The Basic Helix-Loop-Helix Transcription Factor Scleraxis Regulates Cardiac Fibroblast Collagen Gene Expression

by

Leon Masunde Espira

A Thesis submitted to the Faculty of Graduate Studies of

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List of Abbreviations

ACE-I	Angiotensin converting enzyme inhibitor
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
ANG-II	Angiotensin II
ARB	AT_1 receptor blockers
α-SMA	Alpha smooth muscle actin
bHLH	Basic helix-loop-helix
BMP	Bone morphogenetic protein
ChIP	Chromatin immunoprecipitation
COP	Coat proteins
CRP	C-reactive protein
DAPI	4',6-diamidino-2-phenylindole
DOCA	Deoxycorticosterone acetate
ECM	Extracellular matrix
EMSA	Electrophoretic gel mobility shift assays
EPHESUS	Eplerenone's neurohormonal efficacy and survival study trial
ER	Endoplasmic reticulum
ET-1	Endothelin-1
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPC	Golgi-to-plasma membrane carriers
IGF	Insulin-like growth factor
LV	Left ventricle

MI	Myocardial infarction
MMP	Matrix metalloproteinase
NFATc	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
P4-H	Prolyl 4-hydroxylase
PD	Pirfenidone
PDGF	Platelet-derived growth factor
PDI	Protein disulfide isomerase
PIIINP	Procollagen III N-terminal peptide
PINP	Procollagen-1 N-terminal propeptide
PPAR	Peroxisome proliferator-activated receptor
PTM	Post-translational modifications
RALES	Randomized Aldactone Evaluation Study
RAS	Renin-angiotensin system
RV	Right ventricle
SCF	Stem cell factor
SMemb	Smooth muscle embryonic isoform of myosin heavy chain
TGF-β1	Transforming growth factor-β1
TNF-α	Tumor necrosis factor-α
VTC	Vesicular tubular clusters

I Abstract

As the heart compensates for the increased workload placed on it following an insult such as a myocardial infarction, changes in gene expression patterns take place. Concomitantly, fibroblasts and myofibroblasts in the cardiac interstitium start laying down connective tissue e.g., fibrillar collagens. Cardiac function is eventually impaired due in part to an increase in myocardial stiffness that is caused by this elevated connective tissue deposition. Scleraxis is a bHLH transcription factor expressed during mouse embryonic development in areas of connective tissue formation such as tendons and cardiac valves, but its role in collagen synthesis remains unknown.

We investigated the regulation of collagen gene expression by scleraxis, and the expression patterns of scleraxis in cardiac fibroblasts and in the infarcted myocardium. Real-time PCR was used to demonstrate that treatment of rat cardiac myofibroblasts with TGF-β1, a known inducer of fibrosis, caused an up-regulation of scleraxis expression, and that transition of fibroblasts to a myofibroblast phenotype similarly resulted in increased scleraxis expression. The use of adenoviral gene delivery to over-express scleraxis in NIH3T3 fibroblasts led to a significant increase in collagen Iα2 gene expression. Examination of infarcted rat hearts showed a nearly four-fold increase in scleraxis expression in the infarct scar compared to non-infarcted tissue. The expression of fibrillar collagens Iα2 and IIIα1 increased in parallel with scleraxis in the infarcted ventricle, in contrast to the expression of non-fibrillar collagens IVα1 and VIα1 which increased throughout the infarcted heart.

Luciferase assays were also performed in COS7 cells transiently transfected with the human full-length *COLIA2* promoter fused to a luciferase reporter transgene with or

without scleraxis, scleraxis deletion mutants lacking the basic or HLH regions, or the E-box proteins E12 and E47. Transcription of the COLIA2 promoter was directly up-regulated by scleraxis. E12 and E47 did not transactivate the promoter as efficiently. Augmentation of E12 and E47 with scleraxis led to an even stronger transactivation of the promoter. Furthermore, co-transfection with the HLH repressor protein Id2 (which lacks a DNA binding domain) caused a dose-dependent decrease in promoter transactivation compared to scleraxis alone. The scleraxis HLH deletion mutant had a diminished ability to transactivate the promoter, while the basic deletion mutant was completely inactive. The basic deletion mutant of scleraxis also attenuated the transactivation of the promoter by scleraxis, E12 and E47.

The regulation of collagen gene expression in the post-infarct myocardium may provide a mechanism to control the degree of fibrosis, thereby reducing associated morbidity and mortality. The data presented here indicates that scleraxis plays a novel and previously unappreciated role in the regulation of fibrotic gene expression in the heart, and supports the further study of scleraxis as a promising new target for the development of novel anti-fibrotic therapies.

II Introduction

Health Canada lists cardiovascular disease not only as the number one cause of mortality but also as the costliest disease to treat in Canada[1]. Cardiovascular conditions can occur independently, for example following a myocardial infarction (MI), or may result from a chronic condition such as hypertension. However all cardiovascular conditions result in the heart undergoing a number of changes – both at the structural level and at the molecular level – which will eventually interfere with the heart muscle's ability to pump blood to the body's tissues.

One change that the heart undergoes following an insult is the laying down of excess extracellular matrix (ECM) components, a process known as cardiac fibrosis. This process is primarily mediated by cardiac fibroblasts and myofibroblasts, which are normally responsible for laying down and maintaining the heart's ultrastructure[1]. In the clinical context of myocardial damage, one of the key points is the transition of a quiescent cardiac fibroblast primarily responsible for ECM homeostasis to its hypersecretory phenotypic derivative, the cardiac myofibroblast[2]. In the initial post-infarct stages, the synthesis of collagen and other matrix proteins in and around the point of injury is beneficial since it helps stabilize the infarcted area and restores cardiac function[3]. This initial reaction is mediated by a series of cell-to-cell signaling events that lead to the migration of inflammatory and other cell types to the point of injury as well as the creation of a new network of capillaries[3].

As the fibrotic response progresses, the excess layers of ECM material being laid down by the cardiac myofibroblasts begin to interfere with global cardiac function by

[1] http://www.hc-sc.gc.ca/dc-ma/heart-coeur/index-eng.php

stiffening the cardiac myocardium. This puts the myocardium under further stress, exacerbating the condition further, eventually resulting in heart failure.

The importance of the ECM in cardiac function cannot be overstated. The cardiac ECM, far from being merely a static support structure for the heart, is now recognized to play central roles in cardiac development, morphology and cell signaling. Recent studies have better shaped our understanding of the tremendous complexity of this active and dynamic network. In a recent groundbreaking study, Taylor's group used the decellularized cardiac matrix to bio-engineer a heart by repopulating it with neonatal cardiac and endothelial cells[4]. The matrix actively guided cell alignment, enabling the formation of a contractile myocardium capable of stroke work. This work demonstrated that the cardiac ECM clearly plays a significant role in cell migration, maturation and cell-to-cell signaling, and ultimately in dictating the spatial arrangement of constituent cardiac cells to form a functional myocardium. These findings reinforce the idea that the ECM plays an active role establishing cardiac architecture. Therefore an understanding of the ECM and the fibrotic response go hand in hand. By better understanding the exact role and transcriptional control of the ECM macromolecules laid down in the fibrotic response, better anti-fibrotic therapies can be developed.

In a mouse model of acute cardiac failure, we observed that the expression basic helix-loop-helix (bHLH) transcription factor scleraxis was elevated a few days prior to the up-regulation of collagen genes. Scleraxis transcripts are first detected in mouse embryos between day 9.5 and 10.5 post coitum and it is highly expressed in the diaphragm, heart, chest wall and heart valves when cartilage and connective tissues are laid down[5]. Scleraxis mediates tendon development and as such is a marker of tendon

development[6]. To date only a few scleraxis gene targets have been identified. Scleraxis over-expression in the Ros 17/2.8 osteosarcoma cell line led to the increased expression of the cartilage markers aggrecan and type II collagen, and to the suppression of osteoblast markers – type I collagen and alkaline phosphatase[7]. Scleraxis has also been shown to act in tandem with Nuclear Factor of Activated T-cells c (NFATc) to activate the *colIα1* gene in tendon fibroblasts, and is involved in the regulation and maintenance of Sertoli cell function in the testis[8,9].

What role scleraxis may have in regulating cardiac function is not yet known. Although scleraxis is expressed in the developing myocardium, its expression and function in the adult myocardium is unknown. Transforming Growth Factor-β1 (TGF-β1), a well-characterized inducer of fibrosis, enhances the expression of scleraxis in osteoblasts[10]. More recently, it has also been demonstrated that during the patterning process of the proximal process of the murine dentary, scleraxis is co-expressed with the TGF-β type II receptor[11]. The study concludes that TGF-β signaling induces the expression of *Scleraxis* and that the inhibition of TGF-β signaling results in the loss of endogenous *Scleraxis* expression. Considering the central role that TGF-β signaling plays in cardiac fibrosis, it is likely that by acting either in tandem or by being a part of this signaling cascade, scleraxis is also involved in the regulation of cardiac fibrosis.

This thesis focuses on the transcriptional regulation of collagen genes by scleraxis. Reporter assays were also used to investigate the ability of scleraxis to transactivate the *COLIA2* promoter. Real-time PCR was used to examine the effect of TGF-β1 treatment on scleraxis expression in cardiac myofibroblasts.

Our data shows that scleraxis is able to transactivate the collagen 1α2 promoter, both independently and has its effect is augmented when co-transfected with E47, another bHLH transcription factor. Over-expression of scleraxis in NIH-3T3 cells, resulted in a nearly 10-fold increase in collagen 1α2 mRNA levels. We have also shown that treatment of cardiac myofibroblasts with TGF-β1 led to an elevation in scleraxis expression both at the mRNA level and protein level.

III Statement of Hypothesis.

Based on our observations in the mouse model of acute cardiac failure, we hypothesized that **scleraxis is a key regulator of cardiac myofibroblast collagen gene expression.**

IV Literature Review.

1.0 Heart Disease in Canada.

Heart disease is the number one cause of mortality in North America. According to the Heart and Stroke Foundation of Canada, heart disease accounted for roughly 32% of all deaths disease due to disease[II]. In addition, heart disease costs the Canadian economy about 18 billion dollars annually in lost wages, lost productivity, hospital costs and physician visits [III].

Given current demographic trends – a rapidly ageing population and declining birthrate – heart disease will continue to impose a major financial burden on both the Canadian economy and health care system. Finding new treatment methods and improving patient care are therefore both of paramount importance. This will not only reduce the burden on the health care system but should also provide tangible economic benefits.

1.1 Underlying Causes of Heart Disease.

Heart disease is the end result of a number of primary or secondary causes including the hereditary and idiopathic cardiomyopathies as well as of hypertension, coronary artery disease, alcohol abuse and other insults[12]. Whatever the cause, the end result is that the heart is unable to meet the body's metabolic demands. As the heart decompensates and progresses towards end-stage cardiac failure, it begins to remodel.

[II] http://www.heartandstroke.com/site/c.ikIQLcMWJtE/b.3483991/k.34A8/Statistics.htm
[III] http://www.heartandstroke.com/site/c.ikIQLcMWJtE/b.3483991/k.34A8/Statistics.htm

One of the hallmarks of end-stage cardiac failure is the deposition of excess ECM, especially collagen. A better understanding of cardiac myofibroblast biology, the key cell in the process of ECM deposition, would enable us to better understand cardiac collagen production, both at the molecular and protein levels.

1.2 The Post-Infarct Myocardium.

An MI results when the blood supply to the heart is insufficient to meet the heart's oxygen and nutrient needs (ischemia). The ischemic conditions during an MI cause tissue damage that results in the death of cardiomyocytes and the initiation of the wound healing response. The two principal cardiomyocyte death pathways are apoptosis and necrosis. Apoptosis is the predominant form of death immediately after and up to 8 hours following the insult[13]. However, necrosis peaks at between 12 hours to 4 days following the MI[13]. The death of cardiomyocytes results in the release of markers such as creatine kinase, troponin-T, fatty acid binding protein and serum glutamic-oxaloacetic transaminase into the bloodstream[14].

The second phase is an immune response characterized by the inflammatory response and activation of the complement system[14]. Several cytokines such as interleukins IL-6 and IL-8 are released within 12-16 hours of ischemia occuring[14]. There is also a phenotypic switch of monocytes to macrophages at the area of damage[15]. Granulocytes, lymphocytes and plasma cells also migrate into the damaged area and begin the removal of dead myocytes[14]. Granulocytes begin migrating into the infarcted

area as early as 6 hours following the MI, with peak numbers observed about 24-48 hours post-MI[14].

The onset of the third phase of wound healing is marked by the deposition of new ECM proteins, first in the border zone, between the infarcted and non-infarcted tissue and later within the infarcted tissue itself[14]. This third phase begins two to three days after the infarction and results in the formation of granulation tissue, which increases the tensile strength of the infarct and prevents cardiac rupture[14]. The first of the ECM components to be deposited is fibrin, followed by fibronectin and tenascin[16]. Within a few days, myofibroblasts surround the infarcted area and start depositing various fibrillar collagens including types I and III, with complete cross linking occurring a few weeks later. [14]

Collagen deposition is also accompanied by an increase in collagen breakdown in the early phases of wound repair in both the infarct zone and in the surrounding cardiac tissue. Collagen degradation can be detected as early as 40 minutes post-MI[17]. However post-MI collagenolytic activity is restricted to the infarcted area and not the surrounding healthy myocardium[14]. The final phase of cardiac wound healing is preceded by the formation of granulation tissue characterized as a cell-rich tissue containing partially cross-linked collagens, macrophages, blood vessels and myofibroblasts [14]. Granulation tissue is also characterized by neovascularization. In rats for example, within 1 week after infarction, basal coronary flow has been normalized and after 35 days coronary flow at maximal dilatation was almost normalized for the left ventricle[14].

In the final phase of cardiac wound healing, all cells except myofibroblasts disappear, probably through apopotosis[18]. The scar collagen also becomes completely

cross-linked and the resulting scar assumes a permanent nature because of the lack of cardiomyocyte regeneration[14].

Following an insult, changes occur in myocardial structure and function that initially augment cardiac performance, but which over the long term may have a detrimental effect on cardiac function. Collectively these changes are termed myocardial remodeling[12]. In the cardiac myocardium, the myocytes undergo hypertrophy accompanied by disorganization, as well as an increase in wall thickness, which eventually results in wall thinning and dilation due to accompanying apoptosis and necrosis. Concomitantly, changes in cardiac fibroblasts also take place. The fibroblasts actively proliferate and there is increased transformation of quiescent fibroblasts to the hypersecretory myofibroblasts with the accompanying net accumulation of ECM resulting in fibrosis[3]. This fibrosis is initially reparative by replacing areas of myocyte loss with scar tissue. However reactive fibrosis may also occur, marked by diffuse deposition of ECM distal to the point of injury[19].

Functionally, fibrosis has a number of critical consequences for the heart. Increased ECM content results in an increase in mechanical stiffness contributing to diastolic dysfunction[2]. Second, the increased collagen content disrupts the electrical conductivity between myocytes and acts as a substrate for reentrant arrythmogenesis[2]. Third, perivascular fibrosis impairs myocyte oxygen availability, reduces coronary reserve and exacerbates myocyte ischemia by surrounding the intracoronary arterioles[2].

1.3 The Cardiac Fibroblast.

One of the key cells involved in the cardiac remodeling process is the cardiac fibroblast. Cardiac fibroblasts comprise 90% of the non-myocyte cells in the heart and represent two-thirds of the total cells in the heart by number[1]. In the healthy heart, fibroblasts are primarily responsible for the maintenance of ECM homeostasis. They lay down the basement membrane that surrounds the cardiomyocytes[20]. The ECM is organized as a complex three-dimensional mesh of collagen fibers, in which the cardiomyocytes and blood vessels are embedded, providing the heart's ultrastructure[1].

Cardiac fibroblasts are derived from a specific spatiotemporal locus in the developing embryo and during the formation of the primitive embryonic cardiac tube. Extracardiac cells from this locus migrate onto the external surface of the heart. Here these extracardiac cells undergo an epithelial-to-mesenchymal transition (EMT) and invade the developing heart to form the coronary vasculature and cardiac fibroblasts[19]. The final differentiation into cardiac fibroblasts is regulated by programmed sequences of growth factors such as fibroblast growth factor (FGF) and platelet derived growth factor (PDGF)[21].

In response to cardiac injury, cardiac fibroblasts undergo a phenotypic conversion to myofibroblasts as part of the wound healing response. Cardiac myofibroblasts are hypersecretory derivatives of fibroblasts and are the primary mediators of fibrosis in the post-insult heart. These myofibroblasts are phenotypically distinct from fibroblasts in that they express contractile proteins such as α-smooth muscle actin (α-SMA), vimentin and desmin along with significant amounts of fibrillar collagens[22]. However the key distinguishing characteristic from fibroblasts is that only myofibroblasts express the

smooth muscle embryonic isoform of myosin heavy chain (SMemb)[23]. A number of cytokines are known to play key steps in the fibroblast-to-myofibroblast transition including PDGF, TGF-β1 and stem cell factor (SCF)[24 22].

Myofibroblasts are particularly important in the initial wound healing steps when they help maintain the structural integrity of the scar. This ability to help maintain the scar's integrity is believed to be mainly due to the high amounts of α-SMA that myofibroblasts express, thereby helping to contract the wound[25]. Myofibroblast apoptosis over time results in the transition from a granulomatous scar to a mature scar[26]. However, cardiac myofibroblasts are known to persist for years in mature infarct scars, causing further ECM deposition, ultimately with detrimental effects on cardiac function[3]. The activation of myofibroblasts distal to the site of infarct further compounds the negative remodeling process by inducing collagen synthesis and deposition in otherwise healthy areas.

