knock-down the $S1P_1$, $S1P_2$ or $S1P_3$ receptors that are predominantly expressed by CF (refer to Figure 3). Figure 15A demonstrates that $S1P_1$ and $S1P_3$ siRNA did not significantly inhibit myofibroblast transformation, while $S1P_2$ siRNA completely abrogated S1P-stimulated α-SMA expression in a statistically significant fashion. It was determined, in Figure 14B and 14C, that S1P-stimulated α-SMA expression was Rho dependent; therefore, siRNAs were employed in a Rho activation assay to determine which S1P receptor is responsible for Rho activation. Figure 15B shows that, similar to myofibroblast transformation, S1P-stimulated Rho activation was dependent $S1P_2$, but not $S1P_1$ or $S1P_3$.

7. Basal myofibroblast transformation is increased in $S1P_3$ siRNA-treated cells through an inside-out mechanism.

Because it was hypothesized that CF could constitutively produce and release S1P into the cell-conditioned media by the 'inside-out' signaling mechanism (Takabe, Paugh et al., 2008), a series of experiments were conducted to test the effects of siRNAs and SphK1 inhibitors on CF characteristics in the absence of added S1P. Figure 16A shows CF cells transfected with siRNAs without S1P treatment increased basal levels of α-SMA expression. While cells transfected with interference RNAi to

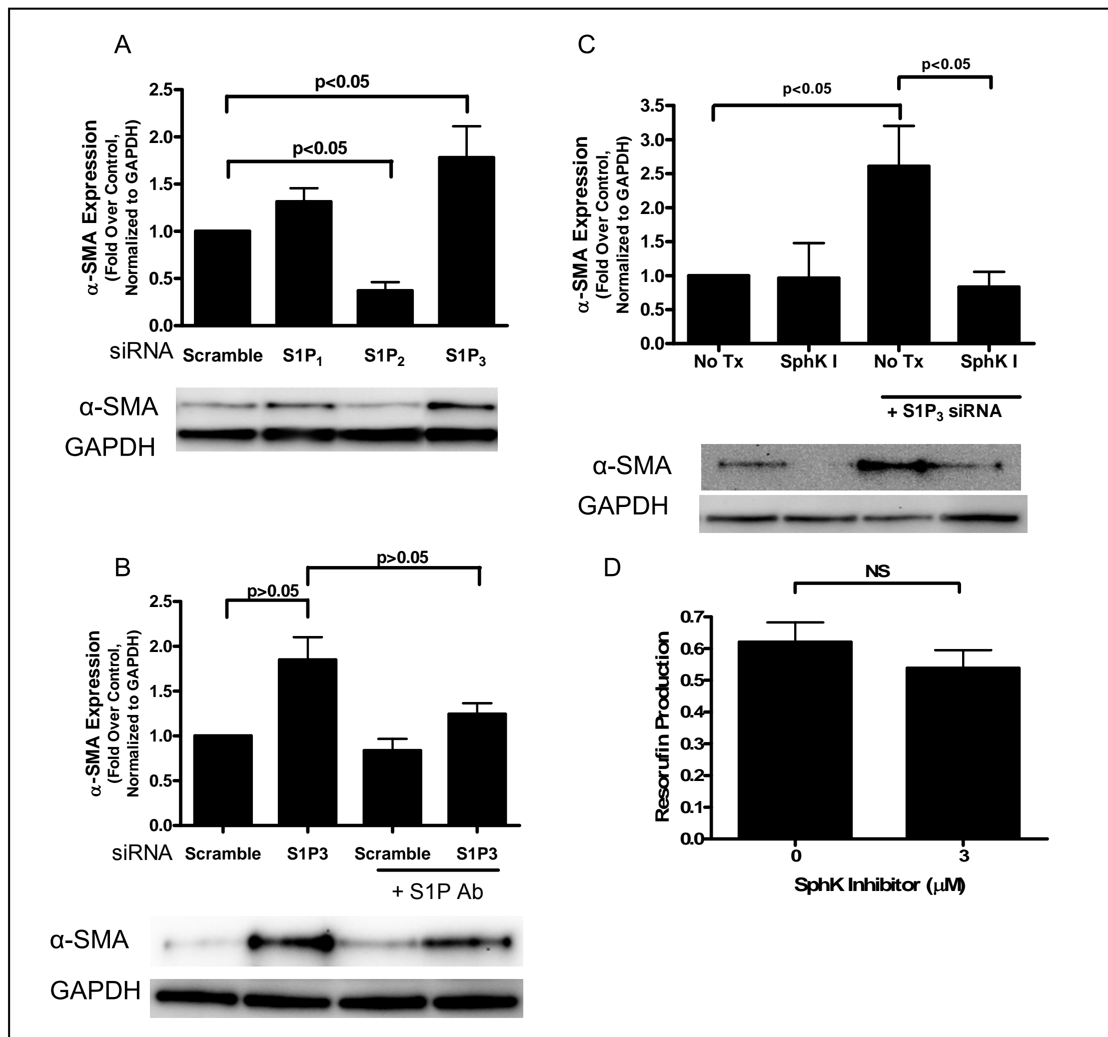


Figure 16. Basal α-SMA expression increased in $S1P_3$ siRNA transfected cells is inhibited with anti-S1P mAb or SphK inhibitor (SphKI). α-SMA expression was measured using immunoblot analysis in CF that were transfected with scramble (control), $S1P_1$, $S1P_2$, or $S1P_3$ siRNA for 48 hr in DMEM/ 1%BSA and then (B) treated with or without anti-S1P mAb (150 μg/ml) for 48hr or (C) with or without 3 μM SphK inhibitor (SphKI) for 48 hr. (D) Mitochondrial dehydrogenase activity (resorufin production) was measured in CF that were serum starved for 48hr and then treated with or without 3 μM SphK inhibitor (SphKI) for 48 hr. Data are represented as fold change over control. Values represent mean ± SEM of at least 4 independent experiments. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

$S1P_1$ had no significant effect on α-SMA expression, $S1P_2$ siRNA significantly and substantially reduced α-SMA expression in the absence of exogenous S1P.

Surprisingly, $S1P_3$ siRNA increased α-SMA expression by 1.8-fold. The increase in the basal α-SMA level, without the addition of exogenous S1P, suggested that S1P produced by CF may be acting on $S1P_2$ in an autocrine and paracrine manner to activate expression, while also inhibiting α-SMA expression via activation of $S1P_3$.

To establish that the inside-out mechanism was operating in CF, we inhibited signaling by two approaches: addition of an anti-S1P mAb to neutralize by molecular absorption any S1P released from cells and, secondly, by addition of a SphK inhibitor which would inhibit endogenous production of S1P by the CF. In Figure 16B, 150 μg/ml of the anti-S1P mAb added after $S1P_3$ siRNA transfection significantly decreased α-SMA expression, suggesting that there was sufficient endogenous S1P in the cell-conditioned medium to activate $S1P_3$ receptors. Likewise, Figure 16C shows that addition of 3 μM of the SphK inhibitor significantly inhibited the increase in α-SMA caused by $S1P_3$ siRNA. As a control, 3 μM of the SphK inhibitor was shown to have no effect on cell viability (Figure 16D).

8. S1P stimulates collagen production by CF in a Rho–dependent manner.

To assess the ability of S1P to stimulate collagen production by CF two methods were used: a collagenase-sensitive [^{3}H]-proline incorporation assay for collagen protein expression and QPCR for collagen Iα1 mRNA expression. Figure 17A demonstrates that only very low levels of S1P (0.1 μM) were required to induce collagen RNA expression, as measured by QPCR. Similarly, Figure 17B shows that S1P induced a dose-dependent increase in collagen protein production as measured by

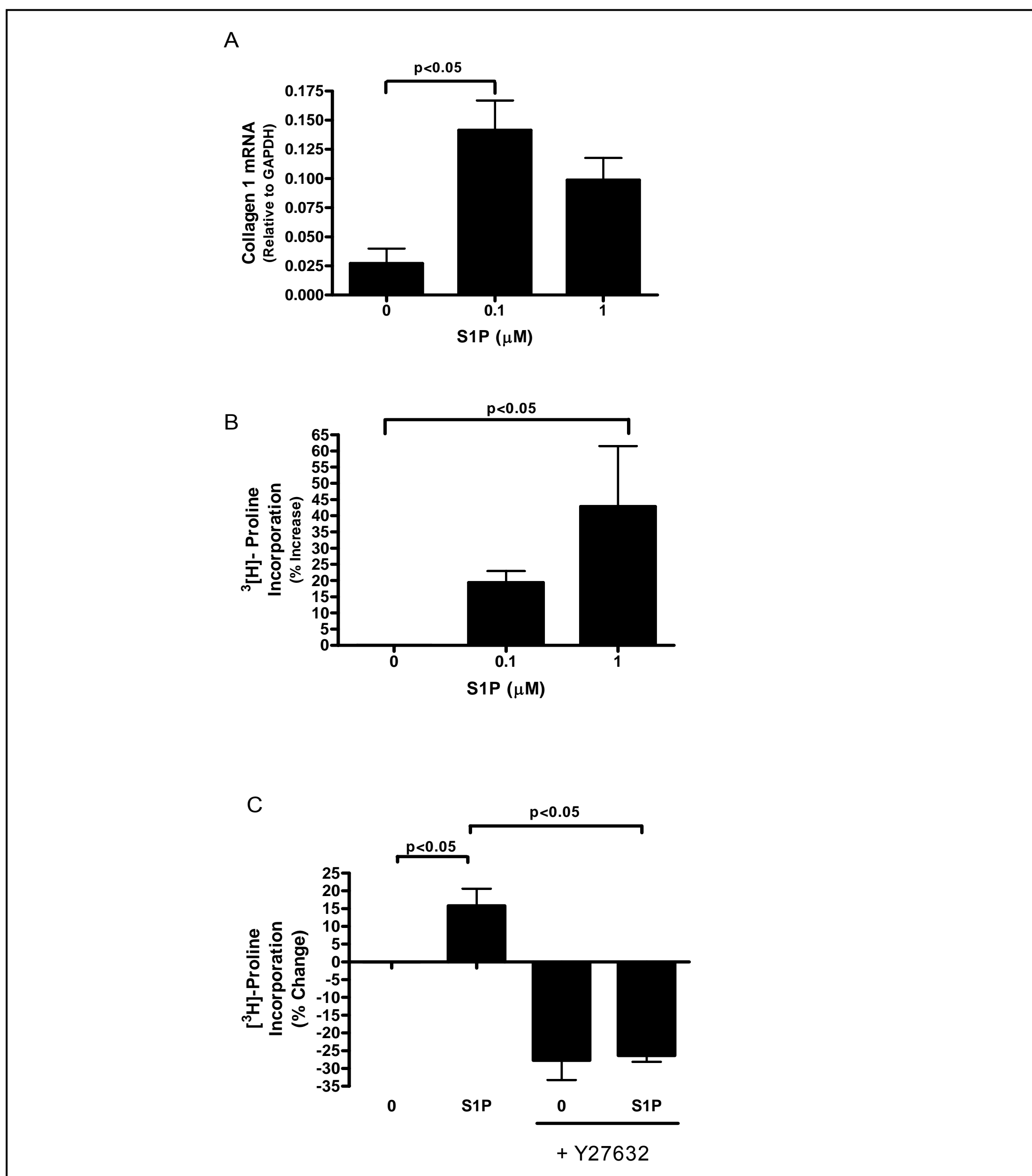


Figure 17. S1P increases collagen production in a Rho-dependent manner in CF. (A) Collagen expression was measured using QPCR in CF that were serum starved for 48 hr and then stimulated for 24 hr with serum-free media alone (control) or 0.1, or 1 μM S1P. Collagen expression was measured using collagenase sensitive [^{3}H]-proline incorporation assay in CF that were serum starved for 48 hr and then stimulated for (A and B) 48 hr with serum-free media alone (control) or 0.1, or 1 μM S1P; and (C) 48 hr with serum-free media alone or 1 μM S1P with or without Y-27632 (Rho Kinase inhibitor). Data are represented as % change from control. Values represent mean ± SEM of at least 4 independent experiments. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

proline incorporation, displaying a 43% in protein level at 1 μM S1P. As seen with α-SMA, the Rho kinase inhibitor, Y-27632, abrogated S1P-induced collagen protein expression (Figure 17C).

