Scleraxis is a mechanoresponsive regulator of the cardiac myofibroblast phenotype

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Abstract

Cardiac fibrosis is the excess deposition of myocardial extracellular matrix components, which increases tissue stiffness and heterogeneity, causing impaired diastolic/systolic function and arrhythmias, and eventually leading to heart failure and death. There are no available treatments for cardiac fibrosis. Myofibroblasts mediate fibrosis, and are characterized by hypersynthesis of collagens, decreased migration, and increased α-smooth muscle actin, which is incorporated into stress fibers, imparting contractility. Scleraxis is a transcriptional regulator of collagen-rich tissues, increased in response to the same stimuli that drive the myofibroblast phenotype, such as cyclic stretch. We show that Scleraxis mediates the conversion of cardiac fibroblasts to myofibroblasts, by increasing myofibroblast marker expression and contraction, and decreasing migration. Additionally, a proximal 1500 bp human *SCLERAXIS* promoter is activated by stretch and is responsive to transforming growth factor-β1. Thus, Scleraxis is a specific mechanoresponsive regulator of the myofibroblast, representing a novel target for the treatment of cardiac fibrosis.

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"Whether you think you can, or you think you can't, you're right."

-Henry Ford

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List of Abbreviations

αSMA	Alpha-smooth muscle actin
ACE	Angiotensin-converting enzyme
AngII	Angiotensin II
AP-1	Activator protein-1
bFGF	Basic fibroblast growth factor
bHLH	Basic helix-loop-helix
BMP	Bone morphogenetic protein
bp	Base pairs
cAMP	Cyclic adenosine monophosphate
CArG	$CC(A/T)_6GG$
CBP	CREB-binding protein
CCN	Connective tissue growth factor, Cysteine-rich protein, and Nephroblastoma overexpressed gene
Cdc42	Cell division cycle-42
CLS-2	Collagenase type 2
COL1A1/1A2/3A1	*Homo sapiens* collagen type 1α1/1α2/3α1
ColIα1/Iα2/IIIα1	*Mus musculus*/*Rattus norvegicus* collagen type 1α1/1α2/3α1
Co-Smad	Common Smad
CREB	cAMP response element-binding protein
CSPG	Chondroitin sulfate proteoglycan
C-terminal	Carboxyl terminal
CTGF	Connective tissue growth factor
DDR1	Discoidin domain receptor-1
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
Dock180	Dedicator of cytokinesis-180
ECM	Extracellular matrix
ED-A Fn	Extracellular domain-A splice variant of fibronectin
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
Egr1/2	Early growth response factor-1/-2
EMT	Epithelial to mesenchymal transition
ERK1/2	Extracellular signal-regulated kinase-1/2
ET-1	Endothelin-1
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
G-actin	Globular actin
GAG	Glycosaminoglycan
GDF	Growth/differentiation factor
GEF	Guanine exchange factor
GFP	Green fluorescent protein
Grb2	Growth factor receptor-binding protein-2
IGF-1	Insulin-like growth factor-1

IL	Interleukin
ILK	Integrin-linked kinase
I-Smad	Inhibitory Smad
JNK	c-Jun N-terminal kinase
KLF15	Krüppel-like factor-15
LAP	Latency-associated peptide
LARG	Leukemia-associated Rho guanine nucleotide exchange factor
LINC	Linker of nucleoskeleton and cytoskeleton complex
LLC	Large latent complex
LOX	Lysyl oxidase
LTBP	Latent TGFβ-binding protein
MAL	Myelin and lymphocyte protein
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
MEK1	Mitogen-activated protein kinase kinase-1
Meox2	Mesenchyme homeobox-2
MI	Myocardial infarction
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase
MOI	Multiplicity of infection
MRTF-A	Myocardin-related transcription factor-A
MSC	Mesenchymal stem cell
MSF	Migration-stimulating factor
NADPH	Nicotinamide adenine dinucleotide phosphate
NFAT	Nuclear factor and activator of transcription
NFκB	Nuclear factor kappa B
Nox4	NADPH oxidase-4
N-terminal	Amine terminal
P/CAF	p300/CBP-associated factor
P0/P1/P2	Passage 0/1/2
PDGF	Platelet-derived growth factor
pFAK	Phosphorylated focal adhesion kinase
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase-B
PVDF	Polyvinylidene fluoride
RECK	Reversion-inducing cysteine-rich protein with kazal motifs
RGD	Arginyl-glycyl-aspartic acid
ROCK	Rho-associated kinase
ROS	Reactive oxygen species
R-Smad	Receptor Smad
SBE	Smad-binding element
SCX	*Homo sapiens* SCLERAXIS
Scx	*Mus musculus/Rattus norvegicus* Scleraxis
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFK	Src-family kinase
SH2	Src homology-2

Shc	Src homology domain-2-containing protein
SLC	Small latent complex
SMEM	Minimum essential media
SMemb	Embryonic non-muscle myosin heavy chain
SnoN	Ski-related novel protein-N
Sos	Son of sevenless
Sox9	Sry-type high mobility group box-9
Sp1	Specificity protein-1
SPARC	Secreted protein acidic and rich in cysteine
SRF	Serum response factor
Syn4	Syndecan-4
TAK-1	TGFβ-activated kinase-1
TCE	TGFβ control element
TGFβ	Transforming growth factor-β
TGIF	TGFβ-induced factor
TIMP	Tissue inhibitor of matrix metalloproteinase
TNFα	Tumor necrosis factor-α
TRE	TGFβ-responsive element
TRPC	Transient receptor potential channel
TSP1/2	Thrombospondin-1/-2
TβRI/II	TGFβ receptor-I/-II
Y397	Tyrosine 397

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1: INTRODUCTION

Cardiac fibrosis is the excess deposition of fibrillar collagens and other extracellular matrix components in the heart. The resulting increase in myocardial stiffness and heterogeneity can result in systolic and/or diastolic dysfunction, cardiac arrhythmias, and eventually, heart failure [1]. The current lack of treatment options for cardiac fibrosis underscores the importance of understanding the molecular mechanisms governing fibrosis. At the cellular level, myofibroblasts are primarily responsible for the development of cardiac fibrosis. In the healthy heart, cardiac fibroblasts are the most numerous cell type, and are necessary for maintaining homeostasis of myocardial tissue through turnover of extracellular matrix components. In response to pro-inflammatory factors released following injury, such as infarct and hypertrophy, cardiac fibroblasts become activated. Upon activation, cardiac fibroblasts proliferate and migrate to the site of injury, where they release enzymes such as matrix metalloproteinases (MMPs) to degrade damaged tissue to allow infiltration of immune cells. Following this inflammatory phase, cardiac fibroblasts convert to myofibroblasts, which initiate the wound healing phase. In comparison to fibroblasts, cardiac myofibroblasts are hypersynthetic for collagens and other matrix components that make up cardiac scar tissue, and exhibit increased expression of key markers including the smooth muscle isoform of α-actin (α-smooth muscle actin/αSMA), which is incorporated into *de novo* cytoskeletal stress fibers [2]. Functionally, these stress fibers impart contractility upon cardiac myofibroblasts, a necessary function in scar maturation and proper collagen cross-linking. Although myofibroblast function is critical to the wound healing process, their persistence in the healed myocardium and continued deposition of collagenous scar tissue

is the basis for the development of cardiac fibrosis [3]. Thus, preventing or reversing the myofibroblast phenotype, *after* proper wound healing has concluded, represents a novel approach to the treatment of cardiac fibrosis.

1.1: Fibroblasts and Cardiovascular Disease

Cardiovascular diseases, including heart attack and stroke, cause nearly 70,000 deaths per year in Canada alone [4]. Cardiac fibrosis is a common result of many of these diseases, the most notable being myocardial infarction (MI) and cardiac hypertrophy. Unfortunately, treatment for cardiac fibrosis is practically non-existent, limited to end-of-life care and/or heart transplant. In addition to the heart, fibrosis affects a host of other organs, in which the stiffening resulting from excess collagen deposition can also be fatal – such as the lungs and kidney. In fact, taken together, scarring and fibrosis account for 45% of all chronic diseases in the Western world [5]. In the last 30 years, short-term survival post-MI has increased, yet so has the incidence of post-MI heart failure, underscoring the need for treatments targeting the detrimental effects of adverse cardiac remodeling [6].

1.1.1: Response to Myocardial Injury

Myocardial infarction is the result of obstructed blood flow (i.e. oxygen and nutrients) to cardiomyocytes resulting in ischemia, usually associated with coronary artery disease and affecting the left ventricle. Within 30 minutes of ischemia, cardiomyocytes suffer irreversible death, resulting in activation of the inflammatory complement system and an acute inflammatory response. Additionally, ischemia/reperfusion induces apoptosis in cultured cardiac fibroblasts [7]. The release of damage indicators and pro-inflammatory cytokines from dead/dying cardiomyocytes and surrounding resident cells (such as

fibroblasts) attracts immune cells (neutrophils, macrophages) to the site of infarct. In response to the acute inflammation following MI, fibroblasts and immune cells release proteolytic enzymes (such as MMPs) and mediators that cause degradation of the cardiac extracellular matrix, allowing for further infiltration of immune cells and engulfment of cellular debris. Due to the limited regenerative potential of the myocardium, the collagen-rich scar produced by fibroblasts (and later myofibroblasts) is required to maintain myocardial integrity by counteracting the drastic loss of cardiomyocytes – thus avoiding left ventricular dilation and cardiac rupture [8]. Four distinct but overlapping phases have been identified in humans following MI [9]. The initial phase, lasting about 2 days, involves cardiomyocyte death through apoptosis and necrosis, the latter of which invokes an early inflammatory response [10]. This inflammatory response, which occurs between 6 to 96 hours post-MI, is considered the second phase of cardiac wound healing, and is characterized by activation of the complement system [11], the release of pro-inflammatory cytokines (such as interleukins) [12, 13], and infiltration of the infarct area by immune cells including neutrophilic granulocytes, white blood cells, and macrophages. These immune cells remove dead cardiomyocytes and initiate matrix degradation [14], and also release growth factors such as transforming growth factor-β (TGFβ), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) to induce the proliferation and recruitment of cardiac fibroblasts to the wounded area [15]. The wound healing or proliferative phase begins within 2 to 3 days of infarct [9], and is associated with inhibition of pro-inflammatory mediators as well as activation of pro-fibrotic pathways [16]. This phase is dependent upon the activation, proliferation, and migration of fibroblasts into the infarcted area and surrounding border zone. These fibroblasts then begin to secrete extracellular matrix (ECM) proteins, beginning with

fibrin, and subsequently other non-collagen constituents such as fibronectin and tenascin [17, 18]. Fibroblasts also release other proteoglycans, glycoproteins, and fibrillar collagens type I and III. In addition to synthesis and deposition of such proteins, fibroblasts also contribute to the wound healing process via degradation of existing ECM through enhanced expression of MMPs, which is kept in check by expression of tissue inhibitors of matrix metalloproteinases (TIMPs)[15].

In addition to acting as chemoattractants for immune cells, inflammatory mediators stimulate resident cardiac fibroblasts and other lineages to differentiate or convert into myofibroblasts, the primary cells responsible for deposition of collagen-rich scar tissue [19]. Within a few days post-MI, myofibroblasts heavily populate the border region of the infarct zone, where their purpose is to produce high quantities of fibrillar collagens type I and III that make up the infarct scar [20]. Initial formation of scar tissue, which occurs within about 4 days post-MI, is also accompanied by the appearance of new blood vessels derived either from pre-existing nearby vessels or endothelial cells recruited to the infarcted area [21]. The events occurring over the following weeks to years after initial scar formation and resolution of the inflammatory phase fall into the maturation phase of cardiac wound healing. Upon initiation of this phase, approximately 2 to 3 weeks post-MI, cardiac myofibroblasts begin to cross-link collagen fibers deposited in the infarct and surrounding border zone, resulting in increased tensile strength and stiffness of the collagen-rich scar. After wound healing has been completed and collagen deposition and synthesis is no longer necessary, the majority of myofibroblasts disappear from the infarct through apoptosis [22]. However, in many cases, myofibroblasts persist in the infarcted region as well as the distal, non-injured myocardium, continuing to inappropriately

deposit fibrillar collagens and contract the tissue. The persistence of myofibroblasts in the healed myocardium, for up to decades after MI, is the basis for the development of cardiac fibrosis [23].

1.2: Extracellular Matrix of the Heart

The ECM of the heart is critical to cardiac structure and function, coordinating transmission of contractile forces produced by cardiomyocytes while preventing over-extension or rupture of the myocardium during ventricular systole [24, 25]. Additionally, the ECM acts as a reservoir of latent cytokines and growth factors that can be made readily available in response to stress and injury, and undergoes dynamic alterations during the different phases of post-MI remodeling [16]. The cardiac ECM is primarily composed of various structural proteins such as collagens and elastins, glycoproteins and glycosaminoglycans (GAGs), non-structural matricellular proteins (Connective tissue growth factor, Cysteine-rich protein, and Nephroblastoma overexpressed gene (CCN) family, secreted protein acidic and rich in cysteine (SPARC), thrombospondins, etc.), and a system of proteolytic MMPs and their inhibitors, TIMPs [15, 26].

1.2.1: Collagens in the Cardiac Extracellular Matrix

Fibrillar collagens type I and III make up about two-thirds of total protein content in humans [25], are the primary non-cellular component of the cardiac ECM, and comprise about 96% of total myocardial collagen content, although this may vary between species [25, 27, 28]. Additionally, the type I and III collagen content of the myocardium increases as rats develop from fetuses into neonates and further into adulthood [29]. Collagens are synthesized and secreted by fibroblasts and myofibroblasts as pro-collagen precursors, which are subsequently converted to mature collagen molecules through proteolytic

cleavage [30]. Cross-linking and assembly of mature collagens into fibers is catalyzed by lysyl oxidase [31]. Collagen degradation, after a half-life of about 80-120 days, is mediated by the enzymatic activity of matrix metalloproteinases (mainly collagenases and gelatinases) [32]. Mature type I collagen fibers, which are the primary determinant of cardiac stiffness [25], are a triple helix composed of two collagen 1α1 chains and one 1α2 chain, transcribed in proportion from the COL1A1 and COL1A2 genes, respectively [33]. Type III collagen is the second most abundant collagen in the myocardium, forming thinner fibers than those of type I collagen, and is composed of three identical collagen 3α1 monomers [25, 34]. In the healthy myocardium, the ratio of collagen type I/III is approximately 2:1 [35], increasing significantly in myocardial diseases associated with adverse ventricular remodeling [36-40]. Though collagen synthesis and cross-linking are critical to post-injury scar formation, excessive collagen deposition by myofibroblasts is the hallmark of cardiac fibrosis, involving scar tissue formation even in remote, non-infarcted areas that contributes to increased myocardial stiffness, development of arrhythmias, and impaired function [9, 41].

1.2.2: Non-Collagen Matrix Components

Next to collagens, glycoproteins are the most abundant component of the cardiac extracellular matrix [29]. These include fibronectin, proteoglycans like fibrillins, versicans, and syndecans, the GAG hyaluronan, and matricellular proteins such as periostin and osteopontin [42]. As for collagens, the distribution and abundance of non-collagen proteins is also altered throughout development [42], with aging [29], and in response to myocardial stress or injury [16]. While collagens increase in the aging rat myocardium, fibronectin and periostin decrease [29]. During the early stages of

inflammation following MI, hyaluronan, and other non-collagen components are also degraded by ECM enzymes, producing pro-inflammatory and chemotactic low molecular weight matrix fragments [43-45]. With degradation of the existing ECM, the provisional matrix produced in its place is fibrin-based and serves as a scaffold for the proliferation and migration of fibroblasts and other cell types [43, 46]. During the proliferative phase of post-MI remodeling, fibroblasts secrete large amounts of fibronectin and hyaluronan, which then serve as the basis for a more organized, cell-derived matrix to replace the fibrin-based provisional matrix [16, 17, 47]. Though matricellular proteins like thrombospondins, osteopontins, periostins, and tenascins are normally not expressed in the cardiac ECM, they play a crucial role following myocardial injury by regulating related cellular functions. Tenascin-C is produced by fibroblasts during the proliferative phase of myocardial repair in the infarct border zone [18, 48]. Its transient up-regulation during this phase appears to be stimulated by a variety of factors, such as tumor necrosis factor-α (TNFα), TGFβ, basic fibroblast growth factor (bFGF), and angiotensin II (AngII). Studies have demonstrated that tenascin-C promotes a de-adhesive state and is associated with recruitment of myofibroblasts to the infarct zone [48, 49]. Thrombospondin-1 (TSP1) is also upregulated in the border zone post-MI, and is involved in inhibiting inflammation and expansion of the infarct zone, likely through induction of TGFβ activation [16, 50]. Additionally, thrombospondin-2 (TSP2) also appears to be important to myocardial repair, as TSP2-null mice exhibit a high incidence of cardiac rupture [51]. Osteopontin, periostin, and SPARC have also been shown to play significant roles in the healing myocardium [16].

1.2.2.1: Fibronectin

Fibronectin is a large multi-domain ECM glycoprotein expressed by many cell types, including fibroblasts. Fibronectin binds cell membrane integrins as well as other ECM proteins, including heparin sulfate proteoglycans, fibrins, and collagens. Through various types of cell and matrix interactions, fibronectin is capable of regulating cell behaviour, and has been shown to play a crucial role in adhesion, growth, migration, and differentiation [52]. For example, fibronectin is critical not only in producing the secondary matrix that replaces the fibrin-based provisional matrix during the proliferative phase of post-MI remodeling (via fibronectin fibrillogenesis), but also in the phenotype conversion of cardiac fibroblasts to myofibroblasts [53], as well as the deposition of type I collagen and TSP1 [54], at least *in vitro*. In general, fibronectin exists as a homodimer of two 250 kDa monomers, each of which contains three types of repeating units (types I, II, and III) [55]. Alternative splicing of type III repeats is the mechanism through which the extracellular domain-A (ED-A) splice variant (also called EIIIA) is produced. ED-A fibronectin (ED-A Fn) expression is a necessary component of fetal heart development, is increased in myocardial injury, and also appears to be necessary for the conversion of cardiac fibroblasts to myofibroblasts [42, 53].

1.2.3: Cardiac Fibroblasts and the Extracellular Matrix

Though previously thought to act simply as the ‘glue’ that holds cardiac tissue together, the last few decades have brought to light the fact that fibroblasts provide far more than structural support to the myocardium. Cardiac fibroblasts also act as autocrine and paracrine modulators of pro-inflammatory and pro-fibrotic chemokines and cytokines, both through releasing these factors and through intracellular signaling pathways [56-58].

Though cardiomyocytes make up the majority of myocardial volume, cardiac fibroblasts are far more numerous, comprising up to 70% of all cardiac cells [59]. The primary function of cardiac fibroblasts is to maintain tissue homeostasis in the myocardium through continual remodeling of the ECM.

1.3: Migration of Cardiac Fibroblasts

Coordinated and directed migration of cardiac fibroblasts to sites of cardiac injury is a critical step in the cardiac remodelling process. Pro-inflammatory cytokines released by injured cardiomyocytes and immune cells, in addition to biomechanical cues, attract cardiac fibroblasts to regions of damaged myocardium. The process of migration begins with a phenomenon known as cell polarization, in which fibroblasts develop distinct regions at the forefront (the leading edge) and rear (the trailing edge) of the cell. Through polymerization of actin monomers at the leading edge, fibroblasts develop protrusions called lamellopodia that extend in the direction of cell movement, while the trailing edge is retracted through a process termed "retrograde actin flow". Cell movement requires dynamic turnover of cell-matrix adhesions to allow detachment of the trailing edge and the formation of new adhesions at the leading edge of the lamellopodia. An array of factors contribute to cardiac fibroblast migration, such as composition and mechanical state of the ECM or substrate, the presence of other cell types and their interaction with fibroblasts, as well as the presence and concentration of various cytokines. As fibroblasts convert to myofibroblasts, the increased strength and size of cell-matrix adhesions is associated with decreased motility, though this is a subject that remains under debate [60-64].

Increased cardiac fibroblast migration is associated with the inflammatory response to myocardial injury. Thus, it is not surprising that a number of injury-related and pro-inflammatory cytokines and chemokines are found to enhance cardiac fibroblast motility *in vitro*. The main modes of action of these cytokines appears to involve activation of MMPs (e.g. MMP9) and/or dissociation of cell-matrix adhesions [65]. AngII stimulates cardiac fibroblast migration through a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-4 (Nox4)- and reactive oxygen species (ROS)-dependent mechanism that results in increased matrix degradation (via MMP activation) and down-regulation of the adhesion protein Reversion-inducing-Cysteine-rich protein with Kazal motifs (RECK) downstream of Rho/Rho-associated protein kinase (ROCK) signaling [66, 67]. Interleukins (IL)-8 , -17, and -18 stimulate similar pathways involving transcription factors activator protein-1 (AP-1) and nuclear factor kappa B (NFκB) that stimulate MMP activity in cardiac fibroblasts, and have been demonstrated to stimulate migration of fibroblasts from non-cardiac tissues as well [68-74]. IL-1β induces cardiac fibroblast migration through a mitogen-activated protein kinase kinase (MEK1)-dependent mechanism that involves up-regulation of MMP9 and is augmented by the addition of the damage-related factor TNFα [75, 76]. IL-1β has also been shown to prevent TGFβ-induced conversion to the cardiac myofibroblast *in vitro* [77]. Adipokines such as leptin and adiponectin stimulate migration in cardiac fibroblasts via MMP activation and cytoskeleton reorganization [3, 78-80] (Figure 1).

The ECM influences cardiac fibroblast migration through both composition and rigidity [81]. The matrix protein fibronectin is an important component of the provisional matrix post-MI, and is both stimulatory for fibroblast migration and chemoattractive for

fibroblasts from various sources [82-85]. As its name would suggest, migration stimulating factor (MSF), a truncated version of fibronectin, is also able to stimulate fibroblast spreading and migration [86]. The large matrix glycoprotein Tenascin-C is transiently upregulated during injury, functioning to reduce cell-matrix adhesions as well as up-regulating MMP production, thereby increasing fibroblast migration [87, 88]. Other matrix proteins such as osteopontin and periostin also regulate cardiac fibroblast migration [89, 90]. Migration of cells in response to changes in substrate stiffness, from a region of lower stiffness to one of increased stiffness, is termed "durotaxis" (Figure 1). A number of *in silico* and *in vitro* models of durotaxis have indicated that fibroblasts preferentially migrate towards regions of higher stiffness [91-95].

Although direct treatment with pro-fibrotic growth factors such as PDGF and TGFβ inhibits cardiac fibroblast migration, these factors can act as chemoattractants. Various studies utilizing Boyden chamber assays, in which chemoattractant is placed opposite cultured cells separated by a porous membrane, found that both PDGF and TGFβ stimulated fibroblast chemotaxis [96-100] (Figure 1). Similar experiments using *in vivo* subcutaneous chambers found that PDGF and TGFβ increased fibroblast chemotaxis [101]. Chemotactic responses of fibroblasts in dermal wounds were also increased with PDGF and TGFβ [102].

Focal adhesion kinase (FAK) is a tyrosine kinase associated with the turnover of adhesions in fibroblasts and other cell types. Evidence for FAK involvement in fibroblast migration is extensive – it promotes the formation of stable lamellopodia [103-105], it facilitates directed motility, and modulates release and retraction of the trailing edge through cooperation with RhoA signaling pathway proteins such as ROCK [106-109].

Studies in FAK-null fibroblasts have demonstrated that this kinase is critical for fibroblast migration, and loss of FAK results in reduced motility due to lack of turnover of cell-matrix adhesions [110] (Figure 1).

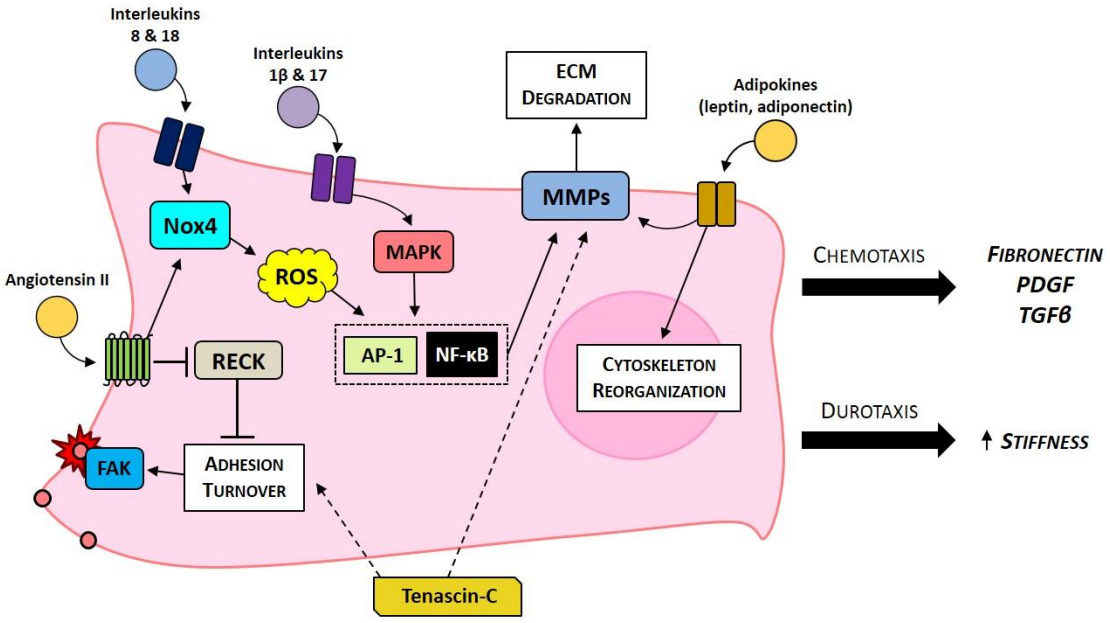


Figure 1: Migration of cardiac fibroblasts. The migration of cardiac fibroblasts requires adhesion turnover, which in turn requires focal adhesion kinase (FAK) activity, cytoskeleton reorganization, as well as degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) to facilitate fibroblast movement through the stroma. Cardiac fibroblasts demonstrate directional migration towards chemoattractants such as fibronectin, platelet-derived growth factor (PDGF), and transforming growth factor-β (TGFβ) in a process called chemotaxis. Cardiac fibroblasts also migrate towards regions of increased stiffness in a process termed durotaxis. Various injury-related modulators induce fibroblast migration via distinct pathways. Binding of angiotensin II, interleukin-8, and interleukin-18 to their respective receptors induces activation of NADPH oxidase-4 (Nox4) and downstream production of reactive oxygen species (ROS). This cascade results in activation of the transcription factors nuclear factor kappa-B (NFκB) and activator protein-1 (AP-1), which cause MMP activation. Angiotensin II receptor binding

also inhibits activity of the adhesion protein reversion-inducing-cysteine-rich protein with kazal motifs (RECK), in turn repressing adhesion. Interleukins-1β and -17 induce NFκB and/or AP-1 activation through a mitogen-activated protein kinase (MAPK)-dependent mechanism. The adipokines leptin and adiponectin induce both MMP activation and cytoskeleton reorganization. The ECM glycoprotein tenascin-C, which is upregulated during myocardial injury, stimulates both adhesion turnover and cytoskeletal reorganization. Other pathways likely contribute to the migration of cardiac fibroblasts, though these remain currently unknown.

