

Transcriptional Regulation of Ski and Scleraxis in Primary
Cardiac Myofibroblasts

by

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Abstract

Transforming growth factor- β_1 (TGF β_1) is a mediator of the fibrotic response through activation of quiescent cardiac fibroblasts to hypersynthetic myofibroblasts. Scleraxis (Scx) is a pro-fibrotic transcription factor that is induced by TGF β_{1-3} and works synergistically with Smads to promote collagen expression. Ski is a negative regulator of TGF β /Smad signaling through its interactions with Smad proteins at the promoter region of TGF β regulated genes. To date, no studies have examined the direct DNA:protein transcriptional mechanisms that regulate Scx expression by TGF β_{1-3} or Ski, nor the mechanisms that govern Ski expression by Scx. We hypothesize that Ski and Scx regulate one another, and form a negative feedback loop that represses gene expression and is a central regulator of the fibrotic response in cardiac myofibroblasts.

Primary adult rat cardiac myofibroblasts were isolated *via* retrograde Langendorff perfusion. First passage (P1) cells were infected with adenovirus encoding HA-Ski, HA-Scx, or LacZ at the time of plating. Twenty-four hours later, cells were harvested for Western blot, quantitative real-time PCR (qPCR), and electrophoretic gel shift assays (EMSA). NIH-3T3 or Cos7 cells were transfected with equal quantities of plasmid DNA for 24 hours prior to harvesting for luciferase, qPCR, and EMSA analysis.

Ski overexpression in P1 myofibroblasts resulted in a reduction in both Scx mRNA and protein levels. Overexpression of Scx had no effect on Ski expression. Luciferase reporter assays demonstrated that Scx was induced by TGF β_1 treatment in a concentration dependent manner. However, ectopic Smad2/3 expression was unable to transactivate the Scx promoter in a luciferase reporter assay. Inhibition of p44/42-

MAPK signaling modestly counteracted the effect of $\text{TGF}\beta_1$ on Scx expression. Scx had no effect on Ski promoter expression, however, both tumor necrosis factor- α (TNF α) and p65 expression repressed the Ski promoter and correlated with reduced Ski mRNA levels.

We conclude that Ski is a repressor of Scx and that Scx expression is partially mediated through a Smad-independent, p44/42-MAPK pathway in cardiac myofibroblasts. Furthermore, this study proposes a role for TNF α /p65 NF- κ B signaling in the regulation of Ski gene expression in the cardiac myofibroblast.

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Dedication

To the love of my life and my best friend, Jessica.

Table of Contents

Abstract.....	i
Acknowledgments.....	iii
Dedication.....	v
Table of Contents.....	vi
List of Tables	x
List of Figures	xi
List of Abbreviations	xiv
Chapter 1: Literature Review.....	1
Prevalence and Epidemiology of Cardiovascular Disease.....	1
Risk Factors for Cardiovascular Disease	2
Cardiac Fibrosis as a Primary Cause of Heart Failure	3
Fibrotic Remodelling and Cardiovascular Disease	3
Wound Healing Following Myocardial Infarction.....	5
The Extracellular Matrix	9
The Cardiac Fibroblast and Myofibroblast	11
Transforming Growth Factor β	14
Smad-Dependent Signaling.....	16
Smad Structure and Regulation	21
Smad-Dependent Mechanisms and Fibrosis.....	23
Smad-Independent Signaling Initiated by TGF β_1	25
TGF β_1 -Mediated p38 Mitogen-Activated Protein Kinases (p38-MAPK) Signaling	25
TGF β_1 -Mediated Phosphatidylinositol 3-Kinase (PI3K)/Akt Signaling	29
TGF β_1 -Mediated Rho/ROCK Signaling.....	33
TGF β_1 -Mediated Extracellular Signal-Related Kinase 1/2 (Erk1/2) Signaling.....	36
Smad-Independent Mechanisms and Fibrosis	39
Tumor Necrosis Factor α	43
Nuclear Factor- κ B Signaling.....	44
TNF α , NF- κ B, and Fibrosis.....	44

Ski.....	46
History and Identification.....	46
Protein Structure.....	48
Transcriptional and Post-Translational Regulation of Ski	50
Ski in Development and Disease	52
Ski and TGF β Signaling	54
Scleraxis	58
History and Identification.....	58
Protein Structure.....	58
Regulation of Scleraxis.....	59
Chapter 2: Overall Study Rationale, Hypothesis, and Aims.....	60
Chapter 3: Transcriptional Regulation of Scleraxis.....	62
Rationale, Hypothesis, and Objectives.....	62
Methodology	63
Animal Ethics	63
<i>In silico</i> Analysis of the Scleraxis Promoter	63
Cloning of the Human Scleraxis Promoter.....	64
Luciferase Assay.....	65
Isolation of Primary Cardiac Myofibroblasts	66
Protein Isolation and Quantification.....	68
Western Blotting.....	69
Generation of Expression Vectors.....	70
Scleraxis.....	70
Ski	73
Cell Fractionation	73
Electrophoretic Mobility Shift Assay (EMSA)	74
Immunoprecipitation	76
Chromatin Immunoprecipitation	77
Quantitative Real-Time PCR.....	80
Inhibition of MEK1/2	82
Rat Model of Myocardial Infarction.....	82
Echocardiography.....	83

Statistics.....	84
Results	84
Ski is a Negative Regulator of TGF β ₁ /Smad Signaling	84
Ski Represses Scleraxis Expression	88
<i>In silico</i> Analysis of the Scleraxis Promoter	90
TGF β ₁ Promotes Scleraxis Expression.....	92
TGF β ₁ Promotes Scleraxis Expression in a Smad-Independent Manner	95
Scleraxis Expression is Modulated by p44/42 MAPK Signaling Pathway	100
Ski Does Not Affect Phosphorylation of MAPK Protein Kinases	107
Ski Interacts with c-Jun to Repress MAPK Signaling.....	109
Myocardial Function in the Post-MI Heart.....	112
MAPK Signaling is Elevated in the Post-MI Heart.....	114
Discussion	119
Conclusions and Future Directions	128
Chapter 4: Transcriptional Regulation of Ski	132
Rationale, Hypothesis, and Objectives.....	132
Methodology	133
Animal Ethics	133
<i>In silico</i> Analysis of the Ski Promoter	133
Isolation of BAC DNA	134
Cloning of the 1.8 kb Human Ski Promoter	135
Isolation of Primary Cardiac Myofibroblasts	142
Cell Culture.....	142
Luciferase Assay.....	142
Protein Isolation and Western Blot Analysis.....	143
Quantitative Real-Time PCR.....	144
Statistics.....	145
Results	145
<i>In silico</i> Analysis of the Ski Promoter.....	145
Scleraxis Does Not Regulate Transcription of Ski.....	146
p65-NF- κ B is a Negative Regulator of Ski Gene Expression	149
Discussion	154

Conclusions and Future Directions	158
Chapter 5: Overall Discussion, Conclusion, and Future Directions	161
Chapter 6: References	165
Appendices.....	215
Appendix A	215
Clustal W of the Mouse, Human, and Rat Scleraxis Proximal Promoter.....	215
Clustal W of the Mouse, Human, and Rat Scleraxis Distal Promoter.....	218
Appendix B	222
Fractionation of Cos7 Cells	222
Electrophoretic Mobility Gel Shift Assay of the Distal and Proximal SBE of the Human Scleraxis Promoter in Cos7 Cells.	223
Appendix C	225
Clustal W of the Mouse, Human, and Rat Ski Proximal Promoter	225
Clustal W of the Mouse and Human Ski Proximal Promoter.....	228
Appendix D	231
Chromatin Immunoprecipitation of the Ski Proximal Promoter	231

List of Tables

Table 1. Previously Reported Smad Binding Elements (SBE).....	18
Table 2. PCR Cycling for Cloning Human Scleraxis into pcDNA3.....	71
Table 3. Electrophoretic Mobility Shift Assay Probes.....	75
Table 4. ChIP Primers.....	79
Table 5. qPCR Primers.....	81
Table 6. qPCR Cycling Conditions.....	81
Table 7. Primer Sequences for the Synthesis of the Human Ski Proximal Promoter.....	136
Table 8. PCR Reaction Composition for the Synthesis of the Human Ski Proximal Promoter.....	137
Table 9. Thermocycling Profile for Amplification of the Human Ski Proximal Promoter from a Bacterial Artificial Chromosome.....	138
Table 10. Nested PCR Reaction.....	140
Table 11. Nested PCR Cycling Conditions.....	140
Table 12. Real-time qPCR Cycling Conditions.....	144
Table 13. Real-time qPCR Primer Sequences.....	145
Table D-1. Ski Proximal Promoter ChIP Primers.....	231
Table D-2. Ski Proximal Promoter ChIP PCR Profile.....	232

List of Figures

Figure 1. Phases of Human Myocardial Wound Healing Post-MI.....	8
Figure 2. Canonical TGF β ₁ /Smad-Dependent Signaling.....	20
Figure 3. Protein Structure of Smad Proteins.....	22
Figure 4. TGF β ₁ /Smad-Independent Signaling: p38-MAPK.....	27
Figure 5. TGF β ₁ /Smad-Independent Signaling: PI3K/Akt.....	32
Figure 6. TGF β ₁ /Smad-Independent Signaling: Rho/ROCK.....	35
Figure 7. TGF β ₁ /Smad-Independent Signaling: Extracellular Signal-Related Kinase 1/2 (Erk1/2).....	38
Figure 8. Protein Structure of Ski.....	50
Figure 9. Ski Mediated Repression of TGF β ₁ /Smad Signaling.....	57
Figure 10. Overall Visual Hypothesis.....	60
Figure 11. Equation for Plating of NIH-3T3 Cells.....	65
Figure 12. Rat MI Randomization Scheme and Surgery Timeline.....	83
Figure 13. Overexpression of Ski in Primary P1 Cardiac Myofibroblasts.....	85
Figure 14. mRNA Expression of Fibrillar Collagens in Ski Overexpressing P1 Myofibroblasts.....	86-88
Figure 15. Scleraxis Protein and mRNA Expression in Ski Overexpressing Myofibroblasts.....	89-90
Figure 16. Conserved SBE of the Mouse, Human, and Rat Scleraxis Promoter.....	91
Figure 17. TGF β ₁ Stimulation of Scleraxis.....	93
Figure 18. Activation of the Human Scleraxis Promoter by TGF β ₁	95

Figure 19. hSmad2/3 Luciferase Assay of the Human Scleraxis Promoter.....	96-97
Figure 20. Electrophoretic Mobility Gel Shift Assay of the Distal and Proximal SBE of the Human Scleraxis Promoter.....	99
Figure 21. Temporal Phosphorylation of p44/42 by TGF β ₁ and its Effect on Scleraxis Protein Expression.....	102-105
Figure 22. U0126 Treatment of P1 Cardiac Myofibroblasts and Scleraxis Expression.....	106-107
Figure 23. Phosphorylation of MAPK Proteins Following Ski Expression.....	108-109
Figure 24. Interactions between Ski, c-Jun, and the Scleraxis Promoter.....	110-111
Figure 25. Echocardiographic M-Mode Images of the Sham Operated and Ligated Left Ventricle.....	113
Figure 26. Cardiac Function in the Post-MI Heart.....	114
Figure 27. <i>In vivo</i> Expression and Phosphorylation of MAPK Proteins Post-MI.....	116-119
Figure 28. Graphic Depiction of the Human Scleraxis Promoter and Putative Transcription Factor Binding Sites.....	126
Figure 29. Proposed Mechanism for the Regulation of Scleraxis by p44/42 (Erk1/2) and Ski.....	129
Figure 30. Formula for Calculating DNA Concentration.....	139
Figure 31. Scleraxis Regulation of the Human Ski Proximal Promoter.....	147
Figure 32. Scleraxis Overexpression in Primary Cardiac Myofibroblasts and Ski Expression.....	148-149

Figure 33. p65/TNF α Mediated Regulation of the Human Ski Proximal Promoter.....	151-154
Figure 34. Schematic Summary for the Transcriptional Regulation of Ski and Scleraxis in the Primary Cardiac Myofibroblast and its Proposed Role in Fibrogenesis...	162-163
Figure A-1. Clustal W of the Mouse, Human, and Rat Scleraxis Proximal Promoter.....	215-217
Figure A-2. Clustal W of the Mouse, Human, and Rat Scleraxis Distal Promoter.....	218-221
Figure B-1. Fractionation of Cos7 Cells.....	222
Figure B-2. Electrophoretic Mobility Gel Shift Assay of the Distal and Proximal SBE of the Human Scleraxis Promoter in Cos7 Cells.....	223
Figure C-1. Clustal W of the Mouse, Human, and Rat Ski Proximal Promoter.	225-227
Figure C-2. Clustal W of the Mouse and Human Ski Proximal Promoter.....	228-230
Figure D-1. ChIP Analysis of the Interaction of the NF- κ B p65 Subunit with the Cos7 Ski Proximal Promoter.....	232

List of Abbreviations

aa	Amino Acid
AP1	Activator Protein 1
ATCC	American Type Culture Collection
ATF2	Activating Transcription Factor 2
α -SMA	α -Smooth Muscle Actin
BAC	Bacterial Artificial Chromosome
bHLH	Basic Helix-Loop-Helix
BMP	Bone Morphogenetic Protein
bp	Base Pairs
BSA	Bovine Serum Albumin
C-Terminal	Carboxyl-Terminal
CAD	Canadian Dollar
cAMP	Cyclic Adenosine Monophosphate
CAMK	Calcium-Calmodulin Dependent Kinase
CAT	Chloramphenicol Acetyltransferase
CBP	CREB-Binding Protein
Cdc42	Cell division cycle-42
ChIP	Chromatin Immunoprecipitation
CK2	Casein Kinase 2
Co-Smad4	Co-mediator Smad4
Col 1 α 1	Collagen type 1 α 1
Col 1 α 2	Collagen type 1 α 2
Col 3 α 1	Collagen type 3 α 1
CoIP	Co-Immunoprecipitation
CTGF	Connective Tissue Growth Factor
CVD	Cardiovascular Disease
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DHD	Dachshund Homology Domain
DN	Dominant Negative
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
ED-A FN	Extra Domain-A Fibronectin
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EMSA	Electrophoretic Mobility Shift Assay
EMT	Epithelial-to-Mesenchymal Transition
EndoMT	Endothelial-to-Mesenchymal Transition
Erk1/2	Extracellular Signal-Regulated Kinase 1/2
ESC	Embryonic Stem Cells
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
gDNA	Genomic DNA

Grb2	Growth Factor Receptor Binding Protein 2
HA	Hemagglutinin
HDAC	Histone Deacetylase
HFpEF	Heart Failure with Preserved Ejection Fraction
HSC	Hepatic Stellate Cells
hScx	Human Scleraxis
hScxP	Human Scleraxis Promoter
hSki	Human Ski
hSkiPP	Human Ski Proximal Promoter
I-Smad	Inhibitory Smad
I.P.	Intraperitoneal
IKK	I- κ B Kinase
IL	Interleukin
ILK	Integrin-Linked Kinase
IPF	Idiopathic Pulmonary Fibrosis
JNK	c-Jun N-Terminal Kinase
kDa	kilodaltons
LAD-CA	Left-Anterior Descending Coronary Artery
LB	Luria Broth
LIMK	LIM Kinase
LAP	Latency Associated Peptide
LTBP	Latent TGF β Binding Protein
LV	Left Ventricle
MCK	Muscle Creatine Kinase
MDCK	Madin-Darby Canine Kidney
MEK1/2	MAP Kinase Kinase 1/2
MIS	Müllerian Inhibiting Substance
MAPK	Mitogen Activated Protein Kinase
MH1	Mad Homology Domain 1
MH2	Mad Homology Domain 2
MI	Myocardial Infarction
MKK	MAPK Kinase
MLF	Murine Lung Fibroblasts
MMP	Matrix Metalloproteinase
MOI	Multiplicity of Infection
mRNA	Messenger Ribonucleic Acid
MRTF	Myocardin Related Transcription Factor
mScx	Mouse Scleraxis
NCBI	National Center for Biotechnology Information
NCoR	Nuclear Corepressor
NF- κ B	Nuclear Factor κ B
NH2-Terminal	Amino-Terminus
NLS	Nuclear Localization Signal
ORF	Open Reading Frame
PAK2	p21-Activated Kinase
PAD	Peripheral Artery Disease

PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PI3K	Phosphatidylinositol 3-Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PPAR δ	Peroxisome-Proliferator Activated Receptor δ
PPRE	PPAR Response Element
PVDF	Polyvinylidene Fluoride
R-Smad	Receptor-Smad
RGD	Arginine-Glycine-Aspartic Acid
RHD	Rel Homology Domain
rhTGF β ₁	Recombinant Human TGF β ₁
rhTNF α	Recombinant Human TNF α
RIPA	Radioimmunoprecipitation Assay Buffer
ROS	Reactive Oxygen Species
rpm	Revolutions Per Minute
SAND	Sp100, Autoimmune Regulator 1, NucP41/75, Deformed Epidermal Autoregulator Factor 1
SARA	Smad Anchor for Receptor Activation
SBE	Smad Binding Element
Scx	Scleraxis
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SE	Standard Error
SGS	Shprintzen-Goldberg Syndrome
ShcA/B/C	Src Homology 2 Containing Protein A/B/C
Ski	Cellular Ski
SLC	Small Latency Complex
SMEM	Spinner-Modified Minimum Essential Media
SMemb	Smooth Muscle Embryonic Myosin Heavy Chain
SMRT	Silencing Mediator for Retinoid and Thyroid Receptors
Smurf2	Smad Ubiquitin Regulatory Factor 2
SOS	Son of Sevenless
Sp1	Specificity Protein 1
SRF	Serum Response Factor
SSXS	Serine/Serine/Any/Serine
TACE	TNF α Converting Enzyme
TAK1	TGF Activating Kinase
T β R	TGF β Receptor
T β RI	TGF β Receptor Type I
T β RII	TGF β Receptor Type II
TBS-T	Tris-Buffered Saline + Tween-20
TGF β	Transforming Growth Factor β

TNF α	Tumor Necrosis Factor α
TNFR1/2	Tumour Necrosis Factor α Receptor 1 and 2
TRAF6	TNF receptor associated factor 6
TSP1	Thrombospondin-1
TTE	Transthoracic Echocardiography
UTR	Untranslated Region
v/v	Volume/Volume
v-Ski	Viral Ski
w/v	Weight/Volume
WCL	Whole Cell Lysate
Wk	Week
WHO	World Health Organization
WT	Wild Type

Chapter 1: Literature Review

Prevalence and Epidemiology of Cardiovascular Disease

Cardiovascular disease (CVD) is a major burden to societies worldwide in terms of morbidity, mortality, and financial costs¹⁻⁴. The World Health Organization (WHO) defines CVD as “disorders of the heart and blood vessels that includes coronary heart disease (heart attacks), cerebrovascular disease (stroke), hypertension, peripheral artery disease (PAD), rheumatic heart disease, congenital heart disease, and heart failure”⁵. Cardiovascular disease is the leading cause of non-communicable disease worldwide and was estimated to be responsible for over 17 million deaths in 2008 and 2012^{1, 6} and is expected to exceed 22 million deaths annually by 2030⁶. The WHO reported that not only was CVD responsible for 37% of all non-communicable disease related deaths⁶, but was directly accountable for 31% of all-cause mortality worldwide¹. Within this population, coronary heart disease (i.e., acute myocardial infarction (MI)) accounted for nearly 46% and 38% of death in males and females, respectively¹.