9. S1P-stimulated collagen production is dependent on signaling through $S1P_2$ but not $S1P_1$ or $S1P_3$.

To determine which receptors are responsible for S1P-stimulated fibroblast-to-myofibroblast transformation, we investigated the effects of siRNA-mediated knock-

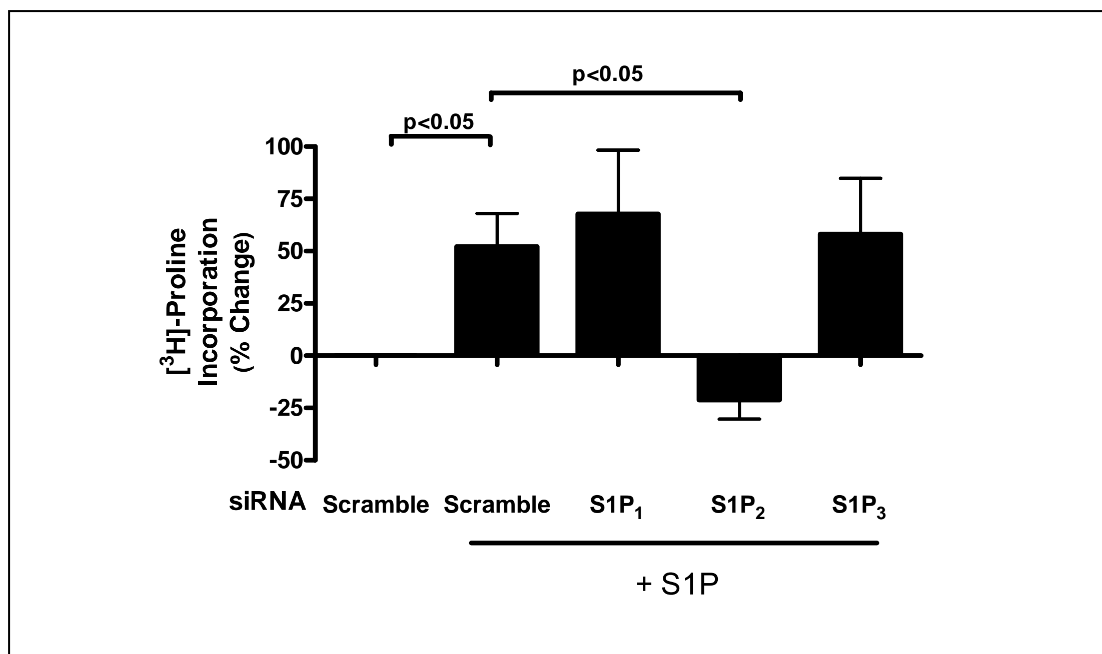


Figure 18. Collagen production is $S1P_2$ dependent. Collagen production was measured by $[^3H]$-proline incorporation in CF that were transfected with scramble (control), $S1P_1$, $S1P_2$, or $S1P_3$ siRNA for 48 hr in DMEM/ 1%BSA and then stimulated for 48hr with serum-free media alone (control) or 1μM S1P. Values represent mean ± SEM of at least 3 independent experiments. Data are represented as % change from control. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

down of the receptors on S1P stimulated collagen expression. The S1P receptors, $S1P_2$ and $S1P_3$, but not $S1P_1$, are known to couple to $G_{12/13}$ which is necessary for Rho

activation and ultimately myofibroblast transformation (Sugden and Clerk, 1997;Anderson, DiCesare et al., 2004). As expected from data using other cell types, Figure 18 shows that knock-down of $S1P_2$ abrogated S1P- stimulated collagen

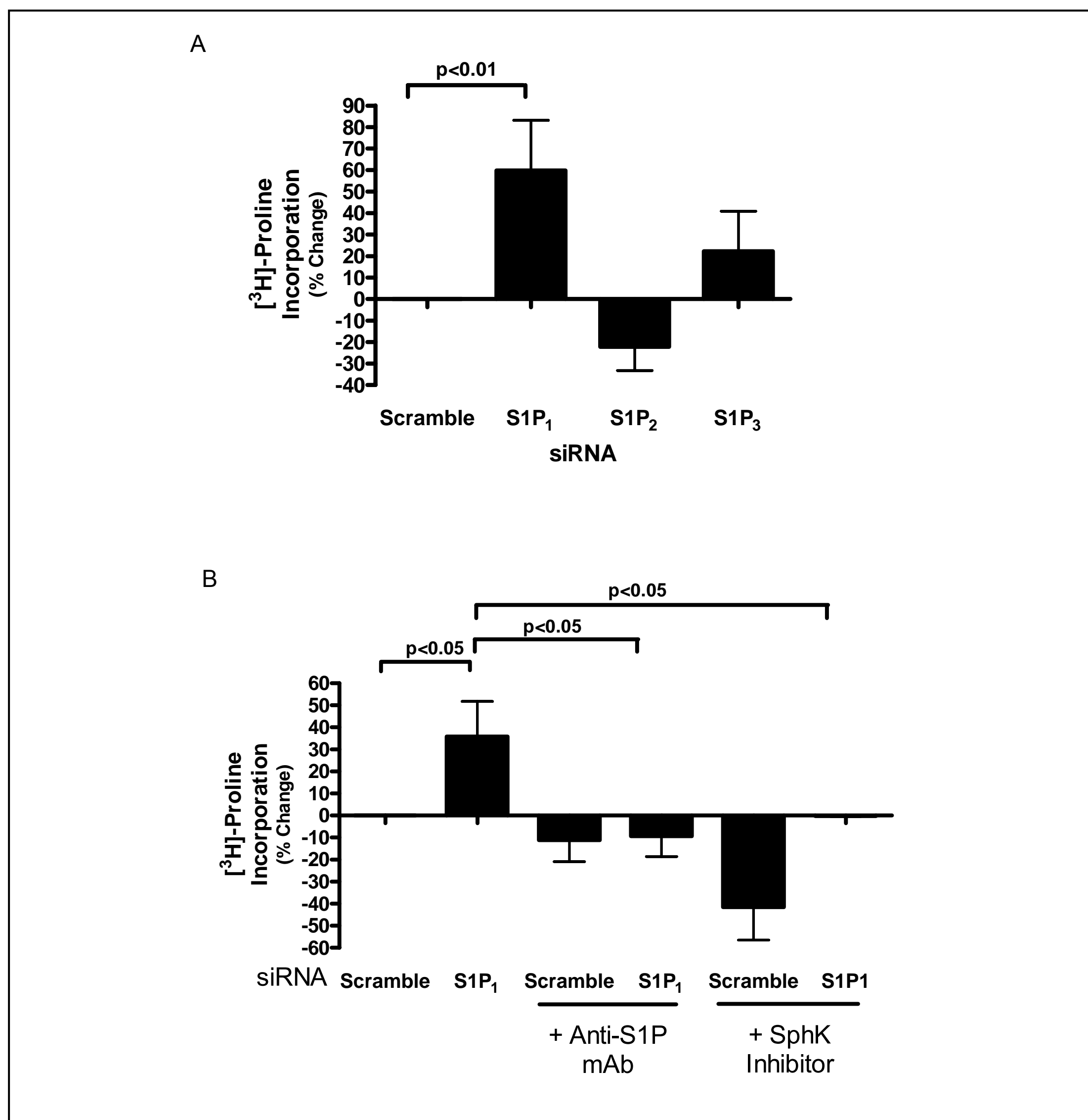


Figure 19. Basal collagen production increased in $S1P_1$ siRNA transfected cells is inhibited with anti-S1P mAb or SphK inhibitor (SphKI). Collagen production was measured by [^{3}H]-proline incorporation CF that were transfected with scramble (control), $S1P_1$, $S1P_2$, or $S1P_3$ siRNA for 48 hr in DMEM/ 1%BSA and then (B) treated with or without anti-S1P mAb (150 μg/ml) for 48hr and with or without 3 μM SphK inhibitor (SphKI) for 48 hr. Data are represented as % change from control. Values represent mean ± SEM of at least 4 independent experiments. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

expression. In fact, $S1P_2$ siRNA decreased proline incorporation below basal levels by 23%.

10. Basal collagen production is increased in $S1P_1$ siRNA-treated cells through an inside-out mechanism.

Given that αSMA expression in Figure 16 was supported by endogenously produced S1P by the inside-out signaling system, the stimulation of collagen protein expression was also investigated. As seen in Figure 19A, CF cells transfected with siRNAs against $S1P_3$ alone, without S1P treatment, increased basal levels of collagen expression. $S1P_2$ siRNA reduced proline incorporation, but the effect was not significant. On the other hand, $S1P_1$ siRNA increased collagen by 60%, an effect that was statistically significant ($p<0.01$). Similar to the case with $S1P_{1,}$ $S1P_3$ siRNA also had a small stimulatory effect that was not statistically significant. The increase in the basal collagen level suggested that S1P produced by CF is acting on $S1P_1$ in an autocrine and paracrine mechanism to inhibit collagen production. To prove this hypothesis we inhibited the inside-out signaling as before by use of both the anti-S1P mAb and us of the SphK inhibitor. Figure 19B shows that 150 μg/ml of the anti-S1P mAb added after $S1P_1$ transfection significantly decreased collagen expression, suggesting that enough endogenously produced S1P was present in the cell-conditioned medium to activate this receptor. Likewise, addition of the SphK inhibitor significantly inhibited the increase in collagen caused by $S1P_1$ siRNA.

11. TGF-β increases sphingosine kinase expression and activity.

TGF-β has been shown to increase SphK1 expression in other cell types (Yamanaka, Shegogue et al., 2004). To investigate if TGF-β has any effects on SphK1 expression in CF, RNA, protein and activity levels were evaluated. Figure

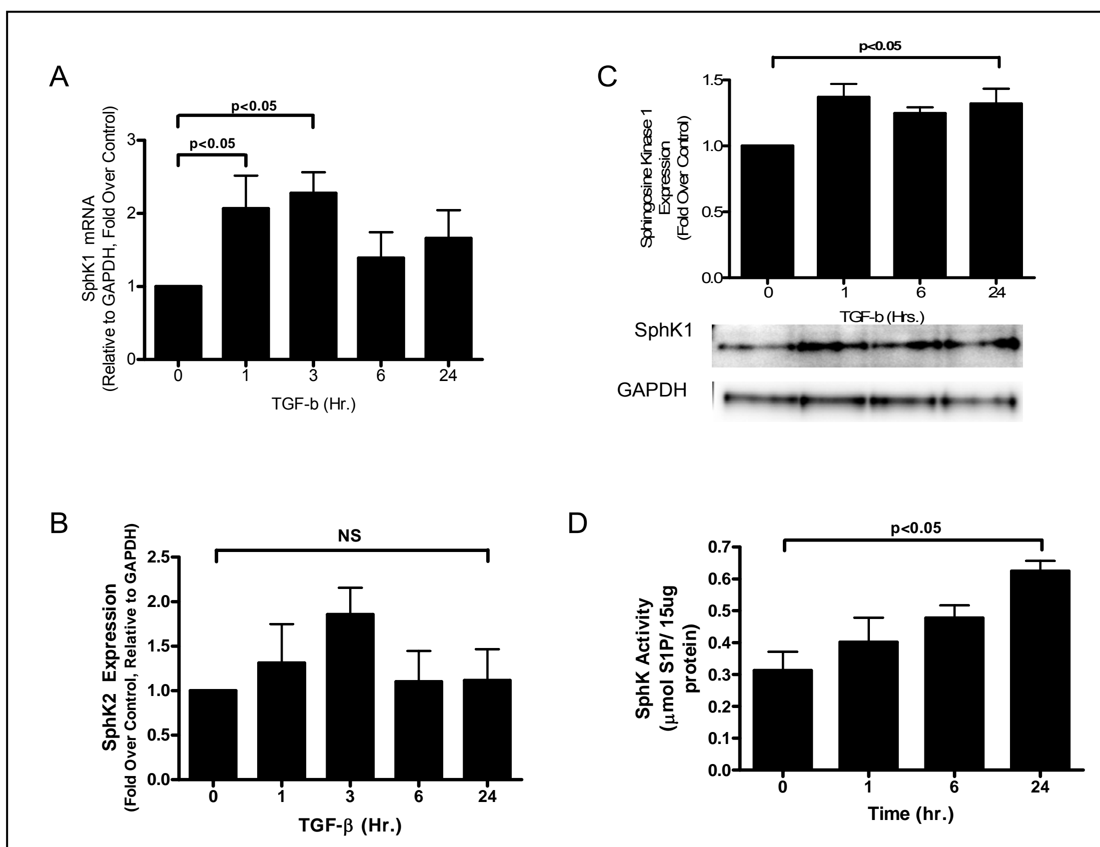


Figure 20. TGF-β increased SphK1 expression and activity in CF. CF were serum starved for 48hr., treated for 0, 1, 3, 6, or 24hr with TGF-β (10ng/ml) and (A and B) RNA was harvest for evaluation of SphK1 or SphK2 expression using QPCR, (C) SphK1 protein expression was measured by immunoblot and (D) SphK activity was measured. Values represent mean ± SEM of at least 4 independent experiments. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

20A shows there was greater than 2-fold increase of SphK1 RNA expression after 1 and 3hr. of TGF-β (10ng/ml) treatment. There was no significant change in SphK2 expression (Figure 20B). Coordinate with the ability of TGF-β to increase SphK1