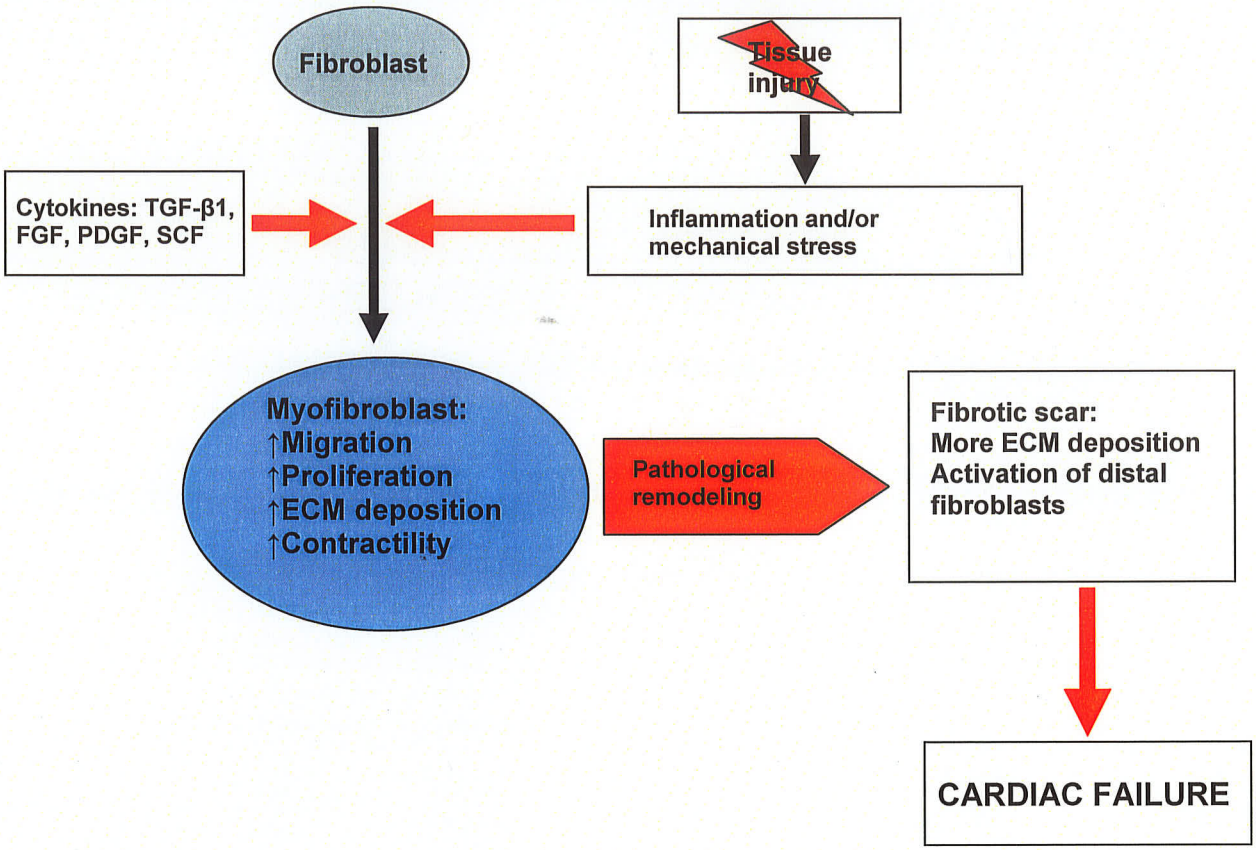

Figure 1. Cardiac remodeling and myofibroblast activation in the post-MI myocardium. Tissue injury or mechanical stress together with cytokine action causes the fibroblast-to-myofibroblast phenotype switch. The presence of myofibroblasts leads to the formation of a fibrotic scar with a concomitant decrease in cardiac function resulting in cardiac failure.

1.4 Clinical Treatment of Fibrosis

The renin-angiotensin system (RAS), through the production and action of angiotensin II (AngII) and its receptors, plays a key role in the compensatory neurohormonal response to myocardial injury. The myocardium contains an endogenous RAS independent of the renovascular RAS[19]. Stimulation with AngII causes cardiac fibroblast proliferation and net accumulation of fibrillar collagen *in vitro* and cardiac fibrosis *in vivo*. These responses are transduced by the AT_1 receptors, whose expression in fibroblasts exceeds its expression in cardiomyocytes[27]. AngII effects are also augmented by the up-regulation of other fibrogenic growth factors like endothelin- 1 (ET-1), TGF-β1 and aldosterone[19].

Large clinical trials have shown that angiotensin-converting enzyme inhibiors (ACE-I) reduce morbidity and mortality, slow progression of established heart failure as well as reduce the risk of cardiovascular events in patients with symptoms of cardiac failure[28 29]. The benefits of ACE-I therapy on cardiac fibrosis and cardiac performance have been shown in limited populations of hypertensive patients. Brilla *et al.* found that six months of treatment with the ACE-I lisinopril reduced cardiac fibrosis and improved left ventricular diastolic function[30]. Schwartzkopff *et al.* reported that treatment of hypertensive patients with the ACE-I perindopril over a period of 12 months caused a significant regression of periarteriolar fibrosis and an improvement in coronary blood flow reserve[31].

Better understanding of the molecular pharmacology of the AT_1 receptor has led to the development of AT_1 receptor blockers (ARB) as an effective alternative to ACE inhibitors. ARBs appear to offer clinical benefits similar to those of ACE-I in heart

failure[32 33]. The antifibrotic actions of the ARB losartan have been examined in two small studies in patients with hypertensive heart disease. 12 months of losartan therapy reduced cardiac fibrosis and serum collagen markers, while in a second series of patients, losartan treatment had the selective benefit of reducing collagen deposition and LV stiffness in more severely fibrotic patients than in patients with non-severe fibrosis[34 35]. However the specific mechanisms of action are not yet clear and whether the collagen deposition was reduced as a result of drug therapy or because of improvements with the patients overall cardiovascular parameters is not yet clear.

The mineralocorticoid aldosterone is also strongly implicated in the fibrogenic response of the myocardium on stimulation with AngII or via AngII-independent mechanisms[36]. In animal models subjected to sodium overload, aldosterone infusion resulted in diffuse fibrosis of the left and right ventricles combined with focal replacement fibrosis. This fibrosis was independent of blood pressure elevation and was reversed by the aldosterone receptor antagonist spironolactone[36]. The Randomized Aldactone Evaluation Study (RALES) clinical trial backed this data up by demonstrating that treatment with spironolactone in heart failure patients receiving an ACE inhibitor resulted in reduced morbidity [37]. These findings were confirmed and extended in the large-scale EPlerenone's neuroHormonal Efficacy and SUrvival Study trial (EPHESUS) of the selective mineralocorticoid antagonist eplerenone in post-MI patients[19]. A sub-study arising from the RALES trial demonstrated that the serum collagen III metabolic marker procollagen III N-terminal peptide (PIIINP) predicts cardiovascular risk and spironolactone therapy normalizes ECM metabolism and the progression of left ventricular dilation[38]. However only patients whose baseline PIIINP levels were above

the median responded to spironolactone therapy as measured by improvement in event free survival.

Endothelin-1 (ET-1) regulates function in cardiac fibroblasts *in vitro* and *in vivo*. Cardiac fibroblasts express a mixed population of ET_A and ET_B receptor subtypes[19]. ET-1 increases cell proliferation, matrix metalloproteinase (MMP) activity and net collagen synthesis in cultured cardiac fibroblasts[39]. AngII-elicited increases in ECM metabolism in cultured fibroblasts are blunted by an ET_A receptor antagonist[40]. ET-1 is also up-regulated in failing left ventricles of human heart failure patients and elevations in serum endothelin concentrations correlate with the severity of heart failure[19]. However following acute MI and in chronic heart failure, ET-1 antagonists have failed to show any benefits[19].

The proven clinical benefit of statins as cholesterol lowering drugs in atherosclerotic disease has been complemented by the finding that they exert pleiotropic effects on a number of cellular signaling pathways. Treatment with statins reduces myocardial remodeling, fibrosis and collagen synthesis in models of myocardial injury[41]. Statins were also found to exert concordant anti-fibrotic and anti-inflammatory actions. Statins may act on cardiac fibroblasts to attenuate inflammatory signaling through reduced prenylation of small GTPases. In this regard, elevated serum concentrations of the inflammatory marker C-reactive protein (CRP) were shown to predict the likelihood of nonfatal MI or fatal coronary events following the initial infarct. Treatment with pravastatin normalized serum concentrations of CRP and reduced the risks of cardiac events[42].

Pro-inflammatory and pro-fibrotic factors play central roles in coordinating the activities of multiple cell types in the injured and failing myocardium. As such these agents provide promising targets for therapeutic development. However, the redundancy and complexity of cytokine signaling pathways has posed a challenge for drug development. Initial drug discovery efforts focused on tumor necrosis factor-α (TNF-α)[43]. Strong experimental evidence indicates an important role for this cytokine in the initial activation of MMPs requisite to myocardial remodeling following an acute MI and the progression to heart failure[43]. However sequestration of TNF-α was ineffective in ameliorating or regressing symptomatic heart failure in a large scale clinical trial[19 43].

Considering the importance of TGF-β as a ubiquitous controller of fibrosis, efforts have been made to intervene directly in the production and activation of this cytokine. Inhibition of TGF-β with neutralizing antibodies, soluble TGF-β receptor: antibody chimeras, or adenoviral-mediated gene transfer of decorin, a TGF-β binding protein, reduce fibrosis in rodent models of pressure overload cardiac hypertrophy, bleomycin-induced pulmonary fibrosis or experimental glomerulonephritis[44 45]. However, systemic inhibition of TGF-β could lead to adverse side effects owing to the pleiotropic actions of this growth factor. Additional strategies to inhibit TGF-β have targeted its interaction with binding partners in the ECM, including latent the TGF-β binding protein or the latency activated peptide in order to the restrict therapeutic modulation of TGF-β to a specific tissue or physiological context[19 46] .

Drugs that act on the enzymatic steps of ECM metabolism present clear-cut therapeutic opportunities to modulate myocardial remodeling by intersecting key functions of the cardiac fibroblast. Extensive research has focused on MMP inhibitors in

the context of pathological remodeling. The rationale to develop MMP inhibitors is based on the demonstrated involvement of MMPs in at least three major aspects of myocardial injury and failure[47 48]. First, the activation of MMPs underlies myocyte slippage, ventricular wall thinning and chamber dilation following an acute MI. Second, chronic MMP activation contributes to the aberrant remodeling of the ECM during the progression to chronic failure. Third, excess MMP activation is a key contributor to instability and rupture of atherosclerotic plaques [19].

Animal studies have investigated the effects of MMP inhibition with pharmacological agents or by gene deletion in transgenic animals in models of cardiac injury and failure. Rohde *et al.* showed that a nonselective pharmacological MMP inhibitor (CP-471 474) reduced left ventricular dilation four days following surgical infarction in mice[49]. However follow up at later time intervals (15 days) post-MI in MMP-9 deficient mice showed defective wound healing with diminished collagen accumulation in the infarct zone and decreased infiltration of macrophages compared to the wild type[50].

The effect of MMP inhibitors on the progression of heart failure has also been examined. Pacing-induced supraventricular tachycardia for three weeks in pigs produces congestive heart failure characterized by left ventricular dilation, increased activities of MMP-1, -2, and -3 and decreased collagen content. Pathological remodeling was attenuated and cardiac function was preserved by treatment with nonselective MMP inhibitor, PD-166 793 [51]. Similar results were obtained with a newer generation MMP inhibitor PGE 7113313 which designed not to inhibit MMP-1, since it is down regulated in chronic human heart failure[52]. It should however be noted that the inhibitors were

administered prior to and throughout the duration of the pathological stimulus rather than after the establishment of congestive heart failure. Preliminary studies in humans lend further support for MMPs as targets in heart disease. A Phase II trial has been completed to test the effect of MMP inhibitor PG 116800 to prevent adverse cardiac remodeling following a first myocardial infarction[19]. The tetracycline derivative Periostat (doxycycline) is the only MMP inhibitor currently approved for clinical use, but its application is limited to periodontal use. Treatment of coronary heart disease patients with Periostat reduced serum inflammatory markers (CRP, IL-1 and IL-6) as well as circulating concentrations of MMP-9[53].

A number of novel antifibrotic/ anti-inflammatory agents are also being explored. One of these is Pirfenidone (PD) 5-methly-phenyl-2(1H) pyridine, which exerts protective actions in animal models of tissue injury and fibrosis. It appears to inhibit fibroblast proliferation and collagen synthesis, potentially through the disruption of TGF-β1 expression and is currently in Phase II trials for idiopathic pulmonary fibrosis and renal tubulointerstitial fibrosis[54 55 56].

Tranilast [N(3,4-dimethoxy cinnamoyl)-anthranilic acid] is a second agent that has shown very promising anti-fibrotic and anti-inflammatory characteristics in experimental studies and early phase clinical trials. Based on its ability to inhibit smooth muscle cell migration and proliferation, this agent has been targeted for therapy to prevent coronary vascular restenosis following angioplasty [57]. Recent studies in the deoxycorticosterone acetate (DOCA)-salt hypertensive rat model show that tranilast blocks myocardial fibrosis and suppresses inflammatory cell inflitration[19]. Like PD, tranilast antagonizes the production and activity of TGF-β[19].

Attention has also recently focused on ligands of the peroxisome proliferator-activated receptors (PPARs) PPARα and PPARγ for their actions on the myocardium. PPARs are nuclear receptors that regulate lipid storage and metabolism and are expressed by multiple cell types in the cardiovascular system, including cardiac myocytes and fibroblasts[19]. PPAR receptors suppress production of inflammatory cytokines, cellular adhesion proteins and chemotactic peptides by inhibiting the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). PPARα and PPARγ ligands are cardioprotective in experimental infarction and prevent interstitial fibrosis, preserve diastolic function and inhibit inflammatory activation in pressure overload cardiac hypertrophy[58 59].

It is still however unclear whether the beneficial of effects of anti-fibrotic therapy are as a result of the inhibition of the fibrotic mechanism or is the reduction of fibrosis a secondary effect resulting from the alleviation of a primary defect like hypertension.

2.0 bHLH Transcription Factors

The transcription of genes in eukaryotic cells is controlled by complex interactions between transcription factors and their specific DNA recognition sequences in target genes. These transcription factors can be divided into a number of groups based on shared sequence and similar structural motifs.

One such motif is the helix-loop-helix structure (HLH), characterized by two α helices connected by a loop[60]. It was first identified in an immunoglobulin enhancer-binding polypeptide as well as in a number of proteins known or suspected to be

transcription factors[60]. The HLH motif is a dimerization domain that mediates homo- and/or hetero-dimerization with other HLH proteins, with adjacent basic amino acid residues that facilitate DNA binding[61].

A related group comprising the bHLH proteins possesses an HLH domain that is adjacent to a short region of basic residues that constitute the actual DNA interaction interface[60]. A second group of bHLH proteins contains an additional dimerization domain – a leucine zipper immediately C-terminal to the HLH. This group is referred to as the bHLHZ proteins[60]. There is a third group of proteins that lack a functional DNA binding domain and therefore act as negative regulators of bHLH proteins[60]. In general one helix is smaller allowing for the loop's flexibility, thereby facilitating dimerization by folding and packing against another helix. The larger helix typically contains the DNA-binding regions[62]. bHLH proteins will typically bind to a DNA consensus sequence known as an E-box, whose sequence is CA*NN*TG where N represents any nucleotide[62].

2.1 Structure and Function of bHLH proteins

There are two broad functional groups into which bHLH proteins can be divided based on their expression patterns. In one group, expression is limited to cells of a particular lineage and these are referred to as class B. Examples of class B bHLH proteins include MyoD, myogenin and scleraxis[60]. The other group is known as class A and are expressed ubiquitously. These proteins, for example the E12 transcription factor, tend to form heterodimers with the class B proteins.[60]

Structurally bHLH proteins consist of a short stretch of hydrophilic residues (often basic) followed by a set of mainly hydrophobic residues located in two short segments (helix 1 and helix 2) which are separated by a non-conserved sequence of variable length (the loop)[63]. Apart from glycine and proline, there are certain other amino acids that are more likely to be present at the boundaries of the helices, referred to as N cap and C cap residues. The most common/preferred N cap residues are asparagine, serine and threonine and are often conserved at the beginning of the helices[60].

During dimerization all the conserved hydrophobic residues from helices 1 and 2 are buried within the core[60]. This results in a parallel left handed four-helix bundle with a stable hydrophobic core. Helix 1 packs against helix 2 of the same unit as well as against helix 2 of the opposing subunit[60]. Extensive van der Waals forces along with electrostatic interactions act to stabilize the four helix bundle[60]. Analysis of the 3 dimensional structures of E47 and MyoD suggests that the minimum length of the interhelical loop is at least four or five amino acids[64]. Most members of the bHLH family have loops in the range of five to twenty four amino acids. Consistent with this, shortening the eight amino acid loop of MyoD to four amino acids abolished DNA binding activity[64].

Since the functions of many bHLH proteins are dependent on hetero/homo-dimerization, a number of internal residues are thought to play an important role in the dimerization process. For example the E47 homodimer may be stabilized by two features. First, an additional turn in helix 1 increases the buried surface of the HLH four-helix bundle, providing for an extra salt bridge between the subunits[65]. Second, glutamine residues form hydrogen bonds[65]. This is a unique feature of E-proteins such as E12 and E47 and contributes to the marked stability of their homodimers as compared to other

bHLH proteins such as MyoD. There is also further evidence to suggest that residues in the loop and in helix 2 can affect the rate at which E47 homodimerizes or heterodimerizes[66].

2.2 DNA Binding by bHLH proteins

The basic domain of bHLH proteins is important for sequence specific DNA binding. Most bHLH proteins bind as dimers to the consensus sequence CA*NN*TG, where *N* represents any base, most commonly either CG or GC[60]. This sequence is known as an E-box. The specificity of the bHLH protein for the two central nucleotides in the consensus sequence depends on the identity of the amino acids close to a key conserved glutamic acid usually at position 32 [60]. The position of these residues relative to one another allows the bHLH homo-heterodimer to bend the DNA towards the minor groove, making several contacts with conserved nucleotides in the binding site[67 68]. The most important of these are the hydrogen bonds from the cytosine and adenine of the CA*NN*TG to the key glutamic acid. This glutamate residue is conserved in all bHLH proteins with known DNA binding activity. Substitution of this glutamate generally results in the abrogation of DNA binding activity[69]. The amino acid residue one helical turn to the C-terminus of the conserved glutamic acid is also an important determinant of the specificity for the 2 internal nucleotide of an E-box[60]. For example, dimers of bHLH proteins that have an arginine at this position prefer CG as the two internal bases (e.g. Myc and Max). In contrast proteins with smaller non-polar residues at this position prefer GC (e.g. MyoD and E12).