In Canada, CVD annually accounts for 20% of all-cause mortality, with coronary heart disease representing approximately half of this population⁷. In the United States, more than 82 million (greater than 1 in 3) Americans are afflicted with at least one type of CVD^{8, 9}. Of these individuals, approximately 92% are hypertensive, and 20% have some form of coronary heart disease of which MI represents half of this population^{8, 9}.

The cost of treatment and care of patients with CVD in Canada exceeds \$20 billion CAD annually^{10, 11}. Medical costs associated with CVD in the United States and the United Kingdom exceed \$180 billion USD (\$189 billion CAD) and €169 billion

Euro¹² (\$272 billion CAD) annually, respectively. Irrespective of the societal impact of cardiovascular disease, the toll on the affected individuals and their families is inestimable.

Risk Factors for Cardiovascular Disease

Cardiovascular disease is a worldwide epidemic afflicting individuals of both sexes, all races, and all social-economic classes. Men are at a higher risk for CVD than women, and low-middle class families have a significantly greater life time risk for developing CVD as compared to the upper classes^{8, 13}. It is important to note that while CVD affects everyone, the risk for developing CVD is disproportionate amongst these groups.

Risk factors for CVD can be broken down into two broad categories that include: i) non-modifiable, and ii) modifiable risk factors¹⁴. Non-modifiable risk factors include congenital malformations, genetic deficiencies⁸, age¹, race, and a family history of CVD^{8, 15-17}. Conversely, modifiable factors include relative physical inactivity⁸, obesity⁸, alcohol, and tobacco^{8, 18} abuse. These factors lead to a spectrum of metabolic and physiological changes that include hypertension, diabetes, and dyslipidemia. In turn, these maladaptive physiological changes result in progressive damage to the systemic and coronary blood vessels due to the development of atherosclerotic plaques. The size and distribution of these plaques within the vasculature will then have an influence on cardiac function and patient mortality.

The majority of CVD cases worldwide can be attributed to modifiable risk factors and thus are largely preventable through proper diet, physical activity, and lifestyle modifications such as quitting smoking and drinking. A common message in

today's popular media focuses on health maintenance and nutrition. It is well established that healthy eating habits and physical activity¹⁹ can delay and/or prevent the onset of CVD and has been demonstrated to significantly reduce one's risk for having a cardiovascular event as they age, even after years of unhealthy eating and physical inactivity. Thus, raising awareness is a popular trend for health organizations to reduce morbidity and mortality due to CVD worldwide. This notwithstanding, simple changes to diet and exercise are insufficient to provide curative solutions to heart failure.

Cardiac Fibrosis as a Primary Cause of Heart Failure

Cardiac fibrosis results from the excessive deposition of extracellular matrix (ECM). Cardiac fibrosis research is a burgeoning field with a large increase in research interest in the past decade. This is not surprising as cardiac fibrosis is a primary cause of heart failure, and despite the recognition of this as a contributor to heart disease, cardiac fibrosis *per se* is essentially currently untreatable, with no specific druggable targets identified for translatable cures. While most current lines of basic cardiovascular science investigations in this field are focused solely on endogenous fibroproliferative mechanisms, the current topic of investigation reflects our focus on proteins with pro-fibrotic (Scleraxis)²⁰ and anti-fibrotic (Ski) properties^{21, 22}. The discovery of Ski's anti-fibrotic role, is at the centre of the current concept to investigate the reciprocal negative regulation between Ski and Scleraxis.

Fibrotic Remodelling and Cardiovascular Disease

Many CVD states have been associated with remodelling of the ECM²³. For the purpose of this discussion, we distinguish between remodelling of the entire heart (i.e. hypertrophy including inappropriate growth of the cardiac myocytes) and remodelling

of the cardiac interstitium (i.e. cardiac fibrosis). For example, systemic hypertension is associated with elevated left ventricular (LV) afterload, which in turn, increases ventricular wall tension and thus ventricular workload. Acutely, the heart is able to temporarily cope with the increased hemodynamic stress with attendant growth i.e., hypertrophy^{24, 25}. However, the chronically overloaded myocardium is unable to maintain compensated forward output with each contraction and thus begins to decompensate^{26, 27 28-30} and ultimately results in heart failure.

A second example of maladaptive remodelling of the heart is that which follows the loss of viable myocardium after a large MI, wherein cardiomyocyte necrosis leads to the activation of resident cardiac fibroblasts, an inflammatory response, and the synthesis of fibrillar collagens²³. Acutely, this process is known as wound healing and is necessary to form a collagen base scar to fill the denuded area and provide tensile strength in the absence of parenchymal cells (cardiomyocytes). The myocardium has essentially no significant intrinsic functional regenerative capacity, and thus without scar formation myocardial rupture and sudden death is inevitable³¹⁻³³.

Despite the many different etiologies of CVD, elevated fibrillar collagen expression is now considered to be a primary contributor to altered cardiac function based on its adverse influence on electrical signal transduction, myocardial stiffness, and impaired cardiac lusitropic function, also known as heart failure with preserved ejection fraction (HFpEF)³⁴⁻³⁷. The wide acceptance of HFpEF by clinicians and researchers as a distinct pathogenic mechanism of heart failure is relatively recent³⁷. The underlying mechanisms responsible for the production and secretion of fibrotic

collagens in the damaged or stressed heart requires more work to identify the underlying molecular mechanisms.

Wound Healing Following Myocardial Infarction

Coronary artery occlusion results in the death of a large number of cardiomyocytes through apoptosis and necrosis. The heart's ability to regenerate functional myocardial tissue is very low, thus the rapid loss of cardiomyocytes poses a significant risk to cardiac survival following an MI³¹. Necrosis of the myocardium is the primary initiator of the inflammatory and reparative process known as wound healing. Myocardial wound healing can be divided into four distinct but overlapping phases (Figure 1)³⁸. These phases include i) death of cardiomyocytes, ii) acute inflammation, iii) cell proliferation, and iv) scar maturation^{23, 39, 40}. There is much debate over whether cardiomyocyte death constitutes a phase of its own or whether it is better placed as the initial event in acute inflammation as myocyte necrosis stimulates the inflammatory response as the cytosolic content of the cell is released into the extracellular space⁴¹. Despite the controversy, all mechanistic pathways are in agreement that inflammation, proliferation, and scar maturation form the core events in the wound healing response.

The first phase of the wound healing process begins immediately following MI and is characterized by cardiomyocyte necrosis and apoptosis (Figure 1)⁴². Hallmarks of this phase include the release of troponin-T, creatine kinase, and serum glutamic-oxaloacetic transaminase into the blood and can be used as biomarkers for the early detection of an MI⁴². Apoptosis tends to peak 6-8 hours post-infarction after which secondary necrosis begins as the surrounding cells are unable to phagotose the large

number of dying cells^{42, 43}. As cell loss progresses, hemostasis occurs at the site of injury to reduce blood loss by the formation of a fibrin clot⁴⁴. With the necrotic loss of cardiomyocytes, the second phase of wound healing begins through activation of the complement system promoting acute inflammation (Figure 1)⁴⁵. This process begins approximately 6 hours post-MI and can continue for up to 6 days⁴². During this time, there is a rapid increase in pro-inflammatory cytokines and chemokines including interleukin-1, -6, and -8 (IL-1, -6, -8), as well as tumor necrosis factor- α (TNF α)^{46, 47}. The primary characteristic of acute inflammation is the recruitment of granulocytes, lymphocytes, and macrophages to the infarcted region. There they remove cellular debris *via* phagocytosis⁴¹. Additionally, the ECM is degraded through increased production of matrix metalloproteinases (MMP) -1, -2, and -9⁴⁸. Removal of necrotic cells and apoptotic bodies in addition to the loss of ECM components sets the stage for phase 3 (the formation of granulation tissue) of the wound healing process (Figure 1)⁴¹. Monocytes and macrophages secrete growth factors including (but not limited to) transforming growth factor β (TGF β), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) to promote fibroblast proliferation, phenocconversion, and migration⁴⁴. Fibroblasts become activated myofibroblasts and migrate towards the area of damage wherein they begin producing and secreting large quantities of ECM. Although it may seem logical that myofibroblasts arise solely from the resident fibroblast population in the heart through the activation process known as phenocconversion, myofibroblasts have been documented to arise from numerous sources including circulating progenitor cells^{49, 50}, cells undergoing epithelial and endothelial-to-mesenchymal transition (EMT and EndoMT respectively)^{44, 51} and

perivascular pericytes⁵². Activation of fibroblasts typically begins on the second or third day post-MI and continues for up to one month following the initial injury⁴⁴. In addition to the formation of granulation tissue, the proliferative phase of wound healing also includes angiogenesis and re-epithelialization to restore tissue perfusion and improve structural strength to the infarct⁵³.

The final stage of post-MI cardiac wound healing is scar formation and expansion of interstitial remodelling of the remnant heart, remote to the infarct scar (Figure 1). These events do not begin for several (2-3) weeks post-MI and may continue for many years following the initial injury⁵⁴. Scar maturation is characterized by collagen cross linking and decellularization through myofibroblast apoptosis and removal by macrophages⁵⁵. Although the majority of myofibroblasts are removed from the scar tissue, there is a subpopulation of myofibroblasts that persists within the scar and continually produces collagen fibers. The chronic expression and deposition of collagen fibres leads to progressive cardiac stiffening and heart failure⁵⁴.

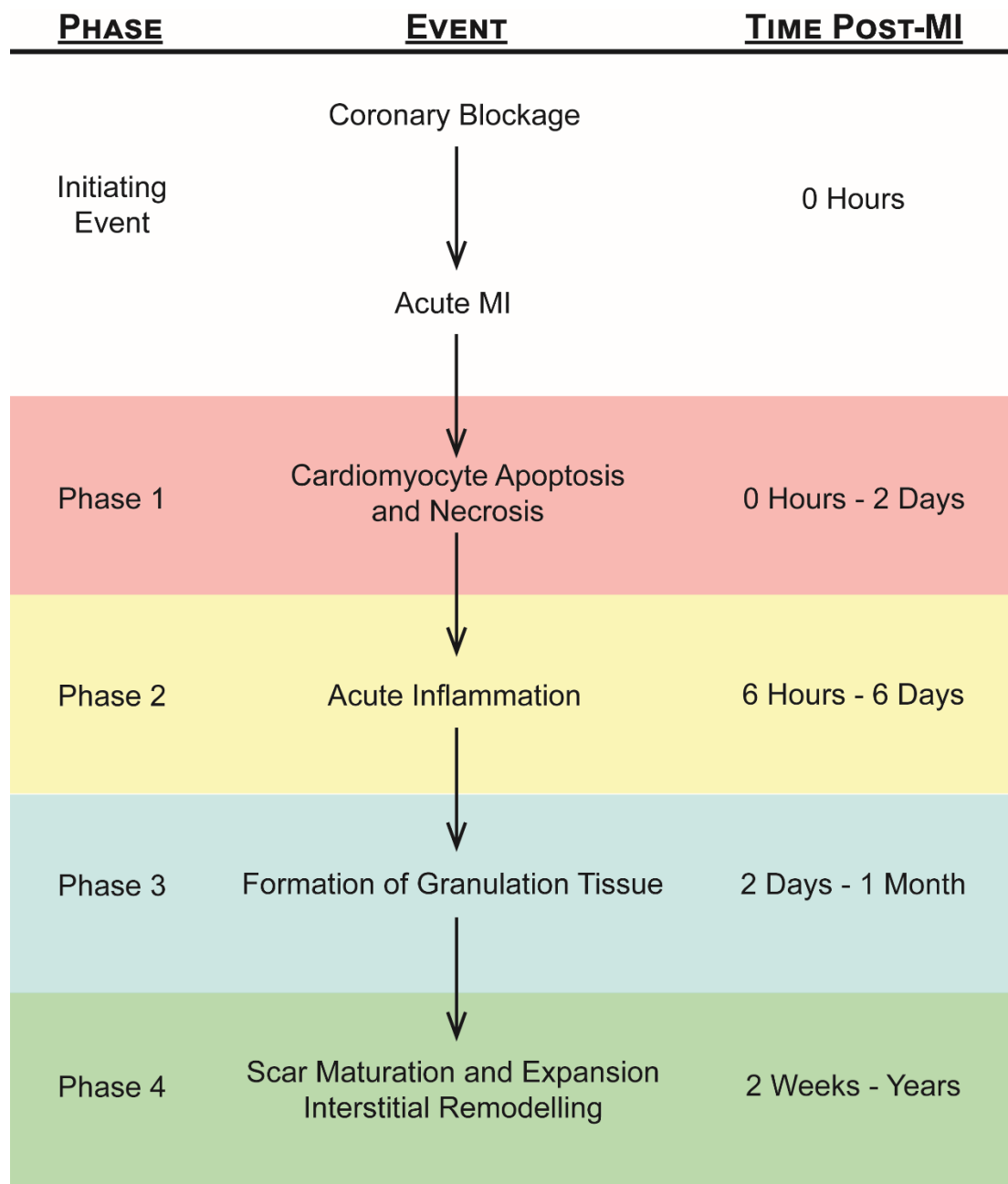


Figure 1. Phases of Human Myocardial Wound Healing Post-MI. Depicted are the four phases of myocardial wound healing that transpire following a myocardial infarction (MI). Note the overlap amongst inflammation, formation of granulation tissue, and scar formation. Note that murine MI models share these landmark events of myocardial wound healing however at a significantly accelerated temporal rate.

The Extracellular Matrix

Traditionally the ECM was thought of as a simple scaffold to hold tissues and cells together. It is now widely accepted that the cardiac ECM is a highly organized network that contains large structural proteins including fibrillar collagens type I and III, proteoglycans, fibronectins, and laminin that provide a framework for which cells can adhere to and may alter intracellular signaling pathways^{20, 44, 56-58}. The primary function of the ECM in the myocardium is to coordinate myocyte-generated contractile forces in addition to preventing overextension or rupture of the myocardium during cardiac contraction and relaxation^{59, 60}. Collagens type I and III account for the majority of the cardiac ECM and help to maintain the myocardial structure and the overall shape of the heart^{24, 60}.

Collagens are synthesized and secreted by cardiac fibroblasts and myofibroblasts as pro-peptides that are cleaved into mature collagen molecules and subsequently cross-linked into fibres by lysyl oxidase in the extracellular space⁶¹⁻⁶³. Fibres of collagen type I account for a majority of the myocardial stiffness and is comprised of two molecules of Col 1 α 1 and one Col 1 α 2 to form a triple helix^{60, 64, 65}. Collagen type III is less abundant than type I in the heart and forms thinner fibres which are comprised of three molecules of Col 3 α 1. Ultimately, myocardial stiffness is a measure of the ratio of type I to type III fibres⁶⁶. Under pathological conditions, this ratio significantly increases and is directly related to the decline in cardiac function and stiffening of the myocardium⁶⁷⁻⁷¹. Pathological hypertrophy of an injured heart is associated with perivascular and interstitial fibrosis of the non-infarcted regions of the heart and replacement fibrosis of necrotic tissues. Ongoing replacement fibrosis

following wound closure may contribute to decompensated heart failure in severe cases.