1.4: Phenoconversion of Cardiac Fibroblasts to Myofibroblasts

Since the initial discovery of myofibroblasts in granulation tissue 40 years ago [111], it has since been determined that these cells are the predominant cell type responsible for scar formation in the majority of tissues, including the skin, lungs, liver, kidney, vasculature, and heart [112]. In the healthy heart, myofibroblast presence is limited to the valve leaflets and is absent from myocardial tissue, but myofibroblasts can be found throughout the heart in the injured myocardium, especially in the border zone of infarct scars [113-115]. Though primarily derived from resident cardiac fibroblasts, myofibroblasts in the heart may also arise from other cell types, including circulating bone marrow-derived fibrocytes, mesenchymal stem cells, pericytes, monocytes, and endothelial or epithelial cells [116]. Myofibroblasts share features of fibroblasts (synthesis of ECM components) and smooth muscle cells (contractility and actin isoform expression). Various cellular features are used to distinguish myofibroblasts from their precursor fibroblasts (Table 3). Transition to the myofibroblast is characterized by increased expression of ED-A Fn [53], αSMA [117], and embryonic non-muscle myosin heavy chain (SMemb) [3, 60, 118, 119], of which αSMA is the most commonly used marker to distinguish myofibroblasts from fibroblasts [120]. However, the phenoconversion process *in vitro* appears to produce an intermediate phenotype, called the proto-myofibroblast, which exhibit increased expression of αSMA without its incorporation into stress fibers, making these cells less contractile than 'fully converted' myofibroblasts [120-124]. Proto-myofibroblasts are characterized by increased expression of ED-A Fn, SMemb, and αSMA, increased levels of focal adhesion proteins, and *de novo* stress fibers composed of β- and γ-actins [3, 60, 119, 121, 122]. Although there is evidence of proto-myofibroblasts during the *in vitro* transition from cardiac fibroblast to

myofibroblast [60], the challenges of identifying these cells and their adhesion structures *in vivo* leaves the question as to whether they exist outside of cell culture models unanswered [120]. With additional stimuli, these proto-myofibroblasts convert further to myofibroblasts, which demonstrate even higher expression of αSMA, which is then incorporated into stress fibers allowing a high degree of cell contractility [120, 122, 123, 125]. Morphologically, myofibroblasts *in vitro* appear larger than fibroblasts and proto-myofibroblasts, with a stellate shape, and have αSMA-positive stress fibers that are anchored at large focal adhesions [122]. Such contractile stress fibers not only allow the production of contractile forces by the myofibroblasts, but also oppose retractile extracellular forces, further promoting the cardiac remodeling process [117, 122]. Myofibroblasts are also hypersynthetic for fibrillar collagens type I and III, the primary components of infarct scars, and exhibit decreased proliferation and migration [121, 126].

Table 1: Distinguishing features of cardiac fibroblasts, proto-myofibroblasts, and myofibroblasts *in vitro*.

	FIBROBLASTS	PROTO-MYOFIBROBLASTS	MYOFIBROBLASTS
Primary function(s) in vivo	Proliferation Migration MMP production	Unknown	Fibrillar collagen synthesis Contraction
Expression of α-SMA	Low, if at all	Intermediate	High
Actin cytoskeleton	β- and γ-actin filaments	β- and γ-actin stress fibers	α-SMA-containing stress fibers
Contractility	Low	Intermediate	High
ED-A Fn and SMemb expression	Low, if at all	Intermediate	High
Cell-matrix attachments	Focal contacts	Developing focal adhesions	Mature, large focal adhesions

1.4.1: Regulation of Phenoconversion

A variety of factors contributes to the temporal and spatial regulation of cardiac fibroblast phenoconversion in the heart. *In vitro* work has identified a number of key players in this process. One major factor that weighs heavily on the clinical relevance of *in vitro* models is the impact of mechanical stress [127, 128]. In the healthy myocardium, cardiac fibroblasts are exposed to external forces of approximately 4 to 18.5 kPa [129-131], but stiffness drastically increases with fibrosis to between 20 and 100 kPa [132]. Additionally, stiffness of the cardiac ECM increases with age; whereas fetal rat ECM has a stiffness of about 10 kPa, this increases to about 18 kPa in neonates, and even further in adult rat hearts, with a stiffness of about 20 kPa [29]. In response to increased stiffness, fibroblasts convert to myofibroblasts, which display increased expression of αSMA, which is subsequently incorporated into *de novo* stress fibers that terminate at focal adhesions [132], imparting increased contractility and stiffness to these cells. *In vitro,* mouse embryonic fibroblasts internally match the stiffness of their surrounding substrate up to approximately 10 kPa, at which point cell spreading is maximized and after which increasing stiffness of the substrate is no longer matched by fibroblasts, inducing formation of stress fibers to accommodate extracellular stress [133, 134]. Since typical plastic tissue culture dishes exhibit stiffness in the range of 10^6 kPa [135], and even flexible membranes used in stretch assays have a stiffness of 930 kPa (FlexCell Internal data sheet, www.flexcell.com), standard culture of primary cells itself is a potent inducer of fibroblast to myofibroblast conversion [136]. Interestingly, a number of studies have shown that the myofibroblast phenotype can be prevented, or even reversed, by reducing the extracellular tension applied to these cells, causing the loss of myofibroblast markers such as αSMA and the dissolution of stress fibers [128, 137, 138]. These studies indicate

that mechanical stress is a critical factor in driving phenoconversion. It is likely that this mechanical effect on phenotype may result from increased Rho/ROCK signaling and activation of myocardin-related transcription factor-A (MRTF-A) that also occurs during ECM remodeling [122, 139]. Cardiac fibroblasts plated on substrates resembling fibrotic myocardium (approximately 40 kPa) exhibited increased phenoconversion compared to those plated on softer substrates (10 kPa). However, with addition of the ROCK inhibitor Y27632 prevented phenoconversion on the stiffer substrate [140]. Another study of NIH-3T3 fibroblasts exposed to mechanical stress implicates FAK as a necessary intermediate during force-induced translocation of MRTF-A to the nucleus, where it activates αSMA transcription, promoting the myofibroblast phenotype [141, 142]. The response of cardiac fibroblasts to mechanical stimulation is discussed further in the Mechanosensing section (1.7).

TGFβ stimulation appears to be the major factor driving cardiac fibroblast conversion to myofibroblasts. ED-A Fn, which is increased with phenoconversion, appears to act in a feed-forward loop by further promoting the myofibroblast phenotype in a TGFβ-dependent manner [53]. Other growth factors, such as PDGF and connective tissue growth factor (CTGF), increased in response to myocardial injury, also contribute to phenoconversion through TGFβ signaling [143-148]. Additional factors influencing phenoconversion include angiotensin II signaling, which appears to converge with canonical TGFβ signaling pathways to promote the myofibroblast phenotype, as well as the mechanosensitive transient receptor potential channel-6 (TRPC6) [149], the vasoconstrictor endothelin-1 (ET-1) [150], ECM components such as hyaluronan [151, 152], microRNAs [153-155], and many others. Inhibitors of the phenoconversion process

include the proto-oncoprotein Ski [156], the homeobox transcription factor mesenchyme homeobox-2 (Meox2) [157], and bFGF [158].

1.5: Transforming Growth Factor-β1

Transforming growth factor-β (TGFβ) belongs to a highly conserved superfamily of cytokines that also includes bone morphogenetic proteins (BMPs), activins, and inhibins. TGFβ exerts pleiotropic effects on a myriad of cell types, regulating processes such as differentiation, proliferation, ECM production, and apoptosis [159]. In mammals, TGFβ exists in three isoforms (TGFβ1, TGFβ2, and TGFβ3) that display similar yet diverse biological effects. All three TGFβ isoforms are involved in EMT during cardiac development [160]. Of these isoforms, TGFβ1 is the most well characterized in regards to cardiac injury and subsequent fibrosis, although there are studies to suggest a role for the other two isoforms in cardiac remodeling. TGFβ1 is released by a variety of cell types, including cardiomyocytes [161, 162], fibroblasts/myofibroblasts, vascular smooth muscle cells, and immune cells [163]. TGFβ2 is also secreted by cardiac fibroblasts [164], is increased in response to MI and norepinephrine-induced cardiac hypertrophy in rats [165], and may be involved in induction of the fetal gene program during remodeling. However, there is a paucity of information regarding the role of TGFβ2 in cardiac fibrosis and related cardiac fibroblast activities. The role of TGFβ3 in the adult myocardium is not well known, though its expression is correlated with increased MMP and TIMP expression following MI in rats [166], and thus TGFβ3 may be involved in the cardiac remodeling process.

TGFβ is secreted as a dimer in an inactive form, bound non-covalently to a TGFβ pro-peptide termed the latency-associated peptide (LAP), which prevents association of TGFβ

with its receptor [167]. The complex formed by TGFβ and LAP is called the small latent complex (SLC). This SLC is in turn bound by latent TGFβ-binding protein (LTBP) [168], forming the large latent complex (LLC) within the endoplasmic reticulum, prior to secretion [169]. LLC is secreted in either soluble or matrix-bound form, the latter of which provides stores within the ECM that can be made readily available upon activation. LTBP is responsible for binding of the LLC to matrix components such as vitronectin and fibronectin [127, 170, 171]. Additionally, LTBP binds cell membrane integrins, providing a link between the cell cytoskeleton and the matrix [172-174]. Activation of latent TGFβ by dissociation from the LLC occurs either via proteolytic cleavage by ECM-degrading enzymes such as MMPs [175-179], or through protease-independent integrin-mediated deformation of the LLC [180, 181]. In the latter mechanism, integrins containing the αv subunit (such as αvβ3, the primary integrin of focal adhesions) bind to LAP via an arginyl-glycyl-aspartic acid (RGD) motif [182, 183], and myofibroblast contraction induces release and activation of the TGFβ molecule, which is then free to associate with its receptor and induce downstream pro-fibrotic signaling events [181, 184].

TGFβ1 is released in response to various clinical pathologies, including hypertension [185], cardiac hypertrophy [161], and following myocardial infarction [166, 186]. In humans, TGFβ1 expression increases in response to cardiac pressure- and volume-overload-induced hypertrophy [187, 188]. In response to MI in rats, TGFβ1 levels increase 3 days post-MI, peaking at 7 days post-MI, and remain consistently elevated through 4 weeks post-MI [189]. Expression of TGFβ1 increases in fibroblasts and myofibroblasts by treatment with angiotensin II, which is elevated in response to cardiac pathology [190-192]. TGFβ1 is a potent driver of fibrosis in the heart and other organs

[193-198], and is critical for phenoconversion of cardiac fibroblasts into myofibroblasts [193-197, 199]. In fibroblasts and myofibroblasts, TGFβ treatment induces increased expression of fibrillar collagens [199-203], CTGF [204], ED-A Fn [205-207], and αSMA [208, 209], as well as inducing stress fiber and focal adhesion formation and subsequent contraction [208, 210-213]. TGFβ promotes cardiac fibroblast proliferation [214, 215], but abrogates proliferation of converted myofibroblasts [121]. TGFβ also contributes to fibrosis through the reduction of MMPs and induction of TIMPs [216-222]. TGFβ1 has also been shown to drive epithelial-to-mesenchymal transition (EMT) both *in vitro* and during embryonic development of the heart [160, 223], a process that may contribute to fibrogenesis in the heart and other organs [224].

1.5.1: Canonical TGFβ Signaling

The canonical TGFβ signaling pathway is initiated by binding of TGFβ1 ligand to a homodimer of type II threonine/serine kinase receptors (TβR-II) at the cell surface [225], recruiting the type I TGFβ receptor (TβR-I) homodimer to form a heterotetrameric receptor complex [226]. TβR-II activates TβR-I through phosphorylation of a glycine-serine (GS)-rich region [227], which provides a docking site for receptor-regulated Smads (R-Smads) 2 and 3, named for their homology to *sma* and *mad* proteins in *Caenorhabditis elegans* and *Drosophila melanogaster*, respectively [159, 228]. Other members of the TGFβ superfamily activate R-Smads 1, 5, and 8. Phosphorylation and activation of R-Smad2 and/or R-Smad3 by TβR-I induces the formation of a complex between Smad2/3 and the common Smad called Co-Smad4 [229-231]. The resultant Smad complex translocates to the nucleus where it acts as a transcriptional regulator of a variety of target genes through binding to a conserved *CAGAC* DNA sequence termed the Smad-binding

element (SBE) [232]. The inhibitory Smad7 interferes with binding of R-Smads to TβR-I, preventing downstream Smad signaling to target genes such as collagens [233, 234] (Figure 2). Different complexes bind DNA with varying affinity and Smads alone are weak transcriptional activators, which is reflected in the ubiquitous nature of their recognition sequence in various gene promoters [136, 235]. Thus, regulation by Smads involves association with other transcription factors and coactivators that may result in either enhanced or repressed target gene expression. Such factors identified in fibroblasts include Scleraxis (Scx) [136] (Figure 2), the cofactor p300 [236-240], the nuclear binding protein Fast-1 [241], p300/cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB)-binding protein (CBP)-associated factor (P/CAF) [242], and specificity protein-1 (Sp1) [243-245], as well as the co-repressors c-Ski [246, 247], Ski-related novel protein-N (SnoN) [248, 249], Krüppel-like factor-15 (KLF15) [250], TGFβ-induced factor (TGIF) [251], and AP-1 [252, 253]. Signaling through the canonical Smad pathway activates a number of cardiac ECM genes, including type I collagen and *CTGF* [136, 231, 243, 254]. Disruption of Smad3 signaling inhibits phenoconversion of fibroblasts to myofibroblasts post-MI [61]. In hypertension-induced heart failure, Smad2 phosphorylation was increased [255]. Additionally, TGFβ1 exerts protective effects in neonatal and adult cardiac fibroblasts exposed to ischemia/reperfusion, preventing apoptosis through both canonical Smad-mediated and non-canonical extracellular signal-regulated kinase-1/2 (ERK1/2) and Akt/protein kinase-B (PKB) pathways [7, 256].

1.5.2: Non-Canonical TGFβ Signaling

TGFβ may also contribute to the development of fibrosis through non-canonical signaling pathways. Studies of the *αSMA* promoter in rat lung fibroblasts have shown that

transactivation occurs through a non-Smad-binding TGFβ-responsive element (TRE). Myofibroblasts derived from dermal wounds required an intact MCAT element for *αSMA* promoter activation [257]. In smooth muscle cells, TGFβ-induced activation of the *αSMA* promoter required two conserved *CC(A/T)$_6$GG* (CArG) boxes and a TGFβ control element (TCE) [258]. Thus it appears TGFβ may be capable of stimulating the myofibroblast phenotype and fibrosis through both Smad-dependent and -independent signaling pathways. Binding of TGFβ to its receptor has been shown to induce non-canonical pathways including MAPK [259], Ras/mitogen-activated protein kinase kinase (MEK)/ERK and associated TGFβ-activated kinase-1 (TAK1) [7, 251, 260, 261], c-Jun amino (N)-terminal kinase (JNK) [262], phosphatidylinositol 3-kinase (PI3K)/Akt, and Rho guanosine triphosphate (GTP)-ase signaling pathways [7, 263] (Figure 2). In addition to regulation of fibrotic genes such as collagens, non-canonical TGFβ pathways may also promote fibrosis through activation of the fibrillar collagen cross-linking enzyme lysyl oxidase (Lox), as inhibition of PI3K, p38-MAPK, ERK1/2, or JNK prevents increased Lox protein levels in cardiac fibroblasts treated with TGFβ1 [262].

1.5.2.1: Phosphatidylinositol 3-kinase/Akt Signaling Pathway

TGFβ1 treatment can induce phosphorylation of Akt in non-cardiac fibroblasts, which is prevented by the use of a dominant negative RhoA mutant and increased with constitutively active RhoA, suggesting that Akt activation occurs downstream of non-canonical TGFβ-induced Rho/ROCK signaling [263, 264]. Similarly, in human glomerular mesangial cells and scleroderma fibroblasts, TGFβ1 treatment rapidly stimulates PI3K and Akt activation to induce *COL1A2* gene expression through increased Smad3 transcriptional activity, indicating cross talk between the PI3K/Akt and Smad

signaling pathways downstream of TGFβ [265, 266]. There is also evidence that PI3K/Akt-mediated cell signaling is involved in EMT and cell migration [263, 267-270] (Figure 2). However, PI3K/Akt activation appears to inhibit expression of *Collagen Iα2* and *Collagen IIIα1* genes in human skin fibroblasts [271], thus it remains unclear how this signaling pathway contributes in general to fibroblast phenotype and function, let alone that of cardiac fibroblasts.

1.5.2.2: Mitogen-Activated Protein Kinase/Extracellular-Signal-Regulated Kinase Signaling Pathway

In addition to phosphorylation of TGFβ receptors at serine and threonine residues, TβR-II is also autophosphorylated in response to TGFβ1 binding in cultured NIH-3T3 fibroblasts [272]]. This results in recruitment of Src homology-2 (SH2) domain-containing proteins such as Src homology domain 2-containing protein (Shc) [273], which then associates with growth factor receptor-binding protein-2 (Grb2) and Son of sevenless (Sos) [274], directly inducing activation of the Ras, Raf, MEK1/2, and ERK1/2 MAP kinases [274, 275] (Figure 2). In cardiac fibroblasts, TGFβ1 treatment induces type I collagen expression through MEK/ERK activation, which is inhibited by cAMP-elevating agents such as forskolin and isoproterenol [260]. There is also evidence that the Ras/Raf/MEK/ERK pathway mediates canonical Smad-mediated TGFβ signaling in various cell types, including cardiac fibroblasts [276], however it remains controversial as to whether signaling through ERK in response to TGFβ1 positively or negatively regulates Smad signaling [277]. In non-cardiac fibroblasts, ERK activation was required for expression of type I collagen [260], though this has yet to be examined in cardiac

fibroblasts. In a fibrosarcoma-derived cell line, JNK signaling, independently of Smad4, was required for induction of fibronectin expression by TGFβ [278].

1.5.2.3: Rho Signaling Pathway

Rho signaling regulates a number of cellular processes, one of the most notable being stimulation of actomyosin contractility. Additionally, Rho signaling has been shown to be involved in fibrosis of various tissues [279, 280], including the myocardium [281, 282]. Rho signaling can also act downstream of TGFβ signaling [283]. A number of studies in various cell types have shown that Rho GTPases such as cell division cycle-42 (Cdc42) and RhoA are required for TGFβ-induced reorganization of the actin cytoskeleton, influencing processes such as stress fiber and lamellopodia formation [284], cell contraction [285], and EMT [286]. There is also evidence of Rho/TGFβ crosstalk during transition of lung and kidney fibroblasts to the myofibroblast phenotype [287, 288]. This likely occurs through induction of the transcription factor MRTF-A in response to ROCK activation. Signaling through ROCK induces organization of cytoskeletal actin into filamentous (F)-actin [289], releasing globular (G)-actin-bound MRTF-A [290], which forms a complex with serum response factor (SRF) [290, 291]. This MRTF-A/SRF complex translocates to the nucleus where it activates transcription of pro-fibrotic genes such as *αSMA* and *Tenascin-C* [139, 292-294], as well as inducing actin cytoskeleton reorganization and myofibroblast phenoconversion (Figure 2). In cardiac fibroblasts, treatment with TGFβ induces MRTF-A nuclear translocation in a ROCK-dependent manner. Additionally, knockdown of MRTF-A expression in mice reduces fibrosis and scar formation following MI or treatment with AngII, exhibiting decreased expression of

Collagen Iα2 and *αSMA* [295]. Thus, Rho signaling appears to be an important non-canonical signaling pathway in TGFβ-induced transition to the cardiac myofibroblast.

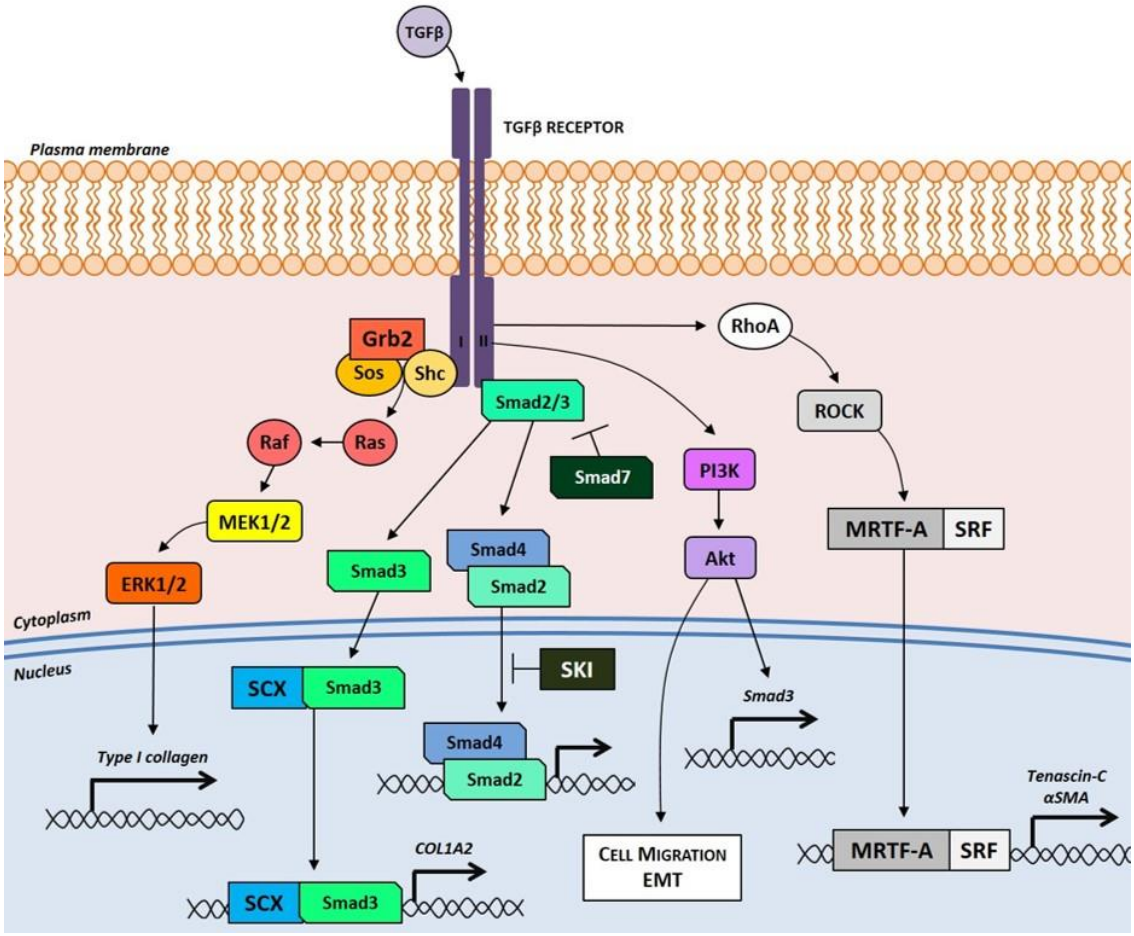


Figure 2: Transforming growth factor-β signaling in cardiac fibroblasts and myofibroblasts. TGFβ binding induces TGFβ receptor dimerization and initiation of various signaling cascades. Canonical TGFβ signaling involves recruitment and activation of R-Smad2/3, which is repressed by inhibitory Smads (e.g. Smad7). R-Smads form complexes with co-Smad4 or other proteins that may enhance (e.g. SCX) or inhibit (e.g. SKI) transcriptional activation of target genes. Non-canonical TGFβ signaling may occur through the mitogen-activated protein kinase pathway, involving recruitment of adaptor proteins Src homology domain 2-containing protein (Shc), growth factor receptor-binding protein-2 (Grb2), and Son of sevenless (Sos). This complex initiates downstream signaling through Ras, Raf, mitogen-activated protein kinase kinase-1/2 (MEK1/2) and

extracellular signal-regulated kinase-1/2 (ERK1/2) to induce transcription of *type I collagen*. Non-canonical signaling also occurs through the phosphatidylinositol-3 kinase (PI3K)/Akt pathway, activating *Smad3* transcription as well as genes involved in cell migration and EMT. Additionally, the RhoA/Rho-associated kinase (ROCK) pathway may be activated through TGFβ receptor activation, resulting in the formation of a myocardin-related transcription factor-A (MRTF-A)/serum response factor (SRF) complex which activates transcription of target genes involved in phenoconversion, such as *tenascin-C* and *α-smooth muscle actin* (*αSMA*). Figure adapted by author from *Roche et al. (2015), Comprehensive Physiology, In Press.*

1.6: Cell-Matrix Adhesions

Cardiac fibroblasts and myofibroblasts play an active role in remodeling their surroundings, and in turn, their structure and function are dependent on the status of their environment and interactions with the extracellular substrate or matrix. The ability of fibroblasts to sense and respond to changes in ECM structure and composition is afforded through cell-matrix adhesions. In fact, unlike non-adhesive cell types such as leukocytes, fibroblasts are so reliant on interaction with their surroundings that cells in suspension or on non-adhesive substrates are incapable of growth and proliferation, even in the presence of growth factors or serum [296]. The adhesions linking cardiac fibroblasts to the ECM are also critical to their ability to contract and migrate - important processes that facilitate cardiac remodeling and wound healing [297]. Cell-matrix adhesions in fibroblasts rely on transmembrane receptor proteins called integrins, which link various ECM components to the actin cytoskeleton. Though integrins are the primary component of both adhesion types, the specific β- and α-isoforms utilized may differ not only between adhesion types, but also depending on cell context cues such as location, matrix composition, and the presence of other cells. Inside the fibroblast, various cytoplasmic proteins are recruited to sites of cell-matrix adhesions to form adhesion complexes that link integrins to the actin cytoskeleton. These include adaptor proteins such as zyxin, talin, vinculin, paxillin, and α-actinin, scaffolding proteins like p130Cas, and associated kinases that interact directly with β-integrins, such as FAK and Src family kinases (SFKs) [298, 299]. It should be noted that although cell-matrix adhesions are critical to fibroblast survival and function, their turnover is necessary for fibroblast migration and is regulated by a complex interplay of signaling pathways, many of which appear to involve FAK.