At present most of the information available on the molecular interactions of bHLH proteins is based largely on the crystal structures of Mac, USF, E47 and MyoD complexed to their respective DNA sites. These four structures share the overall conservation of the bHLH fold, and therefore it is valid to infer that other bHLH proteins behave in the same way. Moreover HLH proteins that do not bind DNA and act as negative regulators of bHLH proteins either do not posses any region with homology to the DNA binding α-helical basic region (e.g. Id and EMC) or contain a helix-destabilization proline residue (e.g. Hairy and Hes proteins)[60]. In contrast, the core hydrophobic residues essential for the formation of the four helix bundle are well conserved in Id and Emc, consistent with the fact that they are still able to form stable dimers with appropriate bHLH partners[60].

2.3 Nuclear Localization and Transactivation Domains of bHLH Proteins

As transcription factors, bHLH proteins must localize to the nucleus. Sequences within the basic domain appear to be sufficient to direct the protein to the nucleus. However, in some bHLH proteins additional sequences have been identified that direct nuclear localization[60]. The existence of two nuclear localization signals that can function independently appears to also be a common feature of bHLH proteins[60]. For example the nuclear import of murine MyoD is mediated by two short sequences present in basic helix 1. These sequences are conserved in all four myogenic bHLH proteins (MyoD, myogenin, Myf4, Myf5) regardless of species[60].

The main task of the transactivation domain is to engage the cell's transcriptional machinery. There are several generic classes of transactivation domains, for example stretches of acidic amino acids or glutamine-rich or serine/threonine rich regions[60]. Based on this, two transactivation domains have been mapped to a conserved region in E12/E47, HEB and Daughterless bHLH proteins that have the potential to form helical structures. Amino acid substitutions in conserved hydrophobic residues within these motifs abolish transactivational activity[70].

2.4 Dimerization Partners and Regulation of bHLH Activity

In general, dimerization between bHLH proteins is fairly specific. It can occur with one or several partners, giving rise to a large number of dimer combinations. However these interactions are not promiscuous and not all bHLH proteins can interact with one another. The specificity of interaction depends on the sequence and hence the three-dimensional structure of the α-helical regions[60].

As noted above, there also exists a distinct class of HLH proteins whose members act as negative regulators of other HLH proteins. These inhibitory proteins have one important feature in common: all lack a functional DNA binding domain[60]. For example Id1 and Id2 lack the basic domain adjacent to the HLH domain. On dimerizing with bHLH proteins like E12/E47, they attenuate their function by sequestering them into dimers that cannot bind DNA. The expression of Id proteins inversely correlates with cell differentiation and it is probable that Id proteins serve as general inhibitors of cellular differentiation[71 72]. In nearly all eukaryotic tissues cell differentiation is associated with

decreased proliferation. Consistent with this, Id proteins (Id1, Id2 and Id3) are rapidly induced by serum stimulation of quiescent human and murine fibroblasts and entry into S-phase is delayed or inhibited by blocking *Id* expression[73 74]. There are however differences in expression patterns of the various Id proteins. Id1 and Id3 are both widely expressed, while Id2 expression is highest during neural development; Id4 expression is up-regulated during murine embryogenesis and is highest in the brain, testis and kidney of adult mice[75 76 77].

There exist a number of mechanisms by which bHLH function can be modulated. The first, is that many bHLH proteins dimerize with more than one partner (including themselves) and the identity of the dimer partner can influence both the DNA-binding specificity and the activity of the bHLH partner[60]. Second, the phosphorylation state of the bHLH protein modulates its affinity for DNA binding and the activity of the activation/repression domain of some dimers[60]. There is also evidence suggesting that the activity of some bHLH partners depends on their ability to recruit accessory partners[60]. This means that the availability of differing monomer subunits within a cell is a major factor in determining which particular types of heterodimer, each with its own particular complement of activities and specificities, will form. As a result, strict spatial and temporal expression of many bHLH proteins is observed during myogenesis, neurogenesis and hematopoiesis.

2.5 Scleraxis

Scleraxis is a bHLH transcription factor that is ahighly specific marker for tendon and ligament progenitors and differentiating cells[7 78 79]. It was originally cloned in a yeast two-hybrid screen for interacting partners of the transcription factor E12[5]. It was also initially shown to specifically bind E-boxes and transactivate a reporter gene under the control of an artificial promoter containing four E-boxes[5]. The ability of scleraxis to bind to oligonucleotides containing E-boxes was augmented by E12. It has been demonstrated using artificial promoter constructs that another class A bHLH transcription factor, E47, also augments scleraxis' ability to bind to E-boxes[80]. However, since these experiments were performed using an artificial promoter, is still unclear whether their findings can be applied in intact cellular systems.

The first gene to be shown to be directly regulated by scleraxis was *aggrecan 1*[7]. It was noted that scleraxis was able to drive the expression of the *aggrecan* promoter with and without the addition of exogenous bHLH transcription factors such as E12 or E47. Since it was earlier shown that heterodimerization with other E-box factors augmented scleraxis activity, it appears that this effect is context specific depending on the cell type and on the E-box consensus sequence.

During murine development, scleraxis is widely expressed at the time of gastrulation around embryonic day 6.0, but thereafter itsr expression becomes restricted[81]. In the sclerotome compartment of the somites, from which the ribs and vertebrae arise, scleraxis expression can be detected as early as embryonic day 9.5[5]. High scleraxis expression is observed in a number of pre-skeletal mesenchymal cells prior to chondrogenesis, but during ossification scleraxis expression levels decrease. Notably,

cells that go on to form the ligaments, tendons and bronchial cartilage show high levels of scleraxis expression[5]. High levels of scleraxis are also noted throughout the pericardium[5]. Furthermore, it has been shown that scleraxis is expressed in the developing chordae tendinae proximal to the papillary muscles of embryonic chick hearts[82].

This data suggests that scleraxis is important in the differentiation and maturation of tissues rich in connective tissue such as tendons and cartilage[78]. Experimental data shows that scleraxis-positive cells form a distinct fourth compartment of the somite during embryogenesis called the "syndetome," separate from the myotome, dermatome and sclerotome regions[79,83]. The cells in this compartment give rise to tendons associated with the axial skeleton and arise spatially between muscle and cartilage progenitors. In fact, there is recent evidence to suggest that tendons and cartilage are alternate fates of a common cell progenitor[84]. Scleraxis is also expressed at the interface between muscles and skeletal primordial in 13.5 day old mouse embryos and then becomes largely restricted to tendons by 15.5 days[85].

In the cardiac context, scleraxis' role remains largely unknown, although it has been cloned from an embryonic heart gene expression library. Scleraxis expression has been detected during valvulogenesis and therefore appears to have a role to play in the development of cardiac valve structures as well as the chordae tendinae[82]. This is supported by a recent report that scleraxis null mice possessed a population of undifferentiated valve precursor cells that continued to express mesenchymal markers[86]. These mice also exhibited altered valve matrix composition including a dramatic increase in tendon-associated collagen type XIV, and presented with gross valve defects. These

observations reinforce the fact that scleraxis is involved in valve development and the ECM changes that occur as it progresses.

To date, several gene targets for scleraxis have been proposed. As mentioned, the first direct gene target for scleraxis to be identified was *aggerecan 1*, a major proteoglycan component of cartilage, which was up regulated in response to scleraxis over-expression in ROS17/2.8 osteoblastic osteosarcoma cells[7 87]. Scleraxis over-expression resulted in increased expression of cartilage markers like collagen II and osteopontin while leading to a decrease in the expression of osteoblast markers collagen I and alkaline phosphatase[7]. In heart valve development scleraxis expression correlated with the expression of collagen II and tenascin[82]. Similarly the differentiation of embryonic stem cells to a chrondrocyte phenotype is marked by the coordinated up-regulation of scleraxis, collagen IIb and aggrecan[88]. In a pluripotent tendon derived cell line, both scleraxis and collagen I were up-regulated, in contrast to what was observed in ROS17/2.8 cells[89]. In sertoli cells scleraxis regulates the expression of transferrin and androgen binding protein[9]. However, it is important to note that in virtually all of the above experiments, the mechanism by which scleraxis regulates these genes was not examined, thus it is unclear whether expression changes directly result from the actions of scleraxis. In contrast, it has recently been demonstrated that the *Col1α1* gene in rat tendon fibroblasts is directly regulated by scleraxis[8]. It was found that scleraxis bound to one of two short promoter elements preferentially as a heterodimer with E47. Collectively, these studies suggest that scleraxis regulates target genes in a context-specific manner, with scleraxis augmenting or attenuating gene expression depending on the cell type.

In an initial attempt to explore scleraxis function using knockout animals, embryos homozygous for a targeted Scx^{KO} allele died in the early stages of embryogenesis, precluding the ability to look at possible cardiovascular phenotypic effects[81]. However Schweitzer et al. produced a novel line of Scx^{KO} animals. They found that the initial attempt at knocking out scleraxis had failed due to the effect of a neomycin-resistance cassette selection marker on the expression of neighboring genes. The entire *Scx* gene is located in the third intron of block of proliferation 1 (*Bop1*), a house-keeping gene required for ribosome biogenesis[90]. It was therefore speculated that because the original Scx^{KO} allele introduced a Neo cassette, it interfered with the transcriptional levels and/or with the splicing of the *Bop1* gene[91]. Schweitzer confirmed this using a Flp/frt approach, demonstrating that leaving the Neo cassette in the mice resulted in embryonic lethality, while excision of Neo permitted the production of live scleraxis-null offspring.

These homozygous *Scx* -/- mice were viable but showed a dramatic disruption of tendon differentiation, that manifested in dorsal flexure of the forelimb paw, limited use of all paws, reduced functionality of the back muscles and the complete loss of the ability to move the tail. It was however noted that *Scx* loss did not affect all tendon categories equally. *Scx* loss was most pronounced in all force-transmitting and intermuscular tendons (though the effects varied in severity); muscle-anchoring tendons and ligaments were not affected. Tendon defects were first noted close to E13.5 in all tendons, coinciding with the condensation and differentiation of all tendon progenitors that result in emergence of discrete tendon morphologies. Scleraxis was also found to have additional roles in tendon formation as all the *Scx* -/- mice were found to have a tendon

matrix that was reduced and disorganized, and tendon cellular organization was disrupted[91].

Even though scleraxis function is not required for the specification of tendon tissues, scleraxis function is critical for the generation of complex tendon structures. The functional components of the mature tendon are highly organized bundles of collagen fibers that transmit forces between muscles and the skeleton[91]. Based on the phenotype seen in the *Scx -/-* animals, scleraxis function is likely related to the incorporation of tendon progenitors into discrete tendons[91]. However little is known about the molecular processes that enable the tenocytes to coordinate the secretion and organization of these matrix structures during tendon genesis[92]. Most tendons in *Scx -/-* mutants displayed a variety of defects. The *Scx -/-* mutants also showed an alteration in their ability to produce the tendon matrix, manifesting in a dramatic decrease in the number of collagen fibers and in their organization within the tendon matrix[91]. Concomitantly, a decrease in the number of tenocytes present as well as a partial loss of the network of cytoplasmic processes that enable the tenocytes to regulate the local secretion and synthesis of collagen fibers was observed[91]. Of particular interest to us was the fact that a loss of collagen I from the diaphragm tendon was observed. The Schweitzer study presented the first demonstration of a tendon differentiation phenotype as well as providing insight into what Scx function and targets may be.

3.0 Collagen

3.1 Introduction

Collagen is most abundant in animal tissues as very long fibrils with a characteristic axial periodic structure[93]. The fibrils provide a major biomechanical scaffold for cell attachment and anchorage for macromolecules allowing the shape of tissues to be defined and maintained[93]. Collagen is distinct from other proteins in that the molecule comprises three polypeptide α-chains which form a unique triple helical structure[93]. Collagen fibril synthesis is a self assembly process but is also sensitive to cell-mediated regulation, especially in young and healing tissues[93].

3.2 Types of Collagens

Twenty-nine collagens numbered I to XXIX have been reported in the literature, although the molecule named collagen XXIX is now thought to be a variant of collagen VI[94]. Their common characteristics include the following: 1) they are transmembrane or extracellular molecules; 2) they are formed by three chains named α chains. For a defined collagen type, one or several α chains can exist and they can assemble into homo or heterotrimers, leading to the production of collagens with various distinct functions; 3) they form a triple helical structure which is determined by a repetition of Gly-X-Y (where X and Y could represent any amino-acid, but X is often a proline and Y a 4-hydroxyproline) triplets in the primary sequence of each α chain[94]. Some of the collagens

have a unique stretch of Gly-X-Y while others have several which are interrupted by non-collagenous sequences[95]. Structural heterogeneities among various collagen types is even broader and includes the existence of alternatively spliced variants for some α chains, the possible formation of hybrid molecules resulting from the assembly of α chains from different collagen types and the addition of covalently-linked glycosaminoglycan chains to some [94].

Collagen molecules can assemble to form several suprastructures and based on this seven different subfamilies have been delineated. The quantitatively major one comprises collagens that are able to aggregate to form fibrils. This sub-family encompasses collagen I, II, III, V, XI, XXIV and XXVII [94]. The diameter and the length of the fibrils vary, but depending on the tissue they reach 500 nm and a few millimeters respectively[94]. To these fibrils, other collagens can be associated and are thus called the Fibril-associated collagens with interrupted triple helices (FACITS) which include collagens IX, XII, XIV, XVI, XIX, XX, XXI, XXII and XXVI [94]. Network forming collagens include collagens IV, VIII and X[94]. Collagen VI forms beaded filaments and collagen VII forms anchoring fibrils between the basal lamina and dermis[94]. Collagens XV and XVIII release an anti-angiogenic non-collagenous fragment called endostatin, while collagens XIII, XVII, XXIII and XXV are transmembrane proteins[94].

3.3 Procollagen Synthesis

When a collagen gene is transcribed, the mRNA is transported from the nucleus through the nuclear pore to a cystolic ribosome for translation into protein[96]. The

endoplasmic reticulum (ER) targeting sequence in the collagen mRNA acts as an attachment location for the ribosome to connect to the rough ER for translation and then translocation of the protein[97]. The collagen mRNA is translated into a procollagen monomer typified by a C-propeptide terminal region, a helical collagenous section and an N-propeptide terminal region[96]. The procollagen molecule is triple helical with each chain containing repeating glycine-X-Y triplets, where X and Y are usually proline and hydroxyproline respectively[98 99].

The procollagen undergoes many post-translational modifications (PTMs) before forming a procollagen triple helix. These modifications include glycosylation of some of the hydroxylysine residues with galactose or glucosylgalactose, and the hydroxylation of about 100 proline residues as well as 5-10 lysine residues to hydroxyproline and hydroxylysine[100 101]. In fact there is a direct relationship between the rate of PTMs and procollagen folding[99]. The PTMs begin as soon as the nascent procollagen chains pass into the cisternae of the rough ER and continue until the protein folds into a triple-helical conformation[101 99].

The C-propeptide region of the procollagen monomer plays an important role in protein folding by acting as a nucleation point[99]. The nucleation point is thought to be formed by a set of intra- and inter-chain disulfide bonds in the C-propetide region that are created by protein disulfide isomerase (PDI)[102 103]. PDI also functions as a chaperone during procollagen biosythesis as the β-subunit of prolyl 4-hydroxylase (P4-H) [99]. In the cellular context many molecular chaperones and enzymes such as PDI and P4-H assist the procollagen folding and trimerization in the ER leading to more efficient and faster folding[95].

3.4 Procollagen Transport

After the protein is folded, the procollagen molecules are transported from the rough ER to the Golgi apparatus[104]. The procollagen molecules are transported in the ER-to-Golgi intermediate compartments (ERGICs), commonly known as vesicular tubular clusters (VTCs), which bud directly from the ER membrane[105 106]. VTCs are characterized by a pleiomorphic shape, increased size compared with individual coated vesicles and the directional movement along microtubules towards the cis-Golgi[106 105]. Through experiments using GFP-tagged procollagen residues, it has been shown that the transport of VTCs requires the action of coat proteins (COP) I and II, although their exact roles in the transport from the ER to the Golgi apparatus has not yet been fully elucidated[107 108]. Typically COP I proteins are present on the VTC membranes and the trans-golgi network (TGN), whereas COP II proteins are localized to the ER membrane buds and vesicles[105]. Evidence available so far indicates that COP II is involved with the budding and transport of procollagen from the ER, while COP I might be involved in a step that occurs shortly after COP II-dependent export[108].

The Golgi apparatus mediates the organized lateral aggregation of procollagen into bundles[104]. The exceptionally long length of the procollagen molecules results in distensions of the Golgi cisternae[107 109]. The morphological changes of the Golgi dimensions during procollagen transport are thought to be caused by changes in the supramolecular organization of the procollagen aggregates[110]. The distortions are caused by the procollagen laterally aggregating into bundles as the cisternae slowly shrink from vesicle expulsion of the lipids from the Golgi membrane[110]. The decrease in lumen volume that follows results in a localized increase in procollagen concentration that

causes a lateral aggregation of the procollagen into tightly bundled parallel arrays. This results in a division of the cisternae between the two opposing bundles of procollagen, creating the secretory vacuoles known as Golgi-to-plasma membrane carriers (GPCs)[110] [109] [111].

3.5 Fibril Formation

There are two distinct models regarding the complex process of forming fibrils from the procollagen bundles released from the Golgi apparatus as GPCs. These are the Kadler and the Birk models, with the major difference being that in the Kadler model, cleavage of the C and N propeptides occurs intracellularly, whereas in the Birk model extracellular proteases cleave procollagen to form tropocollagen[99] [112].