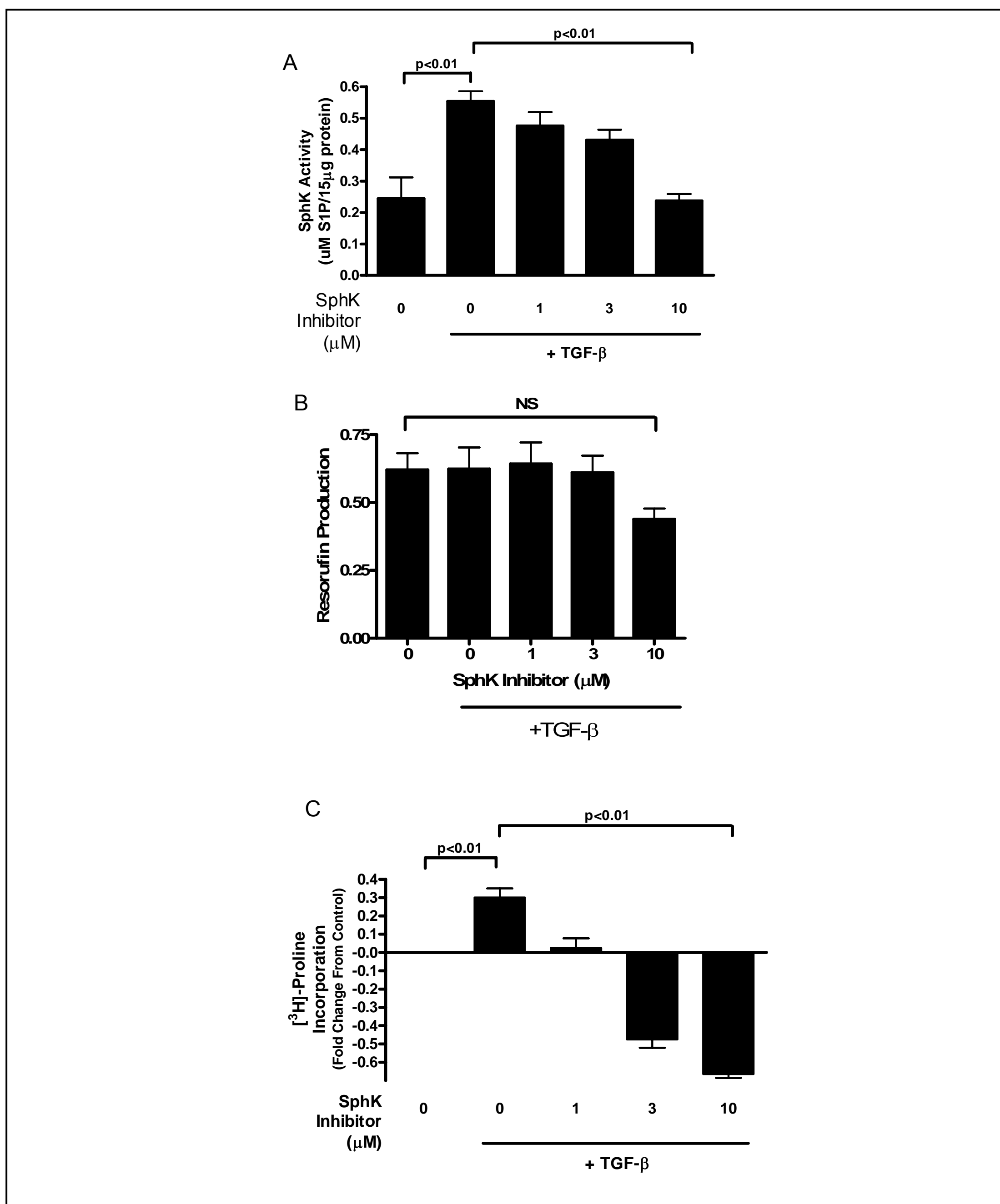


Figure 21. SphK inhibitor inhibited TGF-β-stimulated SphK activity and collagen production. CF were serum starved for 48hr, pretreated for 30min. with 0, 1, 3, or 10 μM SphKinhibitor and treated for 48hr. with or without TGF-β (10ng/ml) and (A) SphK activity was measured, (B) cellular viability (mitochondrial dehydrogenase activity) was measured and (C) Collagen production was measured by [^{3}H]-proline incorporation. Values represent mean ± SEM of at least 4 independent experiments. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

RNA expression (panel A) , there was a significant increase in SphK1 protein expression seen in Figure 20C. In addition to expression, TGF-β induced a significant

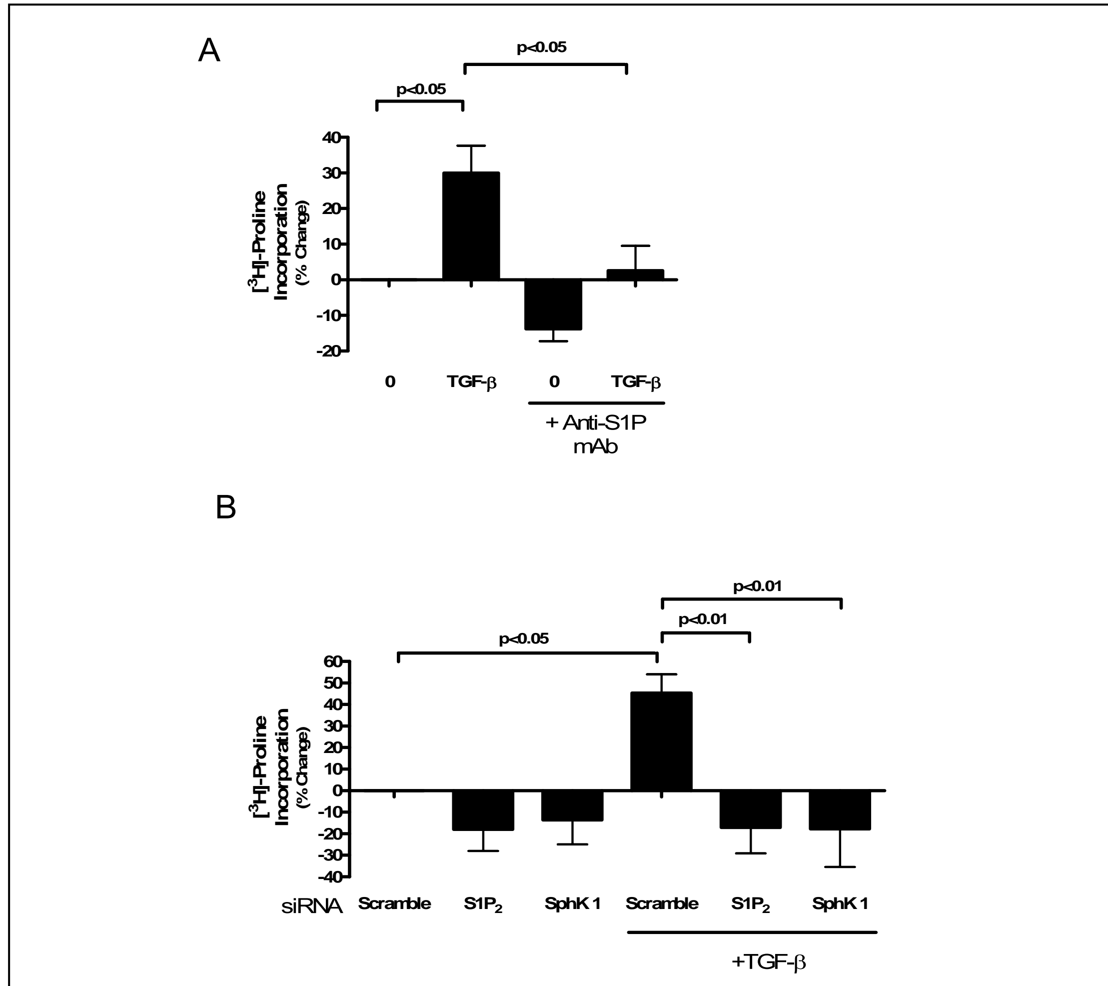


Figure 22. TGF-β-stimulated collagen production is inhibited with anti-S1P mAb or siRNA to $S1P_2$ or SphK1. Collagen production was measured by [^{3}H]-proline incorporation CF that were (A) serum starved for 48hr and treated for 48hr with serum free media (control) or 10ng/ml TGF-β with or without anti-S1P mAb (150 µg/ml) and (B) transfected with scramble (control), $S1P_2$, or SphK1 siRNA for 48 hr in DMEM/ 1%BSA and then treated with or without 10ng/ml TGF-β for 48hr. Data are represented as % change from control. Values represent mean ± SEM of at least 4 independent experiments. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

increase in SphK enzymatic activity that was detectable starting after 6hr of TGF-β treatment and progressed to 24hr (Figure 20D).

12. TGF-β-stimulated increases in collagen expression are sphingosine kinase 1 dependent.

Increased SphK activity seen in Figure 20 could be inhibited with a SphK inhibitor (Figure 21A). As a control, TGF-β in the presence of the SphK inhibitor at all concentrations tested had no significant effect on cellular viability (Figure 21B).

To determine if TGF-β and S1P coordinate to stimulate collagen expression through TGF-β-stimulated increases in SphK activity, a SphK inhibitor was used in a TGF-β-stimulated proline incorporation assay. As shown in Figure 21C, TGF-β-stimulated collagen production was inhibited by the SphK inhibitor in a dose-dependent manner. Likewise as seen in Figure 22A, addition of the anti-S1P mAb inhibited TGF-β-stimulated collagen expression. To further confirm these results, and to demonstrate S1P receptor specificity, siRNAs to SphK1 or $S1P_2$ were employed. Both siRNAs reduced TGF-β-stimulated collagen expression to basal levels. Together these experiments demonstrate that TGF-β-stimulated increases in SphK expression play a role in TGF-β-stimulated collagen expression through endogenous production of S1P.

B. *In vivo* investigation of S1P's effect on fibrosis post-MI

As shown in Part A, CF possess the capability to produce and respond to S1P. Specifically, CF express S1P receptors and kinases and appear to have a large pool of intracellular S1P. CF respond to S1P with increased proliferation, myofibroblast transformation and collagen production. Importantly, TGF-β-stimulated collagen production was SphK and S1P dependent implying that it utilizes an 'inside-out'

mechanism for cellular activation. In addition, plasma represents a concentrated supply of S1P (Yatomi, Igarashi et al., 1997). During the remodeling phase after a MI, CF proliferation, myofibroblast transformation and collagen production contribute to fibrosis and eventual heart failure. Therefore, it is possible that S1P present after a MI could contribute to fibrosis through its direct effects on CF. To test this hypothesis, mice were subjected to MI in the presence of an anti-S1P mAb or control (vehicle or non-specific antibody) and assessed for different fibrotic endpoints.

1. Anti-S1P mAb does not effect inflammation or injury in a post-myocardial infarction in vivo model.

Multiple literature reports have identified SphK and production of S1P as protective during a MI (Karliner, 2004). One would hypothesize, as a result, that removal of S1P during a MI could increase infarct size. The studies in this dissertation are focused on S1P's effects post-MI, when CF are actively making ECM. However, there is some overlap between the initiation of fibrosis and the completion of cell death post-MI (Rodriguez, Lucchesi et al., 2002;Nian, Lee et al., 2004). Therefore, there was a potential that administration of the anti-S1P mAb, which was hypothesized to decrease fibrosis, could concurrently increase infarct size. Increased infarct size would confuse effects on fibrosis because a larger infarction will lead to worsened fibrosis. In order to avoid to test if the anti-S1P mAb had any effect on infarct size, mice were either pre-treated (1hr pre-MI) or post-treated (48hr post-MI) with anti-S1P mAb, subjected to reversible infarction, sacrificed at 4 days post-MI and hearts were evaluated for infarct size. Figure 23 shows that there were no significant

differences in infarct size when measured as a percentage of area at risk when NS Ab and anti-S1P mAb-treated animals were compared.