In addition to collagen, glycoproteins such as fibronectin are an important component of the ECM. Fibronectin is a large, multi-domain ECM glycoprotein that binds cell membrane integrins in addition to other ECM proteins such as fibrins and collagens^{72, 73}. It exists as a homodimer of two 250 kilodalton (kDa) monomers containing repetitive subunits^{72, 74}. Alternative splicing of fibronectin can yield more than 20 different isoforms^{72, 75}. Following cardiac injury, there is an upregulation of the extracellular domain-A splice variant of fibronectin (ED-A FN) that plays an important role in fibroblast activation and wound closure^{76, 77}. Following MI, cardiac fibroblasts secrete fibronectin which serves as a base for a more organized, cell-derived matrix during the wound healing process⁷⁷.

The ECM also plays a prominent role as a cytokine storage depot⁷⁸. Numerous cytokines and growth factors such as TGF β and FGF⁷⁸ are stored in the ECM and can be made available through proteolytic cleavage or mechanical release from their associated latency complexes⁷⁸⁻⁸¹. Storage of such molecules within the ECM allows for a rapid and direct response rather than relying on the rapid production of the necessary cytokine.

Another class of peptides capable of regulating cell phenotype and function are matrikines and matricryptins⁸²⁻⁸⁵. Matrikines are peptides derived from cleaved ECM macromolecules that have the capacity to bind to cell surface receptors and regulate a variety of cellular activities^{83, 86}, whereas matricryptins have been described as “enzymatic fragments of the ECM containing exposed matricryptic sites” not visible in

the parent molecule⁸⁵. Elastins, collagens, and glycoproteins such as thrombospondin-1 (TSP1) and fibronectin have all been identified to be a source for matrikines and matricryptins. Proteolytic cleavage of elastin gives rise to a VGVAPG (valine, glycine, valine, alanine, proline, glycine) repeated motif that has been shown to stimulate MMP-2 expression in human fibroblasts⁸⁷. Thrombospondin-1 is a glycoprotein that produces several matrikines that participate in TGF β activation as well as regulate angiogenesis⁸⁸. Cleavage of fibronectin exposes an RGD domain that is recognized by $\alpha 5 \beta 1$ and $\alpha v \beta 3$ integrins and alters cell adhesion, migration, proliferation, and survival. Due to the low prevalence or absence of matrikines in normal tissues, identifying the presence of matrikines as biomarkers in disease has become a rapidly expanding field⁸⁹.

The Cardiac Fibroblast and Myofibroblast

The heart is made up of numerous cell types including smooth muscle cells, endothelial cells, cardiomyocytes, and fibroblasts⁹⁰. Although cardiomyocytes occupy the majority of spatial volume within the heart, cardiac fibroblasts are the most numerous cell type in the heart. The exact number/percentage of fibroblasts that are found within the myocardium is unknown, but studies estimate that more than 70% of the cells found in the adult heart are of the fibroblast phenotype, although these estimates have been challenged^{90, 91}.

Morphologically, fibroblasts are flat, spindle shaped cells that lack a basement membrane⁹². The fibroblast is found within the cardiac interstitium²⁵ and is derived from a mesenchymal origin during development⁹². Fibroblasts are considered sentinel cells as they are highly responsive to a variety of stimuli *in vitro* and *in vivo* including TGF β ⁹³⁻⁹⁵, angiotensin II⁹⁶⁻⁹⁸, endothelin-1⁹⁹, mechanical stress, electrical signaling,

and seeding at low densities¹⁰⁰. Under normal physiological conditions, fibroblasts are relatively quiescent cells that maintain matrix homeostasis, and play a role in the biochemical, mechanical, and electrical properties of the myocardium^{101, 102}. Pathologically, however, these cells become activated and undergo phenocconversion where they become hypersynthetic and hypersecretory cardiac myofibroblasts. Morphologically, myofibroblasts are spatially larger than their quiescent counterparts¹⁰³. These “activated” cells produce numerous matrix components including α -smooth muscle actin (α -SMA), ED-A FN, non-muscle myosin heavy chain-b (SMemb), vimentin, periostin, TGF β receptors (T β R’s), and fibrillar collagens (type I and III)^{22, 77, 104-109}. It is important to note that a small population of myofibroblasts exists within the healthy heart, however, these cells are isolated to the valve leaflets. Within the valve leaflets myofibroblasts help maintain and preserve valve structure and durability from the constant hemodynamic stress that they are under during ventricular contraction (inotropism) and relaxation (lusitropism)¹¹⁰.

Functionally, myofibroblasts are contractile cells that impart isometric tension within the granulation tissue of the healing heart¹¹¹. This phenomenon has been demonstrated in numerous *in vitro* and *in vivo* models and is considered one of the hallmarks of the myofibroblast phenotype^{111, 112}. Myofibroblast contraction is critical during myocardial wound healing as tensile force distorts the ECM to oppose the retractile forces of the myocardium and promote scar contraction post-MI. Transmission of cellular tension occurs through focal adhesions, which form strong connections with collagen fibres and fibronectin¹¹³ while increased α -SMA expression enhances myofibroblast contraction¹¹⁴. As cardiac myofibroblasts are neither

smooth/skeletal muscle nor cardiomyocytes, it is likely that they use a unique model of cellular contraction. An alternative model proposed by Castella *et al.*¹¹⁵ suggests that myofibroblast contraction is the cumulative sum of intracellular Rho-ROCK signaling and calcium-mediated transients. Myofibroblasts have a spontaneous calcium transient of approximately 100 seconds (220 seconds in quiescent fibroblasts)¹¹⁵ and express critical ion channels including L-type Ca^{2+} channels and the sodium calcium exchanger¹¹⁶ on their cell surface membranes to mediate calcium flux^{116 115}. Additionally, these cells are electrically coupled to cardiomyocytes through connexin-43 and connexin-45¹¹⁷⁻¹¹⁹. In Castella's proposed model, Rho-Rock signaling produces and maintains isometric tension on stress fibres while calcium entry plays a role in further enhancing cell tension and stabilizes the contracture¹¹⁵. These additive events help the cell to stabilize and support the load that it faces and may allow for re-spreading, resulting in multiple cycles of contracture¹¹⁵. Collectively, it is suggested that this may lead to wound contracture at a rate of 1.0 centimeter per month^{111, 115}.

Myofibroblasts are a heterogeneous population that are found within nearly all tissue and organ systems but display marked phenotypic and topographical diversity¹²⁰. Furthermore, it has been recognized that not only do fibroblasts differ in phenotype from tissue to tissue, but fibroblasts isolated from different regions of the same organ tissue have different physiological characteristics¹²⁰. For example, fibroblasts isolated from the superficial and deep dermis regions displayed different matrix protein expression profiles for versican, decorin, collagen type I, and type III thus demonstrating a topographical diversity in matrix protein expression amongst fibroblasts within the same tissue^{121, 122}.

Traditionally, the appearance of myofibroblasts in the post-MI heart comes from the activation of resident cardiac fibroblasts. This hypothesis has recently been supported wherein data showed that two resident fibroblast lineages are responsible for the fibrosis that accompanies pressure overload induced hypertrophy, and that they could be targeted for alleviation of pathological fibrosis¹²³. Through cell lineage tracing, it is now increasingly recognized that myofibroblasts originate from a variety of sources including endothelial and epithelial cells through EndoMT⁵¹ and EMT^{52, 124-126}, pericytes⁵², bone-marrow derived cells^{127, 128} and circulating fibrocytes^{129, 130}. However, there remains much debate regarding the contribution and true pathological importance for these alternative sources for myofibroblasts within the remodelling heart. This notwithstanding, myofibroblasts are important in the infarcted heart for the deposition of matrix proteins to provide structural strength to the healing myocardium to prevent cardiac rupture.

Transforming Growth Factor β

Transforming Growth Factor β (TGF β) is an evolutionary ancient and highly conserved cytokine that plays a critical role in processes ranging from development to pathological wound healing¹³¹⁻¹³⁴. The TGF β superfamily consist of over 30 family members which include activins, nodal, bone morphogenetic proteins (BMP), inhibins, Müllerian inhibiting substance (MIS), and TGF β to name a few^{135, 136}. Mammalian TGF β is found as three distinct isoforms, TGF β_1 , TGF β_2 , and TGF β_3 ¹³⁷. Each isoform arises from a distinct gene locus: in humans TGF β_1 arises from a region of chromosome 19, TGF β_2 from chromosome 1, and TGF β_3 from chromosome 14¹³⁸.

Despite its origin, each cytokine is released to the extracellular space as an inactive dimer due to its non-covalent association with a latency-associated protein (LAP)^{139, 140}. This LAP is produced from the N-terminal region of the TGF β gene product *via* cleavage from the nascent protein to form the small latency complex (SLC). The SLC is bound to the large latent TGF β binding protein (LTBP) *via* a disulfide link in the endoplasmic reticulum to form the large latency complex¹⁴¹. Although the LTBP is not necessary for TGF β latency, it has been implicated in the secretion and storage of TGF β in the ECM¹⁴² through its interactions with fibronectin and cell membrane bound integrins¹⁴³.

Release of active TGF β from the latency complex requires cleavage of the LAP. Cleavage can occur through a variety of mechanisms including direct interaction with proteases (eg, MMP's, plasmin, thrombin)¹⁴⁴⁻¹⁴⁶, interaction with other proteins (eg, TSP1, integrin $\alpha v \beta 6$)¹⁴⁷⁻⁸⁰, reactive oxygen species (ROS)¹⁴⁷⁻¹⁴⁹, low pH^{147, 150}, or shear-mechanical stress¹⁵¹. Mechanical stress induced through myofibroblast contraction has recently been demonstrated to play a significant role in TGF β release¹⁵¹. Integrins containing the αv -subunit bind to the LAP *via* the RGD (arginine-glycine-aspartic acid) motif and myofibroblast contraction has been demonstrated to pull the complex sufficiently to release active TGF β from the matrix^{79, 80}.

Active TGF β_1 signals through a pair of membrane bound serine/threonine kinase receptors known as TGF β receptor type I (T β RI) and type II (T β RII)^{136, 152-155}. Free, active TGF β_1 binds to the T β RII homodimer which then recruits T β RI to form a heterotetrameric receptor complex^{152, 154, 156}. These receptors have an intrinsic affinity for one another as it has been demonstrated that they are capable of spontaneously

forming active complexes when the receptors are overexpressed *in vitro* in the absence of TGF β ¹⁵⁷. Physiologically, binding of extracellular TGF β to T β RII induces receptor autophosphorylation and increases the affinity of T β R's for each other which ultimately leads to T β RI recruitment¹⁵⁷. T β RII then activates T β RI through phosphorylation of serine residues that provide a docking site for receptor-mediated Smad (R-Smad) proteins within the cytosol to induce a Smad-dependent signaling cascade¹⁵⁵. Alternatively, receptor stimulation may lead to activation of several Smad-independent signaling events¹⁵⁸⁻¹⁶⁴, thus highlighting the biological importance of this signaling pathway.

Smad-Dependent Signaling

R-Smads were named due to their significant homology to *sma* and *mad* proteins from *Caenorhabditis elegans*¹⁶⁵ and *Drosophila melanogaster*¹⁶⁶ respectively. The R-Smad family consists of two functional classes of proteins, i) those that induce gene expression, and ii) those that repress gene expression. Those that induce gene expression include R-Smad 1, 2, 3, 5, 8 and co-mediator Smad4 (Co-Smad4) while Smad 6 and 7 are potent negative regulators of TGF β signaling. R-Smads 1, 5, and 8 are linked to BMP-mediated signaling events through T β R's while R-Smads 2 and 3 are associated with TGF β -mediated signaling¹⁶⁷. Co-Smad4 is a common mediator for both BMP and TGF β signaling¹⁶⁷. It forms a complex with phosphorylated R-Smad proteins and together translocate to the nucleus to influence gene expression. Smad 6 and 7 are known as inhibitory Smads (I-Smad) due to their ability to form a negative feedback loop on BMP/TGF β signaling to turn off R-Smad dependent signaling events¹⁶⁸⁻¹⁷¹.

R-Smad proteins are sequestered in the cytoplasm near the plasma membrane in close proximity to the T β R's by the two FYVE-domain (a cysteine rich protein comprised of Fab1, YOTB, Vac1 and EEA1) containing proteins, Hgs¹⁷² and Smad Anchor for Receptor Activation (SARA)¹⁷³. Upon receptor activation, T β RI induces phosphorylation of R-Smad proteins at their SSXS (Serine, Serine, X, Serine where X can be any amino acid (aa)) motifs, at their C-termini^{155, 174}. Phosphorylation of R-Smads releases them from SARA, which allows them to complex with Co-Smad4 and, together, translocate to the nucleus and alter gene expression^{156, 175}. The Smad complex is a transcription factor that is capable of binding directly to DNA by recognizing a specific DNA sequence known as a Smad binding element (SBE)¹⁷⁶. The precise definition of a SBE is an ever evolving one as Smad complexes have been shown to bind to a variety of DNA sequences that vary in composition and length (Table 1). However, all variants of putative SBE seem to contain a conserved CAGAC DNA sequence that is likely key with respect to Smad complexes recognizing specific gene promoters¹⁷⁶.

<u>SBE Sequence</u>	<u>Reference</u>
CAGACA	Chen <i>et al.</i> ¹⁷⁷ Bagchi and Czubryt ²⁰ Ghosh <i>et al.</i> ¹⁷⁸
GTCT or AGAC	Chen <i>et al.</i> ¹⁷⁹ Shi <i>et al.</i> ¹⁸⁰
CAGAC	Chen <i>et al.</i> ¹⁷⁹
CAGA	Dennler <i>et al.</i> ¹⁸¹
GTCTAGAC	Zawel <i>et al.</i> ¹⁷⁶

Table 1. Previously Reported Smad Binding Elements (SBE). A selection of putative SBE that have been previously reported to play a role in directing Smad-mediated gene expression.

Once bound to the promoter region of TGF β regulated genes, the Smad complex can recruit transcriptional co-activators (p300/CBP, P/CAF, Fast1, SMIF)^{20, 182-185} and/or co-repressors (Ski, SnoN, N-CoR, SMRT, mSin3A, HDACs)^{21, 22, 186-188} to either promote or repress gene expression. Cessation of Smad signaling occurs through ubiquitination of Co-Smad4 by Ectodermin/TIF1 γ within the nucleus (Figure 2)^{189, 190}. Once ubiquitinated, Co-Smad4 can be recycled back into the cytoplasm where it is promptly de-ubiquitinated by FAM/Usp9x¹⁹¹ and primed for another round of signaling. Additionally, phosphorylated R-Smads in the nucleus are de-phosphorylated

upon release from Co-Smad4 by PPM1A^{192, 193} and may also be recycled back into the cytoplasm and associate with SARA (Figure 2).

I-Smad proteins differ from R-Smads in that they are not transcription factors. Rather, I-Smad7 serves as a negative feedback mechanism in response to TGF β signaling^{194, 195} by binding to and facilitating the degradation of T β RI, thus inhibiting R-Smad activation¹⁹⁶. Recruitment of Smad ubiquitin regulatory factor 2 (Smurf2), an E3 ubiquitin ligase, leads to ubiquitination of T β RI which targets it for degradation (Figure 2)¹⁹⁷. Ski, an endogenous inhibitor of Smad signaling, recruits a negative regulatory complex to TGF β -regulated promoters to prevent gene expression. The mechanisms associated with this process are discussed in greater detail later in this thesis.