The removal of the C and N propeptides is the last step of procollagen posttranslational modification and is essential for the assembly of collagen fibrils[95]. This cleavage results in a 10,000 fold decrease in the solubility of the collagen triple-helical region, resulting in a decrease in the critical concentration required for fibril assembly[113] [96] [114]. The cleavage of the N and C propeptides results in a rod like form of collagen, called tropocollagen, that can spontanaesously self assemble to form collagen fibrils during fibrillogenesis[115] [116].

Several of the zinc metalloproteinase members of the tolloid family have C-proteinase activity including bone morphogenetic protein (BMP) -1, mammalian tolloid (mTLD) and tolloid like (TLL)-1[114]. The three members of the "A Disintegrin and Metalloproteinase with ThromboSpondin motifs" (ADAMTS) family that have N-

proteinase activity are ADAMTS-2, ADAMTS-3 and ADAMTS-14[93 95 114 117]. The resulting tropocollagen protein is a triple-helical collagen fibril monomer ~300nm in length and 1.5nm in diameter[115 116]. Tropocollagen can spontaneously assemble into highly ordered, string like aggregates known as fibrils which have a characteristic banded pattern when negatively stained and viewed with transmission electron microscopy[95 118].

After the procollagen monomers have been postranslationally modified and folded into the triple helical conformation, the fibril formation process begins. It has been shown that collagen fibril formation begins after the procollagen bundles are extruded from the TGN into secretory vacuoles referred to as GPCs.[111] Typical non-collagen related vacuoles are ~50 to 100 nm in diameter and spherical [119 120]. GPCs on the other hand can range from 300 to 1700 nm in length, but are typically around 500 nm[121 111]. GPCs tend to have a tubular appearance due to aligned procollagen vacuoles[111]. Data obtained by visualizing GPCs in human fibroblasts using antibodies directed against C-propeptide of collagen I, implies that the cleavage of procollagen to the tropocollagen form has not yet occurred before the procollagen exits the Golgi[114].

Based on the Birk model of collagen synthesis, in which procollagen cleavage occurs extracellularly inside a fibril assembly channel, the collagen molecules aggregate extracellularly to form collagen fibril intermediates[112]. Collagen intermediates are semiflexible collagen structures of ~10-30 μm in length[122]. The collagen fibril intermediates form mature fibrils through a series of linear and lateral fusions. Early collagen fibrils are either unipolar or bipolar as designated by monomers in N to C alignment throughout the fibril, or a mid fibril reversal of alignment resulting in a fibril with two C or N terminals[93 123]. The fibril ends are composed of C-telopeptides or N-

telopeptides which are the remains of the cleaved C and N propeptides respectively[93]. Fibril diameter then starts to increase due to lateral fusions of the fibril intermediates[124].

As fibroblasts start synthesizing collagen, their plasma membrane becomes highly convoluted, especially in the early stages of collagen deposition[112 125]. Cross-sections of these areas show collagen fibrils embedded in narrow channels surrounded by cell membrane[112 111]. This causes a compartmentalization of the fibrils into groups of parallel fibers[99]. This is accompanied by an increase in collagen fibril diameter caused by lateral or end-to-end fusion of collagen fibrils[123 111]. This fusion of collagen fibrils may be termed as the second compartmentalization and is characterized by the combination of growing fibrils from multiple channels within a single fibroblast to form a collagen fiber bundle.[112 122 99]

The extracellular fusion of the collagen fibrils in the ECM can increase the length and diameter of the fiber bundles 10 fold[126]. The strength of the resulting extracellular collagenous matrix is determined by two major factors: the formation of intermolecular crosslinks within and between fibrils and the increase in the size of the collagen fibrils[99]. This process is defined as the third compartmental stage in which the growing collagen fiber bundles from two to three adjacent fibroblasts coalesce to form a collagen macroaggregate [112 99]. As the tissue matures, the compartments retract, allowing for further lateral tension of the collagen fibrils and the formation of larger and more mature collagen fibers[99 112].

3.6 Regulation of Collagen Gene Expression

Different well-characterized transcription factors and cytokines have been shown to interact with the promoters and upstream regulatory elements of various collagen genes and are implicated in basal as well as pathological collagen gene expression. This thesis will focus on the TGF-β pathway and the accompanying Smad family of transcription factors because TGF-β treatment is known to upregulate scleraxis expression in cardiac myofibroblasts.

Smad family members have been classified into three subgroups based on their structures and functions[127]. In the first sub-group, TGF-β receptor-dependent R-Smads like Smad2/3 contain N-terminal MH-1 domains, a linker region and C-terminal MH2 domains where the SSXS motif is known to be phosphorylated and activated by serine-threonine kinase receptors[127]. The second sub-group lacks the SSXS motif and includes the co-operative Smad co-Smad4[127]. The so-called anti-Smads like Smad6/7 constitute the third group (also called inhibitory Smads or I-Smads), which lack the MH1 domain and block TGF-β signaling by direct interaction with the TGF-β receptor, thereby blocking receptor mediated phosphorylation of R-Smads[127 128]. MH1 generally helps the Smads interact with DNA and the MH2 domain is involved in protein-protein interaction. Along with the modulation of TGF-β receptor activity and TGF-β signaling, anti-Smad7 may also act as a direct transcriptional regulator of R-Smads[129]. Smad7 acts as a repressor or activator of gene transcription based on the nature of the promoter, and whether it is phosphorylated at Ser249 which is important for its transcriptional activity[129].

The TGF superfamily includes different forms of TGF-β, BMPs and activins. The TGF-β subfamily consists of β1, β2 and β3 isoforms, which are synthesized as large

precursor molecules with propeptides[127]. The peptides are cleaved into mature 25kDa homodimers that are kept inactive by complexing with latent TGF-β binding protein[130]. To become active, the homodimers are proteolytically cleaved by a number of different factors including plasmin or thrombospondin-1[131 132]. Active TGF-β binds to a complex of TGF-β type I and TGF-β type II receptors, resulting in the phosphorylation of the R-Smads Smad2 and 3 by the TGF-β receptor I kinase[130]. The activated R-Smads bind Smad4 and translocate into the nucleus where they activate gene transcription. The Smad3/Smad4 pair binds promoters at the Smad consensus sequence, CAGAC, while the Smad2 containing complex requires a nuclear DNA-binding protein of the Fast family (Fast-1) to bind DNA[133 134]. In adult fibroblasts, Smad3 is essential for the activation of all the major TGF-β responsive genes[130]. On their own Smads are weak transcriptional activators and interact with general transcription factors like p300 and specific basal transcription complex proteins that vary depending on the promoter in question, to form an active transcriptional complex on promoters[135]. The inhibitory Smads (Smad6 or Smad7) can prevent R-Smad activation by competing for binding for Smad2 and Smad3 to the TGFβR1 and by enhancing receptor degradation[136 137]. TGF-β can also feedback inhibit its own action via the transcriptional regulation of Smad7[138]. A recent study by Dupont *et al.* recently showed that is another layer of control in the Smad signaling complex. They demonstrated that Smad4 monoubiquination and deubiquination is another way for cells to set their responsiveness to TGFβ[139]. The loss of the deubiquitinase FAM disabled Smad4 responsiveness, showing that ubiquination, along with phosphorylation acts as a regulatory mechanism for Smad function in cells.

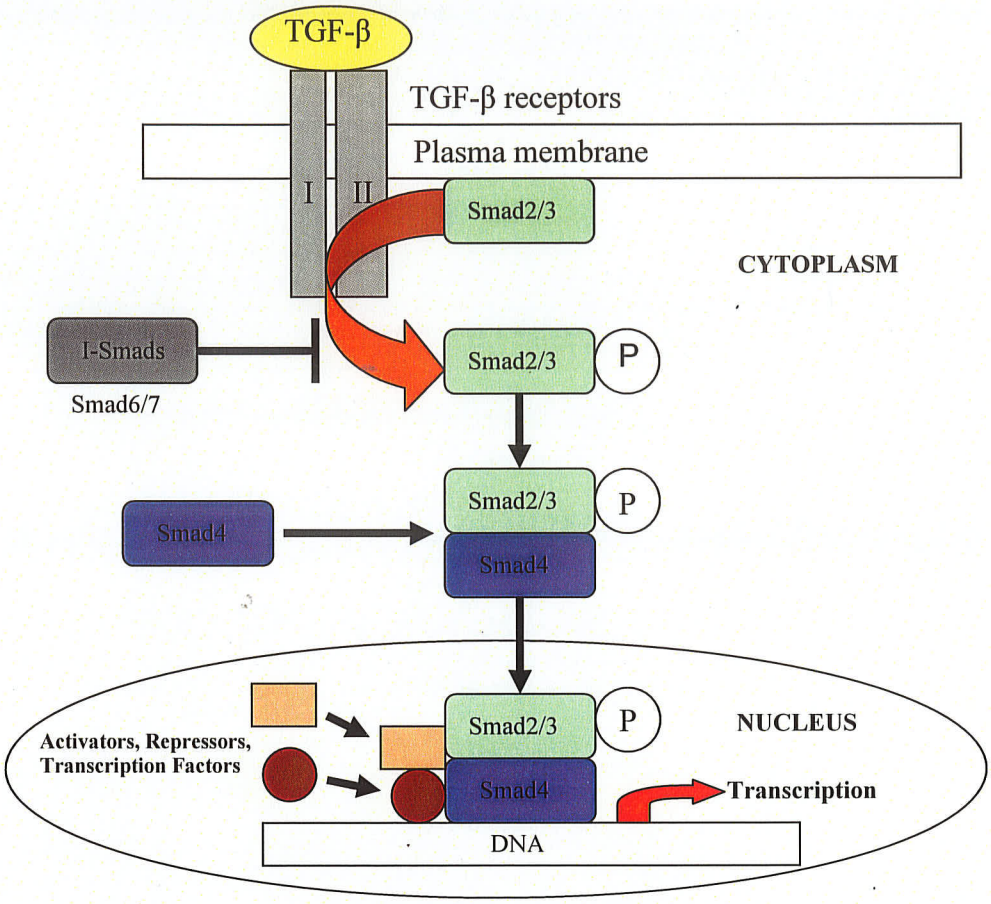

Figure 2. The TGF-β1 signaling pathway in cardiac myofibroblasts. Active TGF-β binds to a complex of TGF-β type I and TGF-β type II receptor complex, causing the phosphorylation of the R-Smads 2 and 3 by the TGF-β receptor kinase. The activated R-Smads then bind Smad4 and translocate into the nucleus where they activate transcription. Their transcriptional regulation can also be modulated by a variety of activators, repressors and transcription factors. The I-Smads-6 or 7 can prevent R-Smad activation by inhibiting their phosphorylation. I-TGF-β receptor I; II- TGF-β receptor II; P- Phosphate group.

V Materials and Methods.

1.0 Materials

Cell culture media (Dulbecco's Modified Eagle Medium (DMEM), DMEM/F12), fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin were purchased from Hyclone (Logan, UT). Tissue culture plates and dishes were supplied by Fisher Scientific (Whitby, ON). Lipofectamine 2000 Transfection Reagent was received from Invitrogen (Carlsbad, CA). The GenElute Mammalian Total RNA Miniprep Kit was received from Sigma-Aldrich (Oakville, ON), while the IQ SYBR Green Supermix kit was purchased from Bio-Rad Laboratories (Hercules, CA). The mouse Anti-HA antibody for immunofluroscence was purchased from Santa Cruz (Santa Cruz, CA). The secondary goat antibody conjugate to Texas Red was received from Invitrogen (Carlsbad, CA). The nuclear counterstain, 4',6-diamidino-2-phenylindole (DAPI) was also purchased from Invitrogen (Carlsbad, CA). Recombinant human TGF-β1 was received from R&D Systems (Minneapolis, MN). The protein assay kit (Comassie Protein Assay Reagent) was purchased from Thermo Scientific (Rockford, IL). Precision Plus Protein Dual Color standards were obtained from Bio-Rad Laboratories (Hercules, CA) and Polyvinylidene difluoride (PVDF) membranes were received from Pall Life Sciences (Pensacola, FL). The Western Blotting Luminol Reagent kit was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and the CL –Xposure Film was received from Pierce Biotechnology (Rocford, IL). The scleraxis antibody was generated by genetic immunization from QED Bioscience Inc (San Diego, CA) and the α-tubulin (12G10) antibody was received from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). Secondary antibodies were purchased from Invitrogen (Carlsbad, CA).

2.0 Methods

2.1 Cell Culture

COS7 and NIH-3T3 cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine. Primary cardiac fibroblasts were isolated from the ventricles of male adult Sprague-Dawley rats weighing between 150-200 grams which were hung on a Lagendorff apparatus and perfused with recirculated Joklik's medium at a flow rate of 5ml/minute at 37°C and then perfused with 0.1% collagenase in SMEM media for 20-25 minutes. Cells were then collected by centrifuging at 2000 rpm for 5 minutes and resuspended in DMEM-F12 supplemented with 10% FBS, 1% penicillin/streptomycin and 1 mM L-ascorbic acid[140]. Animals were treated in accordance with the guidelines of the Canadian Council on Animal Care and the University of Manitoba Animal Protocol Management and Review Committee.

2.2 Luciferase Reporter Assays

COS7 cells were plated in 6-well cell culture plates 24 h prior to transfection and used at ~80% confluence. Each sample was transfected with 500 ng reporter plasmid (human *COLIA2* 3.7 kb promoter driving luciferase, COLIA2-pGL3), 500 ng pCMV-LacZ for normalization of transfection efficiency and 500 ng expression vectors as required (pECE-HA-FLAG-Scx, pECE-ScxΔBD, pECE-ScxΔHLH, pHBAP-E12, pHBAP-E47, pCMV-Id2, pcDNA-MyoD). Empty vector (pECE) was added to each

sample as required to normalize total DNA transfected. Transfection was performed using Lipofectamine 2000 Transfection Reagent as per the manufacturer's instructions. The luciferase activity of each sample was measured on a TD20/20 luminometer (Turner BioSystems, USA). β-galactosidase activity was assayed using a fluorometric assay as described.[141] Scleraxis deletion mutants (pECE-ScxΔBD and pECE-ScxΔHLH) were generated from the parent vector pECE-HA-FLAG-Scx by nested PCR.

2.3 Treatment of NIH-3T3 cells and adult rat cardiac fibroblasts

NIH-3T3 cells were grown on 10 cm cell culture plates to ~60% confluence, then equilibrated for 24 h in DMEM supplemented with 0.5% FBS, 1% penicillin/streptomycin, 1% L-glutamine and 1 mM L-ascorbic acid. Cells were then infected with adenoviruses encoding either CMV-β-galactosidase (AdLacZ) or CMV-HA-Scx (AdScx). A multiplicity of infection of 100 was used throughout these experiments. Total RNA was isolated from cells at 48 h post-infection using the GenElute Mammalian Total RNA Miniprep Kit. Alternatively, cells were treated with 10 ng/ml TGF-β1 (or other appropriate concentrations) for 24 h prior to being harvested for total RNA or protein.

Primary adult rat cardiac fibroblasts were maintained in DMEM-F12 supplemented with 10% FBS, 1% penicillin/streptomycin and 1 mM L-ascorbic acid. TGF-β1 treatment was initiated after incubating cells for 24 h in DMEM-F-12 in the absence of serum. Cells were harvested 24 h after the initiation of treatment for collection of total RNA. For generation of myofibroblasts, primary cardiac fibroblasts were

maintained in DMEM-F12 supplemented with 10% FBS, 1% penicillin/streptomycin and 1mM L-ascorbic acid and passaged one or two times to induce phenoconversion.[142] Total RNA or protein was collected as above.

2.4 Real-Time PCR

Total RNA was isolated from cells using a GenElute Mammalian Total RNA Miniprep Kit as per the manufacturer's instructions. 2 ng RNA was used for real-time PCR reactions, which were performed using the IQ SYBR Green Supermix kit. The primers used are listed in table I. The real-time reactions were performed on a Bio-Rad iQ5 real-time PCR machine using the following thermocycler conditions: an initial denaturing step at 50°C for 10 minutes to establish persistent well factors, a denaturing step at 95°C for 5 minutes, 45 cycles of 10 seconds denaturing at 95°C and 30 seconds of annealing at 60°C (data collection point), 1 minute at 95°C and an annealing step of 1 minutes at 55°C. Melt curve data was collected as follows: 81 cycles of an initial annealing at 55°C followed by a temperature increase of 0.5°C with each succeeding cycle. The cycling and melt curve parameters were consistent with the IQ SYBR Green Supermix kit protocol. The optimal amount of RNA was initially determined by generating standard curves using serial dilutions of RNA, and to obtain PCR efficiency and melt curve data. Data was calculated using the $2^{-\Delta\Delta CT}$ method and was normalized to GAPDH.

Table 1. Real-Time PCR primers

The sequences of forward (Fwd) and reverse (Rev) primers used for qPCR amplification are given below.

Primer	Sequence (5' → 3')
Scleraxis – Fwd	AACACGGCCTTCACTGCGCTG
Scleraxis – Rev	CAGTAGCACGTTGCCCAGGTG
ColIα1 – Fwd	TGCTCCTCTTAGGGGCCA
ColIα1 – Rev	CGTCTCACCATTAGGGACCCT
ColIα2 – Fwd	GTCCCCGAGGCAGAGAT
ColIα2 – Rev	CCTTTGTCAGAATACTGAGCAGC
ColIVα1 – Fwd	AGCGAGATGTTCAAGAAG
ColIVα1 – Rev	TGGACAGTGAGGTACACA
ColVIα1 – Fwd	TCAGAATAGTGATGTGTTCGACGTT
ColVIα1 – Rev	AGCAACATGGATATGGTTCAGAAA
αSMA – Fwd	CGGGCTTTGCTGGTGATG
αSMA – Rev	CCCTCGATGGATGGGAAA
GAPDH – Fwd	TGCACCACCAACTGCTTAGC
GAPDH – Rev	GGCATGGACTGTGGTCATGAG

2.5 Rat cardiac infarct model

Cardiac infarct was induced in adult male Sprague-Dawley rats by coronary ligation as previously described.[143 144] Following anaesthetization with isofluorane inhalation (2-2.5% in a flow of 2 liters/min O_2), an incision was made to the left and parallel to the sternum. The fifth intercostal space was opened with a small retractor to visualize the heart. The left coronary artery was tied with a suture prior to closure of the incision and the animals allowed to recover. Sham operated animals were treated identically, but the left coronary suture was left untied. Animals were sacrificed at 4

weeks after infarct, and hearts dissected to isolate the infarcted left ventricular region itself or the distal (non-infarcted) right ventricle.