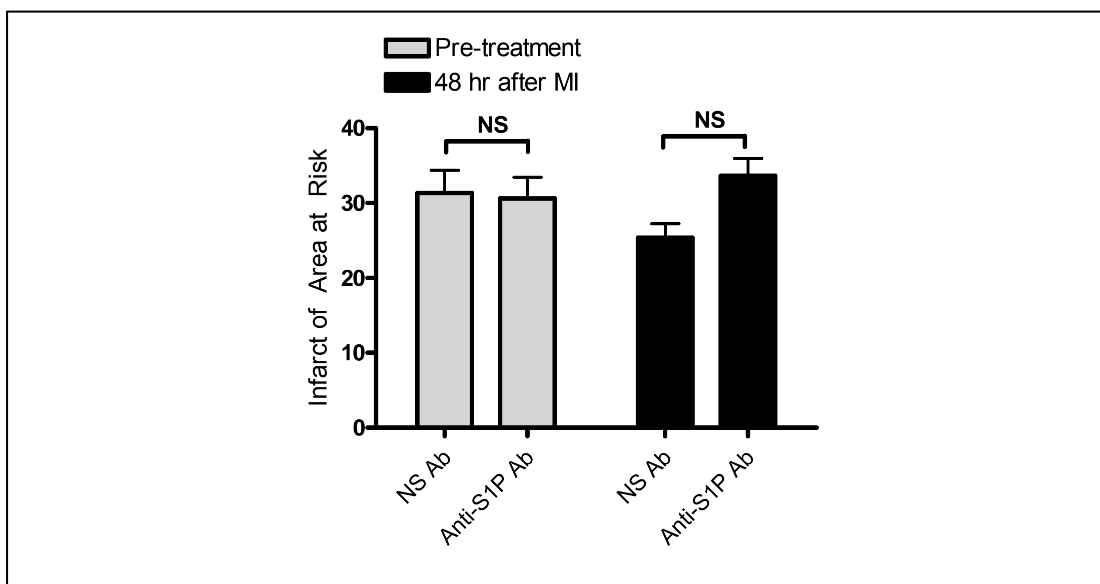


Figure 23. Anti-S1P mAb does not effect infarct size when administered 1hr before or 48hr after reversible MI. Infarct size of area at risk was evaluated using Evan's blue to delineate area at risk and TTC to show infarct size. Values represent mean ± SEM of at least 6 animals for NS Ab and anti-S1P mAb. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

In addition to potential effects on cell death, literature supports a pro-inflammatory role for S1P. For example, SphK activation is associated with degranulation, chemotaxis, and cytokine production from macrophages (Melendez and Ibrahim, 2004). To appreciate effects S1P might have on post-MI inflammation, mice subjected to ischemia/reperfusion were dosed with anti-S1P mAb or control (saline or NS Ab) at 48hr post-MI and then immunostained for neutrophils. As shown in Figure 24, there were no differences between control mice and anti-S1P mAb treated mice with regard to neutrophils infiltration (Figure 24). In conclusion, dosing the anti-S1P mAb, before or 48hr after MI, did not affect infarct size or inflammation. Therefore,

in the following studies animals were administered anti-S1P mAb early as 48hr post-MI to assess S1P's effects on fibrosis.

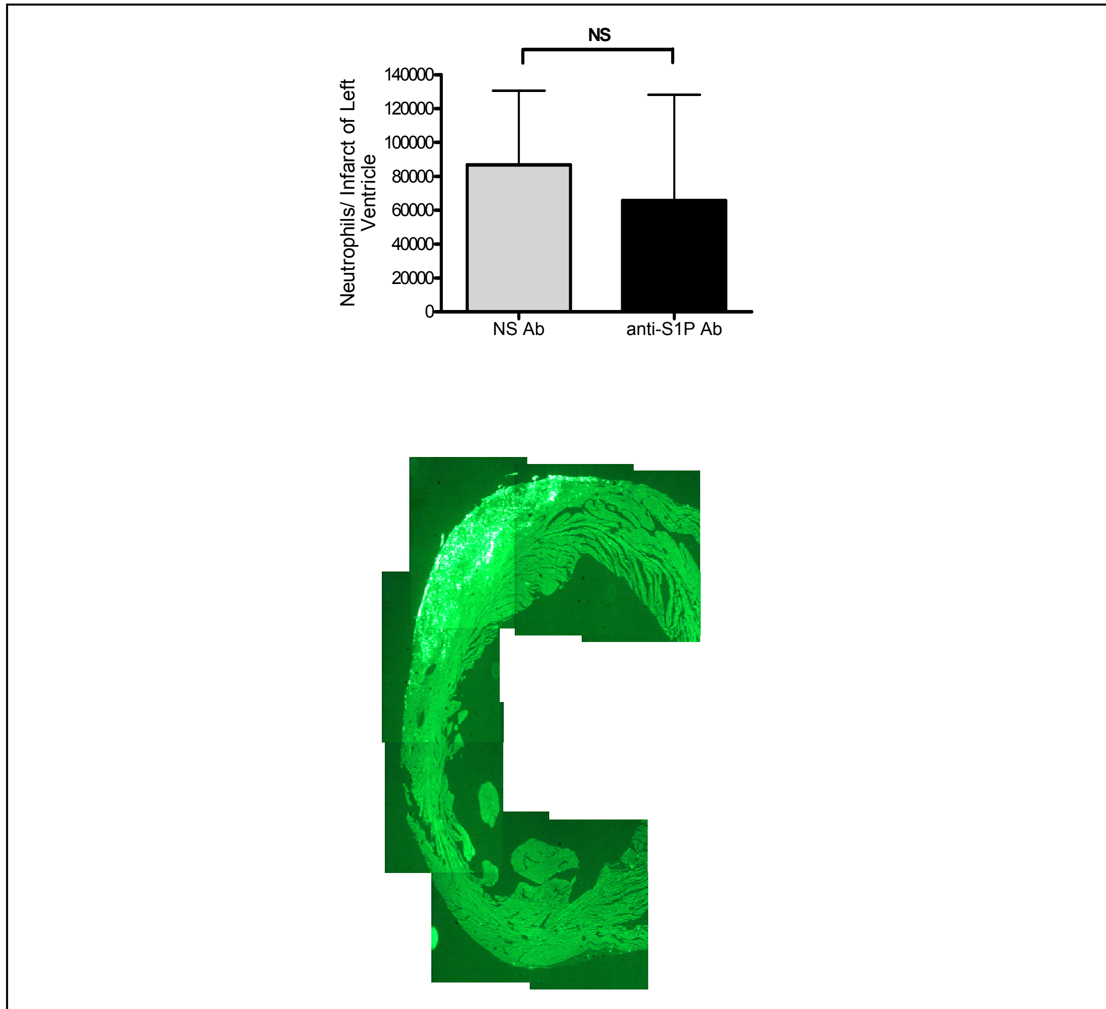


Figure 24. Anti-S1P mAb does not effect neutrophil infiltration when administered 48hr after reversible MI. Neutrophil infiltration was evaluated using neutrophils specific immunostaining and expressed as a percentage of infarct size which was determined in hematoxalin and eosin stained sections from the same hearts. Values represent mean ± SEM of at least 6 animals for NS Ab and anti-S1P mAb. Statistical analyses were performed student's t-test.

2. Anti-S1P mAb Increases 2 week survival post-MI.

Decreased fibrosis could potentiate increased survival. Therefore, we evaluated survival in a large group of mice subjected to permanent MI and dosed with

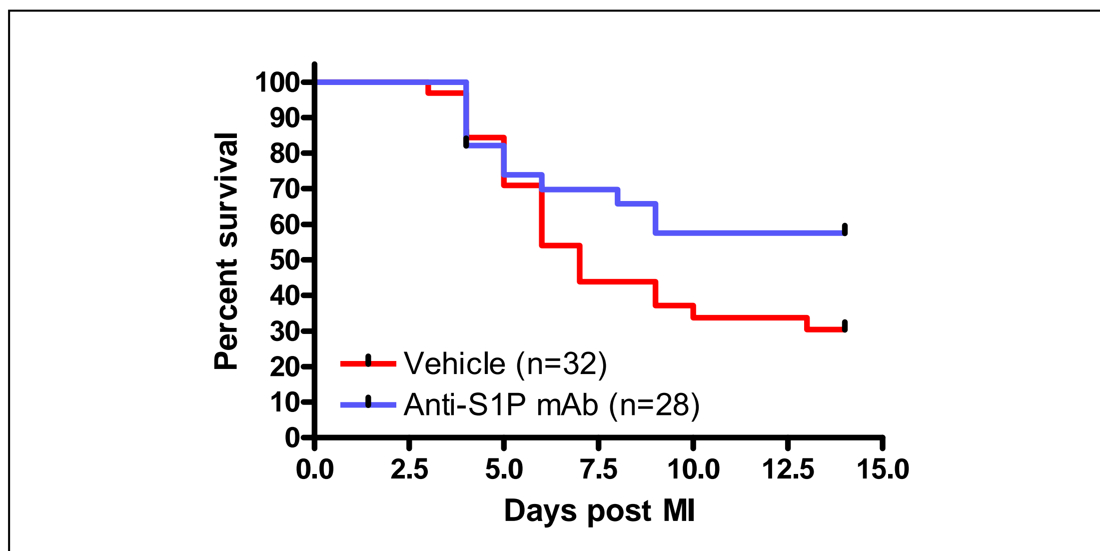
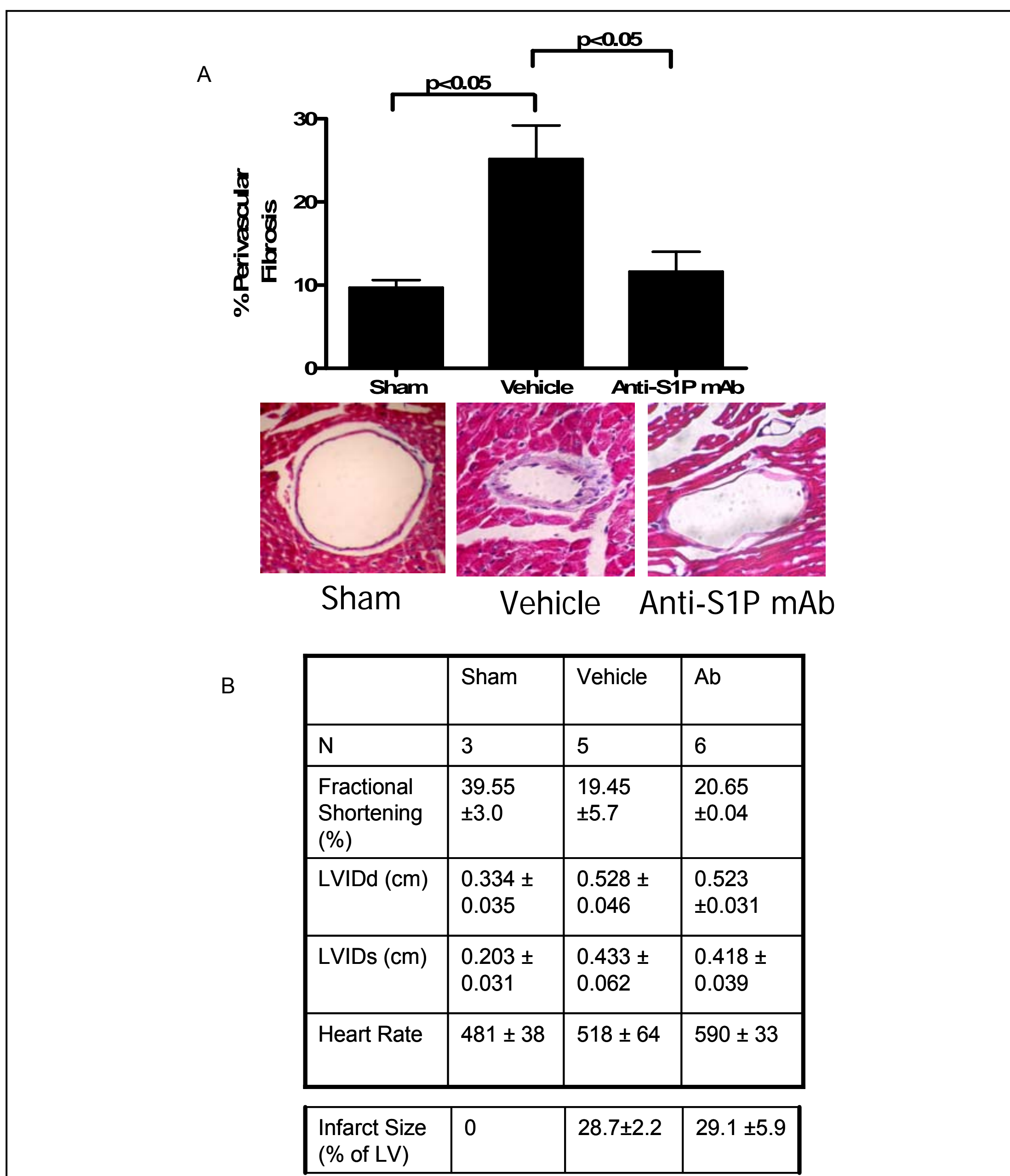


Figure 25. Anti-S1P mAb treatment increased 2 week survival when administered 48hr after MI. Survival was evaluated in large groups of mice subjected to permanent MI and treated with anti-S1P mAb (25mg/kg) or vehicle (saline).

control (saline vehicle) or anti-S1P mAb for two weeks post-MI. Interestingly, 55% of anti-S1P mAb survived two weeks while only 35% of mice from the control group survived.