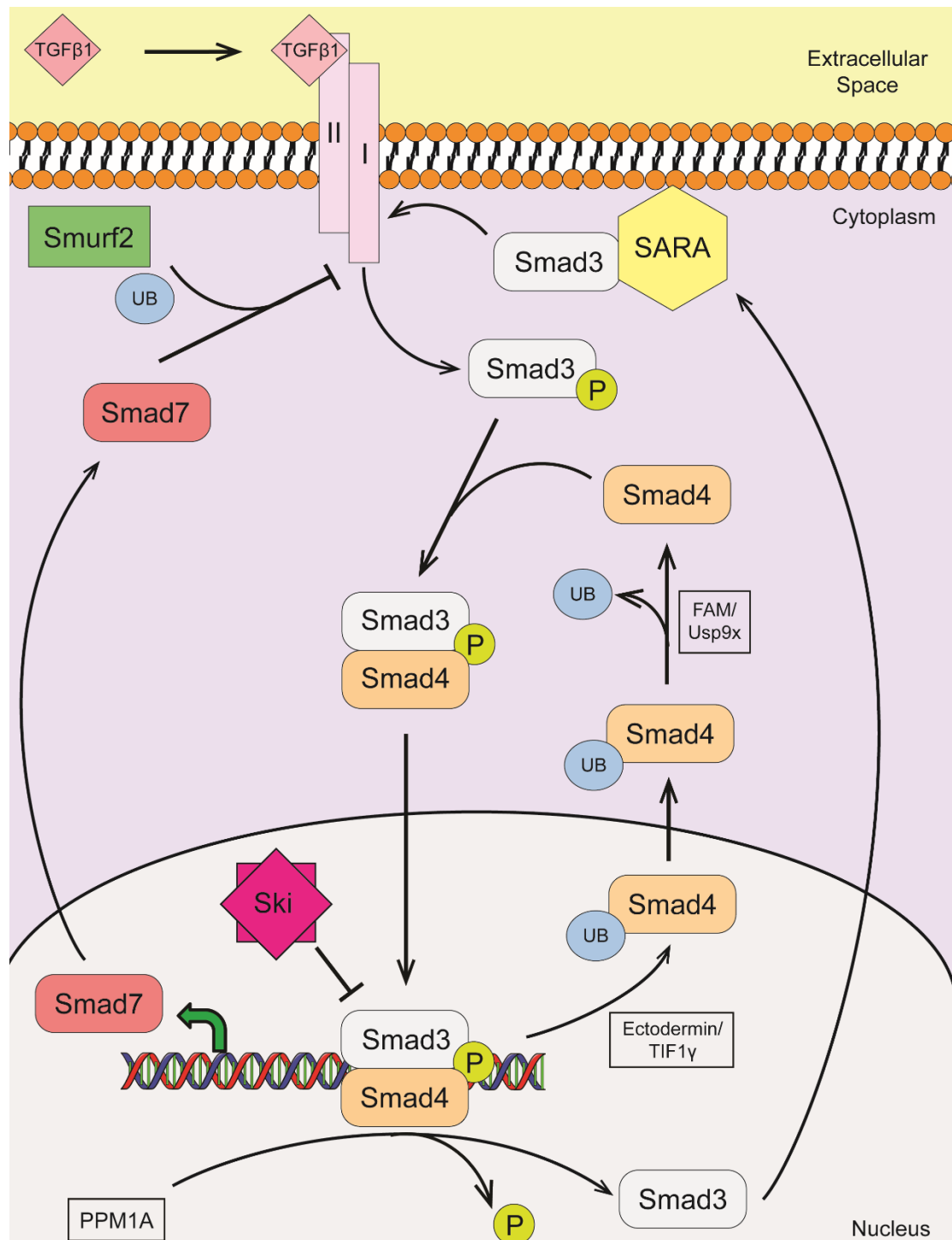


Figure 2. Canonical TGFβ₁/Smad-Dependent Signaling. Extracellular TGFβ₁ binds to its cell surface receptor TβRII and promotes recruitment of TβRI. Formation of a heterotetrameric complex promotes R-Smad phosphorylation by TβRI which releases them from SARA. Phosphorylated R-Smads form an active complex with Co-Smad4 and together translocate to the nucleus where they recognize specific DNA binding sites known as SBE. There the Smad complex promotes gene expression of TGFβ₁

regulated genes such as I-Smad7. I-Smad7 negatively feeds back on TGF β signaling as it is shuttled to the cytoplasm and promotes destruction of T β RI. Dissociation of the Smad complex in the nucleus is mediated by Ectoderm/TIF1 γ through ubiquitination of Co-Smad4. Ubiquitinated Co-Smad4 is shuttled to the cytoplasm where it can be de-ubiquitinated by FAM/Uspx and recycled. Free phosphorylated R-Smads in the nucleus are dephosphorylated by PPM1A and recycled back to the cytoplasm where they re-associate with SARA to be used again. Ski is an endogenous inhibitor of Smad signaling that prevents Smad mediated gene expression at the promoter region of TGF β ₁ regulated genes.

Smad Structure and Regulation

Smad proteins contain two well conserved domains located at either end of the protein. At the N-terminus is the Mad Homology 1 (MH1) domain (Figure 3). It is highly conserved amongst both R-Smads and Co-Smad4 and plays a critical role in Smad nuclear transport and DNA binding¹⁶⁷. The N-terminus of the I-Smads however have little to no similarity to that of R-Smads and Co-Smad4¹⁶⁷. In contrast to the N-terminal domain, the C-terminus contains a Mad Homology 2 (MH2) domain that is well conserved amongst all Smad proteins, including the I-Smads (Figure 3)¹⁶⁷. This region mediates Smad oligomerization, recognizes T β RI's, and interacts with transcriptional co-activators (p300/CBP, AP1)^{20, 182-185} and co-repressors (Ski, SnoN)^{21, 22, 186-188} within both the cytoplasm and nucleus. Between the N- and C-termini lies a linker region that connects the Mad homology domains¹⁹⁸. This region has no identifiable similarity or homology from Smad to Smad whether it is an R-Smad, Co-Smad4, or I-Smad¹⁶⁷. The linker region varies in sequence and length but has been shown to play an important role in Smad regulation and nuclear accumulation^{167, 199, 200}.

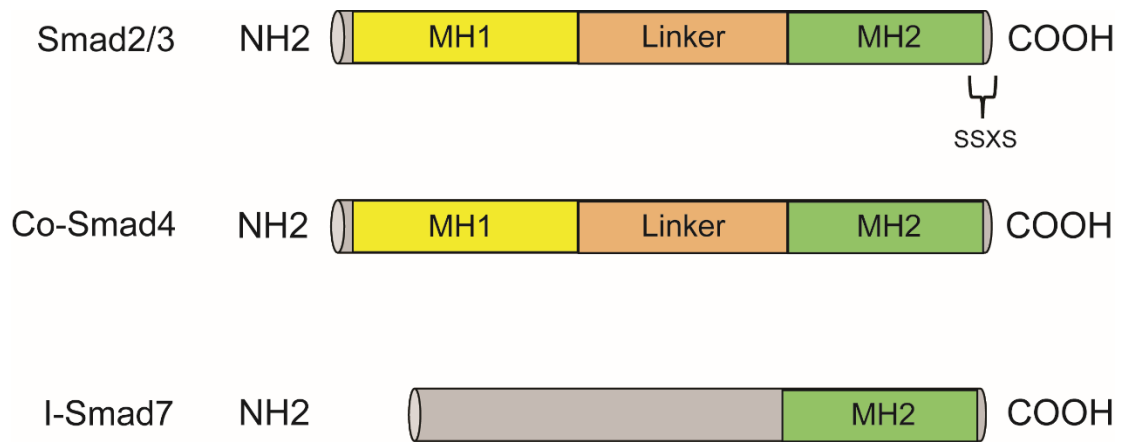


Figure 3. Protein Structure of Smad Proteins. Smad proteins contain regions of both high homology and divergence. All Smad proteins contain an MH2 domain which plays an important role in Smad activation and interaction with other proteins. R-Smads and Co-Smad4 proteins also contain an MH1 that is highly conserved and plays a critical role in nuclear translocation and DNA binding. Additionally, they contain a linker region that can be modified (i.e. *via* phosphorylation) by other proteins (such as p38-MAPK) to regulate R-Smad and Co-Smad4 function. I-Smad7 does not contain a DNA binding domain.

The C-terminus of R-Smads contain several serine residues that are critical to Smad signaling. Specifically, there is an evolutionarily conserved SSXS motif that must be phosphorylated in order to allow Smad oligomeric complexes to propagate the TGF β signal from the cell surface receptor to the nucleus^{155, 156, 167, 198, 201}. T β RI's demonstrate substrate selectivity as TGF β and activin receptors will phosphorylate R-Smad2/3, whereas BMP receptors will phosphorylate R-Smad1/5/8¹⁶⁷. The basis for this receptor selectivity lies in the L45 loop in the T β RI and L3 loop of the R-Smad proteins. The detailed mechanisms of this signaling selectivity, however, goes beyond the scope of this literature review¹⁶⁷.

Detailed analysis of endogenous Smad proteins has revealed that there are more than 10 Smad phosphopeptides suggesting that other kinases may play a role in Smad regulation^{202, 203}. This has been found to be the cause of phosphorylation of serine

residues within the linker region between the MH1 and MH2 domains^{167, 199}. Phosphorylation of these sites is mediated through several different kinases including Extracellular Signal-Related Kinase 1/2 (Erk1/2), p38-MAPK, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), RhoA/ROCK, and calcium-calmodulin dependent kinase (CAMK)^{158-160, 162, 163, 204}. Studies in the late 1990's by Kretzschmar *et al.* demonstrated that R-Smad1/2/3 were phosphorylated by Erk1/2 which led to nuclear exclusion and accumulation of R-Smads in the cytoplasm^{205, 206}. Site-directed mutagenesis of Erk1/2 phosphorylation sites prevented phosphorylation of the Smad-linker region, and rescued the growth inhibitory effects of TGF β ²⁰⁶. CAMK has also been demonstrated to phosphorylate the linker region of R-Smad2 to prevent nuclear translocation²⁰⁴. Furthermore, induction of protein kinase C (PKC) has been demonstrated to promote phosphorylation of the R-Smad3 linker region²⁰³. Interestingly, rather than preventing nuclear translocation, phosphorylation by PKC prevented gene expression by inhibiting the DNA-binding ability of R-Smad3²⁰³. It is clear from these examples that there is considerable cross-talk between the TGF β /Smad-dependent pathway and those pathways regulated by the kinases described above that ultimately play a role in R-Smad localization and function. This only further highlights the importance of TGF β signaling and its significance to cell homeostasis and pathogenesis.

Smad-Dependent Mechanisms and Fibrosis

It has been recognized for some time now that TGF β /Smad-dependent signaling plays a critical role in promoting tissue fibrogenesis through the production of fibrillar collagens and ECM^{60, 196, 207, 208}. In 1999, Poncelet *et al.* isolated the human collagen

1 α 2 promoter and found that promoter expression could be stimulated by TGF β ₁ and R-Smad3 overexpression in human mesangial cells²⁰⁹. A follow up study in 2001 by this group also demonstrated that Specificity Protein 1 (Sp1) is a co-activator of Col 1 α 2 gene expression²⁰⁷. Specifically, they found that Sp1 and R-Smad3 acted in a synergistic manner to promote collagen 1 α 2 expression and could be co-precipitated with one another from cell extracts²⁰⁷. They noted that Sp1 on its own could not affect collagen production suggesting that R-Smad3 was a rate limiting factor in enhancing collagen expression²⁰⁷. More recently, Scleraxis (Scx), a basic helix-loop-helix (bHLH) transcription factor, was also found to act in a synergistic manner with R-Smad3 to promote Col 1 α 2 gene expression^{20, 210}.

In the post-MI heart, there is a significant increase in the amount of R-Smad2/3 phosphorylation which has been shown to coincide with Smad nuclear accumulation²⁰⁸ and collagen deposition^{96, 211}. In a model of parasitic liver fibrosis, there was a significant increase in collagen production and secretion as determined by hydroxyproline incorporation and Masson's Trichrome staining²¹². Furthermore, there was a significant increase in mRNA transcripts for pro-fibrotic markers including collagen type I and III, and α -SMA which could be correlated with increased expression and activation of R-Smad proteins, TGF β ₁ cytokine, and T β R's in those animals with *Clonorchis sinensis* infections²¹². Recently, in a carbon tetrachloride (CCl₄) rat model of hepatic fibrosis, CCl₄ treatment promoted collagen type I mRNA and protein expression which followed the same pattern of increased R-Smad3 expression²¹³. They found that while R-Smad3 promoted collagen expression, R-Smad2 overexpression repressed collagen expression, suggesting that R-Smad2 was protective against

TGF β ₁/Smad-mediated collagen expression and hepatic fibrosis²¹³. This protective effect of R-Smad2 against TGF β ₁/R-Smad3 mediated fibrosis has also been described in a model of renal fibrosis²¹⁴.

Smad-Independent Signaling Initiated by TGF β ₁

In addition to its ability to stimulate Smad-dependant signaling events, TGF β ₁ has been shown to promote several Smad-independent signaling cascades through T β R's including p38 mitogen-activated protein kinase (p38-MAPK)^{152, 159, 164, 215}, Erk1/2^{162, 216-220}, SAPK/JNK^{158, 221}, phosphatidylinositol 3-kinase (PI3K)/Akt^{160, 163, 222-224}, and RhoA/ROCK^{158, 161, 225-228}. In response to TGF β ₁ stimulation, these pathways have been shown to play key roles in multiple cellular and sub-cellular events including EMT, actin polymerization, proliferation, migration, differentiation, collagen production, fibrosis, and regulation of Smad-dependent signaling. The ability of TGF β to induce multiple signaling pathways demonstrates the complexity of TGF β -dependent signaling and the diverse roles that it plays in regulating homeostasis and disease pathogenesis.

TGF β ₁-Mediated p38 Mitogen-Activated Protein Kinases (p38-MAPK) Signaling

Of the various kinases that have been shown to play an important role in TGF β /Smad-independent signaling, p38-MAPK signaling has been extensively investigated. Mammalian p38 is activated by 2 specific upstream kinases, MAPK kinase -3 and -6 (MKK3 and MKK6)^{159, 229-231}. In response to TGF β ₁ stimulation, T β RI induces ubiquitination of the receptor associated TNF receptor associated factor 6 (TRAF6). TRAF6 then associates with and phosphorylates TGF β activating kinase-1 (TAK1) which in turn phosphorylates MKK3/6 (Figure 4)¹⁶⁴. Upon phosphorylation of

p38 by MKK3/6, p38 itself phosphorylates numerous downstream transcription factors including activating transcription factor-2 (ATF2), CHOP, and CREB (Figure 4)¹⁵⁹.

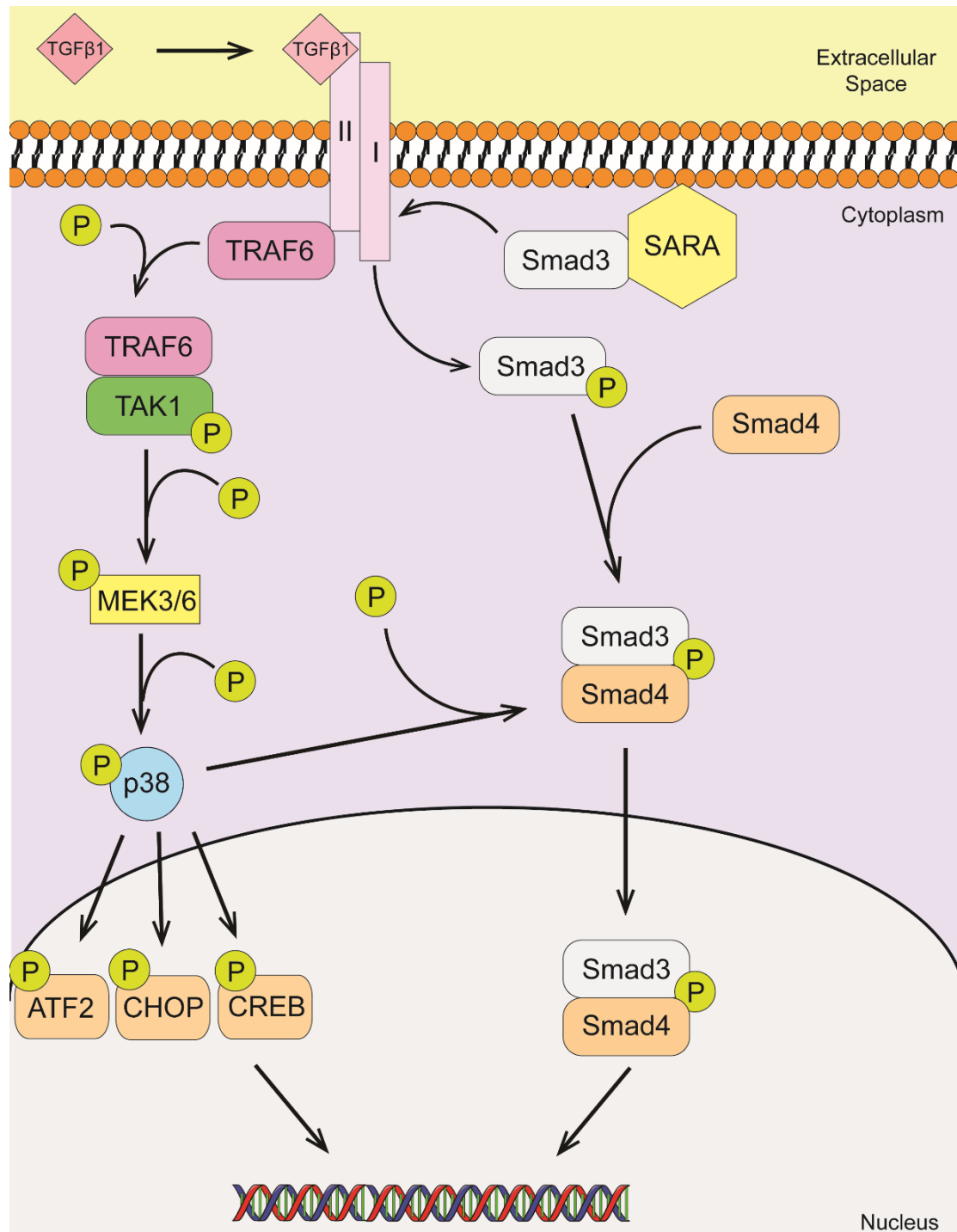


Figure 4. TGFβ₁/Smad-Independent Signaling: p38-MAPK. In addition to its role in stimulating a Smad-dependent pathway, extracellular TGFβ₁ can stimulate a Smad-independent pathway through TβR's. TGFβ₁ promotes ubiquitination of TRAF6 which in turn phosphorylates and forms a complex with TAK1. This activated complex induces phosphorylation of MKK3/6 which activates p38 kinase through phosphorylation. Activated p38 then has the capability to phosphorylate downstream

transcription factors such as ATF2, CHOP, and CREB or the linker region of R-Smad proteins to regulate their function.