1.6.1: Formation of Focal Complexes

Initial formation of cell-matrix adhesion involves the development of weak, nascent adhesions called focal complexes or contacts, which form in response to interaction of fibroblasts with their substrate. Focal complexes are highly dynamic structures with a diameter of less than 1 μm, which employ αvβ3 as their primary integrin component [210, 298]. Generally, focal complex formation appears to occur independently of mechanical tension [300, 301], though studies in NIH-3T3 have found myosin II-generated contractility to be indispensable for focal complex formation on fibronectin-coated substrates [302].

1.6.1.1: Signaling Pathways in Focal Complex Formation

Focal complex formation relies on RhoA signaling, which is also associated with the formation and maturation of stress fibers in myofibroblasts [302]. Several studies have demonstrated the necessity of RhoA signaling in focal complex development and maturation through induction of myosin II-mediated actin cytoskeleton contraction induced by the downstream kinase ROCK [301-303]. Interestingly, the initial stages of adhesion formation involve RhoA down-regulation. This is mediated at least in part by Src-dependent, integrin-mediated phosphorylation and activation of the RhoA inhibitor p190RhoGAP [304, 305]. The RhoA antagonist Rac is another GTPase that can inhibit RhoA in early focal complex formation through direct activation of p190RhoGAP [306], or indirectly through increased production of ROS [307]. FAK may also be involved in this transient RhoA down-regulation, since it forms a complex with p190RhoGAP and p120RasGAP during focal complex formation in fibroblasts [109]. This latter model is further supported by the findings that FAK-null cells show reduced activation and

localization of Rac to focal complexes, and that FAK forms a complex with Src, p130Cas, and dedicator of cytokinesis-180 (Dock180), which is associated with elevated Rac and JNK expression in adhering fibroblasts [308, 309]. Activation and autophosphorylation of FAK at tyrosine 397 (Y397) induces formation of a binding site that allows further FAK phosphorylation and full activation by Src. FAK and Src both mediate the phosphorylation of additional downstream adhesion proteins such as p130Cas and paxillin, facilitating recruitment of adaptor proteins and formation of signaling complexes at sites of focal adhesions [310, 311].

1.6.2: Maturation of Focal Adhesions

Following cell spreading and increased attachment, focal complexes can mature into focal adhesions that are characteristic of myofibroblasts *in vitro* [312] (Table 3). In comparison to their smaller, nascent counterparts, new focal adhesions are stronger and larger, with a diameter of several micrometers, expanding up to 30 μm in fully converted myofibroblasts – these larger focal adhesions are considered super-mature focal adhesions, distinguished by increased expression of *Tensin* [210, 298]. Various factors contribute to regulation of focal adhesion strength and size, including extracellular forces such as substrate stiffness, integrin activation and clustering, expression of other transmembrane receptors such as *discoidin domain receptor 1* (*DDR1*) [313, 314], as well as the localization and interaction of cytoplasmic effector proteins [315]. Mechanical tension has been shown to be one of the more important factors in the maturation of focal adhesions [316, 317], which may originate from inside the cell through actomyosin contractility [318], or externally through the application of extracellular forces [301]. In fact, in myofibroblasts, contractile activity produced by αSMA-containing stress fibers is

required for the maturation of super-mature focal adhesions [300]. Additional stimuli associated with transition to the myofibroblast phenotype also induce focal adhesion maturation, including TGFβ and ED-A Fn [210, 319].

1.6.2.1: Signaling Pathways in Focal Adhesion Maturation

Following transient down-regulation of RhoA during initial adhesion formation, Rac is gradually inhibited, accompanied by an increase in RhoA activation and maturation of focal complexes into focal adhesions [320]. RhoA activation during focal complex maturation permits the establishment of cytoskeletal tension and recruitment of adaptor proteins such as talin, paxillin, vinculin, and α-actinin that augment associations between the fibroblast cell membrane and actin cytoskeleton [321, 322]. FAK also plays a role in focal adhesion maturation that relies on actomyosin contractility – treatment of MEFs with blebbistatin, an inhibitor of contraction, reduces FAK phosphorylation at Y397 and impairs maturation of focal adhesions [323]. A key step in the establishment of these mechanically stable cell-matrix adhesions is the clustering of various integrins, for example, the integrin αvβ3 (found in focal complexes), and its association with the fibronectin receptor integrin α5β1 [298]. Clustering of integrins is correlated with the recruitment and activation (via Y397 autophosphorylation) of FAK to the cell membrane. Subsequently, adaptor proteins are recruited to adhesion sites, allowing association of focal adhesions with stress fibers that form during phenoconversion to the myofibroblast [324].

1.7: Mechanotransduction in Fibroblasts and Myofibroblasts

The ability of cells to respond to mechanical stimuli and translate such forces into biochemical signals is referred to as “mechanotransduction” [325]. Although the heart

beats over 100,000 times per day, the cardiac ECM acts to shield cardiac fibroblasts from constantly changing mechanical conditions, permitting fibroblasts to maintain equilibrium between internal and external forces. However, when the matrix is disrupted, cardiac fibroblasts become exposed to increased mechanical stress, leading to activation of force transduction pathways through mechanosensitive receptors and adaptor proteins that can induce both structural and functional changes. In addition to external forces, internally-produced tension (through actomyosin contractility) will also affect the amount of force experienced by the cell. Different experimental models of stress have been utilized to study mechanotransduction by fibroblasts and other cell types, such as uni-, bi-, or multi-axial extension of cells plated on elastic membranes to around 10-15% of their original length, either through static or cyclic stretch [326], the latter of which is discussed later in this section. Using light microscopy, Haston *et al.* observed that fibroblasts exposed to uniaxial static stretch reoriented in the direction of force application [327]. Single cell and single molecule force application approaches have also been developed that include adhesive pipette tips [301, 328], coated magnetic beads [329], and optical tweezers [330, 331].

The observation that cardiac fibroblasts are capable of phenoconversion on rigid substrates, even in the absence of additional stimuli, indicates a mechanism must exist whereby cells are able to identify physical changes in their environment and respond by eliciting intracellular signals that alter gene expression. In turn, mechanosensitive factors and their downstream messengers are able to alter cell morphology, contractility, migration, and phenotype [332, 333]. Various mechanisms have been proposed to regulate mechanotransduction in fibroblasts, including force-activated ion channels such

as transient receptor potential cation channels (TRPCs) that facilitate influx of ions like calcium and subsequent myofibroblast conversion [149, 334-337]. Experiments in ventricular cardiac fibroblasts provide evidence that TRPCs integrate mechanical and biochemical signals during phenoconversion. Inhibition of TRPV4 prevents TGFβ-induced conversion of cardiac fibroblasts to myofibroblasts. This result may be due to TRPV4-mediated β1 integrin activation, supported by findings that these two transmembrane receptors co-localize at focal adhesions, and that TRPV4 is necessary for integrin activation in endothelial cells [338, 339]. Interestingly, atrial fibroblast phenotype conversion appears to rely on another TRPC, TRPM7, and not TRPV4, which may be due to differences in mechanical environments between the chambers [335]. Another potential mechanotransduction mechanism in fibroblasts is force-induced activation of integrins and subsequent activation of downstream kinases, small GTPases such as RhoA and Rac, and additional signaling pathways such as the ERK pathway [340]. Additionally, direct force transmission may through transcriptional regulators of the Hippo pathway (Yap and Taz), such as occurs in epithelial cells cultured on rigid substrates. Direct force transmission can also occur through the cytoskeleton, altering the shape of the nucleus and chromatin organization. The latter mechanism occurs through the linker of nucleoskeleton and cytoskeleton complex (LINC), which can transmit forces to the nuclear matrix and lamin network of the nuclear membrane, causing changes in chromatin folding and protein-DNA associations [341]. However, these direct force transmission mechanisms have yet to be examined in cardiac fibroblasts.

Focal adhesions are the crux of mechanotransduction in cardiac fibroblasts, and because integrins are the link between ECM and cytoskeleton at these sites, they appear to do the

majority of the heavy lifting when it comes to force sensing [342]. The response to mechanical force is dependent upon the type of integrin activated, which is in turn determined by the substrate. For example, integrin αvβ3 was shown to undergo conformational activation in response to mechanical strain in NIH-3T3 cells plated on fibronectin or fibrinogen, accompanied by increased PI3K/Akt phosphorylation and activation [343]. Force-dependent activation of JNK relies on upstream activation of integrin αvβ3 [343]. However, integrins are not the only cell membrane component that contribute to mechanotransduction in fibroblasts. N-cadherins, the primary component of cell-cell interactions in fibroblasts, also possess mechanosensitive capabilities in fibroblasts through interaction with α-/β-catenin complexes that bind various adaptor proteins such as vinculin, α-actinin, and F-actin itself [344, 345]. Additionally, loss of the mechanosensitive proteoglycan syndecan-4 (Syn4) prevented cardiac fibroblast to myofibroblast transition both *in vitro* and *in vivo*, likely through reduced nuclear factor and activator of transcription-4c (NFAT4c) transcriptional activity [346].

1.7.1: Rho Signaling Pathway in Mechanotransduction

In addition to its activity downstream of TGFβ1 signaling, the Rho signaling pathway has been strongly implicated in mechanotransduction in fibroblasts. Direct force application to integrins is capable of inducing RhoA activation and downstream signaling [139, 347]. Although RhoA activity is initially down-regulated in response to mechanical force, such as culture on stiff substrates [305], sustained adhesion induces activation of several Rho-activating guanine exchange factors (GEFs) [348]. In NIH-3T3 fibroblasts, RhoA signaling and resultant formation of focal adhesions and stress fibers is activated by p115RhoGEF and leukemia-associated Rho guanine nucleotide exchange factor (LARG),

in response to plating on fibronectin-coated plates [349]. Force-induced integrin activation in fibroblasts activates LARG and GEF-H1, another GEF that activates Rho signaling in response to force [347]. RhoA activity also plays a critical role in the assembly and contraction of fibronectin fibrils [350], and thus may be involved in producing this type of provisional matrix in response to increased mechanical stress in fibroblasts during post-MI wound healing.

FAK is also implicated in mechanically induced Rho signaling. In addition to RhoA, FAK is involved in adhesion of fibroblasts to fibronectin matrix as well as being a crucial component in fibronectin matrix contraction [350, 351]. In MEFs plated on fibronectin, FAK forms a complex with p190RhoGEF, upstream of Rho activation [352]. FAK activity activates the GEFs PDZ-RhoGEF, GEF-H1, and LARG [353]. Inhibition of FAK prevents force-induced GEF-H1 induction in fibroblasts, but not that of LARG [347]. Numerous studies have demonstrated that integrin activation and adhesion to fibronectin in fibroblasts induces FAK activation, primarily through phosphorylation at tyrosine 397 (Y397) [354-357]. FAK is a key player in integrin-mediated mechanosensing by cardiac fibroblasts, modulating focal adhesion turnover, migration, and force-induced *αSMA* gene activation via the transcription factor MRTF-A [333, 358]. Myosin-II-induced contractility increases FAK phosphorylation and subsequent recruitment of focal adhesion proteins like paxillin and vinculin [141, 359]. The mechanosensitive scaffolding protein p130Cas can be activated in response to mechanical stress either through kinase (FAK and SFK)-mediated phosphorylation or by direct conformational changes that reveal active domains [360, 361]. p130Cas plays an important role in cell spreading, integrin activation, migration, and focal complex formation [323, 362-364]. Downstream

of FAK, as well as the GTPase Ras, ERK signaling is also implicated in mechanotransduction through Rho signaling, via induction of the Rho-activating factor GEF-H1. MAPK signaling has also been shown to act in fibroblast mechanotransduction through integrins [347, 365, 366], independently of FAK activation [355], indicating the presence of multiple signaling pathways in response to integrin-mediated force sensing in fibroblasts.

1.7.2: Response to Stretch

The myocardial ECM constantly exposes cardiac fibroblasts to mechanical stress *in vivo*, though in the healthy heart, they are 'stress-shielded' from the majority of external forces. In response to increased mechanical strain, such as occurs with degradation of the ECM post-MI, cardiac fibroblasts are exposed to increased extracellular forces [367]. Cyclic stretch experiments are models that attempt to recapitulate the force exposure of fibroblasts in a cyclical manner *in vitro*. However, though there are a number of studies examining the effect of cyclic stretch on cardiac fibroblasts, the regimes vary in substrate used (fibronectin, vitronectin, collagen, etc.), elongation (percent stretch), dimension (uni- versus bi-axial strain), frequency, and duration, all of which potentially affect the response to cyclic stretch. Thus, the effect of cyclic stretch on cardiac fibroblast biology *in vitro* remains relatively unexplored, though most studies implicate cyclic stretch as being an inducer of collagen synthesis and increased contractility in cardiac fibroblasts, suggesting a transition to the myofibroblast phenotype.

In response to low degrees of cyclic stretch (3-10%), primary rat cardiac fibroblasts show increased synthesis and secretion of fibrillar collagens type I and III [368-370], αSMA [371], TGFβ [372], MMP-2, TIMP-2 [373], fibronectin [368], and insulin-like growth

factor-1 (IGF1) [374], as well as p42/44-MAPK activation [375]. The transcription factor NFAT4c, which has been shown to promote the myofibroblast phenotype, was also increased in cardiac fibroblasts exposed to cyclic stretch, in a Syn4- and calcineurin-dependent manner [376]. Cardiac fibroblasts plated on a variety of substrates showed decreased proliferation and increased type I collagen mRNA levels in response to 5% cyclic stretch for 12 hours [375]. Although these responses have not been performed at higher levels of strain (e.g. 15-20%), some of them, like fibroblast orientation, may be strain intensity-independent [377].

Cyclic stretch of non-cardiac fibroblasts also induces increased activation of cellular pathways implicated in cardiac myofibroblast phenoconversion, such as members of the RhoA, MAPK, ERK1/2, and Akt signaling pathways [378, 379]. The RhoA signaling pathway in particular is significantly affected by cyclic stretch. Within 5 minutes of 10% equi-biaxial cyclic strain (0.3 Hz), active RhoA was increased in MEFs. In MEFs and primary embryonic chick skin fibroblasts, 10% cyclic stretch induces increased formation of stress fibers and nuclear translocation of the MRTF-A homolog myelin and lymphocyte protein (MAL), and increased expression of *Tenascin-C* [289]. Fibronectin appears to play a critical role in cyclic stretch-induced Rho signaling, as fibronectin-depleted MEFs showed a significant decrease in RhoA activation, stress fiber formation, and MAL translocation, responses which were rescued by plating on fibronectin-coated membranes [380]. Additionally, integrin-linked kinase (ILK) was necessary for activation of RhoA and subsequent focal adhesion and stress fiber formation, as well as nuclear translocation of MAL in response to 10% cyclic strain of primary embryonic chick skin fibroblasts [294]. The mechanosensitive scaffolding protein p130Cas, implicated in Rho

signaling, is also affected by cyclic stretch in fibroblasts, such that its response involves binding to the focal adhesion protein zyxin, which is activated by ERK in response to stretch. In response to cyclic strain, zyxin relocalizes to stress fibers and is necessary for recruitment of α-actinin to stress fibers in mechanically stretched fibroblasts [381, 382].

1.7.2.1: Focal Adhesion Kinase in Response to Cyclic Stretch

Increased FAK autophosphorylation (Y397) is also induced in response to cyclic strain in non-cardiac fibroblasts, though this has only been demonstrated with very high levels of elongation (120% stretch) and short duration (60 minutes). Uniaxial cyclic stretch (120%, 1 Hz) of 3Y1 fibroblasts induced reorientation perpendicular to the stretch axis and increased expression of *paxillin* and *p130Cas*, increased FAK tyrosine phosphorylation, as well as increased kinase activity of Src within 30 minutes [379, 383, 384]. Additionally, mesenchymal stem cells exposed to 10% stretch (1 Hz) for 30 minutes also showed increased tyrosine phosphorylation (Y397) of FAK [385]. However, after 60 minutes, levels of FAK phosphorylation begin to decline. In airway epithelial cells, 20% cyclic stretch (0.5 Hz) induced a transient increase in FAK phosphorylation (Y397) that decreased to below levels in unstretched cells after 6 hours [386]. Additionally, experiments in FAK-null fibroblasts exposed to 10% cyclic stretch indicate that FAK is not necessary for stretch-induced stress fiber formation, nor for ERK activation. Rather, it appears that these processes are dependent upon stretch frequency, of which 1 Hz appears to be the most potent inducer of stress fibers and ERK induction [387]. Nonetheless, FAK appears to play an important role in fibroblasts exposed to cyclic stretch. In MEFs exposed to 1 hour of 20% uniaxial cyclic stretch (0.25 Hz), loss of FAK resulted in drastically reduced cell viability [388]. These data suggest that FAK phosphorylation at

Y397 may be a transient event that occurs early during the fibroblast response to cyclic stretch, in order to redistribute focal adhesion complexes.

1.8: Myofibroblast Contraction

The ability of myofibroblasts in the heart and other tissues to contract and exert mechanical forces on their surrounding matrices is dependent upon expression of αSMA and its incorporation into stress fibers. It is well known that the mechanical forces produced by myofibroblasts are essential for proper wound healing and tissue remodeling, partly through regulation of cytokine activation and ECM synthesis [2].

1.8.1: Stress Fibers

In non-muscle cells, stress fibers are the primary contractile apparatus, and are composed of large actin bundles anchored at one or both ends by focal adhesions, from which stress fiber formation begins [389, 390]. Stress fibers are cross-linked through an alternating distribution of α-actinin and myosin II, the latter of which is responsible for stress fiber contractility and the development of isometric tension in fibroblasts from various tissues [125, 391-394]. The appearance of stress fibers and incorporation of αSMA protein is characteristic of the transition of cardiac fibroblasts to myofibroblasts, and their development is highly dependent upon cellular forces [395]. In fibroblast-populated collagen gels attached to a tissue culture dish, release of gels and thus cellular tension induces rapid gel contraction and compaction of stress fibers, followed by spontaneous stress fiber disassembly [396-398]. Stress fiber formation in human dermal fibroblasts requires expression of *α4 integrin*, and is further promoted by ED-A Fn [399]. Although stress fiber formation is associated with increased fibronectin expression and the

development of fibronectin fibrils, stress fiber formation and subsequent cell contraction can occur independently of fibronectin and fibronectin fibril assembly [399, 400].

Stress fiber formation and turnover is modulated by two pathways, Rho signaling and myosin light chain kinase (MLCK) [401]. RhoA, which has been shown to bind directly to stress fibers, primarily regulates central stress fibers [358, 402, 403], while MLCK appears to modulate stress fibers near the periphery of the cell [125, 394, 404, 405]. In response to culture on rigid substrates, RhoA is activated, in turn activating downstream effectors ROCK and ROCK2, of which Rho and ROCK2 are critical in the formation, maintenance, and contractility of centrally located stress fibers [403, 404, 406-408].

1.8.2: Mechanisms of Myofibroblast Contraction

Myofibroblasts demonstrate a greater degree of contractility than fibroblasts [128, 409, 410]. Contractility increases with increased mechanical tension, and is correlated with increased *αSMA* expression [128]. Some of the factors that modulate conversion to the myofibroblast phenotype also induce cardiac fibroblast contraction, including TGFβ1 [156, 211, 212, 411], PDGF [123, 411, 412], IGF1 [413], AngII [414], Similarly, anti-fibrotic factors that inhibit the myofibroblast phenotype prevent contraction, such as c-Ski [156].

In cardiac fibroblasts/myofibroblasts, contraction generally occurs via two signaling mechanisms: short, transient contractions are generated through Ca^{2+}-dependent calmodulin/MLCK signaling, and longer, sustained contractions generated via Ca^{2+}-independent RhoA activation of ROCK [407, 415, 416]. Both pathways converge in the phosphorylation of stress fiber-bound myosin light chain (MLC), which allows contraction. In Ca^{2+}-dependent contraction, influx of cellular Ca^{2+} can occur in response

to stretch-activated channels (such as TRPCs). Intracellular Ca^{2+} ions bind and activate calmodulin, in turn activating MLCK, which phosphorylates MLC, inducing contraction. Contraction of stress fibers is disrupted by dephosphorylation of MLC by myosin phosphatase [417-419]. In Ca^{2+}-independent contraction, activation of RhoA through mechanical stimulation and activation of integrins induces activation of downstream ROCK, which can induce not only phosphorylation of MLC, but also inhibit MLC dephosphorylation by myosin phosphatase. Thus, in the latter mechanism, inhibition of myosin phosphatase allows MLC to remain phosphorylated and permits sustained contraction of stress fibers [407, 416] (Figure 3).

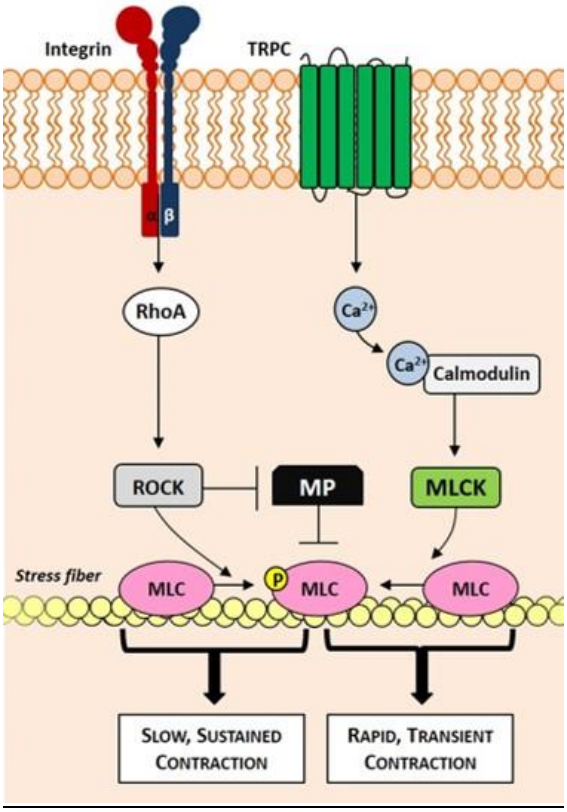


Figure 3: Contraction in cardiac myofibroblasts. In calcium-dependent contraction, stretch-activated membrane proteins such as transient receptor potential channels (TRPCs) transport calcium into the cytoplasm, where it forms a complex with calmodulin. This complex activates myosin light chain kinase (MLCK), resulting in MLC phosphorylation and contraction. Stress fiber contraction is disrupted by myosin phosphatase (MP) dephosphorylation of MLC, resulting in rapid, transient calcium-dependent contraction. Conversely, calcium-independent contraction results from force-activated integrin signaling through the RhoA/Rho-associated kinase (ROCK) pathway, which not only activates MLC phosphorylation, but also inhibits dephosphorylation by MP, thus resulting in slower and more sustained calcium-independent contraction. Figure adapted by author from *Roche et al. (2015), Comprehensive Physiology, In Press.*

1.9: Scleraxis

1.9.1: Basic Helix-Loop-Helix Proteins

SCX is a 201 amino acid protein belonging to the basic helix-loop-helix (bHLH) family of transcription factors, which also includes proteins involved in myogenesis, cardiogenesis, and neurogenesis [420]. The highly conserved bHLH motif was first identified in 1989 in the immunoglobin enhancer-binding murine transcription factors E12 and E47 [421]. The bHLH region is homologous to regions in proteins of the *myc* family, *MyoD*, and the *Drosophila* gene products of *daughterless*, *achaete-schute*, and *twist*. The bHLH motif, made up of approximately 60 amino acid residues, consists of an N-terminal basic motif, and a carboxyl (C)-terminal HLH region consisting of an α-helix, an unstructured loop region, and another α-helix. The amphipathic HLH region of these proteins mediates the formation of hetero- and homodimers between various bHLH proteins [422]. Dimerization of bHLH proteins induces combination of the basic domains to form a bipartite DNA-binding domain, which recognizes the DNA consensus sequence *CANNTG*, also known as an E-box [421, 422]. bHLH proteins were originally divided into 6 classes (I-VI), based on their tissue distribution, dimerization potential, and DNA binding specificity [423]. In the late 1990s, a new classification system of four groups (A-D) was introduced that considers evolutionary relationships and conservation of other motifs, as well as E-box binding and the presence or absence of additional domains [424]. More recently, 2 additional groups (E and F) were added as a result of the advent of high throughput genome sequencing and phylogenetic mapping [425]. Class A bHLH transcription factors, to which Scleraxis belongs, are widely expressed, generally form heterodimers with class B bHLH proteins, and preferentially bind *CAGCTG* and

CACCTG sequences [425-427]. Scleraxis has been shown to bind E12, E47, and more recently, Smad3, of the canonical TGFβ signaling pathway [136, 428]. *In vitro* assays have shown that Scx and its binding partner E47 form a complex with p300 and Sry-type HMG box 9 (Sox9, a major transcription factor regulating chondrogenesis) to transcriptionally activate the *Collagen IIα1* gene promoter via E-box binding [429].

1.9.2: Role of Scleraxis in Development

Olson *et al.* used in situ hybridization experiments to examine *Scleraxis* expression during murine development [426]. Low basal levels of *Scx* throughout the embryo were observed up to 9.5 days post-coitum (d.p.c.), after which increased transcripts were detected in the lateral sclerotome of somites that gives rise to collagen-rich dense connective tissues such as tendons, ligaments, and cartilage [430, 431]. *Scx* has since been identified as a specific marker for tendons and tenocytes [432-434]. In tenocytes, the cells responsible for synthesis and organization of the tendon ECM, Scx and NFATc regulate tenocyte differentiation and production of type I (α1) collagen, the most abundant tendon protein [435]. Expression of *Scx* in tenocytes is potently induced by treatment with TGFβ, and experiments using *TGFβ* and *Scx* knockout mice that show significant impairment in the formation of load-bearing tendons underscores the importance of these proteins in tendon development [436, 437]. Examination of various collagen-rich musculoskeletal tissues demonstrates that *Scx* and *Collagen Ia1* expression are highest in the load-bearing collagen bundles of tendon, as compared to other tendon regions, muscles, cartilage, and bone [438]. Additionally, *Scx* has been implicated in development of the auditory system [439] and Sertoli cells [440]. Work that is more recent has provided evidence for a role of *Scx* in cardiac development. The morphology

of cardiac valves is significantly altered in *Scx*-null mice, with increased valvular thickness, ECM disorganization, alterations in collagen content, and a generalized decrease in proteoglycan content [441, 442].