2.6 Immunofluorescence labeling

Isolated second passage (P2) cardiac fibroblasts were infected with AdScx as described above. 24 h after infection, cells were fixed in 10% formaldehyde in PBS, permeabilized with Triton X-100 and treated with anti-HA antibodies to detect HA-tagged scleraxis following blocking in 10% bovine serum albumin. Goat anti-rabbit secondary antibodies conjugated to Texas Red were used for visualization, with DAPI as a nuclear counterstain. Images were obtained on a Zeiss AxioImage M1 epifluorescence microscope using appropriate filters.

2.7 Genetic Immunization

Scleraxis cDNA was PCR cloned was cloned into the pSecTag A plasmid expression vector (Invitrogen). Purified plasmid DNA was resuspended in sterile phosphate-buffered saline (PBS) at a concentration of 1mg/ml and 4mg was sent to QED Bioscience Inc. for genetic immunization. Two New Zealand White rabbits received 3 injections about 2 weeks apart and approximately 200 ml of antiserum was collected over 4 bleeds.

2.8 Cell lysis and protein assay

Different cell treatment groups were washed 3 times with ice cold 1xPBS and then lysed by the addition of 200ul of RIPA buffer (pH 7.8) which contains 50mM Tris, 1mM EDTA, 1mM EGTA, 150mM NaCl, 0.5% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS) supplemented with 1mM DTT (Dithiothreitol), 1mM PMSF (phenylmethylsulphonyl fluoride) and 1x Complete Protease Inhibitor Cocktail tablets (Roche). Cells were scraped and incubated on ice for 15 minutes and the lysate centrifuged at 14,000 rpm 4 °C for 15 minutes and the supernatant collected and stored at -80 °C. The concentration of each sample was determined using the Comassie Protein Assay Reagent as per the manufacturers instructions.

2.9 Western blot and analysis of target proteins

Cell lysates were mixed Laemmli SDS-loading buffer (Tris HCL (pH 6.8) 375 mM, SDS 9%, Glycerol 50%, Betamercaptoethanol 9%, Bromophenol blue 0.03%) and boiled for 5 minutes. 20ug of protein was separated on a 12% SDS-polyacrylamide by gel electrophoresis (SDS-PAGE). Separated proteins were then transferred electrophoretically onto a 0.45μM polyvinylidene difluoride (PVDF) membrane and blocked with 5% skim milk in phosphate buffered saline with 0.1% Tween 20 (PBST) overnight at 4°C. The membranes were incubated with primary antibodies for 2 hours at a concentration of 1:5000 in 1% skim milk in PBST at room temperature. Secondary antibody labeled with horseradish peroxidase (HRP) was added for 1 hour at room

temperature at a concentration of 1:10000 in 1% skim milk in PBST. The membrane bands were visualized using the Western Blotting Luminol Reagent kit and developed using X-ray film. Equal loading was confirmed by probing with α-tubulin at a concentration of 1:5000.

2.10 Statistical analysis

Statistical significance of results was determined by the Student t-test or one-way analysis of variance using Dunnet's posthoc test, as appropriate. $P \leq 0.05$ was considered statistically significant.

VI Results.

1.0 Scleraxis over-expression using adenoviral gene delivery vectors

P2 adult rat cardiac myofibroblasts were infected with adenovirus encoding HA-tagged scleraxis (AdScx) at an MOI of 100 24 h post infection, cells were fixed using paraformaldehyde and scleraxis was visualized using primary rabbit anti-HA antibodies and goat anti-rabbit secondary antibodies conjugated to Texas red. The control samples were not infected with AdScx. Both sets of samples were counterstained with the nuclear stain DAPI. The AdScx infected samples showed high levels of scleraxis expression in the nucleus compared to the control samples, demonstrating that adenoviral gene delivery is an efficient method for over-expression in cardiac myofibroblasts.

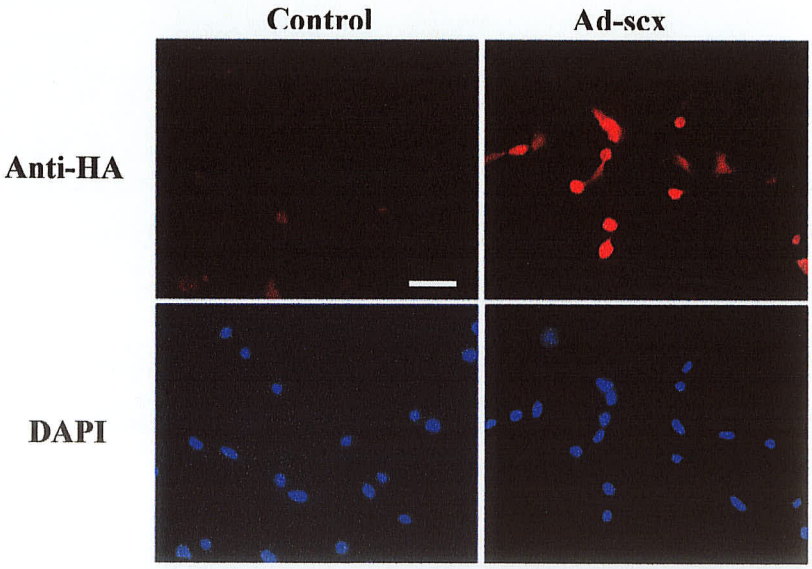

Figure 3. Scleraxis overexpression using adenoviral gene delivery vectors. P2 cardiac myofibroblasts were infected with an adenovirus encoding HA-tagged scleraxis (Ad-scx) and 24h later the cells were visualized on a Zeiss AxioImage M1 epifluorescence microscope. Scale bar, 100 μm.

2.0 Expression of scleraxis in adult rat cardiac fibroblasts and myofibroblasts in response to TGF-β1 treatment

Isolated adult rat cardiac P2 cardiac myofibroblasts were treated with 10 ng/ml TGF-β1 for 24 h. Total RNA was extracted and used for qPCR with primers specific for scleraxis. The results were normalized to GAPDH expression and fold change was calculated using the $2^{-\Delta\Delta Ct}$ method. Scleraxis expression was strongly induced in the TGF-β1 samples as compared to the control samples.

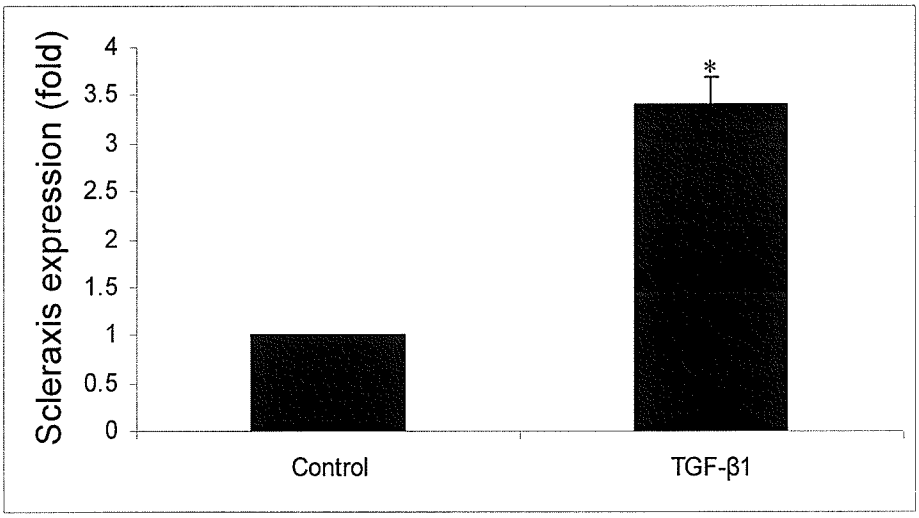

Figure 4. Effect of TGF-β1 treatment on adult rat cardiac P2 myofibroblasts. Total RNA was extracted and used for qPCR with primers specific for scleraxis. The results were normalized to GAPDH expression and fold change was calculated using the $2^{-\Delta\Delta Ct}$ method. Mean ± standard error, n =3. *$p \leq 0.05$ vs. control cells.

3.0 Effect of passage number on scleraxis expression in fibroblasts (P0) and myofibroblasts (P1 and P2)

Fibroblasts convert to myofibroblasts with increasing passage number *in vitro*, with P2 cells most closely resembling *in vivo* myofibroblasts[145]. We therefore examined scleraxis expression as a function of passage number. Total RNA was extracted from

isolated primary adult rat fibroblasts (P0) or first or second passage myofibroblasts (P1, P2). qPCR was performed using primers specific to scleraxis and results normalized to GAPDH expression and P0. Induction of fibroblast-to-myofibroblast conversion led to a greater than 4-fold induction in P2 cardiac myofibroblasts compared to freshly isolated P0 primary cardiac fibroblasts. We also examined the expression of the myofibroblast marker α-SMA, collagen Iα1 (Col Iα1) and collagen Iα2 (Col Iα2). The samples prepared above were subjected to qPCR using primers specific for α-SMA, Col Iα1 or Col Iα2. Results were normalized to GAPDH expression and P0. As expected, expression of α-SMA increased with passage number, as did the expression of the fibrillar collagens: Col Iα1 and Col Iα2.

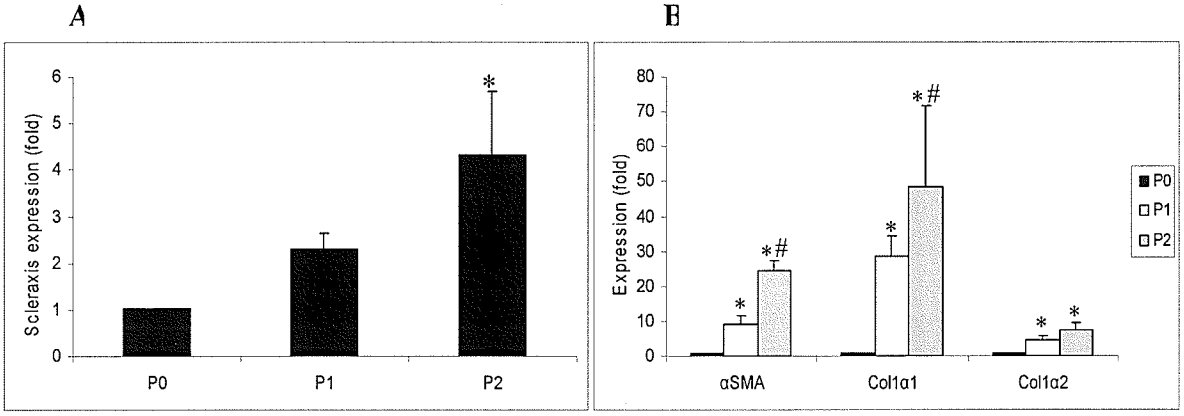

Figure 5. Effect of passage on relative scleraxis expression in fibroblasts (P0 cells) and myofibroblasts (P1 and P2 cells). Total RNA was extracted from isolated primary adult rat fibroblasts (P0) or first or second passage myofibroblasts (P1, P2). qPCR was performed using primers specific to scleraxis. α-SMA, Col Iα1 or Col Iα2 and results normalized to GAPDH expression and P0. **Panel A:** Graph showing the relative amounts of scleraxis mRNA at each passage number. Mean ± standard error, n =3. *p ≤0.05 vs P0 cells. **Panel B:** Graph showing the relative amounts of α-SMA, ColIα1 and Col Iα2 mRNA at each passage number. Mean ± standard error, n =3. *p ≤0.05 vs P0 cells; #p ≤ 0.05 vs P1 cells.

4.0 Scleraxis over-expression stimulates collagen Iα2 gene expression

Infection of NIH-3T3 cells with AdScx at MOI 100 resulted in scleraxis expression levels similar to those obtained with TGF-β1 treatment as determined by qPCR. The results were normalized to GAPDH expression and fold change was calculated using the $2^{-\Delta\Delta Ct}$ method. Using qPCR we also determined that scleraxis was sufficient to induce expression of collagen Iα2, a key component of the predominant cardiac fibrillar type I collagen. Over-expression of scleraxis in NIH-3T3 cells strongly induced expression of collagen Iα2, as did TGF-β1 treatment.

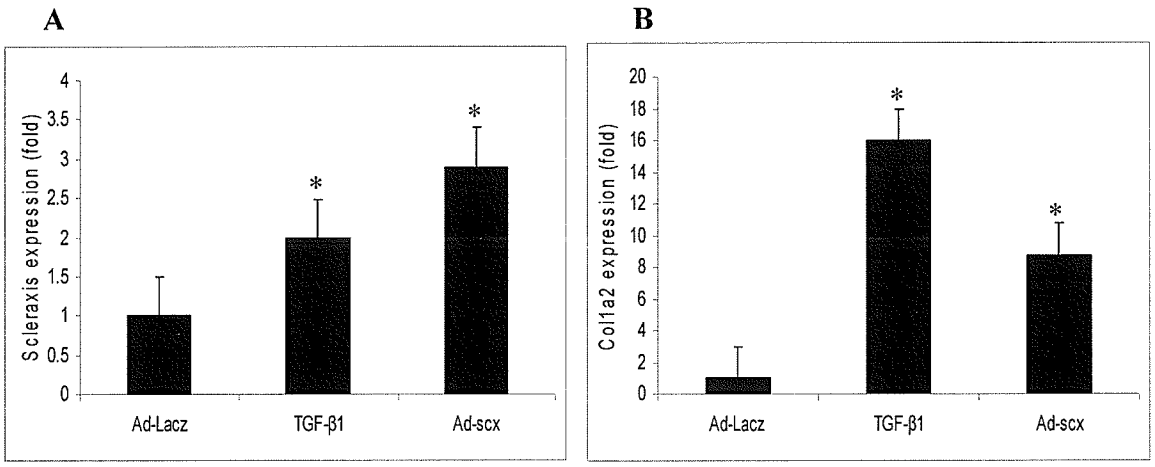

Figure 6. Scleraxis over-expression stimulates fibroblast collagen Iα2 gene expression.NIH-3T3 fibroblasts were infected with adenoviruses encoding LacZ (AdLacZ) or scleraxis (AdScx) at MOI 100, or were treated with 10 ng/ml TGF-β1 for 48 hours. qPCR was performed using primers specific to scleraxis or Col Iα2, and results normalized to GAPDH expression and the AdLacZ infected samples. **Panel A:** Graph showing scleraxis levels after AdScx infection or TGF-β1 treatment. **Panel B:** Graph showing Col Iα2 levels after AdScx infection or TGF-β1 treatment. Mean±standard error, *n=3*. *$p<0.01$ versus AdLacZ.

5.0 Regulation of collagen Iα2 promoter activity by scleraxis

COS7 cells were co-transfected with an empty expression vector (pECE), or with expression vectors for scleraxis, E12, E47, MyoD or Id2, and normalized to co-transfected

β-galactosidase. Scleraxis strongly transactivated the *COL1A2* promoter. The E2A transcription factors, E12 and E47, that have been shown to heterodimerize with scleraxis, also transactivated the promoter, but the effect was augmented by the addition of scleraxis, especially in the case of E47 where cotransfection with scleraxis caused a significant up-regulation in promoter activity compared to E47 alone. In contrast, the related bHLH transcription factor MyoD was unable to significantly transactivate the promoter. The HLH protein Id2, which lacks a DNA binding domain, repressed scleraxis-mediated transactivation of the *COL1A2* promoter in a dose dependent manner.

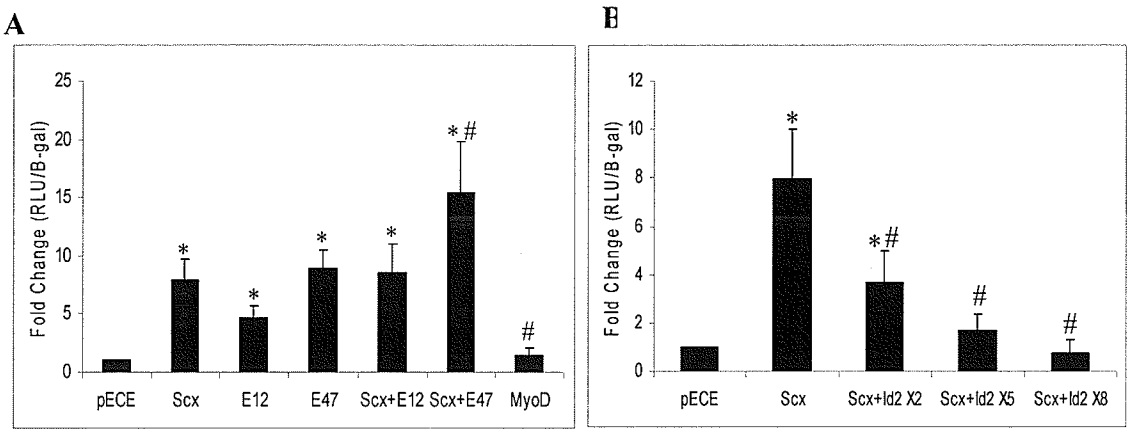

Figure 7. Regulation of collagen Iα2 promoter activity by scleraxis. Panel A: COS7 cells were transiently transfected with an empty expression vector or expression vectors for scleraxis, E12, E47 or MyoD. Luciferase expression levels were normalized to co-transfected β-galactosidase. Mean ± standard error, $n \geq 3$. *p $\leq$0.05 versus reporter. **Panel B:** COS7 cells were transiently transfected with an empty expression vector, or with expression vectors for scleraxis or Id2. Luciferase expression levels were normalized to co-transfected β-galactosidase. Mean ± standard error. *p $\leq$0.05 versus expression vector. #p $\leq$0.05 versus scleraxis alone. $n \geq 3$.