3. Anti-S1P antibody decreases perivascular fibrosis 2 weeks post-MI.

In order to assess the role of S1P in cardiac fibrosis *in vivo,* mice were administered with an anti-S1P mAb 48 hr after a permanent coronary ligation. Two weeks after treatment, the animals were sacrificed and examined for the extent of



	Sham	Vehicle	Ab
N	3	5	6
Fractional Shortening (%)	39.55 ±3.0	19.45 ±5.7	20.65 ±0.04
LVIDd (cm)	0.334 ± 0.035	0.528 ± 0.046	0.523 ±0.031
LVIDs (cm)	0.203 ± 0.031	0.433 ± 0.062	0.418 ± 0.039
Heart Rate	481 ± 38	518 ± 64	590 ± 33
Infarct Size (% of LV)	0	28.7±2.2	29.1 ±5.9

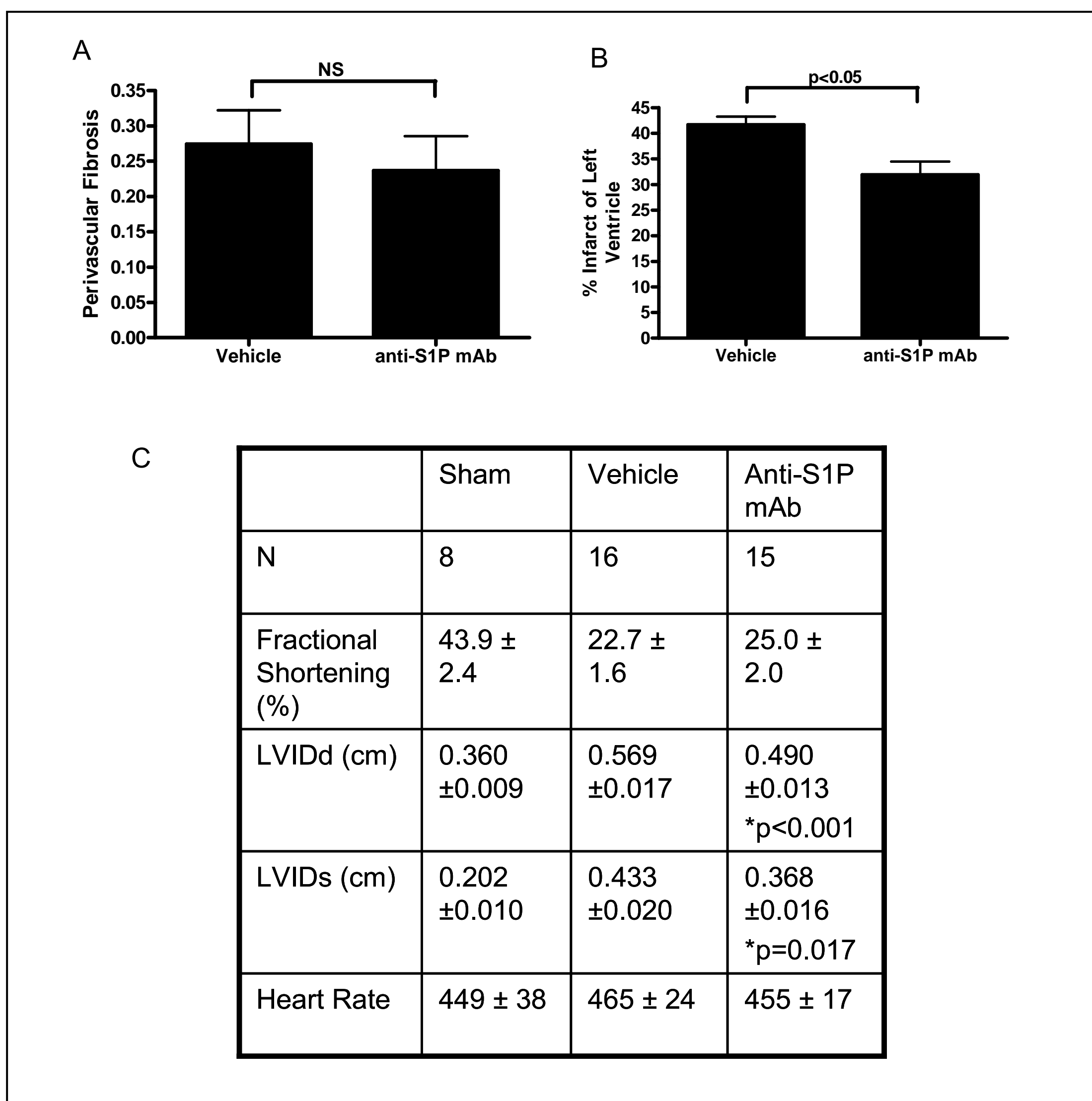
Figure 26. Anti-S1P mAb decreases perivascular fibrosis in mice at 2 weeks post-MI. Perivascular fibrosis was evaluated in the non-infarcted myocardium using Masson's Trichrome. (A) Data are represented as % vessel fibrosis. Representative images demonstrate collagen (blue) around vessels. (B) Echocardiographic parameters measured at 2 weeks and infarct size measured in Masson's Trichrome stained sections. Values represent mean ± SEM of at least 6 animals for vehicle and anti-S1P mAb and 3 animals for sham. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

perivascular fibrosis, one of the earliest fibrotic events post-MI (Weber, Brilla et al., 1993). As can be seen in Figure 26A, significant and substantial perivascular fibrosis was evident surrounding blood vessels in the non-infarcted zone in vehicle-treated animals which was reduced from 25% to 11.5% by the anti-S1P mAb.

Echocardiographic analysis, as seen in Figure 26B, revealed no differences in all parameters tested: heart rate (518±64 for vehicle vs. 590±33 for anti-S1P mAb); LVIDd (0.528±0.046 cm for vehicle vs. 0.523±0.031 cm for anti-S1P mAb); LVIDs (0.433±0.062 cm for vehicle vs. 0.418±0.039 cm for anti-S1P mAb); and fractional shortening (19.5± 5.7% for vehicle vs. 20.65 ± 4% for anti-S1P mAb). Average infarct size was 23.8± 1.9%. Sham mice had no evidence of infarct. Histopathological examination of mouse hearts 2 weeks post-MI did not show significantly detectable interstitial fibrosis.

4. Anti-S1P mAb administered 1 week post-MI Decreased Chamber Dilation and Infarct Size at 2 weeks.

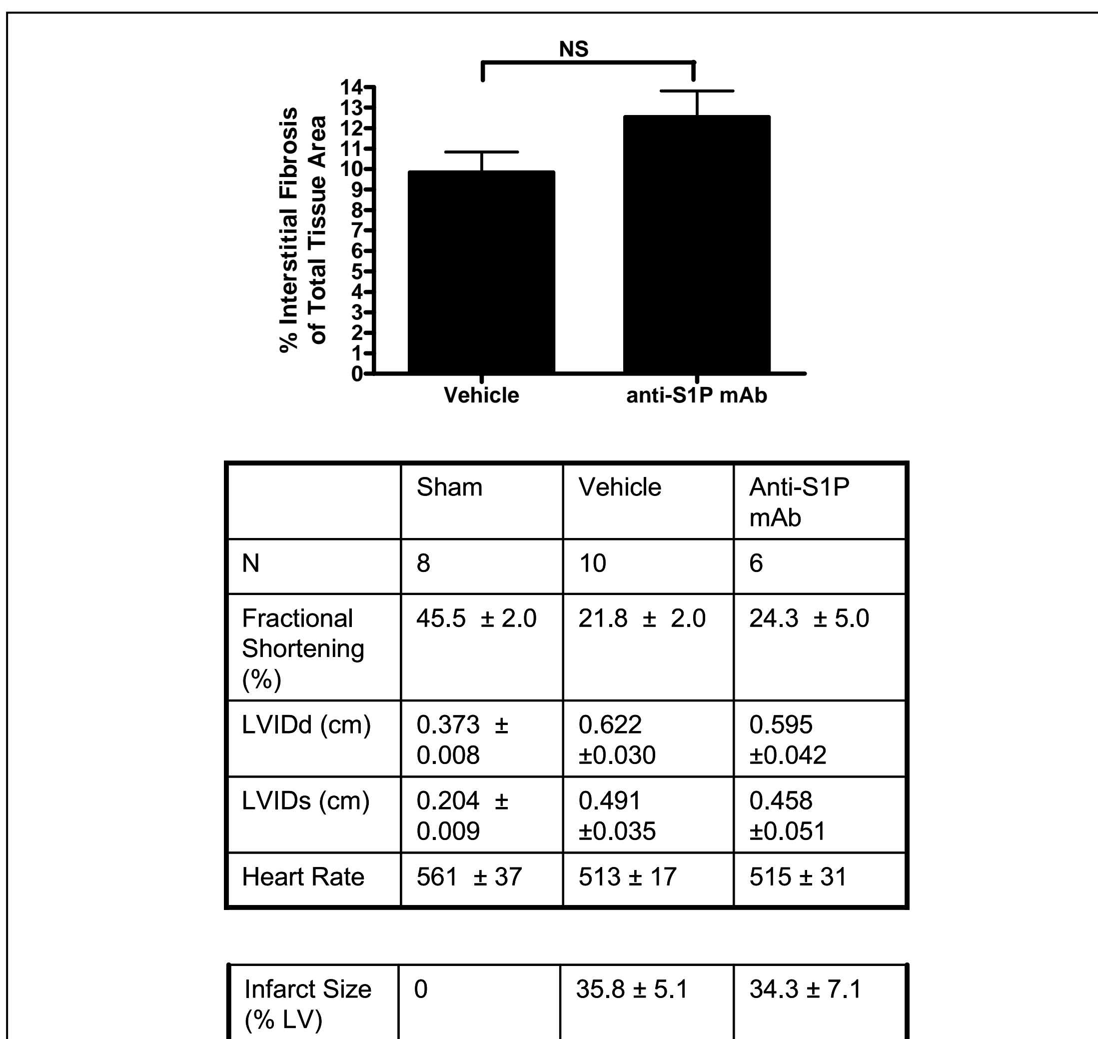
Despite a lack of effect on both inflammation and infarct size when anti-S1P mAb was administered before or 48hr after permanent coronary ligation, an additional fibrosis study was completed with initial anti-S1P mAb injection at 1 week. One week post-MI was chosen because the majority of inflammation and matrix metalloprotease activity is completed by this time and we were not positive that anti-S1P mAb administered at 48hr was without effect on these processes important to long term



	Sham	Vehicle	Anti-S1P mAb
N	8	16	15
Fractional Shortening (%)	43.9 ± 2.4	22.7 ± 1.6	25.0 ± 2.0
LVIDd (cm)	0.360 ±0.009	0.569 ±0.017	0.490 ±0.013 *p<0.001
LVIDs (cm)	0.202 ±0.010	0.433 ±0.020	0.368 ±0.016 *p=0.017
Heart Rate	449 ± 38	465 ± 24	455 ± 17

Figure 27. Anti-S1P mAb decreases infarct size and chamber dilation in mice at 2 weeks post-MI. (A) Perivascular fibrosis was evaluated in the non-infarcted myocardium using Masson's Trichrome. Data are represented as % vessel fibrosis. (B) Infarct size was evaluated in Hematoxylin and Eosin stained sections. Data are represented as % of total left ventricle. (C) Echocardiographic parameters measured at 2 weeks. Values represent mean ± SEM of at least 15 animals for vehicle and anti-S1P mAb and 8 animals for sham. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

resolution of infarct size. When administered one week after permanent MI, the anti-S1P mAb did not affect on perivascular fibrosis (Figure 27A). This indicates that the



	Sham	Vehicle	Anti-S1P mAb
N	8	10	6
Fractional Shortening (%)	45.5 ± 2.0	21.8 ± 2.0	24.3 ± 5.0
LVIDd (cm)	0.373 ± 0.008	0.622 ±0.030	0.595 ±0.042
LVIDs (cm)	0.204 ± 0.009	0.491 ±0.035	0.458 ±0.051
Heart Rate	561 ± 37	513 ± 17	515 ± 31
Infarct Size (% LV)	0	35.8 ± 5.1	34.3 ± 7.1

Figure 28. Anti-S1P mAb dose not effect interstitial fibrosis in mice at 8 weeks post-MI. Interstitial fibrosis was evaluated in the non-infarcted myocardium using Masson's Trichrome. (A) Data are represented as % fibrosis of total tissue area. (B) Echocardiographic parameters measured at 8 weeks and infarct size measured in Masson's Trichrome stained sections. Values represent mean ± SEM of at least 6 animals for each group. Statistical analyses were performed using one-way ANOVA with Bonferroni post-hoc analysis.

presence of anti-S1P mAb between 48hr and 1 week was important for reduction of this form of fibrosis. Surprisingly, however, we found a significant reduction in infarct size (41.7±1.6% for vehicle vs. 31.9±2.6% for anti-S1P mAb) (Figure 27B) and in overall chamber dilation (LVIDd: 0.569±0.017 cm for vehicle vs. 0.490±0.013 cm

for anti-S1P mAb, LVIDs: 0.433±0.020 cm for vehicle vs. 0.368±0.016 cm for anti-S1P mAb) (Figure 27C).