Several elegant studies conducted in the early 2000's demonstrated the importance of p38-MAPK signaling in mediating fibroblast differentiation, migration, and EMT^{159, 232}. Using a T β RI mutant that lacked the ability to activate R-Smads but retained its kinase ability, Yu *et al.* demonstrated that TGF β ₁ stimulation of NMuMG mouse mammary epithelial cells induced prominent phosphorylation of p38 in the absence of R-Smad signaling²³². Furthermore, they showed that p38 alone was sufficient to induce TGF β ₁-mediated apoptosis. They observed that activation of p38 in the absence of R-Smad signaling was unable to promote EMT in these cells, suggesting that input from R-Smad signaling is necessary to induce EMT²³². They confirmed their results by using a kinase deficient T β RI and saw that loss of receptor kinase activity led to complete loss of p38 phosphorylation²³². At the same time, Bakin *et al.* were evaluating the role that p38-MAPK specific inhibitors had on TGF β ₁-dependent p38 signaling¹⁵⁹. As little as 2.0 ng/mL TGF β ₁ led to rapid (15 minutes) induction of p38-MAPK signaling resulting in the phosphorylation of the transcription factor ATF2 and promoted EMT. This was evident through morphological changes in cell shape, actin cytoskeleton structure, and cell migration characteristic of mesenchymal cells¹⁵⁹. Conversely, the p38 specific inhibitors SB203580 and SB202190 prevented the morphological and phenotypic changes in cell structure and ATF2 phosphorylation without affecting R-Smad phosphorylation¹⁵⁹. This data was in line with previous data from this group where they had demonstrated that dominant-

negative mutants of the upstream kinases MKK3/6 impaired TGF β -mediated phosphorylation of p38-MAPK and EMT²³³.

TGF β ₁-Mediated Phosphatidylinositol 3-Kinase (PI3K)/Akt Signaling

TGF β stimulation has also been demonstrated to induce activation of the PI3K/Akt signaling pathway through T β R's in several cell types¹⁶³. Incorporation of ³²P-labelled phosphatidylinositol phosphate (PIP) has been shown to occur within 15 minutes of TGF β stimulation of AKR-2B fibroblasts with a maximal signal occurring within 60 minutes¹⁶³. This response is similar to those seen in previous studies that demonstrated that as little as 2.0 ng/mL TGF β ₁ was able to rapidly induce Akt phosphorylation¹⁶⁰. Immunoprecipitation studies in the late 1990's demonstrated that PI3K was physically associated with both type I and type II T β R's²³⁴. Several independent groups showed that p85, the regulatory subunit of PI3K, was part of a complex that was associated with both T β R's and TGF β ₁ stimulation led to rapid and robust phosphorylation of Akt, a downstream component of PI3K signaling (Figure 5)^{160,234-236}. Expression of a dominant-negative form of p85 (DN-p85) led to a significant reduction in basal Akt phosphorylation levels and TGF β ₁ stimulation of cells expressing DN-p85 demonstrated significantly less reporter activity¹⁵⁹. Functionally, the role of PI3K in cell migration was evaluated using a PI3K specific inhibitor¹⁵⁹. Inhibition of PI3K in mouse tumor cells (which are highly responsive to TGF β ₁ stimulation) using the small molecule inhibitor LY294002 blocked TGF β ₁ mediated cell migration, while having no effect on cell proliferation¹⁶⁰. PI3K/Akt signaling was found to be sufficient to disrupt cell junctions, but did not promote morphological changes in NMuMG epithelial cells associated with EMT¹⁶⁰. Disruption of cell

junctions is an early event in EMT and thus PI3K may be a critical mediator of the early responses to TGF β ₁-mediated EMT. Other events, however, such as cytoskeletal reorganization may not be regulated through PI3K.

The precise role and outcome of PI3K/Akt signaling in response to TGF β ₁ has been inconsistent. The majority of the early studies in this area used epithelial and cancer cells undergoing EMT to evaluate the role of PI3K/Akt signaling^{159, 160, 163}. More recently, Wilkes *et al.* compared several fibroblastic (NIH3T3, Swiss3T3, AKR-2B) cell lines to epithelial (HeLa, Mv1Lu, MDCK) cell types to elucidate the importance of PI3K/Akt-mediated signaling in response to TGF β in a mesenchymal cell type¹⁶³. They found that TGF β stimulation induced phosphorylation of Akt in fibroblastic but not epithelial cell lines. The fact that the epithelial cells demonstrated no Akt activation in response to TGF β differs from that of previous reports in NMuMG and mouse tumor cells and suggests a cell dependent dichotomy for TGF β regulation of PI3K/Akt¹⁶³. With respect to R-Smad signaling, inhibition of PI3K using LY294002 had no effect on TGF β -dependent R-Smad2 phosphorylation or nuclear localization indicating a separation in the pathways¹⁶³. Furthermore, using R-Smad3 null mice (R-Smad3^{-/-}) they found that lack of R-Smad activation had no effect on PI3K/Akt signaling in response to TGF β ¹⁶³. To further elucidate the differences between PI3K/Akt signaling in cells of mesenchymal and epithelial origin, Wilkes and colleagues evaluated the activation of p21-activated kinase (PAK2)¹⁶³. PAK2 has previously been shown to be stimulated in a R-Smad-independent manner in cells of mesenchymal origin but not epithelial cells¹⁶³. Wilkes found that TGF β stimulation of AKR-2B fibroblasts induced PAK2 stimulation and inhibition of PI3K completely blocked PAK2 phosphorylation¹⁶³.

Specific inhibition of Akt had no effect on PAK2 phosphorylation and thus suggests that PI3K may activate 2 independent pathways (Figure 5), Akt and PAK2, and that the PAK2 pathway is required for the transformation and proliferation¹⁶³.

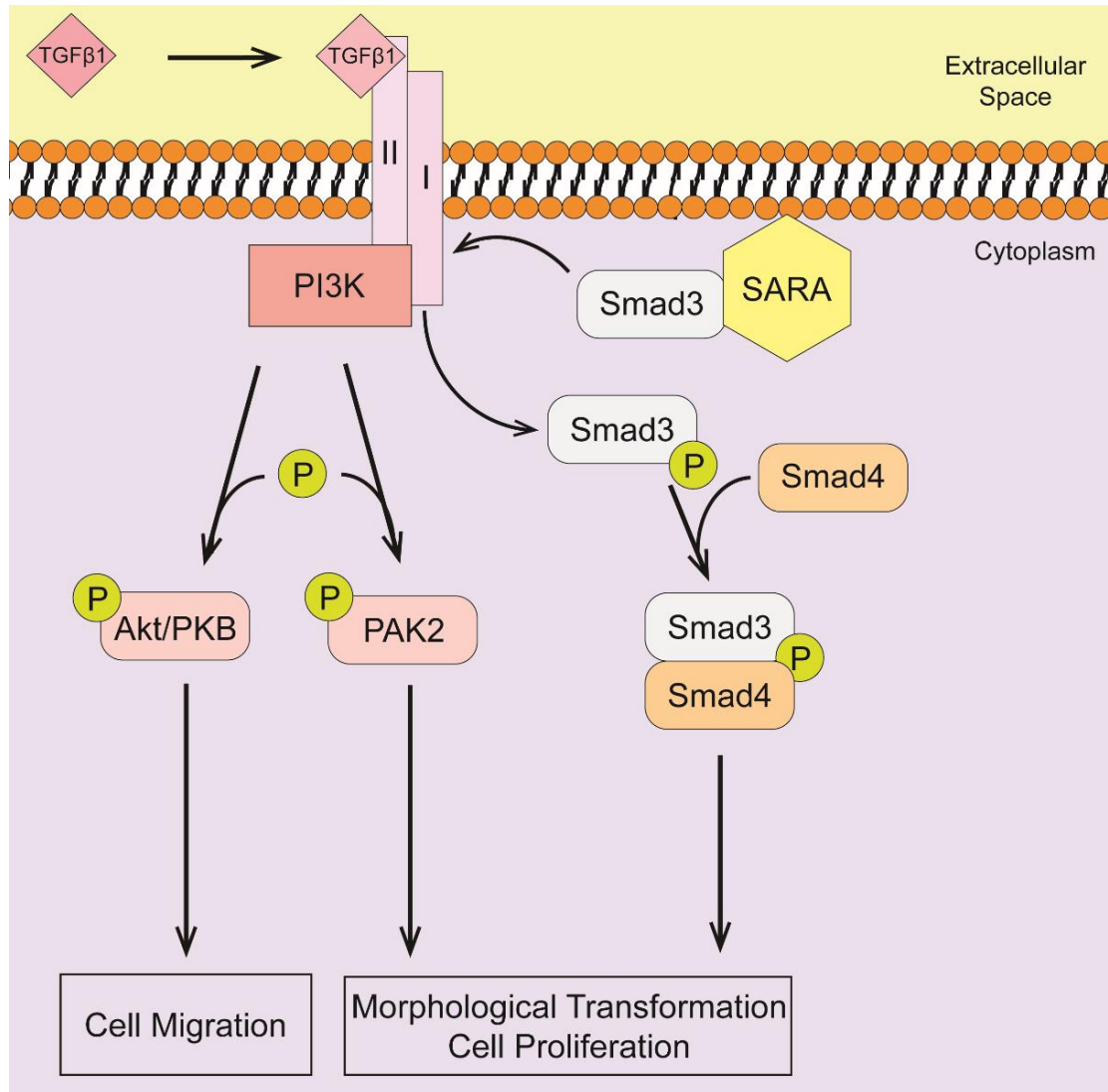


Figure 5. TGFβ₁/Smad-Independent Signaling: PI3K/Akt. In addition to its role in stimulating a Smad-dependent pathway, extracellular TGFβ₁ can stimulate a Smad-independent pathway through TβR's. TGFβ₁ promotes phosphorylation of PI3K which in turn phosphorylates Akt and PAK2. Signaling down an Akt pathway leads to cell migration, whereas signaling through a PAK2 dependent pathway leads to morphological transformation and cell proliferation.

TGF β ₁-Mediated Rho/ROCK Signaling

Rho signaling plays a critical role in mobilization of the actin cytoskeleton and has been shown to be induced by TGF β ₁¹⁶¹. Within 5 minutes of TGF β ₁ stimulation several cell types including NMuMG, Mv1Lu, and primary mouse keratinocytes demonstrate activation of RhoA, which produced a maximal signal of GTP-bound RhoA within 10 minutes¹⁶¹. This lead to activation of ROCK and the formation of stress fibers and a fibroblastic morphology in NMuMG cells following TGF β ₁ treatment¹⁶¹.

Other Rho GTPases, including Rac1 and Cdc42, may be involved in TGF β ₁-mediated signaling through Smad-independent mechanisms. Cells transfected with wildtype (WT) Rac1, dominant-negative Rac1 (DN-Rac1), and Cdc42, however, displayed a phenotype equivalent to that of untransfected cells in response to TGF β ₁, which included cell flattening, membrane ruffles, and stress fibres²²⁵. Conversely, transfection of the dominant-negative Cdc42 (DN-Cdc42) completely inhibited the formation of stress fibers despite TGF β ₁ stimulation²²⁵. This result was recapitulated with WT and DN-RhoA suggesting important roles for Cdc42 and RhoA, but not Rac1 in TGF β ₁-mediated stress fiber formation²²⁵. Further studies using small molecule inhibitors and dominant-negative forms of ROCK were also able to prevent the formation of stress fibers in response to TGF β ₁²²⁵. Inhibition of p38, a downstream effector of Cdc42, also prevented stress fiber formation²²⁵, thus demonstrating the extreme complexity and cross-talk that occurs within TGF β ₁-mediated signaling pathways.

Actin reorganization and stress fiber formation has been found to be independent of R-Smad signaling as dominant-negative Co-Smad4 (DN-Co-Smad4)

had little to no effect on reorganization of the actin filament system in response to $\text{TGF}\beta_1$ ²²⁵. Studies have extended the importance and role of the RhoA/ROCK pathway and indicate that LIM kinase (LIMK) stabilizes F-actin through inhibition of cofilin^{237, 238}, while mDia1 promotes actin assembly (Figure 6)²³⁷. Furthermore, phosphorylation of ROCK has been found to activate myocardin related transcription factor (MRTF) which is then capable of forming a complex with serum response factor (SRF)²²⁶. Together, MRTF/SRF form a transcription factor complex that translocates to the nucleus where it can regulate gene expression in addition to its well documented role in inducing actin cytoskeleton reorganization (Figure 6)²²⁶.

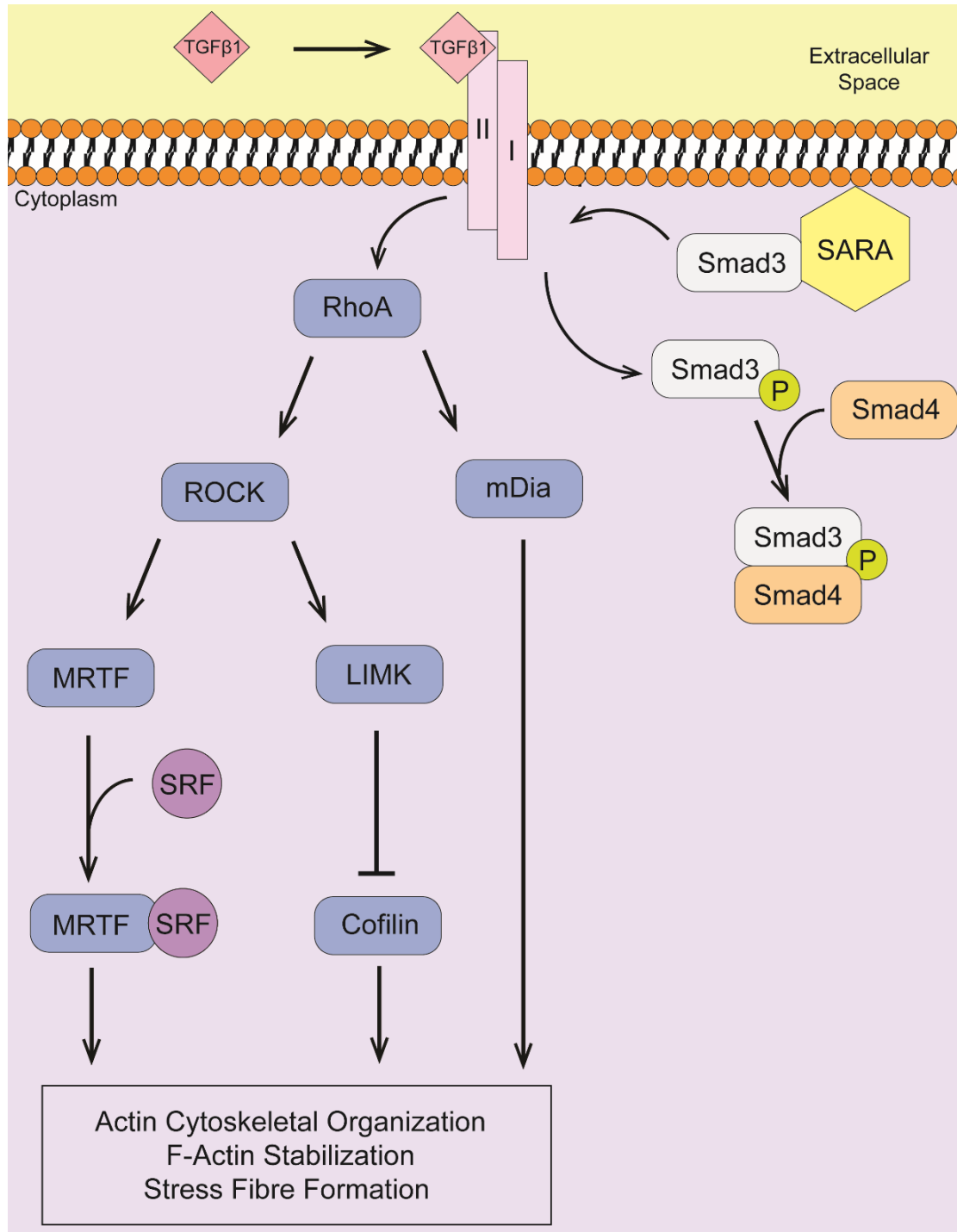


Figure 6. TGFβ₁/Smad-Independent Signaling: Rho/ROCK. Actin organization, stabilization, and stress fibre formation are potently regulated by TGFβ₁ signaling through a Smad-independent pathway. Activation of TβRI promotes the activation of RhoA. RhoA can then induce the activity of both mDia1 and ROCK by phosphorylation. ROCK activation leads to phosphorylation of MRTF and the formation of a complex with SRF. Additionally, ROCK stimulates LIMK which

represses cofilin. Collectively, these pathways lead to actin cytoskeleton organization, F-actin stabilization, and stress fibre formation.