1.9.3: Scleraxis in the Heart

Post-MI remodeling is the precursor for the development of the infarct scar and subsequent cardiac fibrosis. Thus, the levels of proteins that regulate scar development rise early after MI. The more than 3-fold increase in *Scx* levels in the infarct scar of rat hearts 4 weeks post-MI, concomitant with significant increases in levels of type I and III collagens, indicates a role for Scx in the wound healing response [136]. SCX is increased in human myxomatous mitral valves, a condition associated with increased matrix production, and its over-expression in interstitial valve cells induces increased proteoglycan production and the associated myxomatous phenotype [441]. Remodeling heart valves of *Scx*-null mice display decreased proteoglycan content, and *SCX* over-expression in embryonic avian valve precursor cells induces increased levels of chondroitin sulfate proteoglycans (CSPGs), which are known to associate with ECM components such as fibronectin and collagens [441].

Scx is expressed by cardiac fibroblasts, and is increased by *in vitro* passaging of primary rat cardiac fibroblasts, a process which induces conversion to the myofibroblast phenotype. Compared to freshly isolated (P0) cardiac fibroblasts, first passage (P1) proto-myofibroblasts show approximately doubled levels of *Scx* mRNA, alongside more drastic increases in *αSMA* and *collagen Iα2* mRNA. Fully phenotypically converted second passage (P2) myofibroblasts show even greater levels of *Scx* mRNA (more than four-fold that of P0 fibroblasts), again concomitant with increases in *αSMA* and *COL1A2* [136,

443]. Treatment of cardiac fibroblasts with TGFβ, a potent inducer of the myofibroblast phenotype, increases Scx levels [443]. Additionally, Smad3 and Smad7 of the canonical TGFβ signaling pathway are capable of regulating *Scx* expression at both the mRNA and protein level. Over-expression of Smad3, a positive modulator of TGFβ signaling, more than doubles *Scx* mRNA levels in primary rat cardiac fibroblasts, concomitant with increased Scx protein, whereas over-expression of inhibitory Smad7 decreases *Scx* mRNA levels by more than half, and protein levels by about 25%. Over-expression of *Scx* in NIH-3T3 fibroblasts increases *Collagen 1α2* mRNA levels, as does TGFβ1 treatment. *Scx* over-expression in P0 cardiac fibroblasts induces a more than two-fold increase in *COL1A2* mRNA, and *Luciferase* reporter assays showed that Scx directly activates the *COL1A2* promoter [443]. Deletion of either the basic DNA-binding domain or helix-loop-helix protein-protein interaction domain resulted in a significant decrease in the ability of Scx to transactivate the *COL1A2* promoter [443]. Thus, it appears that Scx is directly involved in the pro-fibrotic process following MI, and alongside fibrillar collagens, may potentially regulate other factors involved in the myofibroblast phenotype, though this has not yet been examined.

1.9.4: Role of Scleraxis in Transforming Growth Factor-β Signaling

Numerous lines of evidence demonstrate that TGFβ stimulates *Scleraxis* expression. For example, treatment of mouse muscle with TGFβ1 increases protein levels of Scx and procollagen Iα2. TGFβ treatment shifts developing limb mesoderm from chondrogenesis to fibrogenesis, which is accompanied by Smad-mediated increases in Scx [444]. TGFβ1 treatment also induces *SCX* expression in osteoblastic osteosarcoma cells [445], tendon fibroblasts [446], and tenocytes, the latter of which was dose-dependent and showed

accompanying increases in tendon ECM collagens and biglycan [447]. In NIH-3T3 fibroblasts and avian valve precursor cells, TGFβ2, which increases *Smad2* expression, also induced increased mRNA levels of *Scx*. Additionally, TGFβ2 treatment induced increased mRNA levels of the CSPG *Aggrecan* in a Scx-dependent manner [441]. Both responses to TGFβ2 treatment were inhibited by constitutively active MEK1 (a downstream component of ERK1/2 signaling). In transgenic *ScxGFP* reporter mice, loss of TGFβ2 or TGFβ3 reduces *Green fluorescent protein* (*GFP*) expression in tendons and prevents the development of limb tendons [436]. Scx physically interacts with Smad3, and is down regulated in the tendons of *Smad3*-null mice, alongside decreased levels of type I collagen and *Tenascin-C* [428]. However, *Scx* expression may not require the presence of TGFβ, as injured tendons in mice treated with TGFβ-inhibiting antibodies did not show alterations in *Scx* gene expression [448]. Other growth factors, including early growth response factors-1 and -2 (Egr1, Egr2) [449], IGF1 [450], BMP-12 and -13, [451-453], growth/differentiation factors (GDFs, a subfamily of the bone morphogenetic protein family) [454-459], epidermal growth factor (EGF) [460], FGFs [460-463], and PDGF [464], have also been shown to increase *Scx* expression in a variety of tissues and cell types.

Scx may not just be induced by TGFβ, but may also regulate downstream pro-fibrotic responses. In NIH-3T3 cells, Scx was found to directly transactivate a human *COL1A2* promoter, which was prevented by the inhibitory Smad7. Additionally, Scx and the pro-fibrotic Smad3 were shown to act synergistically to activate the *COL1A2* promoter. However, using a mutant version of *Scx* lacking the DNA-binding domain, TGFβ-induced expression of *COL1A2* is abolished in cardiac myofibroblasts [136]. Thus, Scx does

indeed appear to regulate TGFβ-induced *COL1A2* activation, and potentially other pro-fibrotic or myofibroblast phenotype-related processes.

1.9.5: Response of Scleraxis to Mechanical Force

In addition to growth factors, *Scx* is also responsive to mechanical stimulation. Scleraxis levels increase in the adaptation of adult tendon to increased mechanical loading, alongside increased expression of type I collagen [446, 465, 466]. Similarly, unloading of cultured tenocytes induces a reversible decrease in *Scx* expression [467]. Scx acts synergistically with mechanical force to induce commitment of human embryonic stem cells to the tenocyte phenotype [468, 469].

In a mesenchymal stem cell (MSC) line (C3H10T1/2) exposed to 5% static tension through tethering to bioartifical tendons, *SCX* expression increased more than 5-fold within a week [438]. Comparison of varying static strain (from 0% to 10%) demonstrated a load-dependent increase in *SCX* and *COL1A1* expression. The addition of cyclic stretch (5%, 0.1 Hz) further increased *Scx* expression in these cells, as did increasing the number of repetitions of cyclic stretch. In MSCs, which are able to give rise to cardiac fibroblasts, 10% uniaxial cyclic stretch (1 Hz) for 24 or 48 hours significantly increased mRNA levels of *SCX*, *TENASCIN-C*, *COL1A2* and *COL3A1* in either 2D or 3D cultures [385, 459, 470-472]. However, MSCs exposed to the same regimen with increased stretch (15%) did not show significant changes in the levels of these transcripts [470]. Application of 2 days of 8% uniaxial cyclic stretch (1 Hz) to periodontal ligament cells resulted in increased expression of *SCX* as well as the ECM protein elastin [473]. In human mesenchymal stem cells, 10% cyclic stretch (1 Hz, 3 hours stretch alternating with 3 hours rest for 14 days) induced differentiation to fibroblasts, accompanied by

significant increases in mRNA levels of *SCX*, *TENASCIN-C*, *COL1A2* and *COL3A1* [474]. MSCs subjected to 7 days of cyclic stretch (1% elongation for 30 minutes per day at 1 Hz) significantly augmented cell contractility, increased expression of *SCX*, *ELASTIN*, *COL1A2*, and *COL3A1*, while causing down-regulation of *MMPs-9* and *-13* [475]. The same study found that culturing MSCs in 3D versus 2D collagen environments for 7 days also was sufficient to significantly increase *SCX* expression [475], further supporting the hypothesis that *SCX* expression is sensitive to the forces exerted on these cells.

2: RATIONALE, HYPOTHESIS & OBJECTIVES

2.1: Scleraxis as a Mechanoresponsive Regulator of the Cardiac Myofibroblast

Numerous studies of different tissues and cell types have shown that *Scx* is activated by static and cyclic mechanical strain [385, 438, 446, 459, 465, 466, 470-475]. Additionally, Scx is implicated in the wound healing process post-MI. *Scx*, alongside collagens, are upregulated during post-MI remodeling, and Scx directly regulates transcription of type I collagens [136, 435, 443]. The cardiac remodeling process is mediated by the activities of myofibroblasts, and *Scx* is induced by the same stimuli that drive conversion of cardiac fibroblasts to myofibroblasts, namely TGFβ and its canonical signaling components Smads [136, 444-447], mechanical strain [385, 438, 446, 459, 465, 466, 470-475], and *in vitro* cell passaging [443]. Thus, we hypothesized that *Scx* expression is mechanoresponsive in cardiac fibroblasts, and *Scx* is directly involved in regulating the cardiac myofibroblast phenotype through induction of key markers and functions.

2.1.1: Objective 1 – The Effect of Cyclic Stretch

Our first objective was to determine if expression of *Scx* and characteristic myofibroblast markers is induced by cyclic stretch of cardiac P1 proto-myofibroblasts and P2 myofibroblasts. To achieve this objective, we subjected these cells to cyclic stretch over a 24-hour period and examined mRNA and protein levels of Scx, αSMA, type I and III collagens, ED-A Fn, and SMemb, as well as phosphorylation (Y397) of FAK.

2.1.2: Objective 2 – The Effect of *Scleraxis* Expression

Our second objective was to determine if *Scx* over-expression in cardiac P1 proto-myofibroblasts would result in increased myofibroblast marker expression. For this

objective, we used adenoviral vectors to over-express *Scx*, and examined resulting levels of myofibroblast marker mRNA and protein.

2.1.3: Objective 3 – *Scleraxis* and Contractility

As myofibroblasts are more contractile than their fibroblast precursors, we wished to examine how changing *Scx* expression would affect contractility in both cardiac P1 proto-myofibroblasts and P2 myofibroblasts. To achieve this aim, we subjected these cells to 2D collagen gel contraction assays, examining contractility in the context of *Scx* over-expression, knock down, and also using a dominant negative, DNA binding-deficient version of *Scx*, in the presence and absence of TGFβ1.

2.1.4: Objective 4 – *Scleraxis* and Migration

To determine the effect of *Scx* on migration, which is reduced with phenoconversion of fibroblasts to myofibroblasts. We used a transwell migration assay (with TGFβ1 as a chemoattractant), as well as a wound-healing (scratch) assay, to determine the effect of *Scx* over-expression and knock down on migration of cardiac P1 proto-myofibroblasts.

2.2: The *SCLERAXIS* Promoter

In situ analysis of the proximal 1500 bp of the human *SCX* promoter revealed a SBE of the consensus sequence *CAGACA* 510 bp upstream of the ATG transcriptional start site that is conserved in mouse and rat transcripts, which we have termed SBE1. Additionally, another SBE was found in a more distal region of the human *SCX* promoter, 1231 bp upstream of the transcriptional start site, which was also conserved in mouse and rat, and termed SBE2. Thus we hypothesized that TGFβ1 may activate the proximal 1500 bp region of the human *SCX* promoter directly through Smad binding to one or both of these sites.

2.2.1: Objective 5 – Activation of the *SCLERAXIS* Promoter by TGFβ1

To determine if TGFβ1 treatment was sufficient to activate a proximal 1500 bp region of the human *SCX* promoter, as well as truncated distal (lacking SBE2) and proximal (lacking SBE1) versions of this promoter, we purchased a luciferase reporter vector driven by the 1500 bp human *SCX* promoter (pGL4.10-SCX1500). The vectors containing different versions of the *SCX* promoter were transfected into NIH-3T3 fibroblasts, and changes in luciferase expression in response to varying doses of TGFβ1 were measured.

2.2.2: Objective 6 – Activation of the *SCLERAXIS* Promoter by Cyclic Stretch

To determine if the 1500 bp human *SCX* promoter is responsive to other stimuli, such as cyclic stretch, we subjected transfected NIH-3T3 fibroblasts to 24 hours of cyclic stretch and compared luciferase expression values to those of unstretched controls.

3: MATERIALS & METHODS

3.1: Cell Culture

3.1.1: Primary Adult Rat Ventricular Fibroblast Isolation

Rats were anesthetized with 90 mg/kg ketamine (C44016, Ketaset, Wyeth Animal Health, Canada) and 10 mg/kg xylazine (B3D3, Rompun, Bayer Inc., Canada) to a surgical plane of anesthesia (determined via pedal reflex). Heparin (9041-08-1, Thermo Fisher Scientific, Canada) was injected into the heart (100 U/kg body weight) to prevent blood clotting, and the heart placed into Dulbecco's Modified Eagle Medium/Ham's Nutrient Mixture F-12 (DME/F-12) media (SH3002301, Thermo Fisher Scientific, Canada). The ventricular portion of the heart was transferred to fresh DME/F-12 media and cut into 1 mm^3 cubes, which were transferred to Simple Modified Eagle Medium (SMEM) (11380037, Life Technologies, Canada) and rinsed. In a small volume (less than 1 ml) of 1 mg/ml collagenase type 2 (CLS-2, Worthington Biochemical Corporation, USA) in SMEM, the cubes were finely minced. The slurry was then transferred to a 25 ml Erlenmeyer flask containing 15 ml of 1 mg/ml collagenase type 2, and digested with constant stirring at 37°C/5% CO_2 for 1 hour. After digestion, large particles were allowed to settle and the supernatant was transferred to a 50 ml Falcon tube containing fibroblast feeding media (DME/F-12 + 1% penicillin/streptomycin (SV30010, Thermo Fisher Scientific, Canada), 1 mM ascorbic acid (A5960-25G, Sigma-Aldrich, USA), and 10% heat-inactivated characterized fetal bovine serum (FBS-HI, SH3039603HI, Thermo Fisher Scientific, Canada)). This initial digestion was then centrifuged at 2000 rpm (24°C) for 5 minutes. The supernatant was aspirated and discarded, and the pellet resuspended on ice for 1 minute in 5 ml of Gey's solution (0.13 M NH_4Cl, 1.24 M KCl,

0.06 M $Na_2HPO_4 \cdot 7H_2O$, 0.04 M KH_2PO_4, 1.39 M glucose, 1.03 mM $MgCl_2 \cdot 6H_2O$, 0.28 mM $MgSO_4 \cdot 7H_2O$, 1.53 mM $CaCl_2$, 0.01 M Na_2HCO_3) to lyse and remove red blood cells. After an additional round of centrifugation (2000 rpm, 24°C, 5 minutes), the remaining pellet was aspirated, resuspended in 5 ml of fresh fibroblast feeding medium, and plated on a 10 cm culture dish in fibroblast feeding medium. Fibroblasts were allowed to attach for 3-4 hours before thorough washing with 1X PBS and addition of fresh fibroblast feeding medium. The remaining tissue fragments in the Erlenmeyer flask from the first digest were digested with a fresh 15 ml of 1 mg/ml collagenase type 2 (in SMEM) at 37°C/5% CO_2 for 1 hour with stirring, and extracted following the same procedure as the initial digest.

3.1.2: Cell Culture

Primary rat cardiac fibroblasts (rCFs) plated directly from the digestions were considered P0 fibroblasts, which were passaged to P1 proto-myofibroblasts after no more than 72 hours of culture. P1 proto-myofibroblasts were then passaged to P2 myofibroblasts within 72 hours of plating. Cell counts were determined via haemocytometer and cells serum starved (DME/F-12 + 1% penicillin/streptomycin, 1 mM ascorbic acid) for 24 hours prior to infection. Proto-myofibroblasts (P1) and myofibroblasts (P2) were infected with over-expression adenovirus encoding enhanced green fluorescent protein (AdEGFP) or *Scx* (AdSCX) for 24 hours at 10 MOI, with adenovirus encoding a *Scx* mutant lacking the DNA-binding domain (AdSCXΔDBD) for 24 hours at 50 MOI, or with knockdown adenovirus encoding for LacZ-targeting shRNA (AdshLacZ) or Scx-targeting shRNA (AdshSCX) for 48 hours at 200 MOI, as per prior optimization [136, 443].

3.1.3: Adenoviral Constructs

3.1.3.1: Scleraxis Over-Expression Adenovirus (AdSCX)

Scleraxis cDNA was derived from pECE-HA-FLAG-Scx [476] and sub-cloned into the pACCMVpLpA(-)loxP-SSP shuttle vector (University of Michigan, USA). *In vitro* Cre/loxP-mediated recombination was used to construct recombinant adenoviruses, using cosmid cs360loxP containing the adenovirus type V dl309 genome [477]. *Scx*-expressing viral clones were confirmed by Western blot and propagated in 911 cells. Viral supernatants were clarified via centrifugation and titered via plaque assay, and stored at -80°C [443]. AdSCX experiments were performed at 10 MOI for 24 hours, alongside AdEGFP infection controls [478].

3.1.3.2: DNA Binding Deficient Scleraxis Over-Expression Adenovirus (AdSCXΔDBD)

PCR was used to amplify and sub-clone the SCXΔDBD coding region into pSHUTTLE-CMV transfer vector (AdEasy Vector System, 24007, QBiogene, USA), which was subsequently cloned into BJ5183 bacterial cells to generate replication-deficient adenoviruses [136]. The Adeno-X rapid titer kit (632250, Clontech Laboratories, Inc., USA) was used to determine viral titer. AdSCXΔDBD experiments were performed at 50 MOI for 24 hours, alongside AdEGFP controls [478].

3.1.3.3: Scleraxis Knockdown Adenovirus (AdshSCX)

For rat *Scleraxis* gene knockdown, shRNA sequences were designed using the BLOCK-iT RNAi Advisor program (Life Technologies, Canada). The oligonucleotide pairs were annealed and cloned as per manufacturer instructions into the pENTR/U6 RNAi Entry Vector (K4945-00, Life Technologies, Canada), then sub-cloned into pAd/BLOCK-iT-DEST vector (v49220, Life Technologies, Canada), generating pAd-shScleraxis. Vectors

were packaged to produce AdshSCX in 293A cells (CRL-1573, ATCC, USA). The Adeno-X rapid titer kit (632250, Clontech Laboratories, Inc., USA) was used to determine viral titer. Infection of primary rat ventricular P1 proto-myofibroblasts were infected at varying MOI (50, 100, and 200) for 48 hours for optimization. Infection controls were treated with adenovirus encoding shRNA for LacZ (AdshLacZ) [479]. Knockdown experiments were performed using 200 MOI for 48 hours.

3.1.4: Luciferase Assays

NIH-3T3 fibroblasts (CRL-1658, ATCC, USA) from passage 13-21 were cultured in DMEM High-Glucose (SH30022.01, Thermo Fisher Scientific, Canada) supplemented with 10% HI-FBS and 1% penicillin/streptomycin were seeded in 6-well tissue culture-treated dishes at 2.5×10^4 cells/well and allowed to grow for 48 hours to reach ~75% confluence. Cells were co-transfected (Lipofectamine 2000, Life Technologies, Canada) for 24 hours in Opti-MEM (31985-070, Life Technologies, Canada) with 250 ng of empty Luciferase expression control vector (pGL4.10, E6651, Promega, USA) and 250 ng of either 1500 bp *SCX* promoter in pGL4.10 backbone (pGL4.10-SCX1500, GeneCopoeia, USA), one of two truncated versions – missing either the proximal region (pGL4.10-SCXΔ+78/-829) or the distal region (pGL4.10-SCXΔ-670/-1525) of the 1500 bp promoter region, or empty control vector (pGL4.10). Following transfection, cells were washed with sterile 1X PBS and treated with either serum-starvation medium (DMEM High-Glucose with 1% penicillin/streptomycin) or serum-starvation medium supplemented with either 0, 0.1, 0.5, 1, 5, or 10 ng/ml recombinant human transforming growth factor-β1 (TGFβ1, 240-B-002, CedarLane, Canada). Truncation of the proximal region (+78 to -829, relative to the ATG transcriptional start site of the 1500 bp *SCX*

promoter) was achieved by restriction enzyme digestion with *NcoI* (R3193, New England Biolabs, USA) followed by ligation with T4 DNA ligase (M0202, New England Biolabs, USA). Truncation of the distal region (-670 to -1525) of the 1500 bp *SCX* promoter was achieved via double restriction enzyme digestion with *KpnI* (R3142, New England Biolabs, USA) and *NheI* (R3131, New England Biolabs, USA), followed by formation of blunt ends with T4 DNA Polymerase (EP0062, Thermo Fisher Scientific, Canada) and ligation with Quick Ligation kit (M2200, New England Biolabs, USA). 5 ng of renilla expression vector (pRL was used as a control for transfection. The Luciferase activity of each sample was assayed using Dual Luciferase Reporter Assay System (E1960, Promega, USA) on a Glomax Multi+ Detection System (Promega, USA).

3.2: Western Blotting

To isolate protein, cells washed with 1X PBS were lysed using 5 ml RIPA extraction buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate/SDS) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF, 7110, EMD Millipore, USA), 1 mM of dithiothreitol (DTT), and 1X protease inhibitor cocktail (87786, Thermo Fisher Scientific, Canada). Total protein was extracted from adherent cells on ice using a cell scraper and from pelleted cells by 10X pulse vortex/ice treatment. Cell debris was pelleted from these samples by centrifugation in an Eppendorf 5417C centrifuge at maximum RPM (15,000) for 5 min at 4°C, and the supernatant transferred to a fresh 1.5 ml tube. Protein concentration was determined via Bradford assay (measured at 600 nm wavelength) using Coomassie Blue Protein Assay Reagent (1856209, Thermo Fisher Scientific, Canada) and bovine serum albumin standards (Thermo Scientific, 23209). Samples were prepared

using 6X Laemmli buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol, 10% 2-mercaptoethanol), boiled for 10 min at 100°C, and loaded onto polyacrylamide gels (7% or 12%). Proteins were separated by electrophoresis in 1X SDS-polyacrylamide gel electrophoresis (PAGE) buffer (25 mM Tris-OH, 192 mM glycine, 0.1% SDS) at 150 volts for 60-90 minutes, alongside Precision Plus Protein Western C Standard ladder (161-0376, Bio-Rad, USA). Proteins were transferred to 0.45 µm pore size polyvinylidene fluoride (PVDF) membrane (Immobilon-P, IPVH00010, EMD Millipore, USA) in 1X Towbin's buffer (25 mM Tris-OH, 192 mM glycine, with or without 20% methanol) at 300 mA for 1 hour (4°C) with stirring. Membranes were blocked with 5% non-fat milk powder (NFMP) in PBST (1X PBS + 0.1% Tween-20 (9005-64-5, EM Science, USA)) for 1 hour (24°C) with shaking.

Membranes were probed with primary antibody overnight (4°C) with shaking in 1-3% NFMP-PBST. Primary antibodies used included mouse 12G10 anti-α-tubulin (DSHB, USA), mouse anti-α-smooth muscle actin (αSMA, A2547, Sigma, USA), rabbit anti-type I collagen (CL50141AP-1, Cedarlane, Canada), mouse anti-cellular ED-A fibronectin (MAB1940, EMD Millipore, USA), mouse anti-non-muscle myosin IIB (SMemb, ab684, abcam, USA), rabbit anti-focal adhesion kinase (FAK, AHO0502, BioSource International Inc., USA), rabbit anti-Y397 phosphorylated focal adhesion kinase (pFAK, 44-624, BioSource International Inc., USA), or rabbit anti-Scleraxis (QED Bioscience, Inc., USA). Streptavidin horseradish peroxidase-conjugated secondary antibodies used included goat anti-mouse and goat anti-rabbit (Jackson ImmunoResearch Laboratories Inc., USA). Antibodies were detected using Western Blotting Luminol Reagent (sc-2048, Santa Cruz Biotechnology, USA) and visualized with CL-Xposure blue X-ray film

(PI34091, Thermo Fisher Scientific, USA). Adjusted volume of bands was measured via densitometry (GS-800 Calibrated Densitometer, Bio-Rad, USA) using QuantityOne software (Bio-Rad, USA), and normalized to that of α-tubulin (12G10) as a loading control.

3.3: Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated using GeneJET RNA Purification Kit (K0739, Thermo Fisher Scientific, Canada). One-step qRT-PCR analysis was performed using a Bio-Rad iQ5 iCycler and B-R One-Step SYBR Green qRT-PCR kit (170-8893BR, Quanta Biosciences, USA) with 25 ng RNA and 2 μM each reverse and forward primers (Table 1). Reactions were exposed cycling conditions outlined in Table 2 for all primer sets. Fold changes in mRNA expression were analyzed by the $2^{-\Delta\Delta CT}$ method [480, 481] and normalized to *GAPDH* for loading control.

Table 2: Primers used in 1-Step qPCR analysis. Primers are specific for both rat and mouse transcripts.

AMPLICON	**DIRECTION**	**SEQUENCE (5' → 3')**
α-Smooth Muscle Actin	Forward	CGGGCTTTGCTGGTGATG
	Reverse	CCCTCGATGGATGGGAAA
Collagen 1α1	Forward	TGCTCCTCTTAGGGGCCA
	Reverse	CGTCTCACCATTAGGGACCCT
Collagen 1α2	Forward	GTCCCCGAGGCAGAGAT
	Reverse	CCTTTGTCAGAATACTGAGCAGC
Collagen 3α1	Forward	GGTTTCTTCTCACCCTGCTTCA
	Reverse	GGTTCTGGCTTCCAGACATC
GAPDH	Forward	TGCACCACCAACTGCTTAGC
	Reverse	GGCATGGACTGTGGTCATGAG
Scleraxis	Forward	AACACGGCCTTCACTGCGCTG
	Reverse	CAGTAGCACTGGCGGGAGGTG

Table 3: Reaction conditions for 1-Step qPCR Analysis.