6.0 Scleraxis regulates collagen Iα2 promoter in a DNA and protein binding-dependent fashion

Scleraxis mutants were generated by nested deletion PCR to remove either the DNA-binding basic domain (ScxΔBD) or the helix-loop-helix protein interaction domain (ScxΔHLH). We then performed luciferase assays using the human *COLIA2* promoter. COS7 cells were co-transfected with an empty expression vector (control), or with expression vectors for scleraxis, ScxΔBD or ScxΔHLH and normalized to co-transfected β-galactosidase. Deletion of the DNA-binding basic domain abrogated the ability of scleraxis to transactivate the promoter. In contrast, deletion of the helix-loop-helix motif attenuated but did not eliminate the ability of scleraxis to statistically significantly transactivate the promoter. The ScxΔBD mutant appears to function as a dominant negative regulator of *COLIA2* gene expression. E47 was able to transactivate the COLIA2 promoter; whereas wild type scleraxis had no effect, ScxΔBD attenuated the effect of E47.

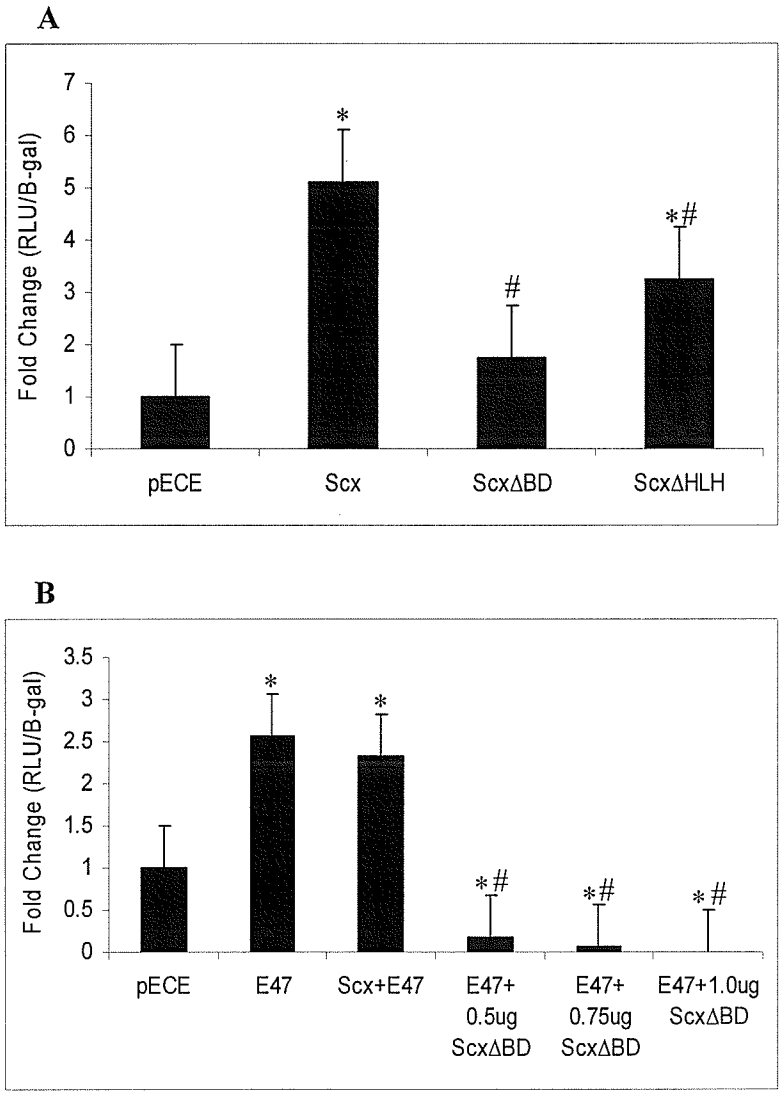

Figure 8. Scleraxis regulates collagen Iα2 promoter transactivation in a DNA and protein binding-dependent fashion. Panel A: COS7 cells were transiently transfected with collagen Iα2 promoter luciferase reporter and a β-galactosidase expression plasmid for normalization. Samples were co-transfected with either an empty expression vector (pECE) or with expression vectors for scleraxis (Scx) or scleraxis basic domain (ScxΔBD) and HLH (ScxΔHLH) deletion mutants. Mean ± standard error, n =3. *p ≤0.05 versus control; #p ≤0.05 versus scleraxis alone. **Panel B:** COS7 cells were transiently transfected with collagen Iα2 promoter luciferase reporter and β-galactosidase expression plasmid for normalization. Samples were co-transfected with either an empty expression vector or expression vectors for the scleraxis basic deletion mutant (ScxΔBD), scleraxis (Scx) or E47. Mean ± standard error, n=3. *p ≤0.05 versus control; #p ≤0.05 versus E47 alone.

7.0 Scleraxis expression is induced in the cardiac infarct scar

Myofibroblasts are known to migrate to the infarcted zone and synthesize large amounts of collagen to help stabilize the damaged myocardium. We therefore assayed scleraxis gene expression post-infarct. Infarction was induced in male Sprague-Dawley rats by left coronary artery ligation. Surgery, but not coronary ligation was performed in the sham animals. Four weeks after infarct, the heart was removed and the infarcted region and non-infarcted right ventricle (RV) was dissected out for preparation of total RNA. Real time RT-PCR was performed using primers specific for GAPDH (control) plus scleraxis, collagen Iα1, collagen Iα2, collagen IIIα1, collagen IVα1 or collagen VIα1. Scleraxis was significantly up-regulated in the infarct scar itself, but not in the distal uninvolved right ventricle or in the left ventricle of the sham control animals. The expression of fibrillar collagens (collagen Iα2 and especially of collagen IIIα1) also increased in the infarct, similar to the pattern for scleraxis expression. In the RV, the expression of collagen Iα2 was not affected, while the expression of collagen IIIα1 was up-regulated. In contrast, the non-fibrillar collagens (collagen IVα1 and collagen VIα1) were elevated throughout the infarcted hearts, with no statistically significant difference between the infarct scar and right ventricle, indicating a generalized induction of this species of collagen post-infarct hearts.

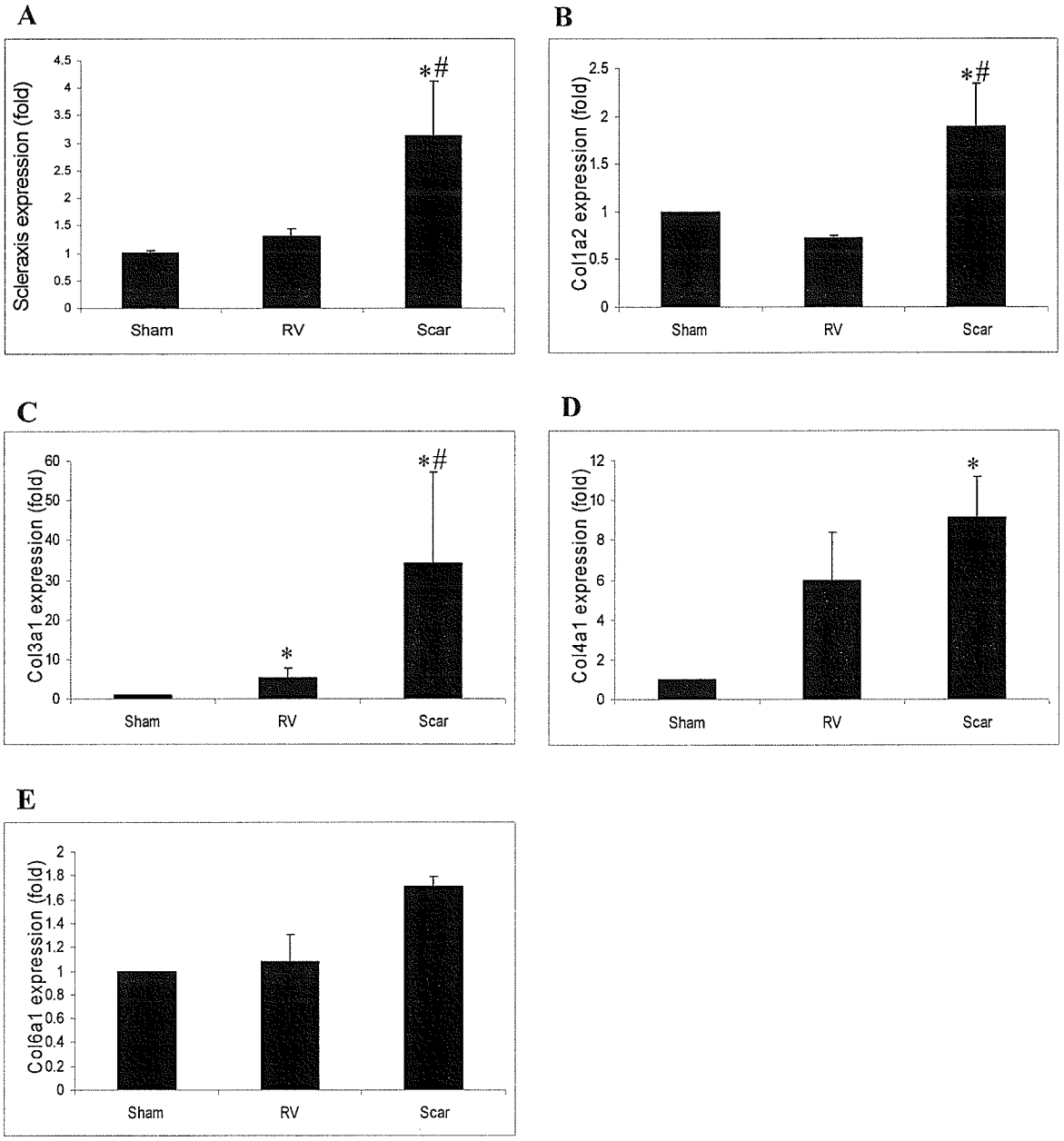

Figure 9. Scleraxis and collagen expression is induced in the cardiac infarct scar. Infarction was induced in male Sprague-Drawley rats by left coronary ligation. Surgery, but not coronary ligation, was performed in sham animals. Four weeks after infarct, the heart was removed and the infarcted LV and non-infarcted RV were dissected out for isolation of total RNA. Real time RT-PCR was performed using primers specific for scleraxis (**Panel A**), collagen Iα2 (**Panel B**), collagen IIIα1 (**Panel C**), collagen IVα1 (**Panel D**), or collagen VIα1 (**Panel E**). Results represent three experiments determined in triplicate, normalized to GAPDH and to the sham sample. Mean ± standard error. *p ≤0.05 versus sham; #p ≤0.05 versus RV.

8.0 Genetic immunization to develop a polyclonal anti- scleraxis antibody

Scleraxis cDNA was PCR cloned into the pSecTag A plasmid expression vector and sent to QED Bioscience Inc. for genetic immunization. As we have shown that TGF-β1 treatment upregulates scleraxis expression (Figure 4), NIH-3T3 mouse embryonic fibroblasts and P2 rat cardiac myofibroblasts were stimulated with TGF-β1 (0.1ng/ml, 0.5ng/ml and 10ng/ml respectively) and the scleraxis antibody was tested using western blotting. We found that the antibody was able to detect endogenous scleraxis in both NIH-3T3 cells and in P2 rat cardiac fibroblasts. As well, a visible increase in scleraxis protein levels was observed following stimulation with TGF-β1, that we were able to detect using the antibody.

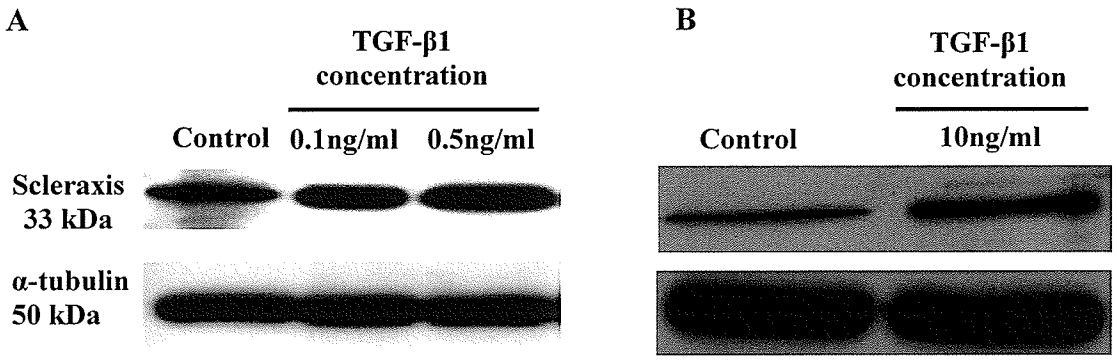

Figure 10. Generation of a scleraxis polyclonal antibody.
Western blotting was used to examine the ability of scleraxis rabbit polyclonal antibody to detect endogenous scleraxis and increases in scleraxis levels after TGF-β1 stimulation in NIH-3T3 mouse embryonic fibroblasts (**Panel A)** and in P2 rat cardiac fibroblasts (**Panel B**). α-tubulin was used to confirm equal loading.

VII Discussion.

The molecular mechanisms responsible for the control of collagen gene expression in cardiac fibroblasts and myofibroblasts are not yet completely characterized. Transcription factors like scleraxis, growth factors like TGF-β1 and signaling proteins like Smads are all parts of a finely choreographed response that begins as the heart remodels. Even though this process is aimed at restoring cardiac function, in many chronic cases it results in fibrosis and cardiac dysfunction, negating its beneficial aspects. It is therefore critical to understand the transcriptional cascades that control fibrosis if effective therapies are to be designed harnessing the ability of collagen deposition to restore cardiac function following a cardiac insult.

Scleraxis as a regulator of cardiac scar fibrillar collagen deposition

This thesis has demonstrated that the bHLH transcription factor scleraxis plays a crucial role in regulating collagen gene expression by cardiac fibroblasts and myofibroblasts, key mediators of cardiac fibrosis. Even though this is not the first time that it has been demonstrated that scleraxis might be involved collagen production, this data strengthens the case that scleraxis is a key player in collagen production through the critical role that it appears to have in the transcriptional machinery of cardiac fibroblasts and myofibroblasts[8 91]. Our data suggests that there is an association between fibroblast collagen expression and scleraxis content. The infarct scar, which is collagen- and myofibroblast-rich, shows a marked increased in scleraxis expression as compared to the non-infarcted right ventricle (Figure 9A). The expression levels of fibrillar collagens

closely mimicked the expression pattern of scleraxis, pointing to a strong correlation between the two (Figure 9B-C). In contrast, the expression of non-fibrillar collagens increased throughout the hearts of infarcted animals (Figure 9D-E). The increase in scleraxis expression levels as fibroblasts phenoconvert into myofibroblasts suggests that scleraxis may regulate the expression of key genes involved in altered ECM expression as the phenotype switch occurs, or may be the key to the whole process by itself (Figure 5A). It is tempting to speculate that scleraxis may be another manner by which the heart's cellular machinery reverts to embryonic isoforms, in order to deal with cardiac trauma.

Scleraxis' role in the TGF-β1-Smad pathway

The recent finding that scleraxis is strongly co-expressed with the TGF-β type II receptor suggests that scleraxis may be part of the TGF-β signaling pathway that is known to mediate the fibrotic response[11 146]. In this regard, it is of interest that TGF-β1 treatment of fibroblasts induces scleraxis expression (Figures 4). Scleraxis may thus represent part of a novel TGF-β1-inducible mechanism for augmenting collagen gene expression following a major cardiac insult. However, it is still unclear whether scleraxis represents a parallel and separate mechanism from Smad proteins for the induction of collagen gene expression, or whether scleraxis works co-operatively with Smads, even though evidence of a direct interaction between Smad proteins and scleraxis has yet to be reported. Scleraxis may however be part of a transcription complex that augments Smad action and as such directly binds Smad proteins. Murchison *et al.* recently reported that in the scleraxis null mice, some tendons exhibited significant structural defects associated with the loss of collagen I expression, while others appeared normal[91]. This would seem

to indicate scleraxis may be required for collagen gene expression in a cell type-specific manner, and its roles in the TGF-β1 pathway may change accordingly. Smad proteins are not potent transcriptional activators, and depending on the cellular and metabolic context, scleraxis may play a key role in augmenting their action and controlling the transcription of collagen genes[147].

Scleraxis interaction with E2A transcription factors and DNA

Our data support a model of regulation of collagen Iα2 expression via a direct interaction between scleraxis and DNA. Scleraxis significantly transactivates the human *COLIA2* promoter (Figure 7). A scleraxis deletion mutant lacking the DNA-binding basic region was unable to transactivate this promoter, indicating that scleraxis interacts with the promoter via a direct interaction with the DNA (Figure 8A). This is similar to what Lejard *et al.* concluded when they demonstrated that scleraxis regulates the collagen Iα1 promoter in tendon fibroblasts, though they did not determine the precise mechanism for this binding[8]. Up to 12 putative E-boxes may be present in the *COLIA2* promoter and will require characterization to reveal exactly how scleraxis regulates this promoter, and which of these E-boxes is vital for scleraxis' ability to transactivate the promoter (Table 2).

The deletion of the HLH protein interaction region resulted in a significant decrease in promoter transactivation, but transactivation was not abolished as was the case with (Figure 8A). Transactivation by the ScxΔHLH mutant was still significantly greater than reporter alone, suggesting that protein-protein interactions are required for full scleraxis activity. Previous reports have indicated that scleraxis heterodimerizes with

the E2A gene products E12 and E47 [5 7 8 9 80]. Indeed scleraxis was initially discovered in a yeast two-hybrid system screen of a mouse library for cDNAs encoding novel cell-type specific bHLH proteins that dimerize with E12[5]. Our data shows that the HLH repressor Id2 inhibits scleraxis transactivation of the *COLIA2* promoter in a dose dependent manner (Figure 7B). However, it has been shown that scleraxis does not directly bind Id2 [80]. Thus, these results suggest that scleraxis may interact with the endogenous E2A protein E47 for full activity, but is also capable of regulating gene expression independently (Figure 7A). This is in line with a previous report that E-box interaction is not necessary for scleraxis-mediated transactivation of the *aggrecan I* gene[7]. Since ScxΔBD repressed E47-mediated transactivation of the *COLIA2* promoter in a dominant negative fashion, it is possible that E47 acts through endogenous scleraxis present in COS7 cells which was competed out by the mutant in our assay (Figure 8B).