TGF β ₁-Mediated Extracellular Signal-Related Kinase 1/2 (Erk1/2) Signaling

TGF β ₁ and TGF β ₂ treatment has been shown to promote Erk1/2 signaling through activation of several adaptor proteins that ultimately lead to a kinase signaling cascade that includes MAP Kinase Kinase 1/2 (MEK1/2) and Erk1/2^{162, 219, 239, 240}. TGF β ₁ treatment of 3T3-Swiss fibroblasts has been shown to directly phosphorylate Src homology 2 containing protein A (ShcA) through T β RI¹⁶². Shc adaptor proteins are key components of receptor tyrosine kinases, but have been shown to be regulated through other receptors, albeit at lower levels^{162, 240}. There are multiple isoforms of Shc (ShcA, ShcB, ShcC), however, ShcA is ubiquitously expressed while ShcB and ShcC are restricted to neuronal tissues¹⁶². Phosphorylated ShcA is able to form a complex with growth factor receptor binding protein 2 (Grb2) and son of sevenless (SOS) (Figure 7)^{162, 241}. It is important to note that while TGF β ₁ can induce complex formation, it does so at a much lower level than observed through activation of tyrosine kinase receptors in response to epidermal growth factor (EGF)^{162, 239}. Formation of the Shc/Grb2/SOS complex ultimately leads to Ras activation by exchanging GDP for GTP and induction of c-Raf, MEK1/2, and Erk1/2¹⁶².

Similar to the relative confusion in the literature with respect to TGF β -mediated PI3K/Akt signaling, there are conflicting reports regarding TGF β 's ability to promote Erk1/2 signaling. TGF β has been reported to both promote and inhibit Erk1/2 signaling. These conflicting results may arise due to the cell type specific responses that are elicited by TGF β , which in turn reflects the pluripotent nature of this factor. To this

end, Suzuki *et al.* undertook a study to investigate the ability of TGF β ₂ to stimulate Erk1/2 signaling in cells of epithelial and fibroblastic origins²¹⁹. They found that stimulation of cells from a fibroblastic origin with TGF β ₂ induced rapid (10 minutes) activation of Ras, whereas epithelial cells displayed no Ras activation even after 240 minutes of stimulation²¹⁹. Furthermore, they found that Ras activation lead to Erk1/2 phosphorylation within 20 minutes of TGF β ₂ treatment and remained active for more than 120 minutes²¹⁹. This prolonged activation of Erk1/2 coincided with phosphorylation and activation of the downstream transcription factor Elk1. These results were confirmed using a dominant-negative Ras (DN-Ras) to show that lack of Ras activation prevented TGF β ₂-induced phosphorylation of Erk1/2 and did not have any effect on TGF β ₂-mediated R-Smad phosphorylation²¹⁹. Conversely, using R-Smad3 deficient MEF cells, they showed that R-Smads played no role in the activation of either Ras or the phosphorylation of Erk1/2 as Elk1 dependent signaling remained unaltered in these cells²¹⁹. Furthermore, they found that inhibition of PI3K and PAK2 activity prevent TGF β ₂-induced Erk1/2 phosphorylation to a comparable extent that was seen with DN-Ras²¹⁹. This may be due to the fact that PI3K is a known effector of Ras^{242, 243}, thus there may be significant cross-talk between both pathways. More recent studies have shown that Ras activation by T β R's is mediated through an adaptor protein complex that contains ShcA/Grb2/SOS as discussed above (Figure 7)^{162, 219}.

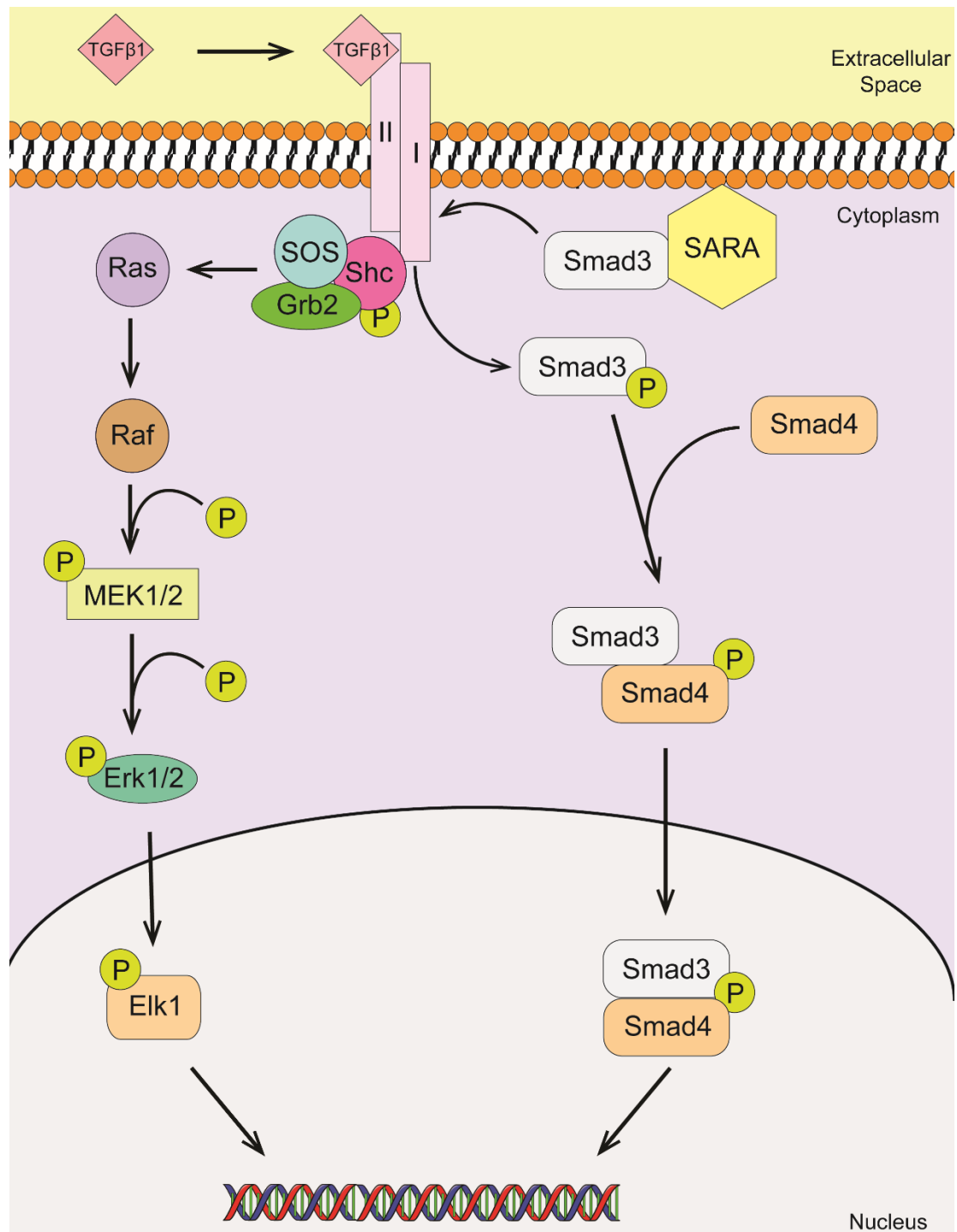


Figure 7. TGFβ₁/Smad-Independent Signaling: Extracellular Signal-Related Kinase 1/2 (Erk1/2). Erk1/2 is a well described TGFβ₁ triggered signal pathway that is Smad-independent. TGFβ₁ stimulation of its receptors leads to the phosphorylation of the adaptor protein Shc. Active Shc forms a complex with Grb2 and SOS which is capable of promoting Ras activation by exchanging GDP for GTP. Activation of Ras leads to a MAPK signaling cascade which ultimately leads to phosphorylation of

Erk1/2 through MEK1/2. Erk1/2 can then phosphorylate transcription factors such as Elk1 to modify gene expression.

Smad-Independent Mechanisms and Fibrosis

There is an abundance of evidence to support the model that TGF β /Smad-independent mechanisms are responsible for increased collagen production and tissue stiffening. In normal skin fibroblasts, TGF β_1 significantly increased collagen 1 α 1 and 1 α 2 gene transcription and protein expression whereas inhibition of PI3K signaling using LY294002 nearly completely inhibited collagen production²²². This effect was found to be due to an inhibition of R-Smad3 phosphorylation which has previously been identified as a key transcription factor required for the induction of collagen transcription²²². Fibroblasts that have been isolated from patients with scleroderma have been shown to synthesize significantly more collagen proteins when compared to normal fibroblasts²²². Similar studies showed that TGF β_1 stimulation of collagen production was inhibited by LY294002. This compound also significantly inhibited collagen production in fibroblasts from scleroderma patients²²². Analogous to the effects of TGF β_1 in dermal fibroblasts, TGF β_1 , but not platelet derived growth factor (PDGF) stimulation of human mesangial cells was shown to promote collagen 1 α 2 gene expression through a PI3K/Akt dependent mechanism²⁴⁴. This again was thought to be due to TGF β_1 's ability to stimulate R-Smad3 phosphorylation whereas PDGF stimulation could not. Inhibition of PI3K/Akt signaling with LY294002 prevented R-Smad3 phosphorylation and collagen 1 α 2 gene expression²⁴⁴, again demonstrating significant cross talk between these two pathways in response to TGF β_1 stimulation.

p38-MAPK signaling has also been linked to the development of tissue fibrosis. Activation of p38 by TGF β ₁ has been shown to promote epithelial injury in idiopathic pulmonary fibrosis and stimulate EMT²⁴⁵. Interleukin-17 (IL-17) has been shown to stimulate airway epithelial cells to synthesize TGF β ₁ and promote autocrine signaling to stimulate EMT through a TGF β ₁-p38-MAPK dependent mechanism. This also resulted in the production of collagen V²⁴⁶. Hepatic stellate cells (HSC) have been shown to play a prominent role in the progression of liver fibrosis characterized by the loss of retinoid content, production of ECM, and formation of a myofibroblast-like phenotype²¹⁵. Furukawa *et al.* demonstrated that TGF β ₁ stimulation of HSC induced phosphorylation of the R-Smad3 linker region and that inhibition of p38-MAPK signaling with the small molecule inhibitor PD169316 diminished the phenocconversion to a myofibroblast phenotype²¹⁵. This effect was found to be independent of Erk1/2 signaling as small molecule inhibitors of MEK1 activity still resulted in R-Smad3 phosphorylation²¹⁵. Additionally, Furukawa's group found that phosphorylation of R-Smad3's linker region was sufficient to promote collagen 1 α 2 gene expression²¹⁵. Furthermore, *in vivo*, normal HSC demonstrated no phosphorylation of the R-Smad3 linker region. Three-weeks after CCl₄ treatment, however, there was an increased number of myofibroblast-like cells which correlated with significant phosphorylation of the R-Smad3 linker region whereas phosphorylation of the C-terminus remained unchanged²¹⁵. Ultimately, it appears from this study that phosphorylation of the Smad-linker region in response to p38-MAPK activation plays a significant role in promoting HSC to become a myofibroblast-like cell type in an *in vivo* model of chronic liver injury²¹⁵.

In cardiac fibroblasts, Erk1/2 signaling has been shown to play a prominent role in the production of ECM and fibrillar collagens²¹⁷. Liu *et al.* demonstrated that TGF β stimulation of primary adult rat cardiac fibroblasts led to significant increases in collagen type I, collagen type III, and α -SMA protein levels as compared to unstimulated controls²¹⁷. They also showed that MEK1 inhibition had no effect on R-Smad2 phosphorylation, yet was able to significantly repress collagen type I and type III production. In an *in vivo* rat unilateral ureteral obstruction model of renal fibrosis, Erk1/2 activation was found to be significantly elevated and corresponded to an increase in both fibrillar collagen and α -SMA expression²¹⁸. Using Madin-Darby canine kidney (MDCK) cells to model polycystic kidney disease, cells treated with TGF β ₁ demonstrated rapid induction of R-Smad, Erk1/2, and p38 pathways reaching a maximal level at 60 minutes²⁴⁷. Pre-treatment of cells with the MEK1 inhibitor PD98059 significantly inhibited the expression of connective tissue growth factor (CTGF), fibronectin, and collagen type I²⁴⁷. Interestingly, pre-treatment with forskolin inhibited TGF β ₁-mediated induction of Erk1/2 but had no effect on R-Smads or p38-MAPK²⁴⁷. Forskolin's ability to induce cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) signaling could prevent TGF β ₁-mediated phosphorylation of Erk1/2 and the downstream production of fibronectin and collagen type I²⁴⁷. These results are in agreement with previously published reports including those studies conducted in cardiac fibroblasts²¹⁷.

In the post-MI heart, ROCK signaling has been demonstrated to be an important player in the activation and phenoconversion of fibroblasts to myofibroblasts. Mice lacking myocardin-related transcription factor-A (MRTF-A) that are subjected to

permanent ligation of the left-anterior descending coronary artery (LAD-CA) were protected from scar expansion as compared to WT animals²²⁶. It was later found that MRTF-A is an important regulator of collagen expression and the myofibroblast expression of smooth muscle cell markers in the post-MI heart²²⁶. Small *et al.* demonstrated that TGF β ₁ induced ROCK phosphorylation which in turn activated MRTF-A and promoted its nuclear translocation²²⁶. Inhibition of ROCK using the small molecule inhibitor Y-27632 prevented MRTF-A nuclear translocation and collagen 1 α 2 expression (mRNA and protein)²²⁶. Notably, there was no difference in animal survival or cardiac function between the groups. Both *in vitro* and *in vivo* studies of idiopathic pulmonary fibrosis (IPF) have shown that TGF β ₁ stimulation of pulmonary fibroblasts promotes phenoconversion to a myofibroblast phenotype, which can be prevented by the drug Fasudil²²⁸. Fasudil has also been shown to play a prominent role in suppressing collagen production in HSC isolated from the livers of male rats²⁴⁸. Additionally, RhoA/ROCK signaling has been shown to be highly activated in patients of human IPF as well as murine models of bleomycin-induced lung injury²²⁸. Perivascular fibrosis has been shown to be suppressed in ROCK^{+/-} haplo-insufficient mice²⁴⁹.

Collectively, these studies into the molecular mechanisms that underpin TGF β ₁-mediated, Smad-independent signaling demonstrate the importance and complexity of the TGF β ₁ pathway. It is clear from these studies that the fibrotic remodelling of tissues during disease have numerous inputs and regulate a vast diversity of signaling pathways. What is more impressive is the fact that all of the pathways described here

can be modulated through a variety of other inputs which are beyond the scope of this literature review.

Tumor Necrosis Factor α

Tumor necrosis factor α (TNF α) was discovered in the mid 1970's as the agent responsible for promoting haemorrhagic necrosis of tumors²⁵⁰. Since its discovery, TNF α has been implicated in numerous disease states and found to be a key component of the inflammatory response. TNF α is synthesized as an inactive 26 kDa pro-peptide that is expressed on the extracellular side of the plasma membrane. Cleavage of TNF α into its active, 17 kDa, soluble form is accomplished by TNF α converting enzyme (TACE - also known as ADAM-17)²⁵¹. In healthy individuals, TNF α is usually undetectable, but is highly elevated in the serum and tissue of individuals with inflammatory disease or infection. While macrophages and monocytes are the primary producers of TNF α in inflammatory disease, numerous other cells such as natural killer cells, cardiac muscle cells, and fibroblasts are all capable of synthesizing TNF α ^{252, 253}.

TNF α signals through one of 2 trimeric membrane bound receptors called TNF α receptor 1 (TNFR1) and 2 (TNFR2)²⁵³. Although, TNFR1 has been shown to be constitutively expressed in most tissues and to be the key mediator of TNF α signaling, TNFR2 is highly regulated and most often found on cells within the immune system²⁵³. Both TNFR1 and TNFR2 contain death domains that can recruit other death domain containing proteins and lead to the activation of caspases, ultimately cumulating in apoptosis. Additionally, TNFR1 has been described as a potent activator of gene expression *via* recruitment of the TNF receptor-associated factors (TRAFs).

Nuclear Factor- κ B Signaling

Nuclear factor- κ B (NF- κ B) is a group of dimeric transcription factors consisting of different combinations of members from the NF- κ B and Rel families. In mammals there are 5 known members to the NF- κ B/Rel family including NF- κ B1/p50, NF- κ B2/p52, c-Rel, RelA/p65, and RelB. These proteins share a Rel homology domain (RHD) that mediates protein-protein interactions, DNA binding, and sub-cellular localization²⁵⁴.

Under basal conditions, I- κ B proteins are bound to NF- κ B dimers. Bound I- κ B masks the nuclear localization signal (NLS) of NF- κ B transcription factors, thus sequestering NF- κ B to the cytoplasm²⁵⁵. Upon TNF α stimulation, I- κ B proteins are phosphorylated by I- κ B kinase (IKK) which leads to its ubiquitination and proteosomal degradation, thus freeing NF- κ B. Free NF- κ B can be further post-translationally modified, i.e. the protein can be phosphorylated, and translocate to the nucleus where it can interact with other transcription factors (such as AP1) and promote gene expression. The NF- κ B transcription factor recognizes the DNA consensus sequence 5' GGGRNYYCC 3' where R is any purine, Y is any pyrimidine, and N is any nucleotide. Collectively these sites are known as κ B sites and they regulate the expression of a wide range of genes.