STEP	DURATION	TEMPERATURE	REPEATS
cDNA Synthesis	10 minutes	50°C	1X
Initial Denaturation	5 minutes	95°C	1X
Denaturation	10 seconds	95°C	45X
Annealing	30 seconds	60°C	45X
Melt Curve	30 seconds	55-95°C	81X

3.4: Cyclic Stretch Assay

Untreated Bioflex flexible silicone elastomer membranes in 6-well plate format (BF-3001U, FlexCell Corporation, USA) were pre-coated with 100 ng/µl human plasma fibronectin (FC010, EMD Millipore, USA) in serum-starvation media (as in section 3.1.2) and incubated at 37°C/5% CO_2 overnight. After washing excess fibronectin from the plates with sterile 1X PBS, cells were seeded in feeding media at either 2.5×10^4 cells/well (for NIH-3T3 fibroblasts and RNA analysis of P1 cardiac proto-myofibroblasts) or 5.0×10^4 cells/well (for protein analysis of P1 cardiac proto-myofibroblasts and all P2 cardiac myofibroblasts). Cells were allowed to attach for 24 hours, followed by serum starvation for 24 hours. Using a FlexCentral FX-4000 FlexerCell Strain Unit (FlexCell, USA), a program of cyclic (1 Hz), biaxial stretch of either 15% (for P1 proto-myofibroblasts and NIH-3T3 fibroblasts) or 20% (for P2 myofibroblasts) elongation was applied to stretch plates for 24 hours, and no elongation applied to unstretched controls in the same apparatus. For RNA analysis, cells were lysed and scraped directly from wells, and for protein analysis, cells were trypsinized, centrifuged (10,600 × g, 23°C, 5 minutes), washed, centrifuged a second time, aspirated, and resuspended in protein lysis buffer.

3.5: 2-Dimensional Collagen Gel Contraction Assay

Compressible collagen gels were prepared prior to cell seeding by combining 7 ml of cold purified bovine collagen solution (PureCol, 3 mg/ml, 5005-B, Advanced Biomatrix, USA) with 2 ml of 5X concentrated DMEM/F-12 (12500-039, Life Technologies, Canada) and 1 ml of filter-sterilized ddH_2O in a 50 ml Falcon tube, and adjusted to pH 7.0 with filter-sterilized 1 M HCl. Bubbles that developed upon mixing were removed by allowing the solution to sit still at 4°C for 1-2 minutes. In a 24-well tissue culture dish, 500 µl aliquots of gel mixture were added to each well. Gels were solidified by incubation at 37°C/5% CO_2 for a minimum of 3 hours prior to seeding cells. Primary P1 proto-myofibroblasts or P2 myofibroblasts were seeded at a density of 3.5×10^4 cells/well in fibroblast feeding media and allowed to attach for 24 hours. Cells were then serum starved for 24 hours and infected with over-expression (AdEGFP or AdSCX, 10 MOI, 24 hours) or knockdown adenovirus (AdshLacZ or AdshSCX, 200 MOI, 48 hours). At time 0 hours, wells were aspirated and treatments added accordingly (either serum-starvation media, feeding media for 10% FBS maximal contraction, or 10 ng/ml recombinant human TGF-β1 in serum-starvation media). Collagen gels were then released from the walls of the well by use of a custom-made stainless steel dowel or medium sterile pipette tip, and pictures taken at 0 and 24 hours with a Canon Rebel xSi digital camera. Contraction was determined by the change in gel area measured at 24 and 0 hour time points (using custom measuregel software, courtesy of Dr. Ian Dixon).

3.6: Transwell Migration Assay

Cells were plated on tissue culture treated sterile polycarbonate 24mm membrane inserts in 6-well format with pore size of 8.0 µm (3428, Corning Life Sciences, USA) at a

seeding density of 3.125×10^4 cells/insert in fibroblast feeding media, and allowed to attach for 24 hours. Following serum starvation for 24 hours, cells were infected with either over-expression or knockdown adenovirus, as above, or left in starvation media (control). At time 0 hours, 10 ng/ml TGF-β1 (in starvation media) was added to the lower chamber of all wells. Serum starvation media was added to the upper chambers to establish a chemoattractant gradient. After 24 hours, inserts were removed and lower chambers washed with 1X PBS, followed by fixation with 4% paraformaldehyde (PFA, in 1X PBS) for 10-15 minutes at room temperature. Following removal of fixative and washing (3X) with 1X PBS, cells were stained with 5% crystal violet for 5 minutes at room temperature, rinsed to run clear with ddH_2O, and air-dried. The number of migrated cells was determined by manual counting of cells stained in a minimum of 5 fields per well at total magnification of 400X, and multiplied by the area factor for each field (6012.5) to represent the total number of cells per well.

3.7: Wound Healing Assay

Primary rat cardiac P1 proto-myofibroblasts were seeded in 6-well tissue culture-treated dishes at 1.0×10^5 cells/well and allowed to attach overnight. Following 24 hours of serum starvation, cells were infected with either AdshLacZ or AdshSCX adenovirus at 200 MOI for 48 hours, or either AdEGFP or AdSCX adenovirus at 10 MOI for 24 hours. A dashed line was made on the underside of each well and a scratch introduced over this line using a medium pipette tip. Cells were washed 3 times with 1X PBS to remove excess cells from wounding, and treated with either starvation media (for control and adenovirus-infected treatments), feeding media, or starvation media containing 10 ng/ml

TGFβ1. Pictures of initial wounds (0 hours), as well as 24 and 48 hour time points were taken using a Nixon CoolPix 995 digital camera at 40X objective.

3.8: Statistical Analysis

Experiments were repeated with a minimum of 3 biological replicates (n=3), such that each replicate was produced from a different source rat (for primary cells). In qPCR experiments, the value for each biological replicate represented an average of three technical replicates (duplicate readings of the same sample). Experiments comparing two samples (i.e. cyclic stretch assays and vehicle vs. TGFβ1 experiments) were analyzed by Students t-test, and those with multiple samples (i.e. migration, contraction, and TGFβ1 dosage experiments) were analyzed by one-way analysis of variance (ANOVA) with Brown-Forsythe test for equal variance and Tukey post-hoc analysis. Results with $p \leq 0.05$ were considered statistically significant.

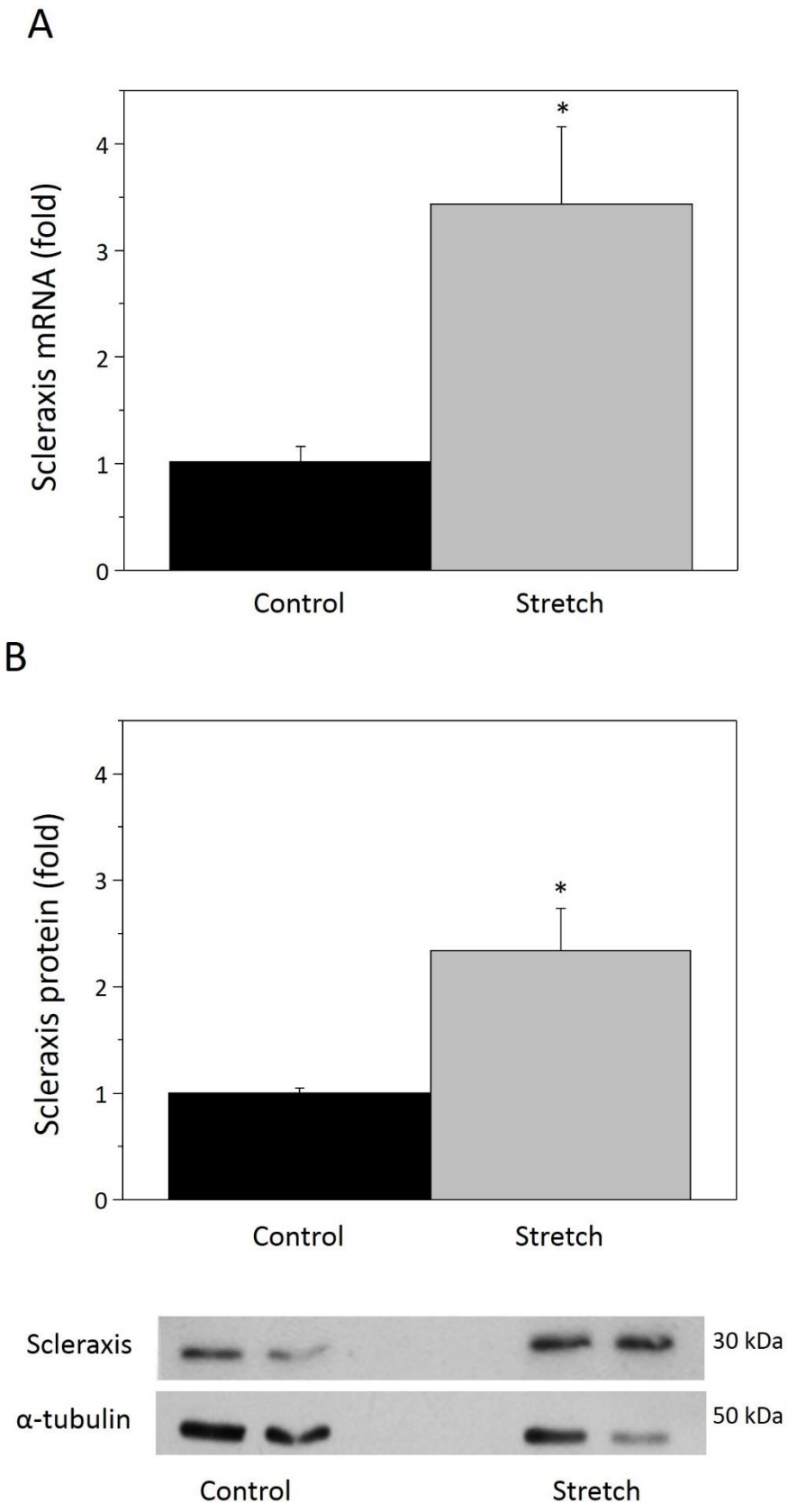
4: RESULTS

4.1: Cyclic Stretch of Cardiac Proto-Myofibroblasts

To determine the effect of mechanical strain on expression of *Scx* and myofibroblast markers, we exposed first- (P1) passage rat cardiac fibroblasts to 24 hours of cyclic stretch at a rate of 1 Hz. Using quantitative real-time PCR (qPCR) and Western blotting, we then examined changes in mRNA and protein levels, respectively, of prototypical myofibroblast markers, fibrillar collagens, and Scx.

4.1.1: Cyclic Stretch Increases *Scleraxis* Expression

Compared to unstretched controls, P1 proto-myofibroblasts exposed to 15% stretch (1 Hz) for 24 hours displayed significantly increased mRNA levels of *Scx* (from 1.00 ± 0.14 to 3.27 ± 0.73, $n=3$, $p \leq 0.05$) (Figure 4A). Similarly, protein levels of Scx were also increased in response to cyclic stretch of P1 proto-myofibroblasts (from 1.00 ± 0.05 to 2.34 ± 0.39, $n=3$, $p \leq 0.05$) (Figure 4B).


A
Scleraxis mRNA (fold)
0
1
2
3
4
*
Control
Stretch
B
Scleraxis protein (fold)
0
1
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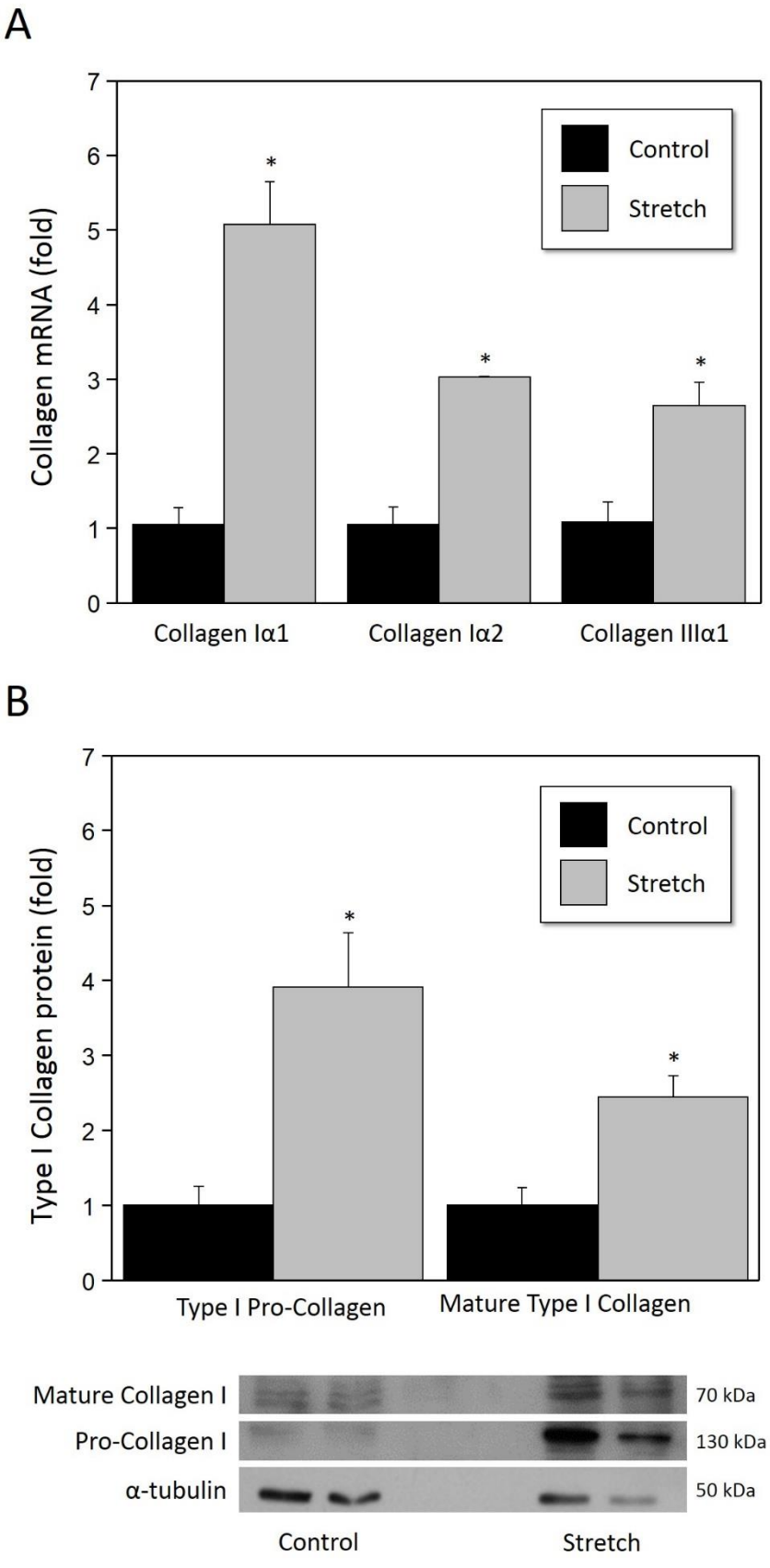
3
4
*
Control
Stretch
Scleraxis
30 kDa
α-tubulin
50 kDa
Control
Stretch

Figure 4: Effect of cyclic stretch on *Scleraxis* expression in cardiac proto-myofibroblasts. mRNA (normalized to GAPDH) (A) and protein (normalized to α-tubulin) (B) levels were measured after 24 hours of cyclic biaxial stretch (15%, 1 Hz) of primary rat ventricular P1 proto-myofibroblasts in serum-free medium. Representative Western Blot images are shown in (B) for Scleraxis and α-tubulin loading control. (*n=3, Student's t-test $p \leq 0.05$, mean ± SEM)

4.1.2: Cyclic Stretch Increases Collagen and Myofibroblast Marker Expression

Concomitant with increased Scx levels in P1 proto-myofibroblasts, cyclic stretch also induced significant increases in mRNA levels of collagen types I and III ($n=3$, $p \leq 0.05$). *Collagen Iα1* mRNA levels increased from 1.00 ± 0.23 to 5.01 ± 0.57, *Collagen Iα2* from 1.00 ± 0.24 to 3.02 ± 0.01, and *Collagen IIIα1* from 1.00 ± 0.27 to 2.60 ± 0.32 (Figure 5A). Similarly, protein levels were significantly increased ($n=3$, $p \leq 0.05$) for both type I pro-collagen (from 1.00 ± 0.25 to 3.91 ± 0.73) and mature type I collagen (from 1.00 ± 0.23 to 2.44 ± 0.29) (Figure 5B). In addition to increasing collagen I and III levels, cyclic stretch also resulted in increased levels of myofibroblast marker expression. *αSMA* expression was increased at both the mRNA (from 1.14 ± 0.42 to 5.44 ± 0.55, $n=3$, $p \leq 0.01$) (Figure 6A) and protein level (from 1.00 ± 0.14 to 2.19 ± 0.60, $n=3$, $p \leq 0.05$) (Figure 6B). Other myofibroblast markers showed a trend towards increased protein levels with cyclic stretch, as determined by Western blotting. Protein levels trended towards an increase for ED-A fibronectin (from 1.00 ± 0.18 to 2.26 ± 1.63), though the difference was not statistically significant for $p \leq 0.05$. However, SMemb protein levels increased significantly ($n=3$, $p \leq 0.05$) with cyclic stretch (from 1.00 ± 0.38 to 5.03 ± 1.77) (Figure 6C). Additionally, the amount of autophosphorylated FAK (pFAK Y397, normalized to total FAK) was significantly ($n=3$, $p \leq 0.05$) decreased to 0.43 ± 0.13 after 24 hours of cyclic stretch, as compared to unstretched controls with protein values of 0.99 ± 0.10 (Figure 7).


A
Collagen mRNA (fold)
0
1
2
3
4
5
6
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Control
Stretch
*
*
*
Collagen Iα1
Collagen Iα2
Collagen IIIα1
B
Type I Collagen protein (fold)
0
1
2
3
4
5
6
7
Control
Stretch
*
*
Type I Pro-Collagen
Mature Type I Collagen
Mature Collagen I
Pro-Collagen I
α-tubulin
70 kDa
130 kDa
50 kDa
Control
Stretch

Figure 5: Effect of cyclic stretch on fibrillar collagen expression in cardiac proto-myofibroblasts. Primary rat ventricular P1 proto-myofibroblasts in serum-free medium were exposed to 24 hours of cyclic biaxial stretch (15%, 1 Hz). mRNA levels (normalized to GAPDH) were measured for collagen Iα1, Iα2, and IIIα1 (A). Protein levels (normalized to α-tubulin) were measured for type I pro- and mature collagen (B). A representative Western blots for type I collagen and α-tubulin controls are shown in (B). (*n=3, students t-test $p \leq 0.05$, **n=3, Student's t-test $p \leq 0.01$, mean ± SEM)

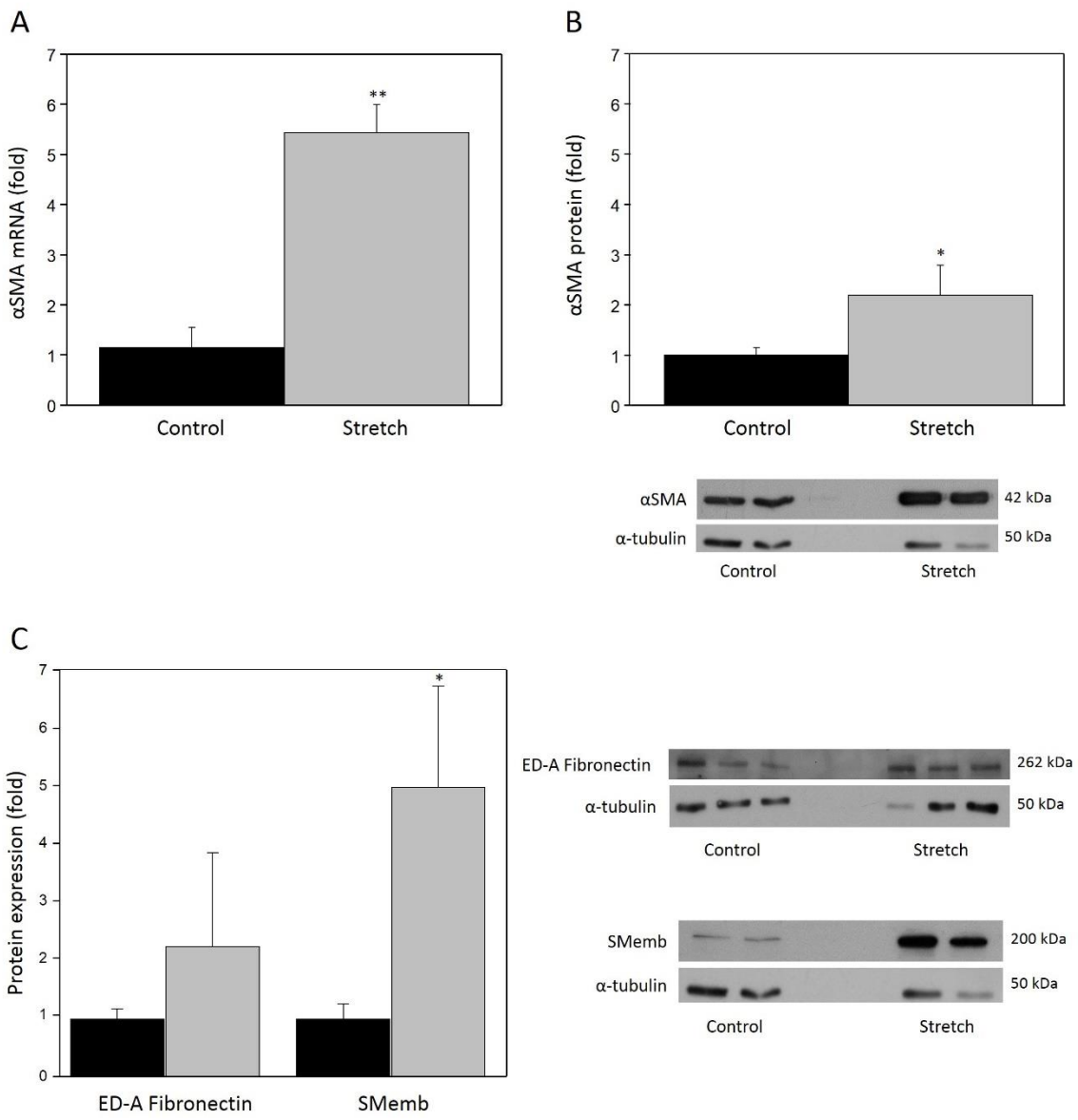


Figure 6: Effect of cyclic stretch on expression of myofibroblast markers in cardiac proto-myofibroblasts. Primary rat ventricular P1 proto-myofibroblasts in serum-free medium were exposed to 24 hours of cyclic biaxial stretch (15%, 1 Hz). mRNA levels (normalized to GAPDH) were measured for α-smooth muscle actin (αSMA) (A). Protein levels (normalized to α-tubulin) were measured for αSMA (B), and the myofibroblast markers ED-A Fibronectin and embryonic non-muscle myosin heavy chain (SMemb) (C). Representative Western Blot images are shown for αSMA (B), and myofibroblast markers

(EC), accompanied by respective α-tubulin controls. (*n=3, students t-test $p \leq 0.05$, **n=3, Student's t-test $p \leq 0.01$, mean ± SEM)

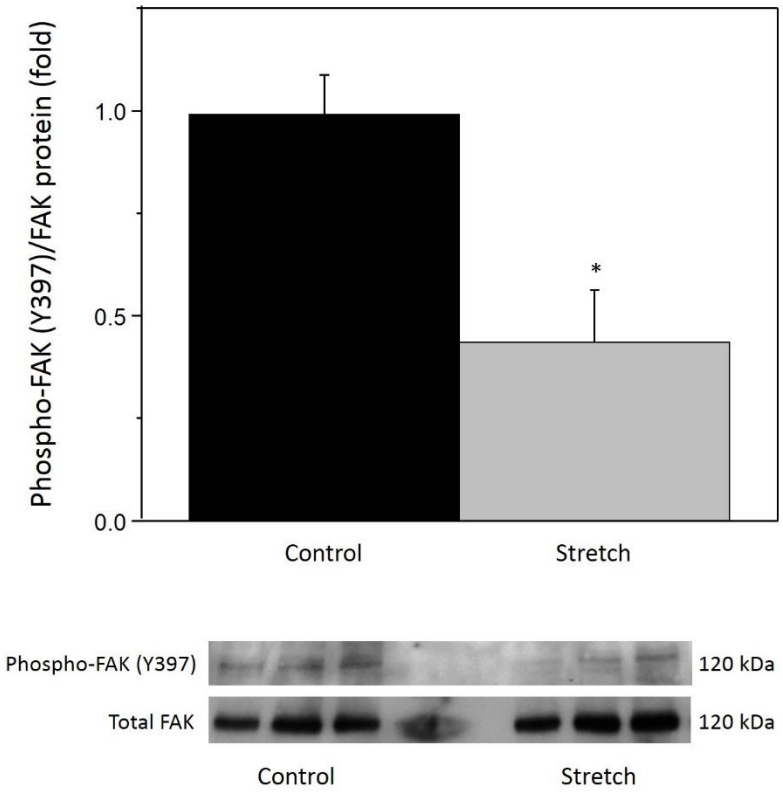


Figure 7: Effect of cyclic stretch on tyrosine 397 (Y397) phosphorylation status of focal adhesion kinase (FAK) in cardiac proto-myofibroblasts. Protein levels of total FAK and phosphorylated FAK (Y397) (normalized to α-tubulin) were measured for serum-starved primary rat ventricular P1 proto-myofibroblasts exposed to 24 hours of cyclic biaxial stretch (15%, 1 Hz). Western blots are shown for phosphorylated FAK (Y397) and total FAK. (*n=3, Student's t-test $p \leq 0.05$, mean ± SEM)

4.2: Cyclic Stretch of Cardiac Myofibroblasts

In P2 cardiac myofibroblasts exposed to 20% cyclic biaxial stretch (24 hours, 1 Hz), *Scx* mRNA levels increased significantly (from 1.00 ± 0.05 to 1.39 ± 0.13, n=5, $p \leq 0.05$) (Figure 8). *Collagen Iα2* mRNA levels were significantly reduced in P2 cardiac myofibroblasts stretched for 24 hours, from 1.00 ± 0.03 to 0.85 ± 0.02 (Figure 8). In P2 cardiac myofibroblasts stretched for 24 hours, mRNA levels of *α-SMA* showed a trend towards increase from 1.00 ± 0.38 to 1.52 ± 0.26, though the difference was not significantly different for $p \leq 0.05$ (Figure 8).