5. Anti-S1P antibody had no effect on interstitial fibrosis post-myocardial infarction.

Results from Figure 26 suggested that anti-S1P mAb decreased fibrosis post-MI, but that animals needed to recover for a longer period of time in order to see interstitial fibrosis. Therefore, animals were subjected to the same surgery and allowed to recover for 8 weeks. Although significant interstitial fibrosis was visible in both control (saline vehicle) and anti-S1P mAb treated mouse hearts, there was no quantifiable difference between the two groups (Figure 28A). Echocardiographic analysis, seen in Figure 28B, revealed no differences in all parameters tested: heart rate (513±17 for vehicle vs. 515±31 for anti-S1P mAb); LVIDd (0.622±0.030 cm for vehicle vs. 0.595±0.042 cm for anti-S1P mAb); LVIDs (0.491±0.035 cm for vehicle vs. 0.458±0.051 cm for anti-S1P mAb); and fractional shortening (21.8± 2.0% for vehicle vs. 24.3 ± 5% for anti-S1P mAb). Average infarct size was 35.05%. Sham mice had no evidence of infarct.

The Results chapter, in part, has been submitted for publication of the material as it appears Gellings Lowe, N; Swaney, JS; Moreno, KM; Sabbadini RA, "Sphingosine-1-phosphate and Sphingosine Kinase are Critical for TGF-β-stimulated Collagen Production by Cardiac Fibroblasts", 2008 in Acta Physiologica. The dissertation author was the primary investigator and author of this paper.

IV. Discussion

Recently, much attention has been placed on the role of S1P in the heart. These studies have focused, in large part, on regulation of cardiac myocyte (CM) survival and function (Karliner, Honbo et al., 2001;Jin, Goetzl et al., 2004;Ochi, Momose et al., 2006;Means, Xiao et al., 2007) but have neglected to examine the effects of S1P on cardiac fibroblast (CF) function and the potential role of S1P in cardiac fibrosis. This dissertation is an initial investigation into this potentially important and un-recognized role for S1P in the heart. The work presented here has demonstrated that S1P has the capability to produce and respond to S1P along with facilitating TGF-β's effects on collagen production. Further preliminary *in vivo* studies suggest that an anti-S1P mAb may be useful in decreasing post-MI fibrosis, but further work will be needed to determine the optimum dosing regimen.

A. *In vitro* determination of S1P's effects on cardiac fibroblasts.

In vitro experiments were designed to test the following hypothesis: S1P activates CF, both on its own and in conjunction with TGF-β. CF were evaluated for the following: expression of S1P receptors and Sphingosine kinases; response to S1P for proliferation, myofibroblast transformation and collagen production; and importance to TGF-β-stimulated collagen production.

Consistent with recent studies (Landeen, Aroonsakool et al., 2007), CF were found to possess both the metabolic machinery for producing S1P, as well as a robust signaling system to respond to extracellular S1P. In our hands, CF expressed $S1P_1$, $S1P_2$, and $S1P_3$ as well as SphK1 and SphK2 by both RTPCR and QPCR. Five S1P

receptors have been identified (Sanchez and Hla, 2004). While $S1P_5$ was not detectable in CF, in initial QPCR analyses, $S1P_4$ was detected. Further investigation revealed that QPCR primers available for $S1P_4$, unlike all other primer sets, did not span an intron-exon boundary. RNA was isolated from CF again including a DNA digestion step. When QPCR for $S1P_4$ was repeated, there was no detectable expression. In conclusion, the initial $S1P_4$ signal detected was due to DNA contamination in RNA samples.

S1P is a pleiotropic signaling molecule that can regulate multiple, sometimes divergent, downstream signaling pathways. S1P receptors differentially couple to three G proteins, G_i, G_q, and $G_{12/13}$ (Sanchez and Hla, 2004), all of which play a role in the observed S1P effects reported in this dissertation. While $S1P_1$ couples exclusively to G_i, $S1P_2$ and $S1P_3$ couple to all three G proteins (Kluk and Hla, 2002). G_i is well known to activate pro-proliferative signaling pathways including ERK (Sugden and Clerk, 1997), whereas G_q primarily binds to and activates PLC-β resulting in increased intracellular Ca^{2+}. This increase is associated with activation of multiple signaling enzymes and, although insufficient on their own to induce proliferation, contribute to its up-regulation most likely through activation of ERK (Radeff-Huang, Seasholtz et al., 2004).

In the studies presented here, S1P induced an increases in proliferation, as measured by BrdU or $[^3H]$-thymidine incorporation. S1P also increased ERK phosphorylation and its translocation to the nucleus, an event linked to activation of immediate early gene expression(Chen, Sarnecki et al., 1992). The G_i inhibitor, pertussis toxin (PTX), significantly reduced the effects of S1P on CF proliferation

(Figure 8A). In addition, the MEK inhibitor, U0126, had a similar effect on proliferation (Figure 8A). These findings suggest that the S1P signals through G_i to activate ERK and thereby increase CF proliferation. Interestingly, there was a notable decrease in proliferation in the presence of PTX or U0126. An important limitation to BrdU incorporation and [^{3}H]-thymidine incorporation is that they measures only DNA synthesis and not actual cellular proliferation. Counting cells per well and measuring mitochondrial dehydrogenase activity in the presence of the inhibitors, suggested that there were no differences in cellular viability. Thus, PTX and U0126 inhibit S1P-induced DNA synthesis, but do not decrease cellular viability. Previous studies have shown that there is a decrease in basal transcription in the presence of U0126(Martin, Farmer et al., 2005). Therefore, it is likely that the control cells were synthesizing DNA, albeit at a slower rate than the S1P-treated cells. In the presence of the inhibitors; however, there was less DNA synthesis than the control cells leading to the observed effect.

All three S1P receptors expressed by CF are known to activate G_i, the G protein shown to increase ERK phosphorylation and proliferation. Further studies were undertaken, using siRNA specific to each receptor, to determine which S1P receptor is responsible for S1P-stimulated proliferation and ERK phosphorylation. None of the siRNAs reduced BrdU incorporation. Likewise, ERK phosphorylation was not effected. Due to the redundancy of the S1P receptors in activating G_i, it was hypothesized that knocking-out any receptor alone is not sufficient to decrease S1P-stimulated proliferation. Therefore, an anti-S1P mAb was employed to molecularly absorb S1P added to the cell culture media thereby inhibiting signaling through all

three S1P receptors. In the presence of an anti-S1P mAb, S1P did not induce a significant increase in proliferation or ERK phosphorylation.

$G_{12/13}$ exclusively activates the small GTPase Rho, which is necessary for myofibroblast formation and function (Sah, Seasholtz et al., 2000). In fact, constitutively active Rho upregulates α-SMA promoter activity and Rho is necessary for stress fiber and focal adhesion formation (Sugden and Clerk, 1997;Anderson, DiCesare et al., 2004). In addition, Rho kinase knock-out mice exhibit dramatic decreases in perivascular fibrosis (Rikitake, Oyama et al., 2005) and the Rho kinase inhibitor (Y-27632) decreases interstitial fibrosis in mice post-MI (Hattori, Shimokawa et al., 2004). Therefore, we hypothesized that S1P stimulates myofibroblast transformation through Rho activation in CF. As predicted, S1P stimulated myofibroblast transformation and collagen production in a dose dependent manner (Figure 13 and Figure 17). Also, S1P-stimulated myofibroblast transformation and collagen production was inhibited by the Rho Kinase inhibitor, Y-27632. Immunostaining for α-SMA seen in Figure 14C, reveals the presence of well developed actin bundles in the presence of S1P. Addition of Y-27632 dissociates actin bundles leaving disorganized actin filaments and ruffled cell edges. Thus, S1P-stimulated myofibroblast transformation and collagen production are Rho kinase dependent and are consistent with literature reports suggesting an important role for Rho in fibrogenic responses.

Reports using fibroblasts from other tissues have demonstrated that the pro-fibrotic function of S1P is dependent on either $S1P_2$ or $S1P_3$ (Keller, Rivera Gil et al., 2007;Serriere-Lanneau, Teixeira-Clerc et al., 2007). Specifically, $S1P_2$ knock-out

mice have reduced hepatic wound healing as quantified by a reduction in the presence of α-SMA expressing hepatic myofibroblasts (Serriere-Lanneau, Teixeira-Clerc et al., 2007). In order to understand which S1P receptors were involved in the observed S1P stimulated Rho activation, α-SMA expression, and collagen production in CF, siRNA knock-down techniques were employed for all three S1P receptors expressed by CF. As seen in Figures 15A and 18, α-SMA expression and collagen production was inhibited by siRNA against $S1P_2$, but not effected by $S1P_1$ or $S1P_3$ siRNAs. Likewise, $S1P_2$ siRNA significantly inhibited Rho activation. Thus S1P-stimulated Rho activation, resulting myofibroblast transformation, and collagen production is $S1P_2$ dependent. Even though $S1P_3$ is capable of activating the same signaling pathways as $S1P_2$, there is a clear divergence in their downstream signaling. In CF only $S1P_2$ is responsible for Rho activation and resulting myofibroblast transformation and collagen production.

An interesting diversity exists in the cellular responses discussed so far. Although untransformed fibroblasts are proliferative and produce only modest levels of collagen, differentiated myofibroblasts are characterized by a low rate of proliferation and produce high levels of collagen types I and III (Grotendorst, Rahmanie et al., 2004). Herein we show S1P activated both cellular responses. A single agonist, such as TGF-β, can generate paradoxical effects on fibroblast function as a result of signaling at the post-receptor level (Grotendorst, Rahmanie et al., 2004). As previously pointed out, S1P receptors differentially couple to three G proteins, (Sanchez and Hla, 2004), all of which play a role in the observed S1P effects. Therefore, it can be hypothesized that, before a fibroblast is transformed towards a

myofibroblast phenotype, S1P signals more through G_i and G_q, to stimulate proliferation. As the fibroblasts transition towards a less proliferative more collagen producing myofibroblast phenotype, $S1P_2$ associated $G_{12/13}$ signaling becomes more dominant and contributes to the further transformation of the cell to a phenotype that is more stationary and more contractile and into a phenotype which expresses collagen.

So far the discussion of the *in vitro* results presented here has focused on CF response to exogenously added S1P. There is a potential; however, that CF make and respond to S1P in an autocrine fashion. In Figure 3, QPCR of isolated cells revealed higher mRNA levels for SphKs in CF as compared to CM. Notably, by comparison to CM, CF expressed much higher levels of SphK1, the isoform of the kinase thought to be responsible for S1P release (Maceyka, Sankala et al., 2005). This result is consistent with work showing that CF have much higher SphK enzymatic activity levels compared to CM (Kacimi, Vessey et al., 2007).

Studies published by Speigel and colleagues suggests that SphK1 is responsible for extracellular release of S1P while SphK2 activity correlates with increased intracellular levels of S1P (Maceyka, Sankala et al., 2005). Others have suggested, from work with mast cells, that SphK1 and the intracellular pool of S1P it creates might be an important source of releasable S1P (Mitra, Oskeritzian et al., 2006). In addition, previous work published by our laboratory demonstrated that CM do not release S1P (Cavalli AL, 2002), which is consistent with the lack of SphK1 expression by CM observed in this study. When combined with data demonstrating the lack of S1P release by CM, the elevated SphK1 expression levels in CF suggests

that CF may be the primary cell type responsible for production of the extracellular pool of S1P in the heart. Thus, S1P released by CF may act in an autocrine fashion to increase CF activity, thereby promoting myocardial fibrosis; or it may act in a paracrine manner to regulate survival and function of CM.

An anti-S1P mAb was used to stain both cardiac tissue sections and isolated CF and CM (Figure 4 and Figure 5). Consistent with the QPCR findings, S1P staining of both cardiac tissue sections and isolated primary CF and CM demonstrate that the CF contain substantial intracellular S1P, likely as a consequence of the robust expression of the kinase. The lack of S1P staining on the plasma membrane, coupled to the punctuate nature of intracellular S1P staining in the CF, suggests that CF may release their complement of S1P to the extracellular space by packaging S1P into exocytotic vesicles rather than by first intercalating the S1P into the plasma membrane prior to export. The Sabbadini lab is currently investigating, in greater depth, the mechanism of S1P release using the anti-S1P mAb as a research tool for immuno electron microscopy. Based on the QPCR and immunostaining results, it was hypothesized that the CF participate in an 'inside-out' signaling mechanism whereby a pool of packaged S1P is release into the extracellular space for autocrine action on the CF(Takabe, Paugh et al., 2008).