TNF α , NF- κ B, and Fibrosis

The role of TNF α in ECM remodelling has been explored, however there is no consensus on the anti- and pro-fibrotic properties of TNF α . However, cell type and topographical location (i.e. dermal fibroblast vs. intestinal fibroblast) appears to be a key determinant of TNF α 's fibrotic properties. As an anti-fibrotic, concentrations of

TNF α ranging from 1.0 – 100 ng/mL, have been shown to inhibit collagen type I, collagen type III, and fibronectin protein and mRNA expression in dermal fibroblasts²⁵⁶⁻²⁵⁸. A study by Kouba *et al.* in 1999 demonstrated that NF- κ B mediates the inhibitory effect of TNF α on collagen synthesis²⁵⁹. Using dermal fibroblasts, Kouba showed that the collagen 1 α 2 promoter contained a critical κ B response element that was regulated by the NF- κ B (p50/p65) transcription factor²⁵⁹. Chloramphenicol acetyltransferase (CAT) assays demonstrated that the critical κ B binding motif was found between -271 and -235 bp of the collagen 1 α 2 gene promoter, that when stimulated with TNF α significantly repressed (80% repression) CAT expression²⁵⁹. Site directed mutagenesis of this site completely abrogated the effect of TNF α , and in fact, lead to a significant (3-fold) induction of collagen expression²⁵⁹. Electrophoretic mobility shift assays (EMSA) extended the CAT studies and demonstrated that TNF α treated dermal fibroblasts resulted in NF- κ B binding to the EMSA probe²⁵⁹. This group went on to show that the p50/p65 NF- κ B complex was responsible for NF- κ B binding to the collagen 1 α 2 promoter *via* EMSA analysis²⁵⁹.

In contrast to studies that support an anti-fibrotic role for TNF α /NF- κ B signaling, there is growing evidence to support a pro-fibrotic role of this cytokine. A study by Theiss *et al.* using murine intestine fibroblasts exposed to TNF α revealed an increase in collagen protein accumulation²⁶⁰. However, they found that TNF α had no effect on collagen 1 α 1 transcript levels. Rather, TNF α was found to significantly reduce collagen degradation through a reduction in MMP2 activity. Furthermore, they demonstrated that TNF α induced Erk1/2 signaling and that loss of TNFR2 resulted in a loss of collagen protein accumulation in murine intestinal myofibroblasts²⁶⁰.

Furthermore, Sullivan *et al.* found that TNF α treatment of primary murine lung fibroblasts (MLF) induced TGF β ₁ mRNA and protein expression through an Erk1/2 dependent pathway^{261, 262}. TNF α treatment of MLFs resulted in a 200 - 400% increase in TGF β ₁ protein levels as indicated by ELISA²⁶². Within the heart, p65 has been shown to localize to myocytes and correlate with increased LV fibrotic remodeling of the myocardium²⁶³. In a murine study of pressure overload, Sun *et al.* demonstrated that mice that lacked TNF α (TNF α ^{-/-}) had reduced fibrosis following aortic banding as compared to WT controls²⁶⁴. Thus it is becoming clear that TNF α has both anti- and pro-fibrotic properties and cell type and origin play a significant role in determining the outcome of TNF α -mediated tissue fibrosis.

Ski

History and Identification

Ski is one of seven members of the Ski-Sno Superfamily of TGF β negative regulators that was first identified as being the transforming agent of the Sloan-Kettering retroviruses²⁶⁵. Other members of this family include SnoN and its alternatively spliced variants SnoN2, SnoA, and SnoI, Fussel-15 and Fussel-18²⁶⁵⁻²⁷⁰. Although both Ski and Sno are ubiquitously expressed in human tissues, their expression levels vary from tissue to tissue²⁶⁶⁻²⁶⁸. Furthermore, unlike Ski and Sno, the expression of Fussel-15 and -18 are restricted to the nervous system^{266, 267}.

The Sloan-Kettering retroviruses are a group of transforming retroviruses that were isolated from cultured chicken embryos²⁷¹. These retroviruses promoted focus formation in chicken embryo cell monolayers, colony formation on soft agar, and could not transform bone marrow cells *in vitro*^{271, 272}. Ultimately, the Sloan-Kettering

retroviruses were shown to contain the viral oncogene Ski (v-Ski)²⁷¹. Additional screens using v-Ski as bait, identified several homologous proteins including a larger version that was later named cellular-Ski (Ski)²⁷¹ as well as a highly similar, Ski-related gene that was later found to be Sno²⁶⁸. Gene analysis of the v-Ski and Ski sequences demonstrated that v-Ski is a truncated isoform of Ski that lacks regions at both the 5' and 3' ends²⁷³. When compared to Ski, v-Ski lacks 76 base pairs (bp) at the 5' end and 879 bp at the 3' end²⁷³. There is also a single base difference at position 1284 that is a cytosine (C) in v-Ski and a thymine (T) in Ski which, at the level of amino acids, results in a missense mutation of an arginine in v-Ski to a tryptophan in Ski²⁷³. However, this substitution appears to play no role in the transforming ability of v-Ski.

Further screens conducted by Suttrave and Hughes identified three distinct sequences of the Ski gene in chickens that were shown to arise due to alternative splicing of the parent gene²⁷³. They characterized these isoforms as one full length Ski that comprised of 7 exons and two shorter versions which lacked either exon 2 or exon 6²⁷³. The notion that Ski was alternatively spliced arose from studies conducted by Grimes *et al.* who also identified a full length version of Ski and one that lacked exon 3²⁷⁴. Grimes and colleagues found that Ski isolated from chickens was actually comprised of 8, rather than 7 exons, as exon 7 was found to encode 2 distinct exons²⁷⁵. In humans, Ski appears to lack exon 2²⁶⁸. Early antibody preparations were synthesized to identify full length Ski at 90 kDa and a 60 kDa form that lacks exon 2. These studies also demonstrated that the 90 kDa Ski is extensively phosphorylated and had a half-life that was approximately 3x longer than that of the shorter 60 kDa isoform²⁷⁶, although both proteins still localized to the nucleus²⁷⁶. A study conducted 20 years later by

Nagata *et al.* showed that Ski is phosphorylated at serine 515, however this had no effect on Ski's stability as mutant Ski (S515A) was as stable as the WT form²⁷⁷. Another study demonstrated that Ski was phosphorylated at amino acid 458 by Akt which resulted in Ski destabilization and proteosomal degradation²⁷⁸. Thus, protein phosphorylation appears to play an important role in Ski regulation however the importance, if any, of S515 phosphorylation has yet to be determined.

Protein Structure

Ski is a highly conserved, evolutionary ancient protein, and a known negative regulator of the TGF β /Smad signaling pathway^{186, 187, 279}. Structurally, Ski and the other members of the Ski-Sno superfamily contain several domains which are critical to their function. Near the N-terminus lies the dachshund homology domain (DHD) (Figure 8) whose function is not fully understood but is thought to play a critical role in mediating the interactions of Ski with transcriptional co-activators and co-repressors²⁸⁰. Some studies have demonstrated that transcriptional co-repressors such as nuclear receptor co-repressor (NCoR), silencing mediator for retinoid or thyroid-hormone receptors (SMRTs), and histone deacetylases (HDACs) are capable of binding to the DHD^{280, 281}²⁸². The crystal structure of human Ski suggests that the DHD contains structural homology to the forkhead/winged-helix family of DNA binding proteins, particularly the retinal determination protein Dachshund²⁸¹. However, the members of the Ski/Sno superfamily do not directly bind DNA themselves so the precise role of the forkhead/winged-helix region is not fully known. Just downstream of the DHD lies the R-Smad2/3 interaction domain while the Co-Smad4 domain lies further downstream of the R-Smad2/3 site (Figure 8)^{279, 282-285}. These two domains are absolutely critical for

Ski's ability to modulate TGF β /Smad signaling and will be discussed in greater detail below. The Co-Smad4 domain is comprised of an unique 88 amino acid C2H2 zinc-binding module that bears structural homology to the SAND domain, named after the sequences of Sp100, Autoimmune regulator 1, NucP41/75, and Deformed epidermal autoregulatory factor 1 homolog^{286, 287}. What is interesting to note is that this C2H2 zinc-binding module is only found in the Ski family of TGF β negative regulators and plays an important role in negatively regulating the TGF β /Smad signal.

Although Ski is found in both the cytoplasmic and nuclear fractions, Ski is primarily a nuclear protein. Nagata *et al.* identified a key motif that may play an important role in the sub-cellular localization of Ski²⁸⁸. Through an *in silico* search of putative nuclear localization signals (NLS) they identified a PRKRKLT (proline, arginine, lysine, arginine, lysine, leucine, threonine) motif that corresponded to amino acids 452-458 of the human Ski protein²⁸⁸. They conducted site-directed mutagenesis and replaced the arginine and lysine residues with asparagine and glutamine (making the aa sequence PNQNQLT) residues, respectively. From this, they observed a significant reduction in the amount of nuclear staining for this mutant form of Ski as compared to WT controls²⁸⁸. The mutation, however, did not result in complete exclusion of Ski from the nucleus, suggesting that some other motifs or factors may play a role in the sub-cellular trafficking of Ski²⁸⁸. This group also found that cytoplasmic Ski was more stable than its nuclear counterpart as determined by pulse-chase assays. How Ski enters the nucleus and the importance of a more stable cytoplasmic construct is largely unknown.

The C-terminal region of Ski is less conserved than the N-terminal but has been shown to play a role in allowing Ski to form homo- and hetero-dimers with other Ski and Sno proteins through a coiled-coiled motif^{289, 290}. To date there have been few studies that have evaluated the abundance and efficiency of Ski:Ski, Sno:Sno, and Ski:Sno homo- and hetero-dimers respectively. However a study conducted by Cohen *et al.* showed that hetero-dimers of Ski:SnoN formed preferentially over all other combinations and was a stronger transforming agent than homo-dimers²⁸⁹.

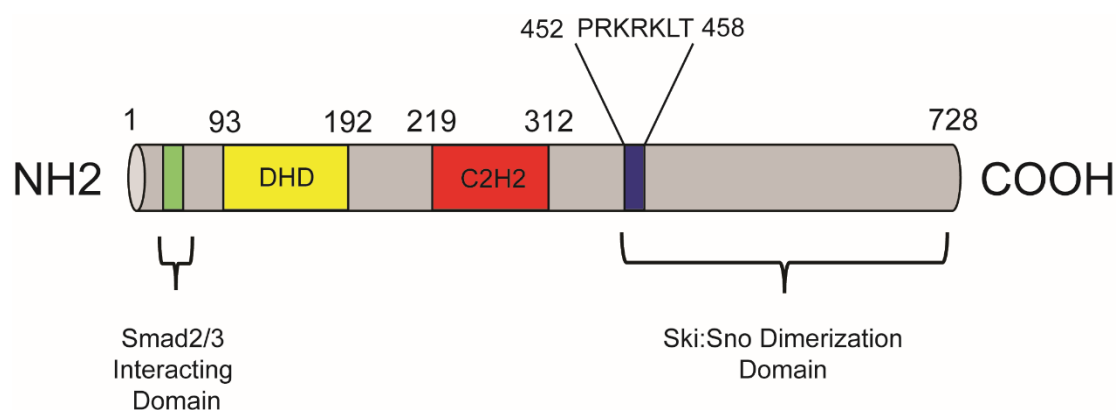


Figure 8. Protein Structure of Ski. The human Ski protein is depicted above. At the N-terminus end of the protein lies a DHD, that plays a critical role in protein:protein interactions, and a R-Smad2/3 interacting domain that is important for Ski's ability to repress TGF β /Smad-dependent signaling. Slightly further downstream lies the unique C2H2 domain that regulates Ski's interaction with Co-Smad4. The C-terminus end of the protein is less conserved than that of the NH2 end. However, the C-terminus plays an important role in Ski:Sno homo- and hetero-dimerization as well as nuclear translocation of Ski (PRKRKLT – nuclear localization signal (NLS)).

Transcriptional and Post-Translational Regulation of Ski

With the advancement of technology since Ski was first discovered²⁷¹, experiments to assess the functionality of gene transcription/regulation and protein post-translational modifications are less daunting. There is little known with regards to

the transcriptional regulation of Ski. There is but one recent study which has provided a look into the transcriptional processes that regulate Ski²⁹¹. In this work, Li *et al.* were able to PCR clone a 2258 bp (-2277 to -19 relative to the ATG start codon) region of the rat Ski promoter from genomic DNA (gDNA) isolated from rat skin fibroblasts²⁹¹. As the nuclear receptor peroxisome proliferator-activated receptor δ (PPAR δ) has recently been suggested to play a role in wound healing^{292, 293} and Ski expression has been suggested to play a role in tissue repair and scar formation^{21, 22, 294, 295} this group aimed to understand the relationship between Ski expression and PPAR δ . Using the GW502526 ligand specific for PPAR δ , they found that PPAR δ increased expression of a luciferase reporter gene construct which contained 2258 bp of the rat Ski promoter²⁹¹. The specific effects of this ligand were confirmed with the use of an irreversible inhibitor (GSK0660) of PPAR δ . *In silico* analysis of the rat promoter revealed 9 putative PPAR response elements (PPRE). Truncations of the 2258 bp promoter identified that a region between -958 and -851 contained a functional PPRE²⁹¹. Site-directed mutagenesis within this region confirmed those studies using truncated promoter constructs and revealed the presence of a functional PPRE²⁹¹. As functionality does not necessarily mean direct DNA:protein interactions, both EMSA and chromatin immunoprecipitation (ChIP) studies were undertaken. In both cases, PPAR δ was found to associate with the DNA at a region encompassing the putative PPRE²⁹¹. This is the first, and only study to date to identify and characterize a functional transcription factor binding site for the induction of Ski transcription. SnoN has been shown to be transcriptionally regulated by TGF β /Smad signaling whereas no such relationship has been identified for Ski²⁹⁶.

Unlike its transcriptional regulation, much more is known regarding the post-translational modifications and function of Ski protein. As discussed above, Ski has been demonstrated to be phosphorylated at several residues²⁷⁷. Although the exact mechanism and importance for each residue is not yet certain, at least one does appear to play a significant role in proteosomal degradation of Ski²⁷⁸. Another method by which Ski may be targeted for proteosomal degradation is through polyubiquitination by the E3 ubiquitin ligase Arkadia^{297, 298}. Through a series of co-immunoprecipitation (Co-IP) studies, Arkadia was found to associate with Ski and co-localize to the nucleus. Nagano *et al.* also found that along with its association with Ski, Arkadia promoted protein ubiquitination and proteosomal degradation as indicated by pulse chase assays²⁹⁷. Moreover, it was found that Arkadia targeted the central region of Ski (aa 211-490) which contains the SAND domain, thus indicating an importance for this region in both protein-protein interactions and protein regulation²⁹⁷. Smurf2 has been identified as another pro-ubiquitinating agent of SnoN but not Ski²⁹⁹. SnoN has been shown to undergo SUMOylation at two lysine residues (K50 and K383) whereas Ski has no such identified regions^{300, 301}. Collectively, these studies demonstrate that despite the structural and functional similarities between these proteins, they are differentially regulated potentially allowing for finer transcriptional and post-translational control of gene and protein expression.

Ski in Development and Disease

Ski has been shown to be ubiquitously expressed in all tissues of the developing mouse as well as cardiac and skeletal muscles of adults and neonates³⁰². In order to better understand the role of Ski in developing and adult tissues, murine knock-in and

knock-out models have been employed³⁰²⁻³⁰⁴. Transgenic mice engineered to overexpress Ski resulted in significantly higher levels of Ski in skeletal muscles which correlated with severe muscle hypertrophy³⁰³. Most notably fast muscle fibres were significantly increased in size due to selective hypertrophy of type IIb muscle fibers whereas there was no change in either type I or IIA fibres³⁰². Despite the significant increase in muscle size, this did not correspond to an increase in muscle strength but rather lead to muscle degeneration^{302, 303}. Thus, these mice had large muscles but could generate little to no power as compared to WT controls³⁰³. To corroborate their functional studies, they examined the cyto-architecture and ultrastructure of muscle fibres using electron microscopy. It was found that mice overexpressing Ski contained less contractile material in the muscle fibres, as well as poor alignment of Z-discs and abnormal mitochondria³⁰³. In addition to increased muscle size, overexpression of Ski has been linked to the growth, invasion, and metastasis of several cancers in humans³⁰⁵⁻³⁰⁹.

In contrast to overexpression studies which demonstrated little to no effect of Ski on murine embryonic development and survival, transgenic studies in which mice lacked Ski (*Ski*^{-/-}) displayed a significantly different phenotype. During normal development Ski mRNA levels are found to increase at embryonic day 8.5-9.5 (E8.5-9.5) which correlates with the migrating neural crest³¹⁰. Ski levels are also elevated at E12.5-15.5 in skeletal muscle correlating with the proliferation of secondary myoblasts and/or differentiation of primary myotubes^{311, 312}. Mice that lack Ski have neural tube defects, cranial mesenchymal defects, as well as a reduction in skeletal muscle mass resulting in perinatal lethality³⁰⁴. Ultimately, perinatal lethality is a result of

exencephaly caused by a failure of the closure of the cranial neural tube during neurulation³⁰⁴.