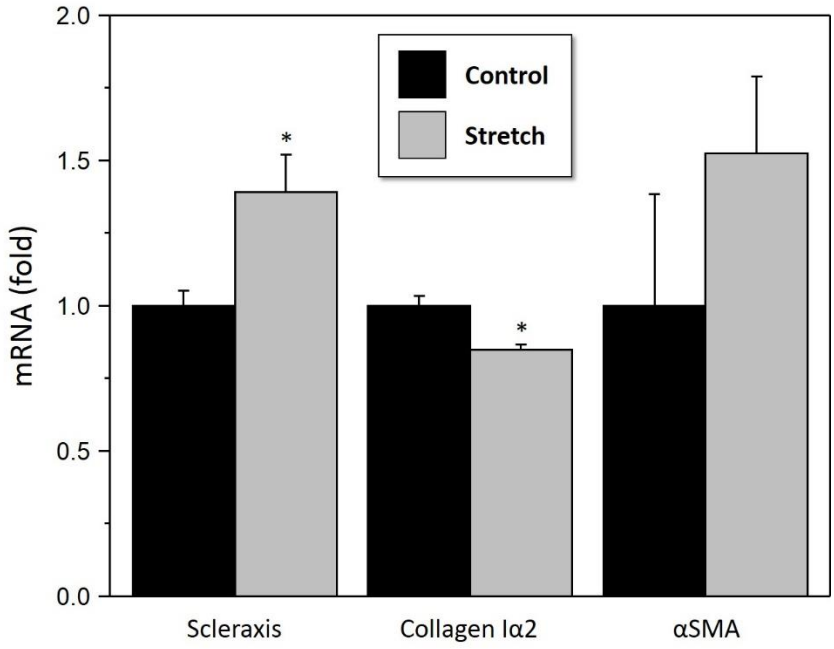


Figure 8: Effect of cyclic stretch on *Scleraxis*, *Collagen Iα2*, and *αSMA* expression in cardiac myofibroblasts. Primary rat ventricular P2 myofibroblasts were subjected to 24 hours of cyclic biaxial stretch (20%, 1 Hz) for 24 hours in serum-free medium. mRNA levels (normalized to GAPDH) were measured for Scleraxis, Collagen Iα2, and α-smooth muscle actin (αSMA). (*n=3, Student's t-test $p \leq 0.05$, mean ± SEM)

4.3: Scleraxis Regulates the Myofibroblast Phenotype

4.3.1: *Scleraxis* Over-Expression Increases Collagen and Myofibroblast Marker Expression

To determine the effect of increased *Scx* levels on the phenotype of P1 primary rat cardiac proto-myofibroblasts, we over-expressed a FLAG/HA-tagged version of *Scx* via adenoviral infection at 10 MOI for 24 hours. Western blot analysis revealed that compared to AdEGFP-infected controls, *Scx* over-expression increased Scx protein levels from 1.00 ± 0.35 to 12.07 ± 1.13 ($n=3$, $p \leq 0.001$) (Figure 9). Protein levels of both type I mature and pro-collagen were also significantly increased ($n=3$, $p \leq 0.01$) with *Scx* overexpression. Pro-collagen type I levels increased from 1.00 ± 0.22 in AdEGFP controls to 3.79 ± 0.56 in AdSCX-infected proto-myofibroblasts. Mature type I collagen protein levels rose drastically from 1.00 ± 0.20 in AdEGFP controls to 10.33 ± 0.20 with *Scx* overexpression (Figure 10A). Additionally, protein levels of ED-A fibronectin were significantly ($n=3$, $p \leq 0.05$) increased (from 1.00 ± 0.28 to 3.23 ± 0.39), as were those of SMemb (from 1.00 ± 0.24 to 2.53 ± 0.10) (Figure 10B).

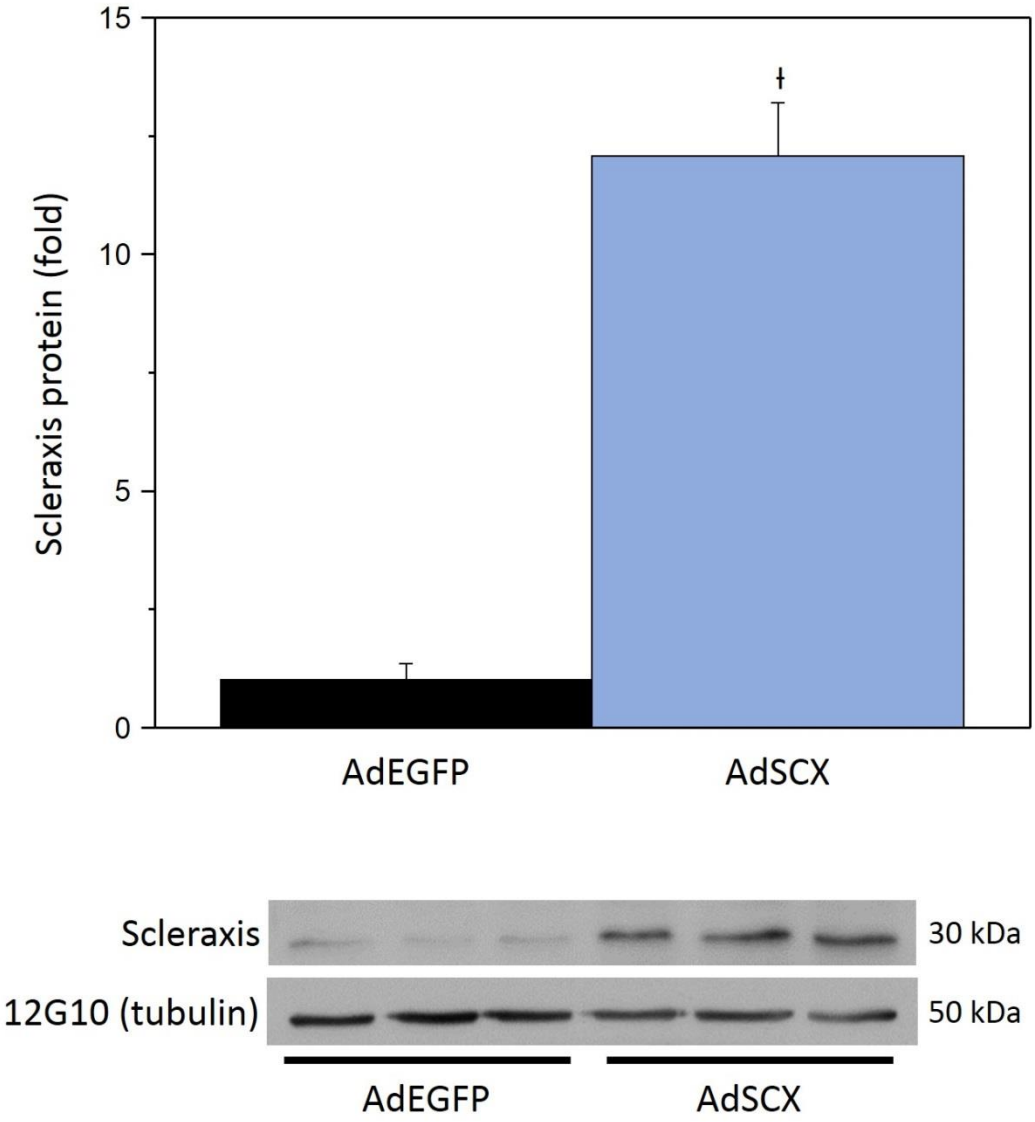


Figure 9: *Scleraxis* over-expression in cardiac proto-myofibroblasts. Protein levels (normalized to α-tubulin) were measured for Scleraxis in primary rat ventricular P1 proto-myofibroblasts infected with *Scleraxis*-expressing adenovirus (AdSCX, 10 MOI) or enhanced green fluorescent protein (*EGFP)*-expressing adenovirus (AdEGFP, 10 MOI) for 24 hours in serum-free medium. (†n=3, Student's t-test $p \leq 0.001$, mean ± SEM)

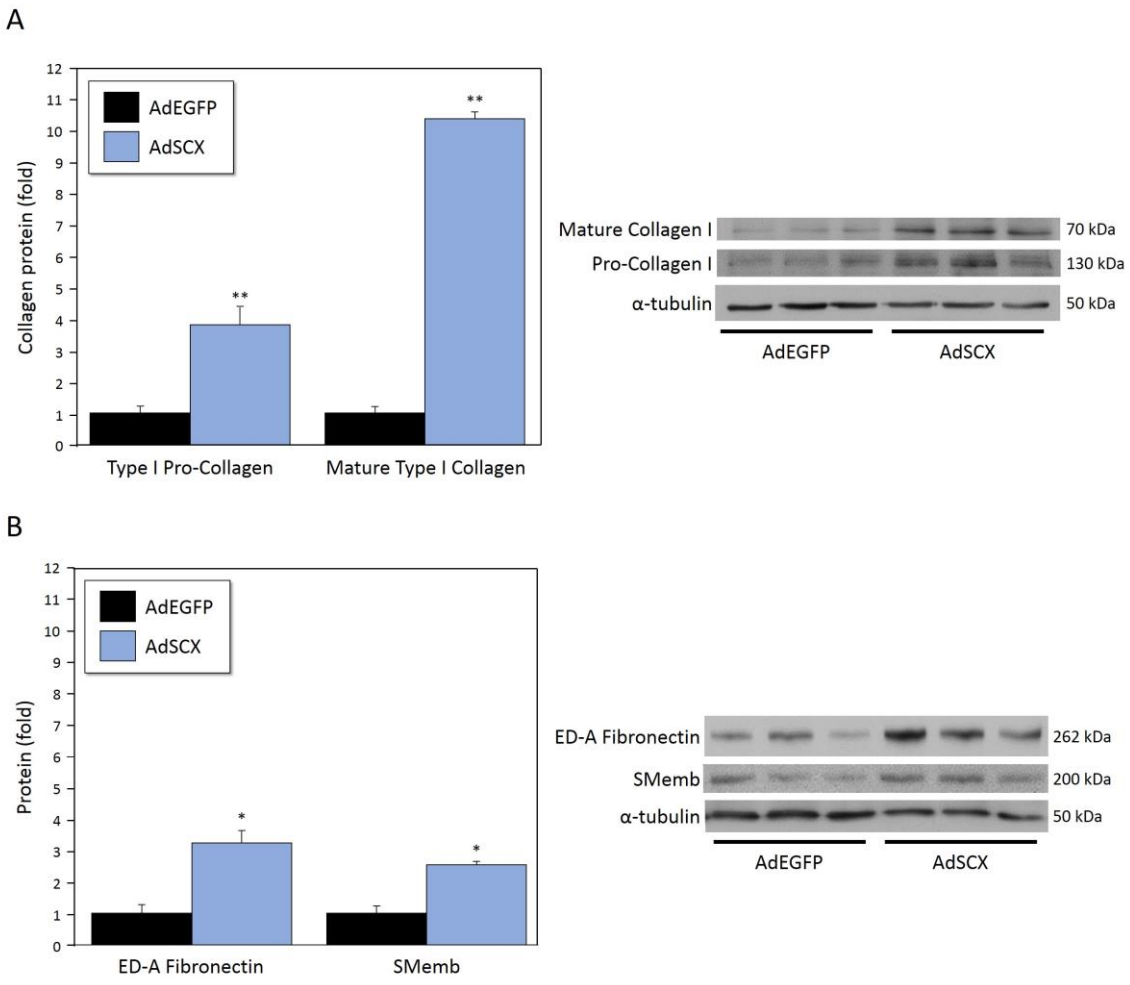


Figure 10: Effect of *Scleraxis* over-expression on collagen and myofibroblast marker protein levels in cardiac proto-myofibroblasts. Primary rat ventricular P1 proto-myofibroblasts were infected for 24 hours with either *Scleraxis*-expressing adenovirus (AdSCX, 10 MOI) or enhanced green fluorescent protein (*EGFP*)-expressing adenoviral control (AdEGFP, 10 MOI) in serum-free medium. Protein levels (normalized to α-tubulin) were measured for type I pro- and mature collagen (A) as well as the myofibroblast markers ED-A Fibronectin and embryonic non-muscle myosin heavy chain (SMemb) (B). Western blots for collagen and myofibroblast markers are shown in (A) and (B), respectively,

alongside α-tubulin controls. (*n=3, Student's t-test $p \leq 0.05$, **n=3, students t-test $p \leq 0.01$, mean ± SEM)

4.3.2: Scleraxis Regulates Cell Contractility

To determine the effect of changes in *Scx* expression on primary rat cardiac proto-myofibroblast (P1) and myofibroblast (P2) contractility, a collagen gel contraction assay was used [119, 156, 411]. As positive controls, cultures were supplemented with either 10 ng/ml transforming growth factor-β1 (TGF-β1), or 10% fetal bovine serum (10% FBS), both of which have been shown to strongly induce fibroblast contraction [211, 212, 411, 482, 483].

4.3.2.1: *Scleraxis* Regulates Contraction of Cardiac Proto-Myofibroblasts

Treatment of proto-myofibroblasts with 10% FBS for 24 hours reduced gel area to 60.95 ± 5.81% of its original size. Using percent contraction (reduction in gel area) induced by 10% FBS as an arbitrary maximum, we found that percent of maximal contraction induced in untreated, serum-deprived control was 10.82 ± 4.82%. Pre-infection with AdSCX induced a significant increase in contractility (51.14 ± 2.88% of maximal contraction, $n=6$, $p \leq 0.05$) compared to the untreated control, whereas pre-infection with control adenovirus (AdEGFP) did not (eliciting only 16.78 ± 5.48% of maximal contraction). The addition of 10 ng/ml TGFβ1 to untreated controls induced contraction (78.20 ± 13.23% of maximum) that was significantly greater than untreated or AdEGFP-infected controls ($n=6$, $p \leq 0.05$), but did not differ significantly from contraction induced by *Scx* over-expression (Figure 11). In combination with 10 ng/ml TGFβ1, AdEGFP elicited contraction of 116.72 ± 1.35% of maximum, significantly greater than contraction induced by untreated, AdEGFP-, or AdSCX-treated proto-myofibroblasts ($n=3$, $p \leq 0.05$) (Figure 11). However, when TGFβ1 treatment was combined with AdSCX infection, proto-myofibroblasts elicited contraction (120.91 ± 8.18% of maximal) that was

significantly greater than that of any other treatments (n=3, $p \leq 0.05$), except for the combination of TGFβ1 and AdEGFP (Figure 11). The use of a dominant negative DNA binding-deficient version of *Scx* (AdSCXΔDBD) caused a trend towards increased contraction (44.02 ± 2.22% of maximum). However, the increase in contractility with AdSCXΔDBD only reached significance in comparison to the untreated control (n=3, $p \leq 0.05$), but not in comparison to AdEGFP-treated control. The contraction observed with AdSCXΔDBD was not significantly different than AdSCX- or TGFβ1-induced contraction (Figure 11). Nonetheless, treatment with the DNA binding-deficient AdSCXΔDBD in combination with TGFβ1 induced a high level of contraction (117.24 ± 5.45% of maximum) that was greater than contraction induced by TGFβ1 treatment alone (n=3, $p \leq 0.05$) (Figure 11).

Conversely, knockdown of *Scx* expression via 48 hour pre-infection with a Scleraxis-targeting shRNA-expressing adenovirus (AdshSCX, 200 MOI) elicited a degree of contraction (7.29 ± 6.02% of maximal contraction) which was not significantly different than that of knockdown LacZ-targeting shRNA-expressing adenoviral controls (AdshLacZ, 3.51 ± 0.08% of maximal contraction). Pre-infection with AdshLacZ did not prevent TGFβ1-induced contraction by cardiac proto-myofibroblasts, with this combination eliciting 49.95 ± 4.24% of maximal contraction. Co-treatment with AdshLacZ and TGFβ1 induced contraction that was significantly greater than that of AdshLacZ or AdshSCX treatments alone (n=3, $p \leq 0.05$) (Figure 12). However, knockdown of *Scx* (AdshSCX) was able to prevent TGFβ1-induced contraction, with the combination eliciting only 11.06 ± 6.69% of maximal contraction, significantly less

contraction than induced by the combination of AdshLacZ and TGFβ1 ($n=3$, $p \leq 0.05$) (Figure 12).

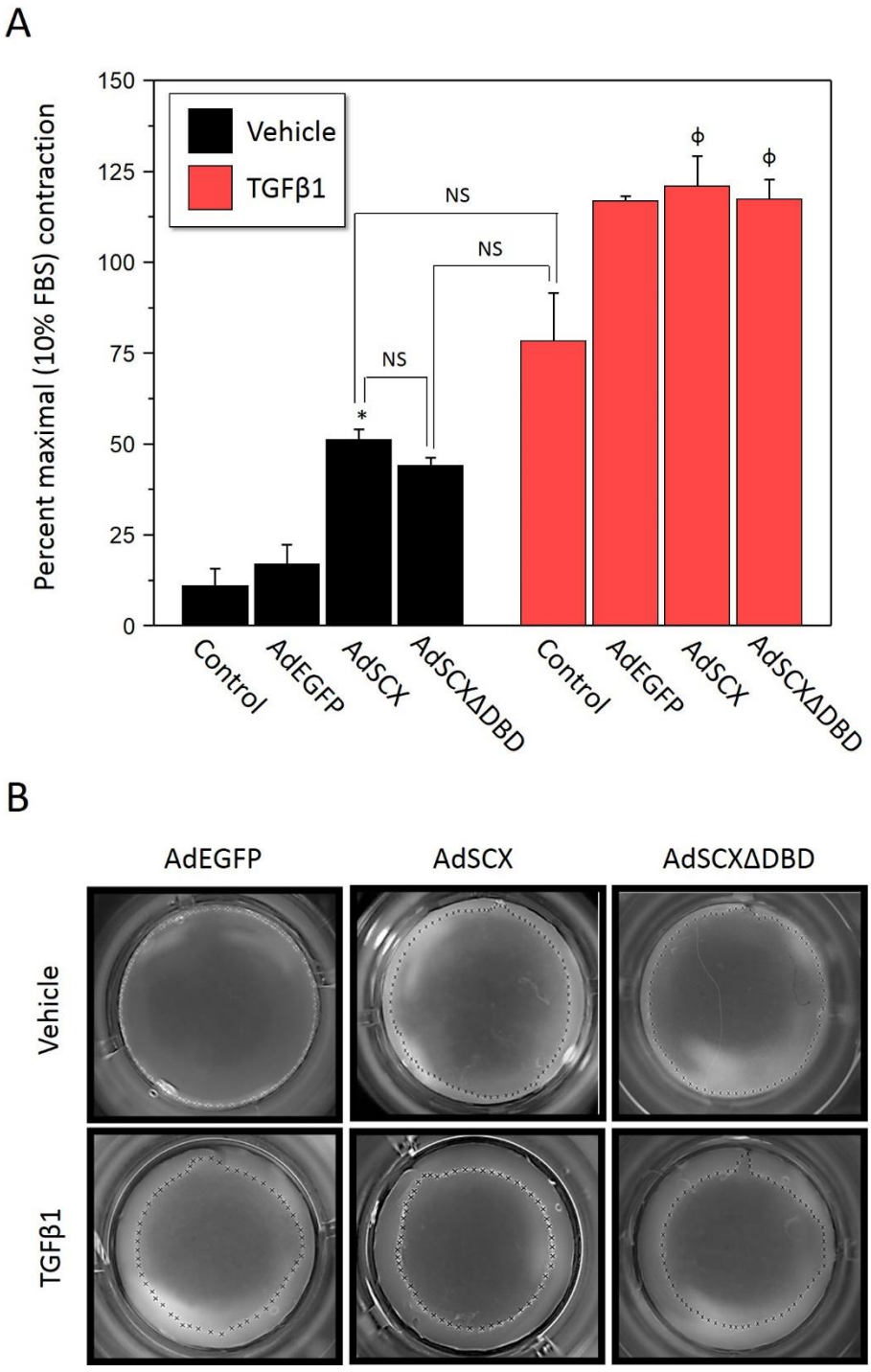


Figure 11: Gel contraction assay of cardiac proto-myofibroblasts in response to *Scleraxis* over-expression. Contraction of compressible collagen gels by primary rat ventricular P1 proto-myofibroblasts after 24 hours of gel release from well walls. Contraction (reduction in gel area) is expressed as a percentage of maximal contraction as induced by 10% FBS

treatment. Contraction was measured for proto-myofibroblast pre-infected for 24 hours in serum-free medium with either *Scleraxis*-expressing adenovirus (AdSCX, 10 MOI), a dominant negative DNA binding-deficient *Scleraxis* mutant-expressing adenovirus (AdSCXΔDBD, 50 MOI), enhanced green fluorescent protein (*EGFP*)-expressing adenovirus (AdEGFP, 10 MOI) as a control for infection, or no virus (uninfected controls). At time 0 hours (gel release from wells), all conditions were subjected to treatment with 10 ng/ml transforming growth factor-β (TGFβ) or vehicle (serum-free medium) (A). Representative gel images at 24 hours are shown in (B) with gel circumference outlined. (*n=3, one-way ANOVA $p \leq 0.05$, compared to control, AdEGFP, AdEGFP + TGFβ1, and AdSCX + TGFβ1; $^{\phi}$n=3, one-way ANOVA $p \leq 0.05$, compared to all treatments except AdEGFP + TGFβ1; NS = not statistically significant; mean ± SEM)

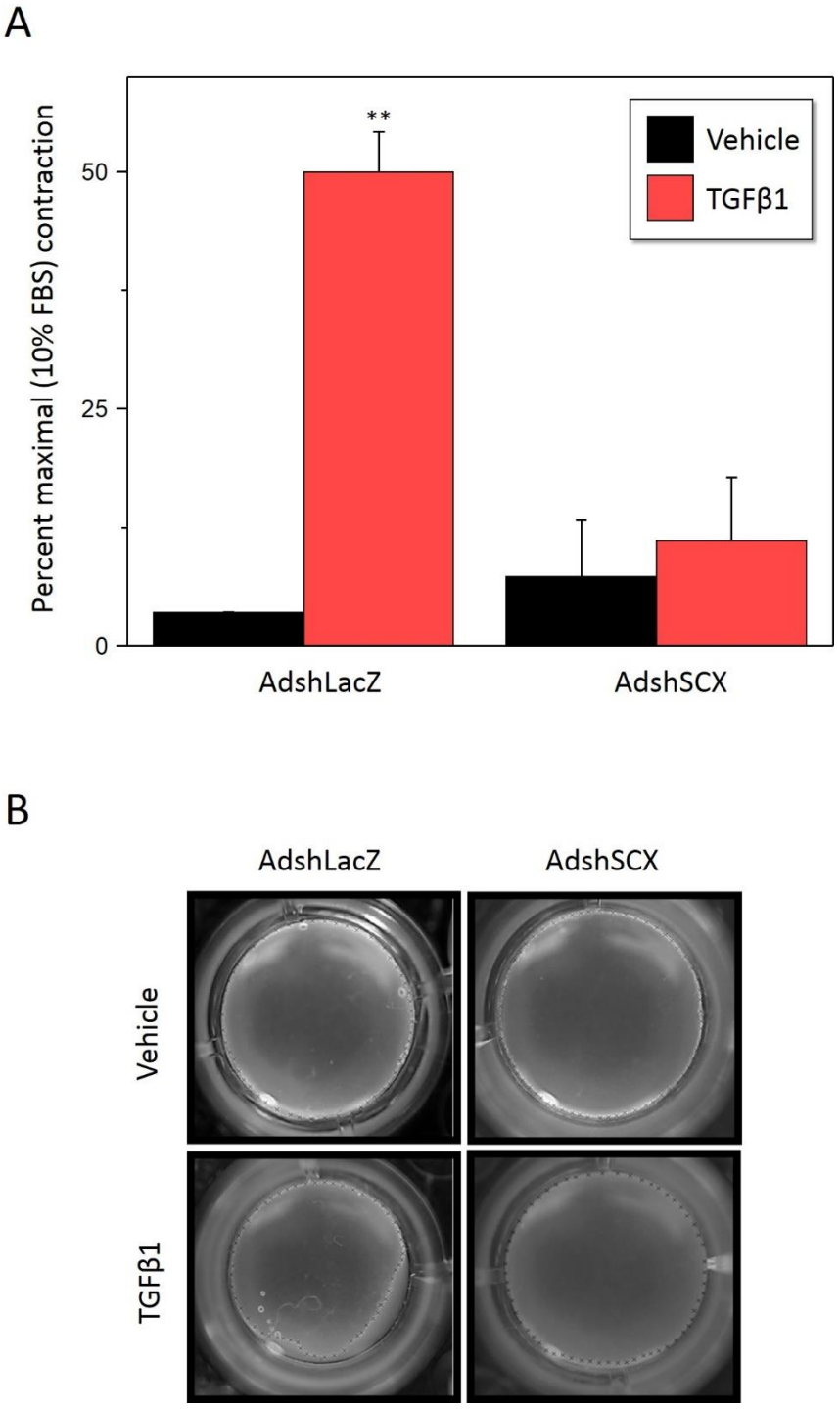


Figure 12: Gel contraction assay of cardiac proto-myofibroblasts in response to *Scleraxis* knockdown. Contraction of compressible collagen gels by primary rat ventricular P1 proto-myofibroblasts after 24 hours of gel release from well walls. Contraction (reduction

in gel area) is expressed as a percentage of maximal contraction as induced by 10% FBS treatment. Contraction was measured for proto-myofibroblast pre-infected for 48 hours in serum-free medium with either Scleraxis-targeting shRNA-expressing adenovirus (AdshSCX, 200 MOI), or LacZ-targeting shRNA-expressing adenovirus (AdshLacZ, 200 MOI) as a control. At time 0 hours (gel release from wells), all treatments were subjected to either 10 ng/ml transforming growth factor-β (TGFβ) or vehicle (serum-free medium) (A). Representative gel images at 24 hours are shown in (B) with gel circumference outlined. (**n=3, one-way ANOVA $p \leq 0.05$, compared to control, AdshSCX, and AdshSCX + TGFβ1 treatments, mean ± SEM)

4.3.2.2: *Scleraxis* Increases Cardiac Myofibroblast Contractility

To determine whether *Scx* could further enhance the contractility of already phenoconverted myofibroblasts, collagen gel assays were performed on primary rat ventricular P2 cardiac myofibroblasts. Treatment of P2 myofibroblasts with 10% FBS caused a reduction in gel size to 63.57 ± 0.98% of the original area, which was defined as maximal contraction. Untreated, serum-starved control P2 myofibroblasts elicited 6.16 ± 5.51% of maximal (10% FBS-induced) contraction. Pre-infection of P2 cardiac myofibroblasts with AdEGFP (50 MOI, 24 hours) induced 16.26 ± 5.59 % of maximal contraction, which was not significantly different from untreated controls. *Scx* over-expression (AdSCX, 10 MOI, 24 hours) induced a significant increase in contractility compared to untreated and AdEGFP controls ($n=3$, $p \leq 0.05$), eliciting 58.16 ± 4.64% of maximal contraction. However, over-expression of the dominant negative DNA binding-deficient version of *Scx* (AdSCXΔDBD, 50 MOI, 24 hours) did not increase contraction beyond control levels, eliciting only 11.31 ± 4.88% of maximal contraction. Treatment with 10 ng/ml TGFβ1 for 24 hours after collagen gel release resulted in a significant increase in percent of maximal contraction (65.76 ± 2.69%) compared to untreated and AdEGFP-infected controls, as well as AdSCXΔDBD treatment ($n=3$, $p \leq 0.05$), but which did not differ significantly from AdSCX-induced contraction (Figure 13).