Several results presented in this dissertation support the hypothesis that CF produce S1P endogenously which acts in an autocrine fashion. First, alteration of S1P receptor expression levels with siRNA yielded increases in α-SMA expression and collagen production (Figures 16A and 19A). It was possible to inhibit these increases through absorption of S1P released into the cell culture media with anti-S1P mAb or

inhibition of endogenous production of S1P with a SphK inhibitor. In addition, use of either of these approaches alone, without S1P receptor knock-down, resulted in decreased basal α-SMA expression or collagen production. This strongly suggests that S1P is present in cell culture media and constitutively activating S1P receptors. When the receptor expression was altered, through knock-down of $S1P_1$ or $S1P_3$, an increase in S1P-stimulated myofibroblasts transformation and collagen production resulted. This suggests that there is a sufficient level of endogenously released S1P to activate S1P receptors in an 'inside-out' paradigm.

Further evidence for the existence of 'inside out' S1P signaling can be found in BrdU assays seen in Figures 10B and 10C. The presence of the anti-S1P mAb in the BrdU incorporation assay increased the basal level of DNA synthesis while an isotype matched non-specific (NS) Ab had no effect. This indicates that the anti-S1P mAb had a specific effect on the assay which cannot be attributed solely to the addition of an antibody. It is possible that the anti-S1P mAb was absorbing endogenously produced S1P thereby prohibiting S1P-stimulated transformation of CF to myofibroblasts and facilitating an increased basal DNA synthesis. It is commonly accepted that myofibroblasts are less proliferative than their fibroblast counterparts. Clearly this necessitates a very delicate balance between a proliferative state and a myofibroblast state; however, it is clear from these studies that S1P signaling is complex and diverse. This would also indicate that the spontaneous myofibroblast transformation seen in Figure 12 requires endogenous production of S1P.

In addition to suggesting that S1P acts by an 'inside out' mechanism in CF, the results presented in Figures 16 and 19 suggest that $S1P_1$ and $S1P_3$ have opposing

effects to $S1P_2$. Knock-down of $S1P_1$ or $S1P_3$ increased myofibroblast transformation and collagen production without addition of exogenous S1P. If signals downstream from these receptors are inhibiting myofibroblast transformation and collagen production then siRNA to these receptors will decrease this inhibitory effect. Figure 29 illustrates this graphically. Therefore, the data indicate that $S1P_2$ is constitutively activated by 'inside-out' S1P signaling to generate basal levels of α-SMA and collagen production; however, these increases are minimal suggesting that $S1P_1$ and/or $S1P_3$ are

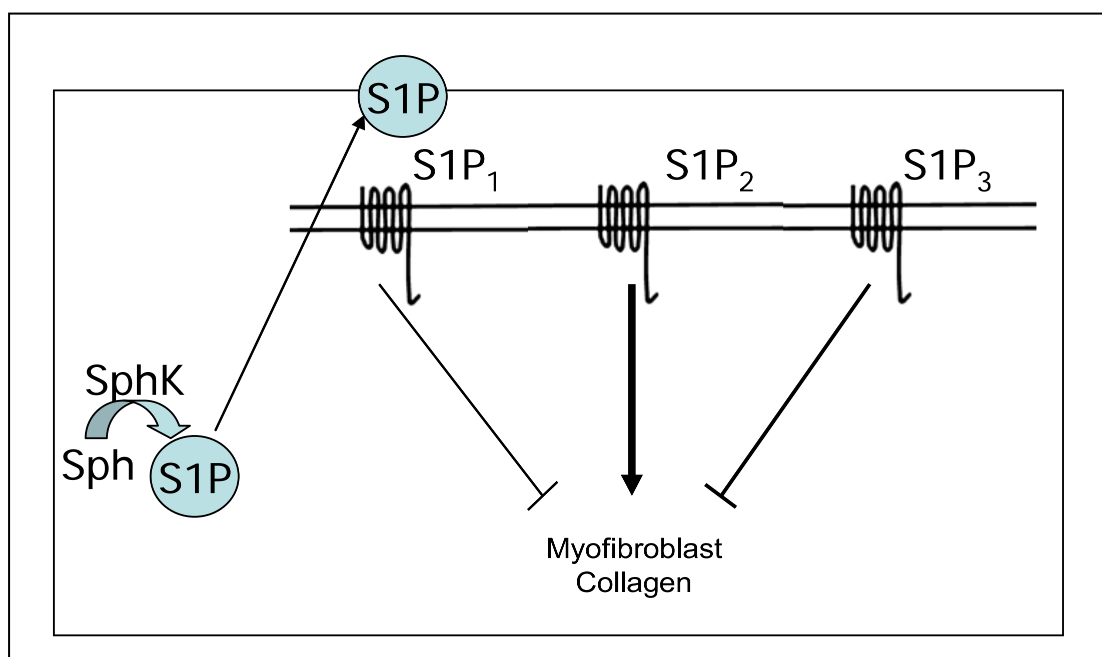


Figure 29. 'Inside-out' signaling in CF. Schematic representation of how 'inside-out' signaling in CF controls myofibroblast transformation and collagen expression through the S1P receptors.

able to counteract $S1P_2$ and keep this basal level of collagen expression to a minimum. When either the $S1P_1$ or $S1P_3$ receptor is knocked-down, the inhibitory effects are minimized and there is a further increase in myofibroblast transformation and collagen production, presumably through $S1P_2$ activation.

Interestingly, this balance of signaling is over ridden when exogenous S1P is added to cells whereby there is a net increase in myofibroblast transformation and collagen production. Changes in the cell culture environment, such as serum starvation, have been shown to increase $S1P_2$ expression (Landeen, Aroonsakool et al., 2007). Therefore, it is possible that the treatment conditions themselves, such as the exogenously added S1P, alter S1P receptor expression patterns thus favoring a pro-collagen producing myofibroblast scenario. One could imagine a situation where S1P treatment increases $S1P_2$ expression while $S1P_1$ and/or $S1P_3$ expression is decreased thereby decreasing their inhibitory capacity resulting in an overall net increase in myofibroblast transformation and collagen production.

Although, this is the first data demonstrating the ability of S1P to potentiate cardiac fibrosis on its own, there is existing evidence to suggest that S1P may facilitate the action of a well-known pro-fibrotic factor: TGF-β. TGF-β is one of the most widely studied and recognized contributors to fibrosis in the heart (Desmouliere, Geinoz et al., 1993) and other tissues (Lin, Zhou et al., 2008). TGF-β increases both production and secretion of collagens (Lijnen and Petrov, 2000) and promotes transformation of fibroblasts and epithelial cells into fibro-contractile myofibroblasts (Elberg, Chen et al., 2008;TA Wynn, 2008). Original work done by Trojanowska and colleagues established a connection between S1P and TGF-β in fibroblasts (Sato, Markiewicz et al., 2003). Using YSR2, the yeast equivalent to SphK, fused to a luciferase reporter, they found that TGF-β stimulated its activity. In addition, TGF-β upregulates SphK1 expression and activity leading to increased expression of tissue inhibitor of mettalloproteinases 1 (TIMP-1), a protein that inhibits ECM degradation

(Yamanaka, Shegogue et al., 2004). Increased expression of TIMP-1 is linked to interstitial fibrosis and diastolic dysfunction in heart failure patients (Heymans, Lupu et al., 2005). Conversely, S1P stimulates expression and release of TGF-β(Norata, Callegari et al., 2005).

In light of these published reports showing cross-talk between S1P and TGF-β in other systems, we sought to investigate the potential for TGF-β/S1P cross-talk in CF. It was found that TGF-β increased SphK1 mRNA, protein and activity. Considering these results, and because TGF-β is such a well-known pro-fibrotic mediator, we next sought to determine if this increase was involved in TGF-β-stimulated collagen production. As seen in Figures 21 and 22, when the TGF-β-stimulated increase in SphK1 expression was inhibited with siRNA or a SphK inhibitor, TGF-β-stimulated collagen expression was abrogated. This suggests that endogenous S1P production is necessary for TGF-β-stimulated collagen expression in CF. In other words, CF are stimulated to express increased levels of SphK1 which produces S1P that then acts in a autocrine manner to increase collagen production.

To further elucidate this idea, we used siRNA knock-down techniques targeted to the specific S1P receptor that we linked to S1P-stimulated collagen expression: $S1P_2$. This also inhibited TGF-β-stimulated collagen expression. Further, the addition of the anti-S1P mAb to TGF-β-treated cells abrogated collagen expression. This demonstrates that neutralizing, by molecular absorption, any endogenously produced S1P from the cell culture media could prevent 'inside-out' S1P signaling. An 'inside-out' mechanism has previously been identified for platelet derived growth factor (PDGF) signaling whereby PDGF stimulated fibroblast migration (Hobson,

Rosenfeldt et al., 2001) and mast cell chemotaxis (Jolly, Bektas et al., 2004). This is the first time that TGF-β has been shown to utilize this type of signaling in association with S1P.

Interestingly, knock-down of SphK1 or $S1P_2$, the anti-S1P mAb, and the SphK inhibitor slightly reduced basal proline incorporation in the absence of either exogenous S1P or TGF-β addition. These results are consistent with those seen for S1P-stimulated α-SMA expression and collagen production, further supporting the hypothesis that endogenously produced S1P maintains a carefully regulated basal level of collagen expression.

The traditional mechanism for TGF-β-stimulated ECM expression is through Smad phosphorylation. However, there are several Smad-independent signaling pathways whereby TGF-β receptor activation induces other signaling pathways including ERK, Rho, cJun N-terminal Kinase and other MAP kinases(Xiao and Zhang, 2008). The exact mechanism of TGF-β-stimulated ECM expression in CF has not been elucidated(Rocic and Lucchesi, 2005). The data presented here demonstrates, for the first time, that TGF-β relies on S1P for induction of collagen production in CF. In addition, activation of Rho kinase and Rho are invoked. However, it should be noted that, in renal mesengial cells, S1P has been shown to cross activate Smad signaling thereby mimicking the effects of TGF-β(Xin, Ren et al., 2004). Therefore, it is still possible that Smad phosphorylation is involved in TGF-β-stimulated collagen production in CF.

Taken together, the *in vitro* data presented in this study suggest that S1P is an important, previously unrecognized, mediator of cardiac fibrosis through its direct

effects on CF and its coordination with TGF-β. This is the first report demonstrating a link between S1P and TGF-β-associated fibrosis in CF.

B. *In vivo* investigation of S1P's effect on fibrosis post-MI

Strong *in vitro* evidence is presented in this dissertation to support the hypothesis that S1P is pro-fibrotic with regard to CF function in cell culture. A series of studies were then undertaken *in vivo* to test if S1P is pro-fibrotic in the heart. For this work, we employed an anti-S1P mAb as a tool to systemically neutralize S1P in mice subjected to surgical myocardial infarction.

Before assessing the role of S1P in cardiac fibrosis *in vivo*, it was necessary to evaluate if the anti-S1P mAb had any effect on infarct size. This is important because the size of the infarct would affect the reparative fibrotic process and become a variable in assessing whether or not S1P influences post-ischemia fibrosis. The large majority of S1P reports involving the heart have demonstrated that SphK and production of S1P protects CM during an MI (Karliner, 2004;Means, Xiao et al., 2007). One would hypothesize, based on these reports, that S1P serves a cardioprotective role and therefore infarcts in anti-S1P mAb treated animals would be bigger than controls. This, however, was not the case. The anti-S1P mAb administered 1hr before or 48hr after MI did not affect infarct size (Figure 23). When considering previous studies in this field, it is important to keep in mind that very different methodologies are being utilized as compared to these studies. Models exploited by Karliner and colleagues employ either a SphK1 knock-out mouse or a

SphK inhibitor (DMS). In the case of Heller Brown and colleagues genetically modified mice are also used, this time knocking-out the S1P receptors. Instead of prohibiting the S1P production and receptor activation well before the infarct or throughout the life of the animal, addition of the anti-S1P allows for normal S1P production and signaling up until immediately before or significantly after the infarction. This suggests that in a wild-type animal the presence of S1P during infarction does not affect the size of the injury. One could hypothesize that conditional knock-outs of SphK1 or the S1P receptors would generate the same result.

In addition, there were no differences between control mice and anti-S1P mAb treated mice with regard to neutrophils and macrophage infiltration. Given literature reports supporting a pro-inflammatory role for S1P(Melendez and Ibrahim, 2004), this too was a surprising result. This is the first time, however, that S1P's role in cardiac inflammation has been evaluated. These results lead to the conclusion that S1P does not influence inflammation or infarct size post-MI.