Scleraxis induction of collagen Iα2 expression

The over-expression of scleraxis is sufficient to up-regulate collagen Iα2 expression in NIH-3T3 murine fibroblasts (Figure 6). These results support our hypothesis that scleraxis is a key regulator of cardiac type I collagen expression and fibrosis by fibroblasts and myofibroblasts. Although we did not assay for collagen Iα1 in this case, others have shown that scleraxis directly regulates the expression of collagen Iα1 in conjunction with NFATc[8]. Since mature collagen type I fibrils consist of two strands of collagen Iα1 and one strand of Iα2, it may be assumed that scleraxis is involved in the overall regulation of type I collagen. Scleraxis null mice exhibit significant defects in force transmitting and intermuscular tendons, suggesting that

scleraxis is not only sufficient to regulate collagen gene expression, it is also required in some cell types. It has also been reported that specific collagens within cardiac valves exhibit altered expression in scleraxis null mice, further suggesting that the collagen genes regulated by scleraxis are cell type-dependent[86].

Cardiac fibrosis remains an unsolved clinical problem for many patients. As Ott *et al.* so elegantly demonstrated the cardiac ECM is crucial for cell patterning, differentiation and alignment to form a contractile myocardium. As the fibrotic process is a derangement of normal ECM production, developing strategies to control the fibrotic process is vital if patient outcomes are to be improved. Direct intervention into the expression of collagen genes may provide such a mechanism to reduce the degree of fibrosis and the associated morbidity. By controlling the deposition of this ECM macromolecule, we may able to control scar formation and the stabilization of the wounded myocardium. Although the precise role of scleraxis in cardiac fibrosis remains to be fully characterized, our data indicate that scleraxis is a previously unappreciated regulator of cardiac collagen synthesis, and may present an important new target for anti-fibrotic therapies.

Table 2. Putative E-Boxes within the collagenIα2 promoter.

Italicized sequences match those that have previously been demonstrated to bind scleraxis with high affinity. Positions are relative to the transcriptional start site of the collagen Iα2 gene.

Position	Sequence	Position	Sequence
-3264	*CATGTG*	-1585	CACTTG
-2813	CAGCTG	-794	*CAGGTG*
-2715	CAAATG	-709	CACCTG
-2655	CATTTG	-441	CAAGTG
-2229	CATATG	-423	*CAGGTG*
-1835	CAAGTG	-125	CAGCTG

VIII Conclusions.

1. P2 cardiac fibroblasts, which synthesize large amounts of collagen, express more scleraxis than do P0 cardiac fibroblasts which exhibit relatively low collagen synthesis.
2. Scleraxis is highly expressed in the collagen- and myofibroblast- rich cardiac infarct scar, but not the non-infarcted right ventricle.
3. Collagen Iα2 and collagen IIIα1 expression increased in parallel with scleraxis in the infarcted ventricle, in contrast to the expression of non-fibrillar collagens IVα1 and VIα1 which increased throughout the infarcted heart.
4. TGF-β1 treatment of myofibroblasts induces scleraxis expression.
5. Over-expression of scleraxis results in the potent induction of collagen Iα2 gene expression.
6. Regulation of the collagen Iα2 expression requires direct interaction between scleraxis and DNA as evidenced by the fact that scleraxis transactivated the human *COLIA2* promoter, whereas a scleraxis mutant lacking the DNA-binding basic region was unable to transactivate the promoter.
7. Protein interaction is required for full scleraxis activity as deletion of the HLH protein-interaction region of scleraxis resulted in a decrease of promoter transactivation, but to a lesser degree than that caused by the deletion of the DNA-binding basic region.
8. The HLH repressor Id2 inhibits scleraxis transactivation of the *COLIA2* promoter in a dose dependent manner, suggesting that scleraxis may interact with E2A proteins E12 and/or E47 for its full activity.

9. The dominant negative manner in which the ScxΔBD mutant repressed E47-mediated transactivation of the *COLIA2* promoter, suggests that endogenous scleraxis present in COS7 cells may be necessary for E47 action or that the ScxΔBD sequesters E47 from the promoter independently of endogenous scleraxis .

IX Future Directions.

The exact functional niche that scleraxis might occupy in the adult myocardium is still unknown. Scleraxis is considered a tendon marker and is used to track tendon development. Only of late has the focus shifted to other functions of scleraxis. Loss-of-function studies are the most interesting direction to pursue at this moment. Studies in scleraxis knockout mice will reveal if scleraxis is required for the fibrotic response in a remodeling heart. Cardiac infarcts can be induced in adult mice and real time-PCR used to examine collagen gene expression. Histological assessments of serial cryosections of the infarct region should shed more light on the structure of the ECM in these mice. Performance assessments using 2D echocardiography will complement the histological data, showing how changes in ECM composition affect cardiac function. Cardiac fibroblasts isolated from knockout animals will be used for proliferation, migration and gel contraction assays, giving us a better idea what role scleraxis might have in inducing and maintaining myofibroblast phenotype and morphology.

To complement studies in knockout animals, studies using shRNA can also be carried out. We have already generated an adenovirus shRNA vector (AdshScx) that we will use to infect P2 myofibroblasts prior to their stimulation by TGF-β1. This would allow us to demonstrate that scleraxis is required for collagen synthesis in response to TGF-β1 treatment. As an alternative to the shRNA approach, we could generate an adenovirus encoding the ScxΔBD mutant that lacks the DNA binding domain. This would allow us to block collagen synthesis and demonstrate a role for scleraxis in this process.

As noted earlier we have identified 12 putative E-boxes in the human *COLIA2* promoter using *in silico* analysis. But it is unclear which of the 12 sites mediate scleraxis regulation of the promoter, although 3 of these E-boxes match sequences that have already been reported to bind scleraxis in other contexts. Deleting progressively larger promoter sections and performing luciferase assays and comparing the reporter results with wild type promoter results we will enable us to determine which region of the promoter is most important for gene transactivation. We can then generate oligonucleotides to E-boxes in each promoter section and perform electrophoretic gel mobility shift assays (EMSAs) to determine the specific scleraxis binding site(s).

We have also successfully generated an antibody against scleraxis using genetic immunization (Figure 10). We could employ it in a number of different ways. Histological examination of serial cryosections taken from a rat model of MI would confirm our real-time PCR data that scleraxis is up-regulated in the scar region. Chromatin immunoprecipitation (ChIP) assays using the antibody would confirm the binding of endogenous scleraxis *in vivo* to E-boxes identified using EMSAs.

It would also be interesting to explore what if any role scleraxis may have in the TGF-β1 pathway, in particular how it interacts with the Smad family of proteins. This could be done through the use of co-immunoprecipitation assays that would pull down scleraxis and any potential partners involved in the TGF-β1-mediated collagen response. Western blots using the anti-scleraxis antibody would allow us to confirm at the protein level that scleraxis levels change in response to TGF-β1 treatment. To confirm whether over-expression of the R-Smads leads to changes in scleraxis levels and *vice versa*, westerns blots could be done.

X Methodological Limitations of the Current Study.

This study relied heavily on the use real-time RT-PCR to look at changes in gene expression levels. Even though our laboratory has established a reliable SyBr green method for real-time PCR, the changes we see at the mRNA level may not always translate into changes at the protein level. This might especially be the case for collagen levels, as collagen molecules will undergo multiple processing steps before forming into fibrils. To overcome this we could use a commercial kit to assay for procollagen-1 N-terminal propeptide (PINP) in cell media, which is a measure of secreted collagen. The use of western blots to confirm real time-PCR data would strengthen some of the key conclusions of this thesis.

Loss-of-function studies might also be able to provide a better insight into the role that scleraxis plays. The reliance on over-expression of scleraxis, be it using adenoviral delivery methods or through the use of transfection reagents, may mask the physiological roles that scleraxis may play. The commencement of studies using the scleraxis null mice and the use of the shRNA against scleraxis should address this limitation and begin to address the exact role of scleraxis in the myocardium, something that this thesis laid the groundwork for.

XI References.

1. Booz, G.W. & Baker, K.M. Molecular signalling mechanisms controlling growth and function of cardiac fibroblasts. *Cardiovasc Res* **30**, 537-543(1995).

2. Long, C.S. & Brown, R.D. The cardiac fibroblast, another therapeutic target for mending the broken heart? *J. Mol. Cell. Cardiol* **34**, 1273-8(2002).

3. Sun, Y. & Weber, K.T. Infarct scar: a dynamic tissue. *Cardiovasc Res* **46**, 250-256(2000).

4. Ott, H.C. et al. Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nat Med* **14**, 213-221(2008).

5. Cserjesi, P. et al. Scleraxis: a basic helix-loop-helix protein that prefigures skeletal formation during mouse embryogenesis. *Development* **121**, 1099-1110(1995).

6. Perez, A.V. et al. Scleraxis (Scx) directs lacZ expression in tendon of transgenic mice. *Mechanisms of Development* **120**, 1153-1163(2003).

7. Liu, Y. et al. Overexpression of a Single Helix-Loop-Helix-type Transcription Factor, Scleraxis, Enhances Aggrecan Gene Expression in Osteoblastic Osteosarcoma ROS17/2.8 Cells. *J. Biol. Chem.* **272**, 29880-29885(1997).

8. Lejard, V. et al. Scleraxis and NFATc Regulate the Expression of the Pro-{alpha}1(I) Collagen Gene in Tendon Fibroblasts. *J. Biol. Chem.* **282**, 17665-17675(2007).

9. Muir, T., Sadler-Riggleman, I. & Skinner, M.K. Role of the Basic Helix-Loop-Helix Transcription Factor, Scleraxis, in the Regulation of Sertoli Cell Function and Differentiation. *Mol Endocrinol* **19**, 2164-2174(2005).

10. Liu, Y. et al. Sclerotome-related helix-loop-helix type transcription factor (scleraxis) mRNA is expressed in osteoblasts and its level is enhanced by type-beta transforming growth factor. *J Endocrinol* **151**, 491-499(1996).

11. Anthwal, N., Chai, Y. & Tucker, A.S. The role of transforming growth factor-beta signalling in the patterning of the proximal processes of the murine dentary. *Dev. Dyn* **237**, 1604-13(2008).

12. Swynghedauw, B. Molecular Mechanisms of Myocardial Remodeling. *Physiol. Rev.* **79**, 215-262(1999).

13. Haunstetter, A. & Izumo, S. Apoptosis : Basic Mechanisms and Implications for Cardiovascular Disease. *Circ Res* **82**, 1111-1129(1998).

14. Cleutjens, J.P. et al. The infarcted myocardium: simply dead tissue, or a lively target for therapeutic interventions. *Cardiovasc. Res* **44**, 232-41(1999).

15. Frangogiannis, N.G., Smith, C. & Entman, M.L. The inflammatory response in myocardial infarction. *Cardiovasc Res* **53**, 31-47(2002).

16. Willems, I.E.M.G., Arends, J. & Daemen, M.J.A.P. Tenascin and fibronectin expression in healing human myocardial scars. *The Journal of Pathology* **179**, 321-325(1996).

17. Cleutjens, J.P. The role of matrix metalloproteinases in heart disease. *Cardiovasc. Res* **32**, 816-21(1996).

18. Willems, I.E. et al. The alpha-smooth muscle actin-positive cells in healing human myocardial scars. *The American Journal of Pathology* **145**, (1994).

19. Brown, R.D. et al. The cardiac fibroblast: therapeutic target in myocardial remodeling and failure. *Annu. Rev. Pharmacol. Toxicol* **45**, 657-87(2005).

20. Eghbali, M. & Weber, K.T. Collagen and the myocardium: fibrillar structure, biosynthesis and degradation in relation to hypertrophy and its regression. *Molecular and Cellular Biochemistry* **96**, 1-14(1990).

21. Kalluri, R. & Neilson, E.G. Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest.* **112**, 1776–1784(2003).

22. Powell, D.W. et al. Myofibroblasts. II. Intestinal subepithelial myofibroblasts. *Am J Physiol Cell Physiol* **277**, C183-201(1999).

23. Frangogiannis, N.G., Michael, L.H. & Entman, M.L. Myofibroblasts in reperfused myocardial infarcts express the embryonic form of smooth muscle myosin heavy chain (SMemb). *Cardiovasc Res* **48**, 89-100(2000).

24. Petrov, V.V., Fagard, R.H. & Lijnen, P.J. Stimulation of Collagen Production by Transforming Growth Factor-beta1 During Differentiation of Cardiac Fibroblasts to Myofibroblasts. *Hypertension* **39**, 258-263(2002).

25. Tomasek, J.J. et al. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat. Rev. Mol. Cell Biol* **3**, 349-63(2002).

26. Zhang, H. & Phan, S.H. Inhibition of Myofibroblast Apoptosis by Transforming Growth Factor beta 1. *Am. J. Respir. Cell Mol. Biol.* **21**, 658-665(1999).

27. Gray, M.O. et al. Angiotensin II stimulates cardiac myocyte hypertrophy via paracrine release of TGF-beta1 and endothelin-1 from fibroblasts. *Cardiovasc Res* **40**, 352-363(1998).

28. Sleight, P. Angiotensin II and trials of cardiovascular outcomes. *Am J Cardiol* **89**, 11A-16A; discussion 16A-17A(2002).

29. Fox, K.M. Efficacy of perindopril in reduction of cardiovascular events among patients with stable coronary artery disease: randomised, double-blind, placebo-controlled, multicentre trial (the EUROPA study). *Lancet* **362**, 782-8(2003).

30. Brilla, C.G., Funck, R.C. & Rupp, H. Lisinopril-Mediated Regression of Myocardial Fibrosis in Patients With Hypertensive Heart Disease. *Circulation* **102**, 1388-1393(2000).

31. Schwartzkopff, B. et al. Repair of Coronary Arterioles After Treatment With Perindopril in Hypertensive Heart Disease. *Hypertension* **36**, 220-225(2000).

32. Abstract 3465: Meta-analyses Of Mortality And Morbidity Effects Of Angiotensin Receptor Blockers In Patients With Chronic Heart Failure. (2007).

33. Kantipudi, S. & Khasnis, A. Effects of Candesartan on Mortality and Morbidity in Patients With Chronic Heart Failure: The CHARM-Overall Programme. *Congestive Heart Failure* **10**, 114-116(2004).

34. Lopez, B. et al. Usefulness of Serum Carboxy-Terminal Propeptide of Procollagen Type I in Assessment of the Cardioreparative Ability of Antihypertensive Treatment in Hypertensive Patients. *Circulation* **104**, 286-291(2001).

35. Diez, J. et al. Losartan-Dependent Regression of Myocardial Fibrosis Is Associated With Reduction of Left Ventricular Chamber Stiffness in Hypertensive Patients. *Circulation* **105**, 2512-2517(2002).

36. Stowasser, M. New Perspectives On The Role Of Aldosterone Excess In Cardiovascular Disease. *Clinical and Experimental Pharmacology and Physiology* **28**, 783-791(2001).

37. Pitt, B. et al. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. *N Engl J Med* **341**, 709-17(1999).

38. Zannad, F. et al. Limitation of Excessive Extracellular Matrix Turnover May Contribute to Survival Benefit of Spironolactone Therapy in Patients With Congestive Heart Failure : Insights From the Randomized Aldactone Evaluation Study (RALES). *Circulation* **102**, 2700-2706(2000).

39. Guarda, E. et al. Effects of endothelins on collagen turnover in cardiac fibroblasts. *Cardiovasc Res* **27**, 2130-4(1993).

40. Cheng, T. et al. Involvement of reactive oxygen species in angiotensin II-induced endothelin-1 gene expression in rat cardiac fibroblasts. *J Am Coll Cardiol* **42**, 1845-54(2003).

41. Bauersachs, J. et al. Improvement of Left Ventricular Remodeling and Function by Hydroxymethylglutaryl Coenzyme A Reductase Inhibition With Cerivastatin in Rats With Heart Failure After Myocardial Infarction. *Circulation* **104**, 982-985(2001).

42. Ridker, P.M. et al. Inflammation, Pravastatin, and the Risk of Coronary Events After Myocardial Infarction in Patients With Average Cholesterol Levels. *Circulation* **98**, 839-844(1998).

43. Feldman, A.M. et al. The role of anticytokine therapy in heart failure: recent lessons from preclinical and clinical trials? *Med Clin North Am* **87**, 419-40(2003).

44. Isaka, Y. et al. Gene therapy by transforming growth factor-beta receptor-IgG Fc chimera suppressed extracellular matrix accumulation in experimental glomerulonephritis. *Kidney Int* **55**, 465-75(1999).

45. Kuwahara, F. et al. Transforming Growth Factor-{beta} Function Blocking Prevents Myocardial Fibrosis and Diastolic Dysfunction in Pressure-Overloaded Rats. *Circulation* **106**, 130-135(2002).

46. Hori, Y. et al. Anti-latent TGF-beta binding protein-1 antibody or synthetic oligopeptides inhibit extracellular matrix expression induced by stretch in cultured rat mesangial cells. *Kidney Int* **53**, 1616-25(1998).

47. Creemers, E.E. et al. Matrix Metalloproteinase Inhibition After Myocardial Infarction: A New Approach to Prevent Heart Failure? *Circ Res* **89**, 201-210(2001).

48. Li, Y.Y., McTiernan, C.F. & Feldman, A.M. Interplay of matrix metalloproteinases, tissue inhibitors of metalloproteinases and their regulators in cardiac matrix remodeling. *Cardiovasc Res* **46**, 214-24(2000).

49. Rohde, L.E. et al. Matrix Metalloproteinase Inhibition Attenuates Early Left Ventricular Enlargement After Experimental Myocardial Infarction in Mice. *Circulation* **99**, 3063-3070(1999).

50. Ducharme, A. et al. Targeted deletion of matrix metalloproteinase-9 attenuates left ventricular enlargement and collagen accumulation after experimental myocardial infarction. *J Clin Invest.* **106**, 55–62(2000).