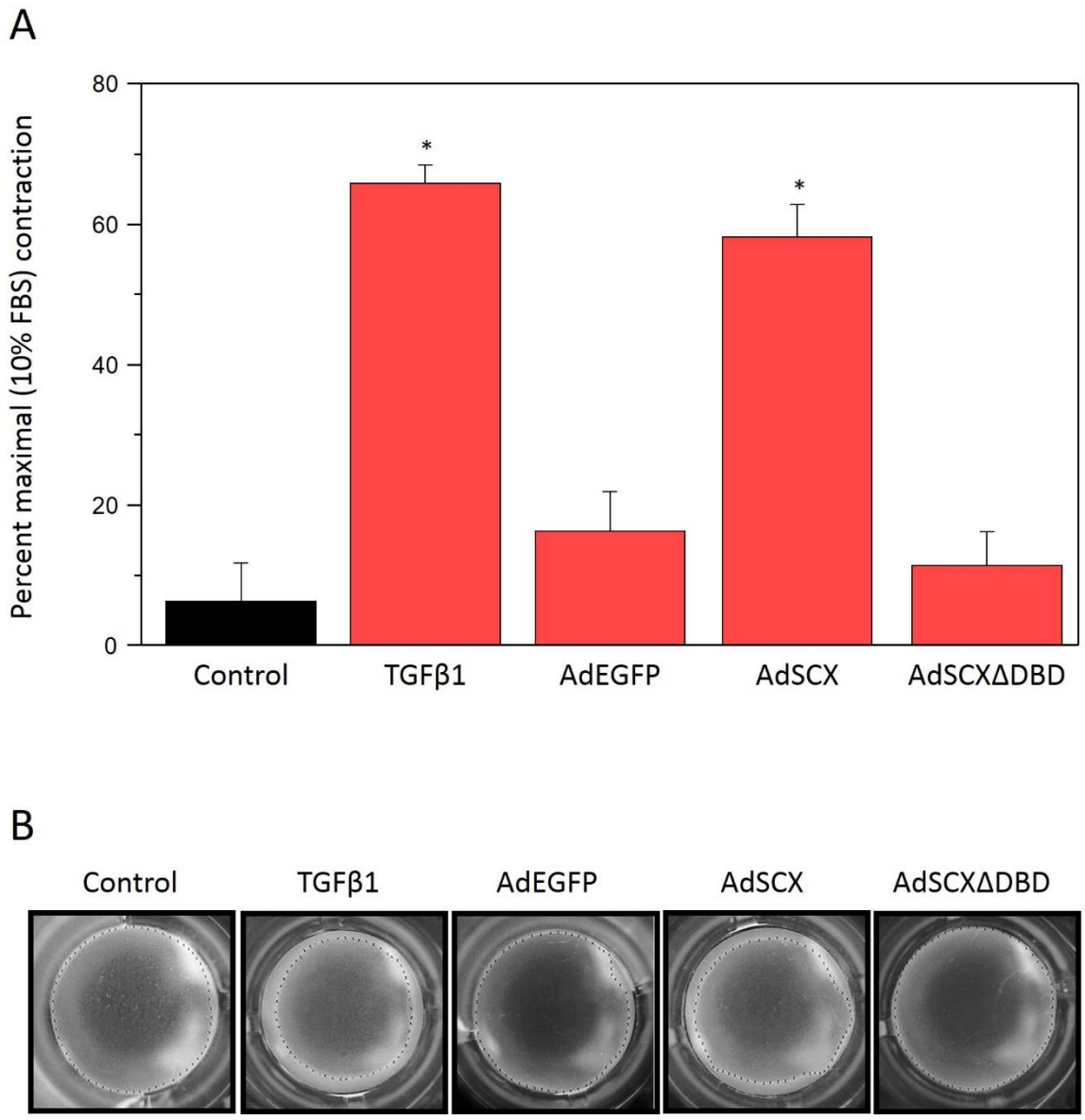


Figure 13: Gel contraction assay of cardiac myofibroblasts in response to *Scleraxis* over-expression. Contraction of compressible collagen gels by primary rat ventricular P2 myofibroblasts after 24 hours of gel release from well walls. Contraction was measured for myofibroblast pre-infected for 24 hours in serum-free medium with either *Scleraxis*-expressing adenovirus (AdSCX, 10 MOI), a dominant negative DNA binding-deficient *Scleraxis* mutant-expressing adenovirus (AdSCXΔDBD, 50 MOI), enhanced green

fluorescent protein (*EGFP*)-expressing adenovirus (AdEGFP, 10 MOI) as a control for infection, or no virus (uninfected controls). At time 0 hours (gel release from wells), untreated myofibroblasts were treated with 10 ng/ml transforming growth factor-β (TGFβ) (A). Representative gel images at 24 hours are shown in (B) with gel circumference outlined. (*n=3, one-way ANOVA $p \leq 0.05$, compared to control, AdEGFP, and AdSCXΔDBD, mean ± SEM)

4.3.3: *Scleraxis* Regulates Migration of Cardiac Proto-Myofibroblasts

4.3.3.1: Transwell Migration Assay

To determine the effect of changes in *Scx* expression on cardiac proto-myofibroblast migration, we performed a modified Boyden chamber (transwell) migration assay using 20 ng/ml TGFβ1 as a chemoattractant. After 24 hours, $1.56 \pm 0.24 \times 10^5$ cells had migrated into the lower chamber of the untreated well. Pre-infection with control AdEGFP virus did not significantly increase migration, with $1.68 \pm 0.18 \times 10^5$ cells having migrated into the lower chamber. AdSCX infection significantly decreased migration, exhibiting only $0.75 \pm 0.15 \times 10^5$ migrated cells. Infection with control knockdown virus, AdshLacZ, resulted in migration of $1.46 \pm 0.21 \times 10^5$ cells, which did not differ significantly from untreated or AdEGFP controls. Knockdown of *Scx* with AdshSCX infection significantly increased the number of migrated cells to $2.37 \pm 0.08 \times 10^5$ (Figure 14A).

4.3.3.2: Wound Heal Assay

To visualize the effect of *Scx* expression on migration, we performed a wound healing assay by creating a scratch in a confluent monolayer of P1 cardiac proto-myofibroblasts. After 24 hours, it can be seen that untreated control, AdEGFP-, and AdshLacZ-infected controls have a similar influx of proto-myofibroblasts into the wound area (Figure 8B). *Scx* over-expression (AdSCX) produces fewer cells within the wound, whereas knockdown (AdshSCX) appears to increase migration, even in comparison to controls (Figure 14B).

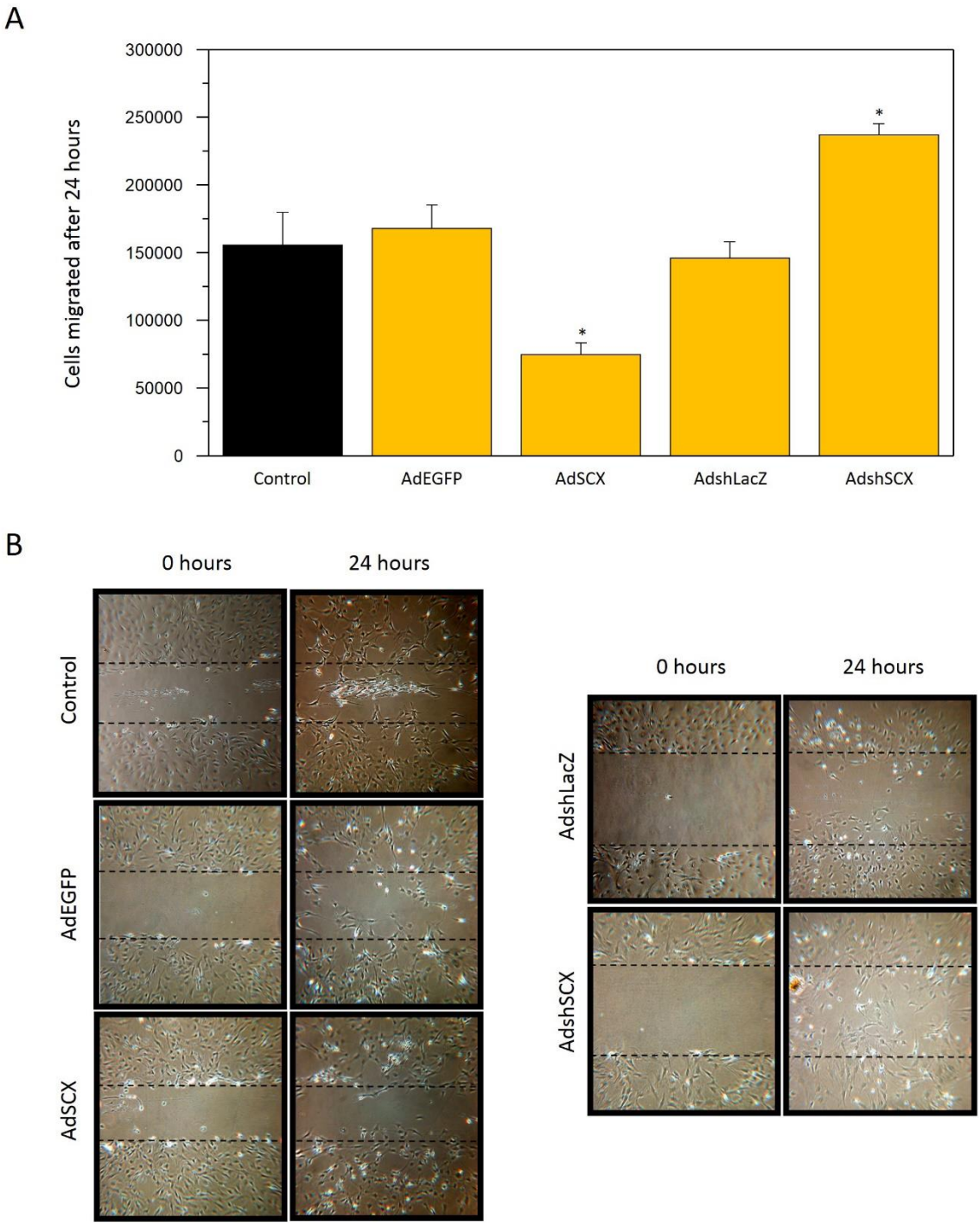


Figure 14: Migration of cardiac proto-myofibroblasts. Primary rat ventricular P1 proto-myofibroblasts were pre-infected in serum-free medium either for 24 hours with *Scleraxis*-expressing adenovirus (AdSCX, 10 MOI) or enhanced green fluorescent protein (*EGFP*)-expressing control adenovirus (AdEGFP, 10 MOI), or for 48 hours with Scleraxis-targeting

shRNA-expressing adenovirus (AdshSCX, 200 MOI) or LacZ-targeting shRNA-expressing adenovirus (AdshLacZ, 200 MOI). At time 0 hours, 20 ng/ml transforming growth factor-β1 (TGFβ1) was added to the lower chamber of modified Boyden chambers as a chemoattractant. The number of migrated cells was measured in the lower chamber after 24 hours for transwell assays (A) and wound healing assays were utilized to visualize differences in migration (B). (*n=3, one-way ANOVA, $p \leq 0.05$, mean ± SEM)

4.4: Activation of the Proximal 1500 bp Human *SCLERAXIS* Promoter

4.4.1: Response to Cyclic Stretch

4.4.1.1: Cyclic Stretch Activates the Proximal 1500 bp *SCLERAXIS* Promoter

To determine whether the *SCX* promoter is directly activated by factors that promote myofibroblast conversion, NIH-3T3 fibroblasts were transfected with a reporter vector containing the proximal 1500 bp region of the human *SCX* promoter upstream of a luciferase reporter gene within a pGL4.10 backbone (pGL4.10-SCX1500) (Figure 15A). 24 hours after transfection with pGL4.10-SCX1500, 15% cyclic stretch (1 Hz, 24 hours) of NIH-3T3 fibroblasts induced a significant increase ($n=3$, $p \leq 0.05$) in luciferase expression (3.18 ± 0.39-fold) as compared to unstretched controls (1.00 ± 0.37-fold), indicating stretch is sufficient to activate the 1500 bp human *SCX* promoter (Figure 15B).

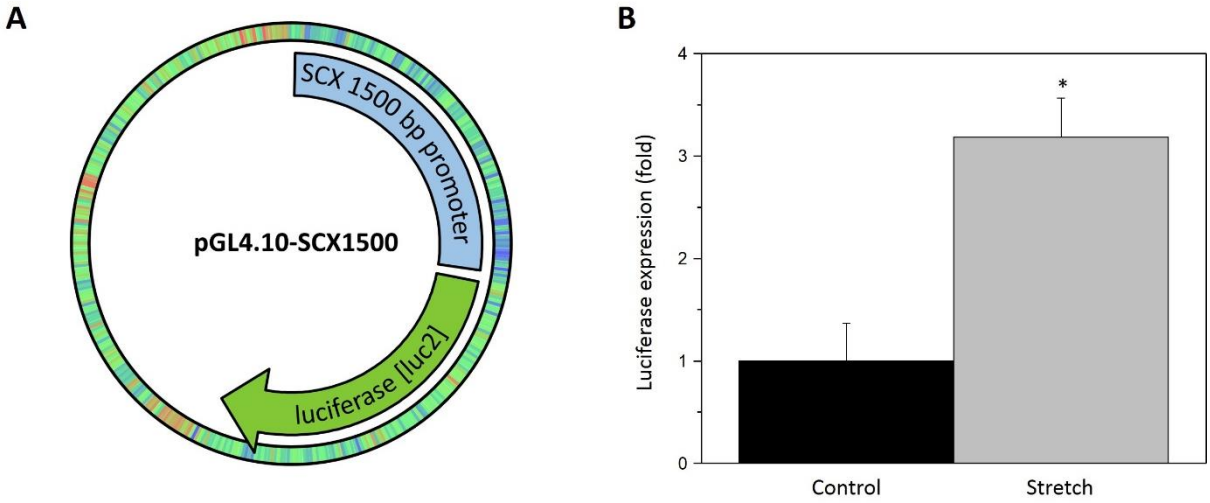


Figure 15: Effect of cyclic stretch on luciferase expression by the 1500 bp human *SCLERAXIS* promoter. NIH-3T3 fibroblasts were transfected with a luciferase reporter vector containing the proximal 1500 bp human *SCLERAXIS* promoter region (pGL4.10-SCX1500) (A) for 24 hours, followed by 24 hours of cyclic biaxial stretch (15%, 1 Hz). Luciferase activity (normalized to renilla luciferase) was measured for stretched and un-stretched controls (B). (*n=3, Student's t-test $p \leq 0.05$, mean ± SEM)

4.4.2: Response to Transforming Growth Factor-β1

4.4.2.1: TGFβ1 May Activate the 1500 bp SCLERAXIS Promoter in a Dose-Dependent Manner

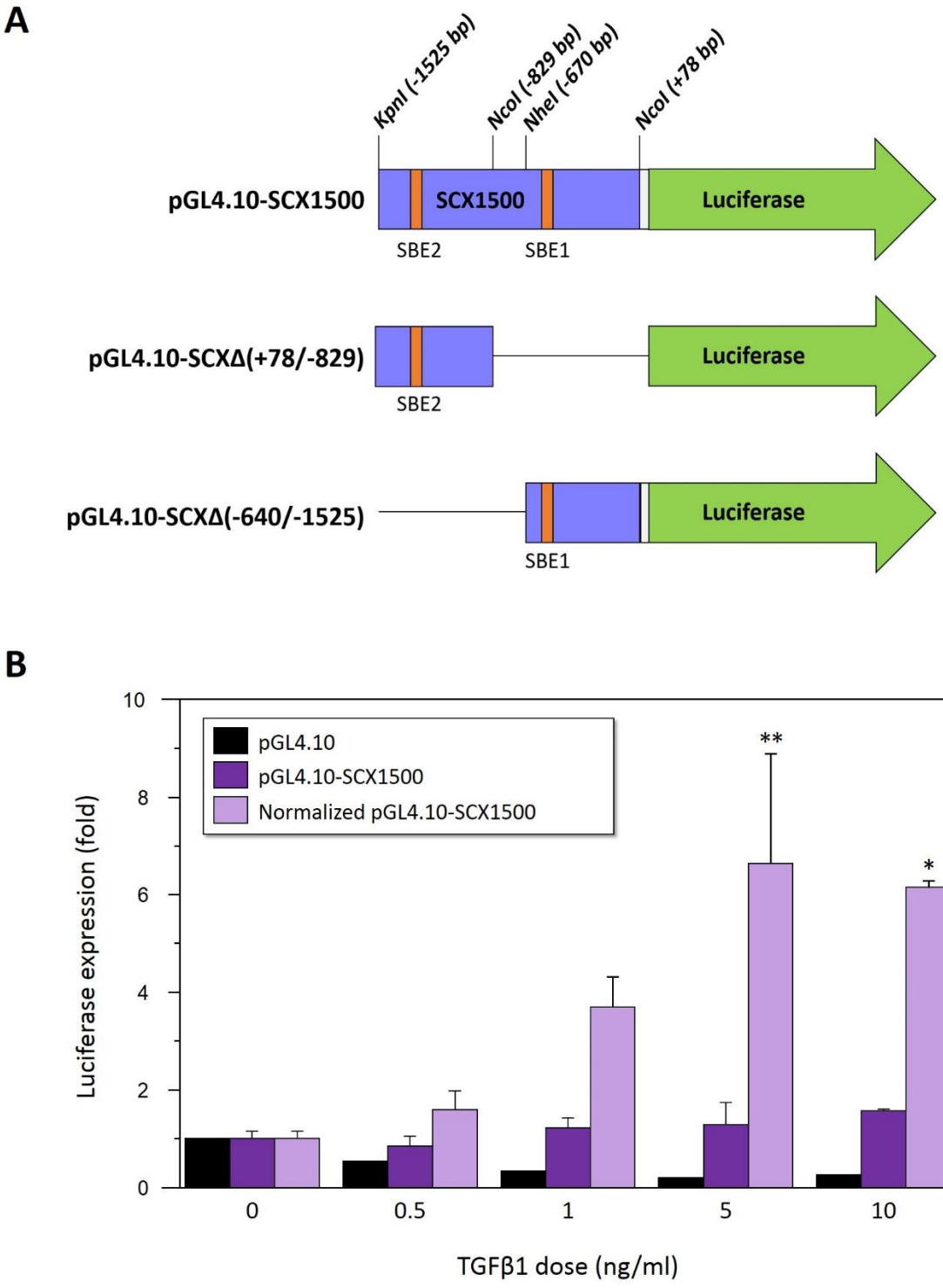
To determine the effect of the pro-fibrotic cytokine TGFβ1 on the proximal 1500 bp *SCX* promoter (Figure 16A), we performed luciferase assays in response to varying doses of TGFβ1. NIH-3T3 fibroblasts transfected with 500 ng of empty pGL4.10 control vector displayed decreased luciferase expression with increasing doses of TGFβ1. Normalized to pGL4.10-transfected cells in the absence of TGFβ1, pGL4.10-transfected cells treated with 0.5 ng/ml TGFβ1 for 24 hours displayed 0.53-fold luciferase expression. Treatment with 1 ng/ml induced 0.33-fold expression, with 5 ng/ml luciferase expression was decreased to 0.19-fold, and even further decreased to 0.25-fold with 10 ng/ml TGFβ1 (Figure 16B). Conversely, NIH-3T3 fibroblasts transfected with the pGL4.10-SCX1500 vector displayed a trend towards increased luciferase expression levels, though these differences were not statistically significant. Compared to cells in the absence of TGFβ1 (1.00 ± 0.15-fold expression), treatment with 0.5 ng/ml TGFβ1 slightly decreased expression (0.84 ± 0.20-fold). However, luciferase expression driven by pGL4.10-SCX1500 increased in a dose-dependent manner; with 1 ng/ml TGFβ1, expression was 1.22 ± 0.20-fold that of untreated controls, with 5 ng/ml TGFβ1, expression increased to 1.29 ± 0.44-fold, and with 10 ng/ml TGFβ1, expression was 1.57 ± 0.03-fold (Figure 16B). If luciferase activity of pGL4.10-SCX1500 is normalized to that of empty pGL4.10, however, there is a significant increase in expression at doses of 5 and 10 ng/ml TGFβ1. Normalized values, compared to untreated (0 ng/ml TGFβ1) controls (1.00 ± 0.15-fold expression), are increased with increasing dosage. Treatment with 0.5 ng/ml TGFβ1

induces 1.58 ± 0.38-fold luciferase expression, and with 1 ng/ml TGFβ1, pGL4.10-normalized expression is increased further to 3.70 ± 0.62-fold, though these expression levels do not differ significantly from that of untreated control (Figure 16B). However, pGL4.10-normalized luciferase expression of pGL4.10-SCX1500-transfected fibroblasts treated with 5 ng/ml TGFβ1 (6.63 ± 2.25-fold) is significantly greater than that of 0 and 0.5 ng/ml TGFβ1 treatments (Figure 16 B). Additionally, treatment with 10 ng/ml TGFβ1 produces similar luciferase expression levels (6.15 ± 0.14-fold), which are significantly greater than that of untreated (0 ng/ml TGFβ1) controls (Figure 16B).

4.4.2.2: The effect of TGFβ1 is Dependent on *SCLERAXIS* Promoter Length

To determine whether specific regions of the proximal 1500 bp *SCX* promoter are responsible for its responsiveness to TGFβ1, we removed the proximal and distal regions of the promoter within the pGL4.10-SCX1500 vector via restriction enzyme digestion. We analyzed luciferase expression of a construct lacking the proximal 829 bases and the ATG transcriptional start site of the 1500 bp *SCX* promoter, which was designated pGL4.10-SCXΔ(+78/-829), as well as a construct lacking the distal 634 bases of the promoter, designated pGL4.10-SCXΔ(-640/-1525). As with TGFβ1-dose experiments performed with the full-length promoter (pGL4.10-SCX1500), luciferase expression decreased in empty vector (pGL4.10)-transfected control treated with 10 ng/ml TGFβ1, compared to untreated control (Figures 16C and 16D). In pGL4.10-SCX Δ(+78/-829)-transfected cells, luciferase expression did not appear to change with TGFβ1 treatment (1.04 ± 0.12-fold) compared to vehicle-treated cells (1.00 ± 0.11-fold). However, if these expression values were normalized to those of empty pGL4.10-transfected readings, treatment with TGFβ1 significantly increased luciferase *e* expression to 4.09 ± 0.48-fold,

compared to vehicle-treated controls (Figure 16C). In cells transfected with the proximal version of the *SCX* promoter (pGL4.10-SCXΔ(-640/-1525)), TGFβ1 treatment significantly decreased luciferase expression to 0.38 ± 0.09-fold, compared to vehicle-treated controls (1.00 ± 0.05-fold). Again, when normalized to pGL4.10 expression values, the response differs. Normalized expression of TGFβ1-treated cells transfected with the pGL4.10-SCXΔ(-640/-1525) vector trended towards increase to 1.48 ± 0.36-fold that of vehicle-treated controls, though the difference was not statistically significant (Figure 16D).