It is important to note that, in order to test if the anti-S1P mAb had any effect on infarct size, a reversible ischemia model was used. In a permanent ischemia model, which was used for fibrotic studies, the ligature is permanently tied and the entire ischemic area will become infarcted. This is ideal for fibrotic studies because a large infarct will generate larger stress on the heart resulting in potent pro-fibrotic effects. In a reversible ischemia model the infarct is defined by two areas: the area at risk and the infarcted area. The area at risk is the entire area that was ischemic during the ligation. The infarcted area is that portion of the area at risk that had CM necrosis and apoptosis as a result of the ischemic period. In a reversible ischemia model, unlike a

permanent ischemia model, there are many CM in the area at risk that may or may not die. These CM can possibly be saved using an intervention. As a result, it is possible to quantify changes in infarct size enabled by drug intervention in a reversible ischemia model, but not in a permanent ischemia model.

The initial long-term study completed, Figure 25, was a survival study including a robust number of animals (32 of saline vehicle and 28 of anti-S1P mAb). In two weeks approximately twice as many animals in the anti-S1P mAb treated group survived as compared to vehicle. Interestingly, necroscopic observations determined that the majority of the animals that died had areas of rupture in their hearts. One might conclude that the anti-S1P mAb decreased fibrotic CF functions leading to increased diastolic compliance. It follows that the control animals then had decreased compliance due to a larger presence of CF and ECM and were more susceptible to cardiac rupture. This survival study did not include echocardiographic analysis. Interestingly, however, when echocardiographic parameters were assessed at two weeks (Figures 26 and 27), anti-S1P mAb-treated animals displayed decreases in chamber dilation parameters both at diastole and systole (LVIDd and LVIDs). These dimensions attained significance when the anti-S1P mAb was administered 1 week post-MI. In conclusion, decreases in fibrosis may have diminished the heart's necessity to grow in compensation for its inability to eject blood.

Two fibrotic models were tested in these studies. Perivascular fibrosis, present around blood vessels, was evaluated 2 weeks post-MI. Anti-S1P mAb treated animals had significant decreases in this parameter and a lack of detectable interstitial fibrosis. To evaluated potential differences in interstitial fibrosis, animals were sacrificed at 8

weeks post-MI. Despite the robust presence of interstitial fibrosis, there were no differences between anti-S1P mAb and vehicle controls.

In all the studies presented here the anti-S1P mAb was administered intraperitoneal (IP) at 25mg/kg every other day. The anti-S1P mAb was not toxic when dosed up to 50mg/kg for 7 consecutive days and it was shown to be efficacious in inhibiting cancer progression when dosed every other day at 25mg/kg(Visentin, Vekich et al., 2006). Therefore, a similar dosing regime was utilized in the present studies. The cancer studies, however, evaluated the effects of the systemically administered anti-S1P mAb on orthotopic tumors in mammary fat pads and a subcutaneous xenograft. In contrast, the studies presented here rely on the anti-S1P mAb's ability to access the heart. There is a possibility that the method of administration (IP) or the dose level chosen (25mg/kg) was not sufficient to access the heart and therefore there is a lack of effect on interstitial fibrosis. In addition, in a permanent MI model the anti-S1P mAb may have had no access to the infarcted area until it was revascularized. This could be another limitation to the dosing of the anti-S1P mAb. Cardiac fibrosis radiates out from the infarcted region so the anti-S1P mAb may not have had access to the infarct zone early enough to slow the potent pro-fibrotic signals coming from that region.

Interestingly, the only difference in fibrosis was perivascular fibrosis two weeks post-MI. The lack of quantifiable interstitial fibrosis at this time point is consistent with previous studies examining myocardial fibrosis at early time points after MI, wherein 2 weeks post-MI is too early for interstitial fibrosis to manifest itself (Weber, 1997). It is possible that effects on perivacular fibrosis may be due to easier

access to anti-S1P mAb. The perivascular space, located directly around blood vessels could have been effect by the absorption of anti-S1P mAb due to its location so close to the blood compartment. Interestingly, the differences in perivascular fibrosis were lost by 8 weeks post-MI. One could speculate that, at that point of advanced fibrosis, the effect of interstitial fibrosis on the perivascular space would far out weigh any beneficial effects of easier access to the anti-S1P mAb.

Two dose initiation time-points were chosen to test the anti-S1P mAb's ability to slow post-MI fibrosis: 48hr and 1 week post-MI. The 48 hr time point was chosen to allow for near-complete resolution of the myocardial infarction (MI). The majority of CM apoptosis occurs by day 3 (Rodriguez, Lucchesi et al., 2002). The 1 week post-MI time point was chosen because it takes approximately 1 week for the majority of inflammation and metalloprotease activity to take place(Vanhoutte, Schellings et al., 2006). It appeared to be undesirable to dose later than that because myofibroblasts have entered the healing infarct and started secreting ECM by then (Vanhoutte, Schellings et al., 2006). It is possible that these choices of dosing regime were not ideal to address fibrotic effects of S1P without avoiding other effects. In other words, it is possible that 48hr was too soon, but 1 week was too late. It would seem that initiating the treatment at the appropriate time is very important to attaining the best effect. If the initial fibrotic events, including laying down of granulation tissue and early proliferation of fibroblasts, are not sufficiently slowed, then in the long term there may not be a significant inhibition of fibrosis. Therefore, dosing too late is undesirable. In contrast, if S1P had adverse effects on healing processes not evaluated, such as angiogenesis of the infarct area, then dosing too early might have

compromised the revascularization of the necrotic tissue. Further studies are necessary to test other dose initiation time points.

In conclusion, no effect of the anti-S1P mAb on interstitial firbosis was observed and there are two potential explanations: (1) insufficient level of anti-S1P mAb was administered to access the cardiac tissue and/or (2) anti-S1P mAb administration was not initiated at the best time. To address these explanations further studies could be conducted testing other doses of anti-S1P mAb and other time points of antibody administration. Because no toxicity has been observed up to 50mg/kg, attempting this level of anti-S1P mAb administration could be more effective in inhibiting fibrosis. Further, time points for dose initiation should include 72hr, when all CM apoptosis is likely complete. In addition, it would be useful to examine S1P's effects on post-MI angiogenesis. Because S1P potently stimulates angiogenesis in other systems(Limaye, 2008), it is important to evaluate if the anti-S1P mAb slowed revascularization of the infarcted area. This could adversely affect the infarct healing process by limiting nutrient and oxygen supply to surviving CM in the border zone around the infarct, yielding their eventual death and a net increase in fibrosis.

Alternatively, MI may not be the ideal model to test the *in vivo* effects of S1P on fibrosis. Aortic banding, would increase left ventricular pressure, results in robust interstitial fibrosis but would not cause a myocardial infarction. The anti-S1P mAb may show greater efficacy in this model if its lack of effects is due to confounding effects on the MI itself. Interestingly, S1P increases CM hypertrophy (Robert, Tsui et al., 2001) and therefore the anti-S1P mAb may have more than one beneficial effect in the aortic banding model.

Another limitation to these studies is that which is typical to translating *in vitro* results to *in vivo* models. Cell culture experiments allow a scientist to use a relatively clean system to test an agonist, such as S1P. *In vivo*, however, there are a multitude of other factors involved. In the case of the healing infarct, this includes other cells such as inflammatory cells, cardiac myocytes, and endothelial. Likewise, many other growth factors, cytokines and chemokines are released during the course of infarct healing which could counteract the effect of S1P through activation of their own signaling mechanisms. Although it seems clear from the *in vitro* data that S1P plays a role in CF function, it is possible that eliminating S1P alone is not sufficient to potentiate a quantifiable decrease in interstitial fibrosis.

There is currently a large unmet need to address the issues of heart failure from the standpoint of remodeling. While much attention has focused on reducing the size of an infarct during an active MI, many patients present with fully-developed MIs. These patients are in the early stages of remodeling and inflammatory events. Current clinical therapies which reduce cardiac fibrosis are limited to angiotensin converting enzyme inhibitors (Fraccarollo, Galuppo et al., 2003) and aldosterone blockade (Hayashi, Tsutamoto et al., 2003). To address the full impact of cardiac fibrosis, new approaches to therapy are needed. The data presented in this study suggest that S1P may be an important, previously unrecognized, mediator of cardiac fibrosis through its direct effects on CF. Not only is S1P found at high levels in blood (300-700nM) (Yatomi, Igarashi et al., 1997), but it's enzymatic production by cells in the cardiac microenvironment suggest that it may be a direct contributor to cardiac fibrosis. While extracellular S1P appears be cardioprotective prior to an ischemic

event(Karliner, 2002), it may be deleterious during the post-MI remodeling period. If true, neutralizing S1P with the anti-S1P mAb may have therapeutic benefit in the treatment of cardiac fibrosis but only after the infarct has been resolved. While this work has demonstrated, in a limited way, the *in vivo* ability of the anti-S1P mAb to mitigate early fibrotic events, further studies will be required to assess its long-term benefit.

V. Appendices

A. Cell culture solutions

1. DMEM media without antibiotics (Cellgro, cat. no. 10-013-CM)

2. DMEM media

DMEM containing 200 U/ml penicillin, 200 µg/ml streptomycin (diluted from 100X stock, Cellgro, cat no. 30-002-CI) and 5 µg/ml amphotericin B (diluted from 250 µg/ml stock, Cellgro, cat no. 30-003-CF)

3. 10% FCS DMEM media

DMEM supplemented with 10% FCS containing 200 U/ml penicillin, 200 µg/ml streptomycin (diluted from 100X stock, Cellgro, cat no. 30-002-CI) and 5 µg/ml amphotericin B (diluted from 250 µg/ml stock, Cellgro, cat no. 30-003-CF)

4. Minimal media

DMEM media supplemented with 1 mg/ml bovine serum albumin (BSA) containing 200 U/ml penicillin, 200 µg/ml streptomycin (diluted from 100X stock, Cellgro, cat no. 30-002-CI) and 5 µg/ml amphotericin B (diluted from 250 µg/ml stock, Cellgro, cat no. 30-003-CF)

B. Antibodies and dilutions

1. Primary antibodies

   a. α-Smooth Muscle Actin (mouse IgG2a monoclonal): obtained from Sigma (catalog no. A5228). Used at 1/500 for immunofluoresence and 1/5000 for immunoblot.

   b. ERK (p-44/42 MAP Kinase) (rabbit polyclonal): obtained from Cell Signaling Technologies (catalog no. 9102). Used at 1/20 for immunofluoresence and 1/1000 for immunoblot.

   c. Phospho-ERK (phosphor-p-44/42 MAP Kinase) (rabbit polyclonal): obtained from Cell Signaling Technologies (catalog no. 9102). Used at 1/1000 for immunoblot.

   d. SphK1 (rabbit polyclonal): obtained from Abgent (catalog no. ap7237c). Used at 1/500 for immunoblot.

   e. GAPDH (rabbit monoclonal): obtained from Cell Signaling Technology (catalog no. 2118). Used at 1/1000 for immunoblot.

2. Secondary antibodies

a. Anti-mouse- HRP (goat): obtained from Santa Cruz Biotechnologies (catalog no. sc-2031). Used at 1/5000 for immunoblot.

b. Anti-rabbit HRP (goat): obtained from Santa Cruz Biotechnologies (catalog no. sc-2030). Used at 1/3000 for immunoblot.

c. Anti-mouse FITC (goat): obtained from Jackson ImmunoResearch Laboratories (catalog no. 115-095-100, 0.5mg). Supplied as solid, resuspended in 0.45ml distilled water and 0.45ml glycerol and stored at -20 °C. Used at 1/500 for immunofluoresence.

d. Anti-rabbit FITC (goat): obtained from Jackson ImmunoResearch Laboratories (catalog no. 711-095-152, 1.5mg). Supplied as solid, resuspended in 0.4ml distilled water and 0.4ml glycerol and stored at -20 °C. Used at 1/500 for immunofluoresence.

C. Buffers and Solutions

1. Tris $CaCl_2$-N-ethylmaleimide buffer

0 mM Tris-HCl (pH 7.6), 5 mM $CaCl_2$, 0.02% NaN_3 (sodium azide), 2.5 mM N-ethylmaleimide (NEM)

2. SphK Assay Buffer

20mM Tris-HC1, pH 7.4; 1mM EDTA; 0.5 mM deoxypyridoxine; 1.5mM sodium fluoride; 0.7% 2-mercaptoethanol; 1mM sodium orthovanadate; 10µg/ml leupeptin, aprotinin and trypsin inhibitor; 8.64 mg /ml 3-glycerolphosphate; 0.4mM phenylmethylsulfonyl fluoride (PMSF); and 25% glycerol

3. Lysis buffer

50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 1% Triton X-100, Protease inhibitor cocktail (Sigma catalog no. P-8340), 1:100 dilution

4. Carbonate buffer

100mM $NaHCO_3$, 33.6 mM Na_2CO_3, pH 9.5

5. PBS

100mM Na_2HPO_4, 20 mM KH_2PO_4, 27 mM KCl, 1.37 mM NaCl, pH 7.4

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