51. Spinale, F.G. et al. Matrix Metalloproteinase Inhibition During the Development of Congestive Heart Failure : Effects on Left Ventricular Dimensions and Function. *Circ Res* **85**, 364-376(1999).

52. King, M.K. et al. Selective Matrix Metalloproteinase Inhibition With Developing Heart Failure: Effects on Left Ventricular Function and Structure. *Circ Res* **92**, 177-185(2003).

53. Brown, P.D. Ongoing trials with matrix metalloproteinase inhibitors. *Expert Opin Investig Drugs* **9**, 2167-77(2000).

54. Iyer, S.N., Gurujeyalakshmi, G. & Giri, S.N. Effects of Pirfenidone on Transforming Growth Factor-beta Gene Expression at the Transcriptional Level in Bleomycin Hamster Model of Lung Fibrosis. *J Pharmacol Exp Ther* **291**, 367-373(1999).

55. Raghu, G. et al. Treatment of Idiopathic Pulmonary Fibrosis with a New Antifibrotic Agent, Pirfenidone . Results of a Prospective, Open-label Phase II Study. *Am. J. Respir. Crit. Care Med.* **159**, 1061-1069(1999).

56. Fukagawa, M. et al. Chronic progressive interstitial fibrosis in renal disease--are there novel pharmacological approaches? *Nephrol. Dial. Transplant.* **14**, 2793-2795(1999).

57. Holmes, D.R. et al. Results of Prevention of REStenosis with Tranilast and its Outcomes (PRESTO) Trial. *Circulation* **106**, 1243-1250(2002).

58. Wayman, N.S. et al. Ligands of the peroxisome proliferator-activated receptors (PPAR-gamma and PPAR-alpha) reduce myocardial infarct size. *FASEB J.* **16**, 1027-1040(2002).

59. Diep, Q.N. et al. PPAR alpha activator fenofibrate inhibits myocardial inflammation and fibrosis in angiotensin II-infused rats. *J Mol Cell Cardiol* **36**, 295-304(2004).

60. Littlewood, T.D. & Evan, G.I. *Helix-loop-helix Transcription Factors*. 164(Oxford University Press: 1998).

61. Lodish, H. et al. *McGill Lodish 5E Package - Molecular Cell Biology & McGill Activation Code*. (W. H. Freeman: 2003).

62. Chaudhary, J. & Skinner, M.K. Basic Helix-Loop-Helix Proteins Can Act at the E-Box within the Serum Response Element of the c-fos Promoter to Influence Hormone-Induced Promoter Activation in Sertoli Cells. *Mol Endocrinol* **13**, 774-786(1999).

63. Goldfarb, A.N. & Lewandowska, K. Nuclear redirection of a cytoplasmic helix-loop-helix protein via heterodimerization with a nuclear localizing partner. *Exp Cell Res* **214**, 481-5(1994).

64. Goldhamer, D.J. et al. Embryonic activation of the myoD gene is regulated by a highly conserved distal control element. *Development* **121**, 637-49(1995).

65. Ellenberger, T. et al. Crystal structure of transcription factor E47: E-box recognition by a basic region helix-loop-helix dimer. *Genes Dev* **8**, 970-80(1994).

66. Hainzl, T. & Boehm, T. A versatile expression vector for the in vitro study of protein-protein interactions: characterization of E47 mutant proteins. *Oncogene* **9**, 885-91(1994).

67. Fisher, D.E., Parent, L.A. & Sharp, P.A. Myc/Max and other helix-loop-helix/leucine zipper proteins bend DNA toward the minor groove. *Proc Natl Acad Sci U S A.* **89**, 11779–11783(1992).

68. Wechsler, D.S. & Dang, C.V. Opposite orientations of DNA bending by c-Myc and Max. *Proc Natl Acad Sci U S A.* **89**, 7635–7639(1992).

69. Fisher, F. & Goding, C.R. Single amino acid substitutions alter helix-loop-helix protein specificity for bases flanking the core CANNTG motif. *EMBO J.* **11**, 4103–4109(1992).

70. Martelli, F. et al. MyoD induces retinoblastoma gene expression during myogenic differentiation. *Oncogene* **9**, 3579-90(1994).

71. Li, L. & Olson, E.N. Regulation of muscle cell growth and differentiation by the MyoD family of helix-loop-helix proteins. *Adv Cancer Res* **58**, 95-119(1992).

72. Alemá, S. & Tató, F. Interaction of retroviral oncogenes with the differentiation program of myogenic cells. *Adv Cancer Res* **49**, 1-28(1987).

73. Barone, M.V. et al. Id proteins control growth induction in mammalian cells. *Proc Natl Acad Sci U S A.* **91**, 4985–4988(1994).

74. Hara, E. et al. Id-related genes encoding helix-loop-helix proteins are required for G1 progression and are repressed in senescent human fibroblasts. *J Biol Chem* **269**, 2139-45(1994).

75. Biggs, J., Murphy, E.V. & Israel, M.A. A human Id-like helix-loop-helix protein expressed during early development. *Proc Natl Acad Sci U S A* **89**, 1512-6(1992).

76. Ellmeier, W. & Weith, A. Expression of the helix-loop-helix gene Id3 during murine embryonic development. *Dev Dyn* **203**, 163-73(1995).

77. Riechmann, V. & Sablitzky, F. Mutually exclusive expression of two dominant-negative helix-loop-helix (dnHLH) genes, Id4 and Id3, in the developing brain of the mouse suggests distinct regulatory roles of these dnHLH proteins during cellular proliferation and differentiation of the nervous system. *Cell Growth Differ* **6**, 837-843(1995).

78. Schweitzer, R. et al. Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. *Development* **128**, 3855-66(2001).

79. Brent, A.E., Schweitzer, R. & Tabin, C.J. A somitic compartment of tendon progenitors. *Cell* **113**, 235-48(2003).

80. Carlberg, A.L., Tuan, R.S. & Hall, D.J. Regulation of Scleraxis Function by Interaction with the bHLH Protein E47. *Molecular Cell Biology Research Communications* **3**, 82-86(2000).

81. Brown, D. et al. Dual role of the basic helix-loop-helix transcription factor scleraxis in mesoderm formation and chondrogenesis during mouse embryogenesis. *Development* **126**, 4317-4329(1999).

82. Lincoln, J., Alfieri, C.M. & Yutzey, K.E. Development of heart valve leaflets and supporting apparatus in chicken and mouse embryos. *Developmental Dynamics* **230**, 239-250(2004).

83. Pourquie, J.D.A.O. Welcome to Syndetome: A New Somitic Compartment. *Developmental Cell* **Vol 4**, 611-612(2003).

84. Brent, A.E., Braun, T. & Tabin, C.J. Genetic analysis of interactions between the somitic muscle, cartilage and tendon cell lineages during mouse development. *Development* **132**, 515-528(2005).

85. Asou, Y. et al. Coordinated expression of *scleraxis* and *Sox9* genes during embryonic development of tendons and cartilage. *Journal of Orthopaedic Research* **20**, 827-833(2002).

86. Levay, A.K. et al. Scleraxis Is Required for Cell Lineage Differentiation and Extracellular Matrix Remodeling During Murine Heart Valve Formation In Vivo. *Circ Res* **103**, 948-956(2008).

87. Watanabe, H. et al. Mouse cartilage matrix deficiency (cmd) caused by a 7 bp deletion in the aggrecan gene. *Nat Genet* **7**, 154-157(1994).

88. Nieden, N.I.Z. et al. Induction of chondro-, osteo- and adipogenesis in embryonic stem cells by bone morphogenetic protein-2: Effect of cofactors on differentiating lineages. *BMC Dev Biol.* **5**, 1(2005).

89. Salingcarnboriboon, R. et al. Establishment of tendon-derived cell lines exhibiting pluripotent mesenchymal stem cell-like property. *Exp Cell Res* **287**, 289-300(2003).

90. Strezoska, Z., Pestov, D.G. & Lau, L.F. Functional Inactivation of the Mouse Nucleolar Protein Bop1 Inhibits Multiple Steps in Pre-rRNA Processing and Blocks Cell Cycle Progression. *J. Biol. Chem.* **277**, 29617-29625(2002).

91. Murchison, N.D. et al. Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. *Development* **134**, 2697-2708(2007).

92. Kannus, P. Structure of the tendon connective tissue. *Scand J Med Sci Sports* **10**, 312-20(2000).

93. Kadler K et al. Collagen fibril formation. (1996).

94. Hubert, T. et al. Collagens in the developing and diseased nervous system. *Cell Mol Life Sci* (2008).

95. Kadler, K.E. et al. Collagens at a glance. *J Cell Sci* **120**, 1955-1958(2007).

96. Bulleid†, L.M.H.A.N.J. Protein-specific chaperones: The role of hsp47 begins to gel. *Current Biology* **Vol 10**, R912-R915(2000).

97. P Walter, A. & A E Johnson Signal Sequence Recognition and Protein Targeting to the Endoplasmic Reticulum Membrane. (2003).

98. van der Rest, M. & Garrone, R. Collagen family of proteins. *FASEB J.* **5**, 2814-2823(1991).

99. Banos, C.C., Thomas, A.H. & Kuo, C.K. Collagen fibrillogenesis in tendon development: Current models and regulation of fibril assembly. *Birth Defects Research Part C: Embryo Today: Reviews* **84**, 228-244(2008).

100. Myllyharju, J. Intracellular Post-Translational Modifications of Collagens. *Collagen* 115-147(2005).

101. Torre-Blanco, A. et al. Copolymerization of normal type I collagen with three mutated type I collagens containing substitutions of cysteine at different glycine positions in the alpha 1 (I) chain. *J. Biol. Chem.* **267**, 4968-4973(1992).

102. Wilson, R., Lees, J.F. & Bulleid, N.J. Protein disulfide Isomerase Acts as a Molecular Chaperone during the Assembly of Procollagen. *J. Biol. Chem.* **273**, 9637-9643(1998).

103. Lamandé, S.R. & Bateman, J.F. Procollagen folding and assembly: the role of endoplasmic reticulum enzymes and molecular chaperones. *Semin Cell Dev Biol* **10**, 455-64(1999).

104. Cho, M. & Garant, P.R. Role of microtubules in the organization of the Golgi complex and the secretion of collagen secretory granules by periodontal ligament fibroblasts. *The Anatomical Record* **199**, 459-471(1981).

105. Martínez-Menárguez, J.A. et al. Vesicular tubular clusters between the ER and Golgi mediate concentration of soluble secretory proteins by exclusion from COPI-coated vesicles. *Cell* **98**, 81-90(1999).

106. Bannykh, S.I. & Balch, W.E. Membrane Dynamics at the Endoplasmic Reticulum-Golgi Interface. *J. Cell Biol.* **138**, 1-4(1997).

107. Pelham, H.R. Traffic through the Golgi apparatus. *J. Cell Biol.* **155**, 1099-1102(2001).

108. Stephens, D.J. & Pepperkok, R. Imaging of procollagen transport reveals COPI-dependent cargo sorting during ER-to-Golgi transport in mammalian cells. *J Cell Sci* **115**, 1149-1160(2002).

109. Bonfanti, L. et al. Procollagen traverses the Golgi stack without leaving the lumen of cisternae: evidence for cisternal maturation. *Cell* **95**, 993-1003(1998).

110. Leblond, C.P. Synthesis and secretion of collagen by cells of connective tissue, bone, and dentin. *The Anatomical Record* **224**, 123-138(1989).

111. Canty, E.G. et al. Coalignment of plasma membrane channels and protrusions (fibripositors) specifies the parallelism of tendon. *J Cell Biol* **165**, 553-63(2004).

112. Birk, D. & Trelstad, R. Extracellular compartments in tendon morphogenesis: collagen fibril, bundle, and macroaggregate formation. *J. Cell Biol.* **103**, 231-240(1986).

113. Hojima, Y. et al. Cleavage of type I procollagen by C- and N-proteinases is more rapid if the substrate is aggregated with dextran sulfate or polyethylene glycol. *Anal Biochem* **223**, 173-80(1994).

114. Humphries, S.M. et al. Active negative control of collagen fibrillogenesis in vivo: Intracellular cleavage of the type I procollagen propeptides in tendon fibroblasts without intracellular fibrils. *J. Biol. Chem.* M708198200(2008).

115. Prockop, D.J. et al. The biosynthesis of collagen and its disorders (first of two parts). *N Engl J Med* **301**, 13-23(1979).

116. Parkinson, Kadler & Brass Self-assembly of rodlike particles in two dimensions: A simple model for collagen fibrillogenesis. *Phys Rev E Stat Phys Plasmas Fluids Relat Interdiscip Topics* **50**, 2963-2966(1994).

117. Hurskainen, T.L. et al. ADAM-TS5, ADAM-TS6, and ADAM-TS7, Novel Members of a New Family of Zinc Metalloproteases.General features and genomic distribution of the ADAM-TS family. *J. Biol. Chem.* **274**, 25555-25563(1999).

118. Plumb, D.A. et al. Collagen XXVII Is Developmentally Regulated and Forms Thin Fibrillar Structures Distinct from Those of Classical Vertebrate Fibrillar Collagens. *J. Biol. Chem.* **282**, 12791-12795(2007).

119. Schekman, R. & Mellman, I. Does COPI go both ways? *Cell* **90**, 197-200(1997).

120. Rothman, J.E. & Wieland, F.T. Protein sorting by transport vesicles. *Science* **272**, 227-34(1996).

121. Brown, D.L. et al. The GRIP Domain is a Specific Targeting Sequence for a Population of trans-Golgi Network Derived Tubulo-Vesicular Carriers. *Traffic* **2**, 336-344(2001).

122. Birk, D. et al. Collagen fibril bundles: a branching assembly unit in tendon morphogenesis. *Development* **107**, 437-443(1989).

123. Graham, H.K. et al. Identification of collagen fibril fusion during vertebrate tendon morphogenesis. The process relies on unipolar fibrils and is regulated by collagen-proteoglycan interaction. *J Mol Biol* **295**, 891-902(2000).

124. Birk, D.E. & Zycband, E. Assembly of the tendon extracellular matrix during development. *J Anat.* **184**, 457–463(1994).

125. Canty, E.G. et al. Actin Filaments Are Required for Fibripositor-mediated Collagen Fibril Alignment in Tendon. *J. Biol. Chem.* **281**, 38592-38598(2006).

126. Birk, D.E. & Mayne, R. Localization of collagen types I, III and V during tendon development. Changes in collagen types I and III are correlated with changes in fibril diameter. *Eur J Cell Biol* **72**, 352-61(1997).

127. Ghosh, A.K. Factors Involved in the Regulation of Type I Collagen Gene Expression: Implication in Fibrosis. *Experimental Biology and Medicine* **227**, 301-314(2002).

128. Hu, P.P., Datto, M.B. & Wang, X.F. Molecular mechanisms of transforming growth factor-beta signaling. *Endocr Rev* **19**, 349-63(1998).

129. Pulaski, L. et al. Phosphorylation of Smad7 at Ser249 does not interfere with its inhibitory role in TGF-beta-dependent signaling, but affects Smad7-dependent transcriptional activation. *J. Biol. Chem.* M011019200(2001).

130. Leask, A. TGF-beta, cardiac fibroblasts, and the fibrotic response. *Cardiovasc Res* **74**, 207-212(2007).

131. Crawford, S.E. et al. Thrombospondin-1 is a major activator of TGF-beta1 in vivo. *Cell* **93**, 1159-70(1998).

132. Lyons, R. et al. Mechanism of activation of latent recombinant transforming growth factor beta 1 by plasmin. *J. Cell Biol.* **110**, 1361-1367(1990).

133. Zawel, L. et al. Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol Cell* **1**, 611-7(1998).

134. Liu, B. et al. FAST-2 Is a Mammalian Winged-Helix Protein Which Mediates Transforming Growth Factor beta □Signals. *Mol. Cell. Biol.* **19**, 424-430(1999).

135. Leask, A. & Abraham, D.J. TGF-beta signaling and the fibrotic response. *FASEB J* **18**, 816-27(2004).

136. Nakao, A. et al. Identification of Smad7, a TGF-beta inducible antagonist of TGF-beta signalling. *Nature* **389**, 631-635(1997).

137. Ogunjimi, A.A. et al. Regulation of Smurf2 ubiquitin ligase activity by anchoring the E2 to the HECT domain. *Mol Cell* **19**, 297-308(2005).

138. von Gersdorff, G. et al. Smad3 and Smad4 mediate transcriptional activation of the human Smad7 promoter by transforming growth factor beta. *J Biol Chem* **275**, 11320-6(2000).

139. Dupont, S. et al. FAM/USP9x, a deubiquitinating enzyme essential for TGF-beta signaling, controls Smad4 monoubiquitination. *Cell* **136**, 123-35(2009).

140. Raizman, J.E. et al. The participation of the Na+-Ca2+ exchanger in primary cardiac myofibroblast migration, contraction, and proliferation. *J. Cell. Physiol* **213**, 540-51(2007).

141. Waser, M. et al. Regulation of Calreticulin Gene Expression by Calcium. *J. Cell Biol.* **138**, 547-557(1997).

142. Wang, B. et al. Regulation of collagen synthesis by inhibitory Smad7 in cardiac myofibroblasts. *Am J Physiol Heart Circ Physiol* 00910.2006(2007).

143. Wang, B. et al. Decreased Smad 7 expression contributes to cardiac fibrosis in the infarcted rat heart. *Am J Physiol Heart Circ Physiol* **282**, H1685-1696(2002).

144. Ju, H. et al. Effect of AT1 receptor blockade on cardiac collagen remodeling after myocardial infarction. *Cardiovasc Res* **35**, 223-232(1997).

145. Freed, D.H. et al. Emerging evidence for the role of cardiotrophin-1 in cardiac repair in the infarcted heart. *Cardiovasc Res* **65**, 782-792(2005).

146. Khan, R. & Sheppard, R. Fibrosis in heart disease: understanding the role of transforming growth factor-β1 in cardiomyopathy, valvular disease and arrhythmia. *Immunology* **118**, (2006).

147. Inman, G.J. Linking Smads and transcriptional activation. *Biochem J.* **386**, E1(2005).