A
KpnI (-1525 bp)
NcoI (-829 bp)
NheI (-670 bp)
NcoI (+78 bp)
pGL4.10-SCX1500
SCX1500
Luciferase
SBE2
SBE1
pGL4.10-SCXΔ(+78/-829)
Luciferase
SBE2
pGL4.10-SCXΔ(-640/-1525)
Luciferase
SBE1
B
pGL4.10
pGL4.10-SCX1500
Normalized pGL4.10-SCX1500
Luciferase expression (fold)
10
8
6
4
2
0
**
*
0
0.5
1
5
10
TGFβ1 dose (ng/ml)

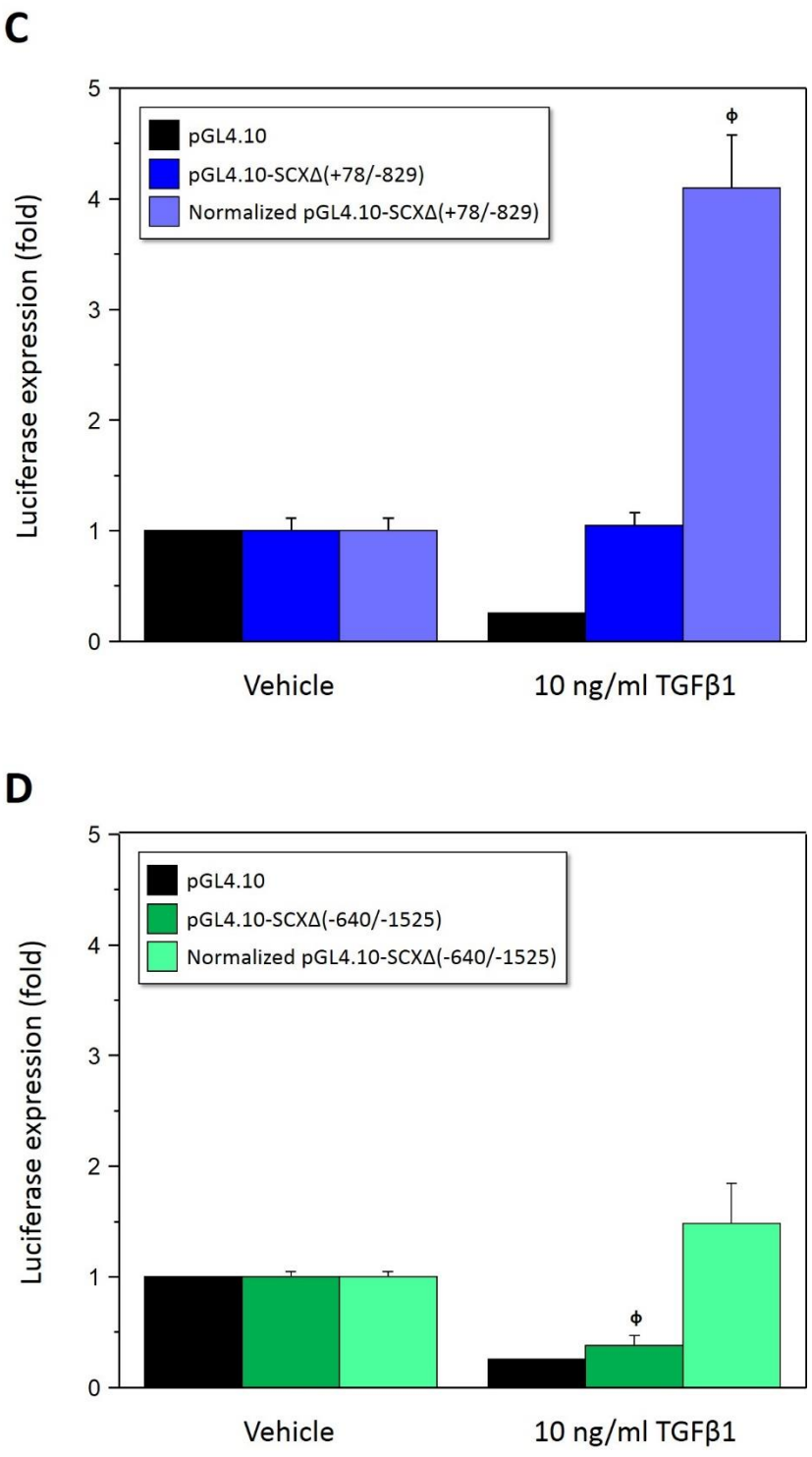


Figure 16: Effect of TGFβ1 on luciferase activity of different *SCLERAXIS* promoter constructs. NIH-3T3 fibroblasts were transfected for 24 hours with different luciferase

constructs, containing either the full proximal 1500 bp human *SCLERAXIS* promoter (pGL4.10-SCX1500) with two Smad-binding elements (SBEs) conserved between human, rat, and mouse genomes, or one of two truncated constructs (A). The distal promoter construct [pGL4.10-SCXΔ(+78/-829)] lacks the proximal *SCLERAXIS* promoter region and SBE1. The proximal promoter construct [pGL4.10-SCXΔ(-670/-1525)] lacks the distal region of the *SCLERAXIS* promoter and SBE2 (A). Following transfection, varying doses of transforming growth factor-β1 (TGFβ1) were added to pGL4.10-SCX1500-transfected cultures, and 10 ng/ml TGFβ1 added to cells transfected with either pGL4.10-SCXΔ(+78/-829) or pGL4.10-SCXΔ(-670/-1525). Luciferase activity (normalized to renilla) was measured for pGL4.10, pGL4.10-SCX1500 (B), pGL4.10-SCXΔ(+78/-829) (C), and pGL4.10-SCXΔ(-670/-1525) (D). Luciferase activity values normalized to pGL4.10 activity are also shown for pGL4.10-SCX1500 (B), pGL4.10-SCXΔ(+78/-829) (C), and pGL4.10-SCXΔ(-670/-1525) (D). (*n=3, one-way ANOVA, $p \leq 0.05$ compared to 0 ng/ml TGFβ1, **n=3, one-way ANOVA, $p \leq 0.05$, compared to 0 and 0.5 ng/ml TGFβ1, $^{\phi}$n=3, Student's t-test $p \leq 0.05$, mean ± SEM)

5: DISCUSSION

5.1: Response of Scleraxis to Cyclic Stretch

Cardiac fibroblasts respond to cyclic stretch by increasing expression of myofibroblast marker genes such as *αSMA* [289], TGFβ [372], fibrillar collagens type I and III [375], and *Fibronectin* [368]. In non-cardiac fibroblasts, cyclic stretch increased the formation of stress fibers, which are characteristic of the myofibroblast transition [289, 294, 381, 382]. Additionally, the transcription factor NFAT4c, which has been shown to cooperate with Scleraxis in the activation of the *Collagen Iα1* gene in tendon fibroblasts [435], is also increased in cardiac fibroblasts exposed to cyclic stretch [376]. mRNA and protein levels of *Scx* and fibrillar collagens type I and III increase in response to different regimes of cyclic stretch in different cell types [366-368, 373, 383, 436, 457, 468-473]. The majority of studies examined have been performed in MSCs, which have been shown to spontaneously differentiate into fibroblasts in culture and share similar properties such as surface markers and the ability to differentiate into varying cell lineages [484, 485]. Most of the MSC studies utilize cyclic stretch regimes similar to ours, though some have utilized lesser degrees of elongation (1 – 10%) and longer duration (48 hours to 7 days) [385, 438, 459, 470-472]. However, one experiment was performed under nearly identical conditions in MSCs (15% stretch, 24 hours, 1 Hz, with uniaxial as opposed to biaxial stretching), which actually showed a decrease in mRNA levels of *SCX* [470]. This discrepancy could be due to a number of factors, which may include the orientation of stretching (in one dimension, as opposed to two in our experiments), and the cell type used. Thus, it appears that in fibroblast-like cells, cyclic stretch induces *SCX* expression,

with which our findings in cardiac proto-myofibroblasts and myofibroblasts agree (Figures 4 and 8).

15% equi-biaxial cyclic stretch (24 hours, 1 Hz) of cardiac P1 proto-myofibroblasts was found to significantly increase levels of *Scx* mRNA and protein (Figures 4 and 8), as well as fibrillar collagens I and III (Figures 5 and 8) and myofibroblast markers αSMA, ED-A Fn, and SMemb (Figures 6 and 8). These findings indicate that cyclic stretch promotes the generation of cardiac myofibroblasts through mechanical force, and Scx is involved in this process. The observation that *Scx* mRNA levels were also significantly increased in P2 myofibroblasts (Figure 8), yet to a lesser degree than in proto-myofibroblasts (Figure 4), indicates a strong sensitivity of *Scx* expression to cyclic stretch, even in already fully converted myofibroblasts. However, even though stretch was increased to 20% elongation in P2 myofibroblasts, the trend towards increased *αSMA* mRNA levels was not significant, and in fact, *Collagen Iα2* showed a significant decrease (Figure 8). This lack of responsiveness of cardiac myofibroblasts to cyclic stretch may be due to their phenotype, in that these cells express increased levels of αSMA and fibrillar collagens, and thus may be resistant to further transcriptional activation of these genes. That is to say, myofibroblasts may be "maxed out" in their ability to respond to cyclic stretch by increasing collagen and myofibroblast marker expression. Additionally, there is an obvious increase in the level of experimental error between biological replicates when examining *αSMA* responses to cyclic stretch in P2 myofibroblasts (Figure 8). It is possible that the increased error may be due to an increase in cell seeding density in cardiac myofibroblasts, as reports have shown that increasing cell density tends to decrease myofibroblast marker expression, especially that of *αSMA* [486, 487]. Thus, the greater

density of cardiac myofibroblast cultures in stretch assays may have itself altered *αSMA* expression, independently of stretching. Additionally, myofibroblasts are larger than fibroblasts [122], which may also be a factor that needs to be considered when making comparisons of the responses of these two cell phenotypes in response to cyclic stretch.

The observation that tyrosine phosphorylation of FAK at Y397 is decreased with 24 hours of cyclic stretch in cardiac proto-myofibroblasts (Figure 7) appears at first glance to conflict with reports in non-cardiac fibroblasts, indicating increased Y397 phosphorylation. However, upon closer inspection, one notices that these reports are temporally limited (i.e. less than 6 hours) [379, 383-385], and in fact FAK phosphorylation only begins to decline after 6 hours [386]. The response of FAK phosphorylation to longer-term stretch (e.g. 24 hours or more) has not been examined previously. The implications of its decreased phosphorylation herein (Figure 7) are likely related to the apparent effect of cyclic stretch on the myofibroblast phenotype. Since FAK is involved primarily in turnover of focal adhesions [110, 358], and stretch promotes the myofibroblast phenotype, which exhibits stronger and larger focal adhesions [298], it is not surprising that FAK phosphorylation (and in turn, its kinase activity) would be down-regulated by this stimulus. This is further supported by reports that the focal adhesion proteins zyxin and α-actinin are recruited to stress fibers with cyclic stretching of mouse embryonic fibroblasts [381, 382].

The mechanisms through which mechanical forces stimulate myofibroblast phenoconversion are not well characterized. For αSMA, numerous lines of evidence indicate that stretch-induced transcriptional activation occurs through the Rho signaling pathway and actin reorganization-mediated translocation of MRTF-A to the nucleus, and

is dependent upon fibronectin [289, 380]. However, there are no studies examining the transcriptional regulation of *Scx* in response to stretch in cardiac fibroblasts and their derivatives. Thus, examining reporter gene activity in response to cyclic stretch was an important experiment in determining the mechanoresponsive capabilities of the *Scx* promoter. It is clear that cyclic stretch in cardiac proto-myofibroblasts potently activates the proximal 1500 bp human *SCX* promoter (Figure 15).

The mechanism by which this activation occurs remains entirely unknown. A few different pathways have been implicated in cyclic stretch-mediated responses in fibroblasts. Various members of the MAPK pathway appear to be activated by cyclic stretch in cardiac [375] and non-cardiac fibroblasts [289, 378, 379], and plays a central role in stretch-mediated responses in lung fibroblasts [488]. p38-MAPK can activate transcription of target genes through translocation to the nucleus and subsequent activation of the AP-1 transcription factor [489], which binds the consensus sequence *TGA(C/G)TCA* [490, 491]. We noted that this site (*TGAGGTCA*) is also found in the human *SCX* promoter, 1415 bp upstream of the ATG start site, though it is not conserved in mouse and rat genomes. Additionally, the RhoA pathway is activated in MEFs exposed to cyclic stretch in a fibronectin-dependent manner [289, 380], and thus is also a candidate for stretch-induced activation of *SCX*. *Tenascin-C*, which is also upregulated by cyclic stretch, as well as *Collagen XI*, contain a conserved putative 'stretch-responsive' sequence in their promoter regions that has been implicated in shear stress-induced responses in endothelial cells [492, 493]. In examining the *SCX* promoter, we also observe this sequence, *GAGACC*, within 100 bp upstream of the transcriptional start site, conserved among rat, mouse, and human sequences. Within a short stretch-responsive

region of the *Tenascin-C* promoter [493], there is also a canonical NFκB response element with the sequence *GGGRNYYYCC* (in which R is a purine, Y is a pyrimidine, and N is any nucleotide) [494]. In cardiomyocytes, induction of *Tenascin-C* by cyclic stretch is NFκB-dependent [495]. Interestingly, this site is also found in the human *SCX* promoter, 1084 bp upstream of the transcriptional start site, though this is not conserved in rat or mouse transcripts. Thus, there are a few putative transcription factors that may be involved in cyclic stretch-mediated transcriptional activation of the human *SCX* promoter.

5.2: Induction of Cardiac Myofibroblast Markers by Scleraxis

It has been well established that no single marker is sufficient for the identification of myofibroblasts, or even fibroblasts [116]. Common myofibroblast marker genes such as *ED-A Fn*, *SMemb*, and *αSMA* are also expressed in smooth muscle cells, which are morphologically similar to myofibroblasts in their shape and organization of stress fibers [496-498]. Additionally, expression of these proteins is also increased in the intermediate proto-myofibroblast phenotype [122]. The incorporation of αSMA into stress fibers is a defining feature of the myofibroblast [499, 500], and thus increased *αSMA* expression alone should not be used to define the myofibroblast phenotype. Thus, examining levels of these three proteins in concert, in addition to αSMA incorporation into stress fibers, provides a relatively effective means for defining fibroblasts, proto-myofibroblasts, and myofibroblasts *in vitro*. Infection with *Scx* over-expression adenovirus (AdSCX) for 24 hours dramatically increases Scx protein levels in cardiac proto-myofibroblasts (Figure 9), and results in significant increases in the myofibroblast markers ED-A Fn and SMemb (Figure 10). Other experiments performed by our lab have demonstrated that αSMA and its incorporation into stress fibers is also increased with *Scx* over-expression (48 hours) in

these cells (Bagchi and Czubryt, data not shown). Additionally, experiments in *Scx* knockdown mice show decreased levels of these same markers (Bagchi and Czubryt, data not shown). Thus it is apparent that Scx is required for expression of these key myofibroblast markers in cardiac proto-myofibroblasts. We also observe an increase in protein levels of pro and mature type I collagen in response to *Scx* over-expression for 24 hours (Figure 10). The drastic increase in collagen I synthesis by proto-myofibroblasts in response to *Scx* overexpression, in concert with increased myofibroblast markers (Figure 10), indicates *Scx* is capable of driving phenoconversion of cardiac proto-myofibroblasts to myofibroblasts.

5.3: Scleraxis Regulates Cardiac Myofibroblast Function

We have demonstrated here that the changes induced by Scx are not limited to alterations of gene expression, and that rather these changes have functional implications in cardiac proto-myofibroblasts and myofibroblasts. Increased contractility is a hallmark of the myofibroblast, and we have demonstrated that *Scx* over-expression in both proto-myofibroblasts (Figure 11) and myofibroblasts (Figure 13) increases this function. *Scx* over-expression in proto-myofibroblasts increases contractility to similar levels as treatment with the pro-contractile cytokine TGFβ1, regardless of DNA-binding capability (Figure 11). The blunted, yet still significant, response of myofibroblasts to TGFβ1 treatment or *Scx* over-expression (Figure 13) is likely indicative of the already increased contractile ability of these cells. In this context, the contractile ability of myofibroblasts in response to additional stimuli may not be as drastically increased as these cells already possess an abundance of stress fibers and their capability to contract in response to these discrete stimuli may become ‘maxed-out’ at this level of phenotypic conversion. In proto-

myofibroblasts, Scx further augments the contractility induced by TGFβ1 (Figure 11), which may be either through up-regulation of TGFβ1 signaling or some other TGFβ1-dependent mechanism. AdEGFP treatment also appears to augment TGFβ1-induced contractility in primary ventricular proto-myofibroblasts, and though the increase is not significant compared to TGFβ1 alone, contractility in response to the combination of AdEGFP and TGFβ1 is also not significantly different than that of AdSCX and TGFβ1 or AdSCXΔDBD and TGFβ1 (Figure 11). The relationship between potential off-target adenoviral effects and TGFβ1 treatment in proto-myofibroblast contractility may be elucidated by increasing the number of replicates in this experiment. However, we also observed that *Scx* was required for TGFβ1-induced contractility – knockdown of *Scx* prevented contraction by proto-myofibroblasts treated with TGFβ1 (Figure 12). Interestingly, this effect appears to be independent of the DNA-binding capability of Scx. With over-expression of the dominant negative, DNA binding-deficient version of *Scx*, TGFβ1 was still able to induce proto-myofibroblast contraction to the same level as full-length *Scx* in combination with TGFβ1 (Figure 11). These results suggest that Scx may mediate TGFβ1-induced contraction through interaction with other cofactors, independently of DNA binding. There are a few candidate proteins that may participate in this cooperative endeavour. These include Smad3, which has been shown to bind directly to Smad3 and regulate tendon matrix organization [428], as well as acting in concert with Scx to synergistically regulate *Collagen Iα2* gene expression [136], and NFATc, which cooperates with Scx in activation of the *Collagen Iα1* gene in tendon fibroblasts [435]. There also exists the possibility of an intermediate gene through which Scx, in cooperation with another cofactor, may regulate expression. For example, *αSMA* expression has been shown to directly correlate with the contractile ability of

myofibroblasts [123], and preliminary data from our lab has suggested that Scx may directly regulate its transcription (Bagchi and Czubryt, data not shown). Whether this occurs in cooperation with another transcription factor remains undetermined.

Interestingly, unlike in proto-myofibroblasts, which contracted to a similar degree regardless of the DNA-binding ability of Scx, the dominant negative AdSCXΔDBD treatment failed to induce contraction in cardiac myofibroblasts (Figure 13). This is indicative of a different, DNA binding-dependent mechanism through which Scx induces contraction in these fully converted myofibroblasts. Perhaps augmentation of contraction in myofibroblasts, which already have increased contractile ability, requires a direct transcriptional mechanism through which Scx may act. The promoter of the *αSMA* gene indeed contains a number of E-boxes to which Scx may potentially bind. However, because this experiment was not repeated in myofibroblasts in the presence of TGFβ1, the question still remains as to whether Scx DNA binding ability is required for contraction, or is simply able to further increase this function in cardiac myofibroblasts.

In addition to regulating contraction, *Scx* expression also regulates the migratory ability of cardiac proto-myofibroblasts, as is shown both quantitatively (via transwell migration assay) and qualitatively (via wound-healing assay) (Figure 14). Because TGFβ1 acts as a chemokine for fibroblasts, control treatments (untreated, AdEGFP-, and AdshLacZ-infected) exhibited some migration of proto-myofibroblasts through the porous membrane of the well insert. However, *Scx* over-expression drastically inhibited the ability of these cells to migrate through the membrane, and appears to diminish the number of cells migrating into the scratch produced in the wound-healing assay (Figure 14). Conversely, knockdown of *Scx* expression enhances the ability of proto-myofibroblasts to migrate

towards TGFβ1 and into the scratch area (Figure 14). This supports the notion that Scx promotes the myofibroblast phenotype, which is less migratory than its precursors due to increased adhesion to its substrate via focal adhesions. This is in agreement with the finding that cyclic stretch induces *Scx* expression concomitant with decreased FAK phosphorylation (and thus decreased focal adhesion turnover) in proto-myofibroblasts (Figure 7). These results also suggest that not only does Scx induce phenoconversion, but a lack of Scx may, in fact, induce *reversion* of proto-myofibroblasts, since migration was increased following *Scx* knockdown (Figure 14), rather than simply remaining at similar levels as control treatments. Overall, these functional studies further implicate Scx in driving the cardiac myofibroblast phenotype. We propose a model whereby Scx promotes phenotypic conversion of cardiac proto-myofibroblasts to myofibroblasts, and inhibits the reversion of proto-myofibroblasts to the fibroblast phenotype (Figure 17). Although changes in *Scx* expression induce alterations in myofibroblast marker expression and cell behaviour (contraction and migration), in many cases the question arises as to whether the effect is via a direct transcriptional mechanism or requires the involvement of other transcriptional regulators.

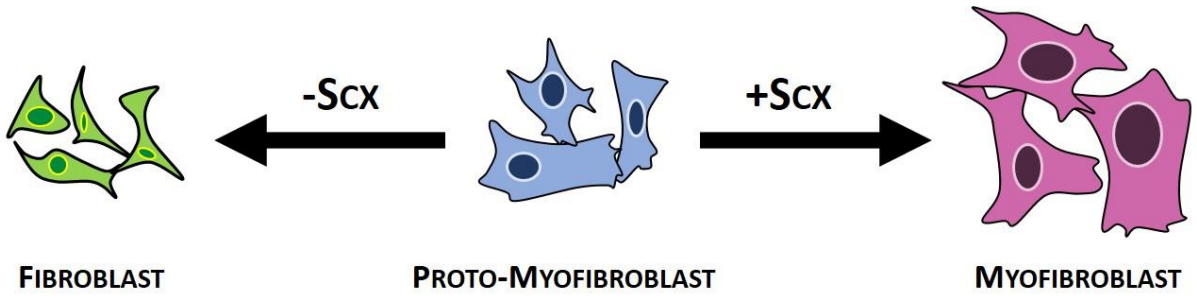


Figure 17: Proposed model of Scleraxis regulation of the cardiac proto-myofibroblast phenotype. We propose that Scleraxis expression promotes conversion of proto-myofibroblasts to myofibroblasts, and inhibition of Scleraxis promotes reversion of proto-myofibroblasts to fibroblasts. Though the role of Scleraxis *in vivo* remains yet to be elucidated, it may play a role in driving the development of myofibroblasts following myocardial injury, as well as the maintenance of the myofibroblast phenotype in cardiac fibrosis.

5.4: Effect of Transforming Growth Factor-β1 on Expression of the Human *SCLERAXIS* Promoter

Though renilla luciferase is used as an internal control for transfection efficiency in most luciferase assays, empty (i.e.: promoter-less) 'backbone' vectors are often used to demonstrate background levels of luciferase expression. However, responsiveness of the empty vector may be affected by various treatments applied to cells. In the case of pGL4.10, NIH-3T3 cells transfected with pGL4.10 display decreasing luciferase signal (normalized to renilla) in response to increasing TGFβ1 dose (Figure 16). Increasing TGFβ1 dose in NIH-3T3s transfected with pGL4.10-SCX1500 trends towards increased luciferase expression (normalized to renilla) (Figure 16). However, the changes in luciferase expression by pGL4.10-SCX150 in response to increasing TGFβ1 dose are modest and not statistically significant. When luciferase values for pGL4.10-SCX1500 are normalized to those of empty pGL4.10, however, the difference in luciferase activity becomes significant for doses of 5 and 10 ng/ml, compared to vehicle alone (0 ng/ml TGFβ1) (Figure 16). This agrees with our previous data demonstrating increased *Scx* expression in response to TGFβ1 treatment [441].

There are a few possible interpretations of this phenomenon. One is that TGFβ1 represses expression by pGL4.10 through one mechanism, mechanism "A", yet increases expression from pGL4.10-SCX1500 via the *SCX* promoter by another mechanism, mechanism "B". If this were the case, then it would appear TGFβ1 activates the *SCX* promoter in a dose-dependent manner. However, there also exists the possibility that TGFβ1 represses expression by pGL4.10 through mechanism "A", and by inserting the *SCX* promoter into this vector, we have abolished this mechanism, independently of any

function through the promoter itself. In this case, we would not conclude that TGFβ1 is activating the *SCX* promoter. Additionally, it is possible that transfection efficiency is altered by use of the pGL4.10 vector – by analysis of luciferase values, independently of renilla (data not shown); it appears as though TGFβ1 does not affect pGL4.10, but TGFβ1 increases expression of pGL4.10-SCX1500. However, when normalized to renilla, this effect is negated by differences in transfection efficiency. The same issue arises when examining the response of truncated promoter vectors pGL4.10-SCXΔ(+78/-829) and pGL4.10-SCXΔ(-670/-1525). When pGL4.10 expression values are not considered, the distal version of the promoter, pGL4.10-SCXΔ(+78/-829) does not appear to be affected by TGFβ1 treatment, but if normalized to pGL4.10 values, luciferase expression by the distal promoter significantly increases (Figure 16). Similarly, raw luciferase values (normalized to renilla) for the proximal promoter vector, pGL4.10-SCXΔ(-670/-1525) are decreased with TGFβ1 treatment, but appear unaffected, or even slightly increased, when these values are normalized to those of empty pGL4.10 (Figure 16). Thus, although the effect of TGFβ1 on the *SCX* promoter cannot be clearly elucidated through these data, it does appear that TGFβ1 does affect activation of the 1500 bp human *SCX* promoter, and may differentially affect various regions (i.e.: proximal vs. distal). Repeating these experiments in the context of a different empty vector, or in the presence of inhibitors of the TGFβ1 signaling pathway (such as Smad7), may shed light on the true nature of the relationship between TGFβ1 and the 1500 bp *SCX* promoter.

6: CONCLUSION

In summary, we have demonstrated the following:

- Cyclic stretch increases levels of Scx in both cardiac proto-myofibroblasts and myofibroblasts, and drives conversion of proto-myofibroblasts to myofibroblasts
- *Scx* overexpression increases proto-myofibroblast collagen synthesis and myofibroblast marker expression
- *Scx* expression is sufficient to induce contraction and is required for TGFβ1-induced contraction in cardiac proto-myofibroblasts
- The DNA binding domain-deficient Scx mutant is capable of inducing contraction in cardiac proto-myofibroblasts, but not fully converted myofibroblasts
- *Scx* expression induces contraction in cardiac myofibroblasts in a DNA binding-dependent manner
- *Scx* inhibits cardiac proto-myofibroblast migration, and knockdown of *Scx* increases migration
- Cyclic stretch activates the proximal 1500 bp human *SCX* promoter
- The 1500 bp human *SCX* promoter responds to TGFβ1 treatment, which is altered by its truncation either in the proximal or distal regions

The results shown here provide evidence sufficient to conclude that the bHLH transcription factor Scleraxis is indeed a novel mechanoresponsive regulator of the cardiac myofibroblast phenotype *in vitro*, through regulation of key myofibroblast markers and cellular functions.

7: SIGNIFICANCE & FUTURE DIRECTIONS

Adverse cardiac remodeling occurs in response to nearly all cardiovascular diseases, and progressively worsens over time. Although cardiac fibrosis is a pretext for heart failure, there remain no treatments that directly target the root cause of fibrosis - the function and persistence of myofibroblasts. In contrast, the majority of treatments are aimed at assuaging symptoms associated with the underlying disease (such as hypertension) [501]. There is evidence that treatment of patients with hypertensive heart disease with the angiotensin-converting enzyme (ACE) inhibitor lisinopril modestly regresses cardiac fibrosis independently of changes in blood pressure and left ventricular hypertrophy [502], suggesting there may be a direct effect on myofibroblast function. Other ACE inhibitors, such as enalapril, have also been shown to regress cardiac fibrosis in rats [503-506], and a clinical trial in hypertensive patients with LV hypertrophy has shown mild regression of cardiac fibrosis with enalapril treatment [507]. Other common drugs utilized in the treatment of cardiovascular disease have shown promising effects on cardiac fibrosis, yet there remains no standard regime for reducing cardiac fibrosis in heart failure patients, and treatment is limited to end-of-life care or transplant [508]. By understanding the factors that regulate the myofibroblast phenotype, we may discover novel, and highly specific, regulators of this phenotype that can be pharmacologically targeted to develop clinically relevant treatments for the inhibition and/or reversal of the myofibroblast phenotype. As the functions of the myofibroblast are critical to proper wound healing following cardiac injury, the timing of treatment must be carefully determined to allow this process and prevent the induction of subsequent adverse cardiac remodeling. Though there have been treatments aimed at targeting the myofibroblast phenotype, many of these, such as inhibition of *TGFβ1* or *αSMA* expression, will undoubtedly affect other cell

types and tissues, due to the pleiotropic nature of these factors [508, 509]. Conversely, a cardiac-specific approach targeting *SCX* expression or activity may improve clinical outcomes in patients with cardiac fibrosis, in the absence of significant negative side effects. Although preliminary evidence from our lab indicates *Scx* is up regulated in response to other factors, such as CTGF and AngII (Bagchi and Czubryt, data not shown), there remains a paucity of information regarding the transcriptional mechanisms affecting *Scx* expression both *in vitro* and *in vivo*. Additionally, Scx appears to play an important role in heart valves, and the possibility exists that targeting *SCX* in the myocardium may negatively affect valve function. Further study is needed into the direct transcriptional mechanisms regulating *SCX*, and elucidation of potentially unwanted side effects that may arise from targeting this transcription factor. There also remains a number of unanswered questions regarding the role of Scx not only in myofibroblasts (such as its effect on proliferation, apoptosis/survival, etc.), but in other cardiac cells. Before proceeding to *in vivo* studies, it will be important to note whether changes in *SCX* expression affect cardiomyocytes and their response to stress, as well as smooth muscle cells – this kind of information will be crucial in predicting the response of the heart to changes in *SCX*, in both physiological and pathological settings.

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