In humans, spontaneous mutation of the Ski gene leads to the production of a malfunctioning protein and results in a severe developmental disorder known as Shprintzen-Goldberg Syndrome (SGS). Patients that present with SGS display a phenotype resembling those afflicted with Marfan's and Loeys-Dietz syndrome³¹³⁻³¹⁵. Specifically, SGS patients present with severe skeletal and craniofacial abnormalities including dolichocephaly, proptosis, micrognathia, dolichostenomelia, pectus chest deformity, joint contractures and hypermobility³¹⁶. Patients also display intellectual disabilities as well as heart and brain abnormalities similar to that of Marfan syndrome^{313, 317, 318}.

Shprintzen-Goldberg Syndrome is an autosomal dominant mutation that most often arises due to a spontaneous mutation within exon 1 which typically, but not always, corresponds to the R-Smad interaction domain^{313, 316, 317 319}. In fact, the R-Smad binding domain has been identified as a hot spot for spontaneous, *de novo* mutations in Ski resulting in SGS³¹⁹. The prevalence of SGS is not known as the condition is extremely rare. The life span of an individual with SGS is reduced to an age of approximately 40, similar to that of a patient with Marfan or Loeys-Dietz syndrome. Typically, death is related to the underlying structural abnormalities that accompany SGS such as structural heart defects.

Ski and TGF β Signaling

Ski has been primarily identified as a nuclear protein that plays a significant role in the regulation of TGF β /Smad signaling^{22, 186, 283, 287, 288, 295}. Ski interacts with R-

Smad2/3 through its N-terminal domain and with Co-Smad4 through its SAND-like domain forming a “wedge” within the Smad-complex²⁸⁷. Within the nucleus, Ski inhibits TGF β /Smad signaling by forming a complex with R-Smads at the promoter region of TGF β regulated genes where it recruits transcriptional co-repressors (NCoR and HDACs) to inhibit gene expression³²⁰. Furthermore, Ski stabilizes binding of the Smad-complex to DNA to prevent further binding of active Smad complexes³²¹. This model of TGF β negative regulation by Ski was described by Suzuki *et al.* as the disrupting bridge hypothesis (Figure 9A)³²¹. In addition to its ability to recruit transcriptional co-repressors, Ski also competes with the co-activator p300/CBP for binding to Smad complexes to prevent the formation of a co-activating complex¹⁸⁶.

Although Ski has been shown to interact with DNA while in complex with R-Smad and Co-Smad proteins, it alone cannot bind DNA^{187, 322-324}. Ski can only interact with DNA when within a Smad-complex. Thus, as opposed to being described as a transcription factor, Ski rather acts as an adaptor or scaffolding protein that mediates protein-protein interactions to modulate gene expression. In an interesting set of studies, Ueki *et al.* inserted mutations into the R-Smad3 binding site of Ski and tested its ability to repress TGF β ₁/Smad signaling³²⁵. They found that despite disrupting the R-Smad3 binding site of Ski, mutant Ski was still capable of repressing TGF β ₁/Smad dependent signaling³²⁵. These results demonstrate that as long as Ski is capable of interacting with one of R-Smad3 or Co-Smad4 it is able to repress TGF β ₁/Smad-dependent signaling.

As described earlier in this literature review, Ski has been found to contain a putatively functional NLS that, when mutated, can cause Ski to accumulate within the

cytoplasm²⁸⁸. Despite the change in sub-cellular localization, cytoplasmic Ski was still capable of inhibiting TGF β ₁ signaling to a similar extent to that of WT, nuclear localized Ski²⁸⁸. Site-directed mutagenesis of the NLS in combination with the R-Smad and Co-Smad4 regulatory regions found that this inhibitory effect was due to Ski's ability to interact with R-Smad proteins within the cytoplasm²⁸⁸. Thus, Ski inhibited the nuclear translocation of the Smad complex and prevented TGF β ₁/Smad mediated gene expression (Figure 9B). Cytoplasmic retention of the Smad complex represents a novel second mechanism by which Ski can antagonize TGF β /Smad signaling.

In a third model of Ski mediated repression of TGF β /Smad signaling, Prunier *et al.*, showed that cytoplasmic Ski can promote the formation of an inactive R-Smad/Co-Smad4 complex independently of TGF β signaling that prevents phosphorylation of Smad2/3 (Figure 9C)³²⁶. To date, the precise mechanism by which Ski is sub-cellularly localized and functions is not well understood. What is clear is the diverse and redundant ability of Ski to inhibit TGF β /Smad-dependent signaling thus highlighting the importance of Ski in developmental and pathological processes.

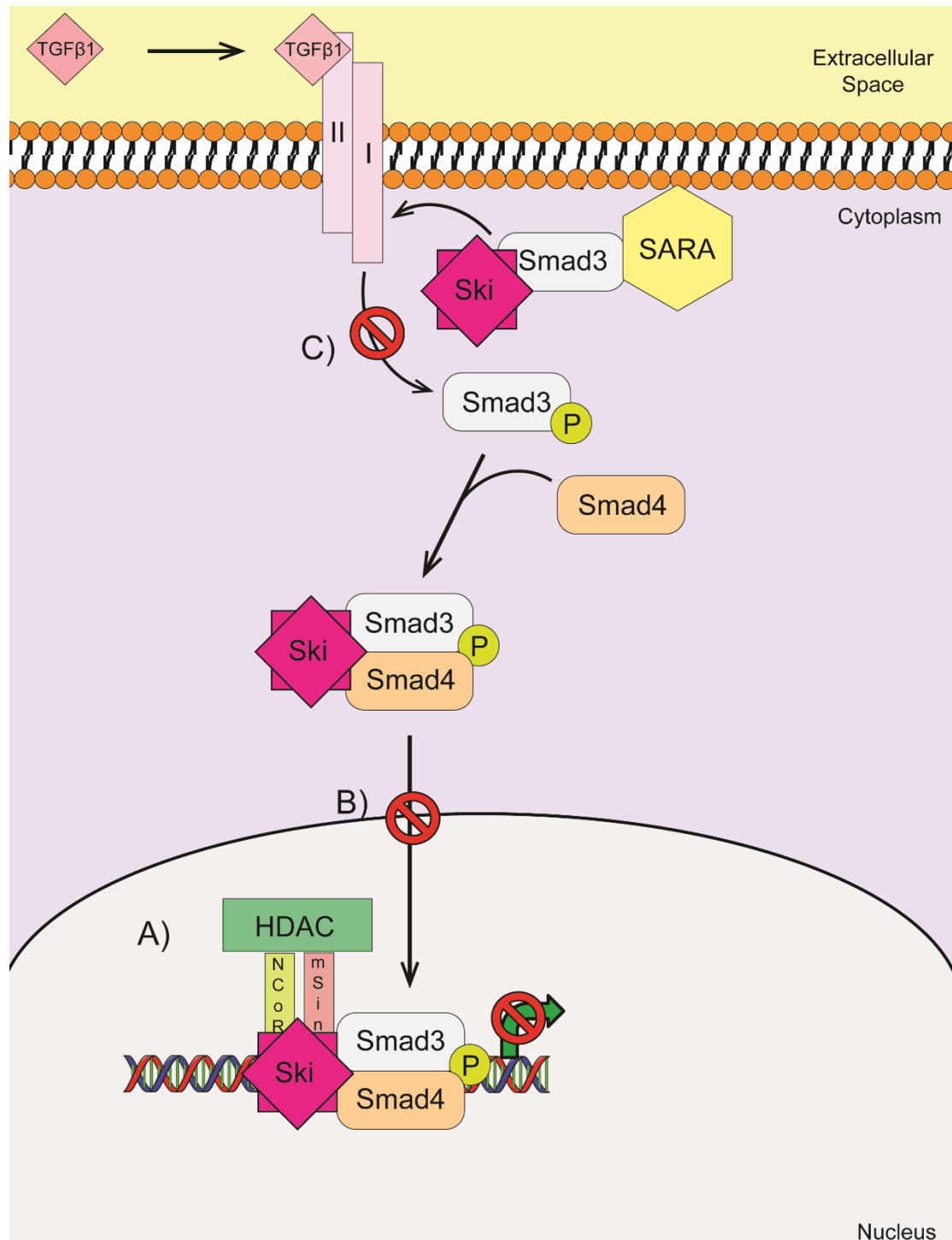


Figure 9. Ski Mediated Repression of TGFβ₁/Smad Signaling. A) Ski is primarily a nuclear protein. Within the nucleus Ski can inhibit Smad dependent signaling by forming an inhibitory complex with the R-Smad/Co-Smad complex and stabilize them while bound to DNA. Ski then recruits a transcriptional inhibitory complex that includes NCoR, mSin3a, and HDACs to inhibit gene transcription. Ski can also be found within the cytoplasmic fraction of cells. Although its function has been

extensively described in the nucleus, Ski can repress TGF β ₁/Smad signaling from the cytoplasm in two ways. First **B**) Ski can form a complex with the Smad complex and prevent nuclear translocation. Second **C**) Ski can prevent R-Smad phosphorylation at the level of the T β RI and prevent R-Smad complex formation at the initiating step.

Scleraxis

History and Identification

Scleraxis (Scx) is a 201 aa class “A” basic helix-loop-helix transcription factor (bHLH) that binds to the E-box consensus sequence CANNTG (cytosine, adenine, any (N), any (N), thymine, guanine)³²⁷⁻³²⁹. Scx was first identified as an important regulator in the embryonic development of collagen-rich connective tissues including tendons, ligaments, and cartilage and has become an important marker of tendons and tenocytes³³⁰⁻³³³. Tenocytes are cells that are responsible for the synthesis and secretion of tendon ECM³³⁴. Scx has been demonstrated to be an important regulator of collagen 1 α 1 in tenocytes whose expression is induced by TGF β ³³⁴. Studies using Scx null mice have shown that the loss of Scx significantly impairs the formation of load-bearing tendons and exemplifies the importance of Scx during embryonic development^{335, 336}. More recent studies have described a role for Scx in development of the auditory system, Sertoli cells, cardiac valves, and the progression of fibrogenic cardiac disease³³⁷⁻³⁴⁰.

Protein Structure

As mentioned above, Scx is a member of the bHLH transcription factors and shares many of the same structural features as other members of this family. The bHLH protein family is divided into 6 categories (A through F) that classifies the proteins based on their evolutionary relationships, conserved motifs, and E-box binding

properties³²⁸. Class A bHLH proteins to which Scx belongs, typically form hetero-dimers with class B bHLH proteins and preferentially bind to *CAGCTG* and *CACCTG* E-box consensus sequences^{341, 342}. The bHLH motif is comprised of an N-terminal basic region and a C-terminal HLH region containing an α -helix, an unstructured loop, followed by another α -helix³⁴¹. The amphipathic HLH region of these proteins mediates the formation of homo- and hetero-dimers with other bHLH proteins such as E12 and E47^{327, 328, 343}. Relatively little else is known regarding Scx protein structure and motifs, however, recent data from the laboratory of Dr. Michael Czubryt provided evidence to suggest that Scx is phosphorylated by casein-kinase2 (CK2)³⁴⁴.

Regulation of Scleraxis

Little is known with regard to the transcriptional regulation of Scx. To date, there is no known direct transcriptional activator of the Scx gene. Several reports have evaluated the relationship between TGF β and Scx and have shown TGF β to promote Scx protein expression in numerous cell lines and models^{20, 337, 345}. TGF β_3 is known to promote Scx expression in embryo-derived stem cells isolated from equine tendons³⁴⁵. TGF β_2 has been shown to promote Scx expression in fibroblasts isolated from murine heart valves³³⁷, while TGF β_1 has been associated with increased Scx expression in primary cardiac fibroblasts isolated from mouse and rat hearts^{20, 210}. Although all of these studies provide a correlative-link between TGF β stimulation, Smad signaling and Scx expression, no study to date has explored the direct DNA:protein transcriptional mechanisms that link this signal and effector protein.

Chapter 2: Overall Study Rationale, Hypothesis, and Aims

Central Hypothesis: As Ski exerts anti-fibrotic properties and Scx is a known pro-fibrotic effector protein, we propose the existence of a regulatory balance between these proteins. We believe that this relationship is critical in maintaining matrix homeostasis in the uninjured heart, which is disrupted following a large MI. Specifically, we believe that Ski and Scx form a transcriptional negative feedback loop that represses gene expression and is a central regulator of collagen expression (Figure 10).

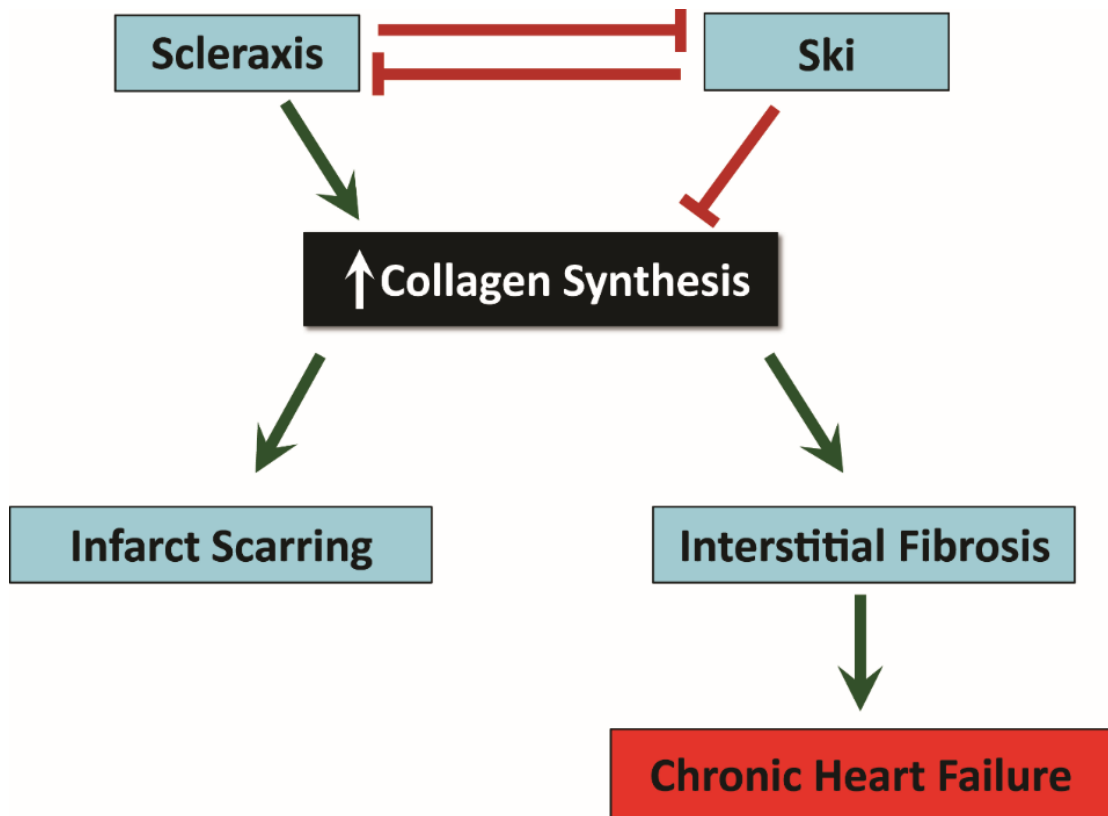


Figure 10. Overall Visual Hypothesis. Ski and Scx form a negative feedback loop on one another that regulates gene expression. In the chronic post-MI setting, we believe that this balance is tipped in favour of Scx, which promotes repression of Ski transcription leading to remodelling of the cardiac matrix. With TGF β ₁ signaling unchecked by Ski, there is a significant increase in expression of fibrillar collagens and matrix proteins that ultimately leads to interstitial fibrosis and heart failure.

Part 1: Transcriptional Regulation of Scleraxis

Aim 1: To determine whether the human Scx promoter (hScxP) is targeted by Ski protein.

Hypothesis: Overexpression of Ski in primary cardiac myofibroblasts will repress Scx gene transcription through its ability to complex with Smad proteins and form an inhibitory complex.

Aim 2: To determine whether R-Smad proteins directly target the hScxP to induce Scx gene expression.

Hypothesis: Smad protein complexes are able to bind to putative Smad binding elements (SBE) in the hScxP and promote Scx gene expression following TGF β ₁ treatment.

Part 2: Transcriptional Regulation of Ski

Aim 3: To determine whether the bHLH transcription factor Scx directly targets the human Ski proximal promoter (hSkiPP) and negatively regulate gene expression.

Hypothesis: Overexpression of Scx in primary cardiac myofibroblasts will repress Ski transcription through its ability to bind to the promoter region of Ski at putative E-box binding (CANNTG where *N* is any nucleotide) domains.

Chapter 3: Transcriptional Regulation of Scleraxis

Rationale, Hypothesis, and Objectives

Ski is a potent anti-fibrotic agent. Its primary mechanism of action is to inhibit TGF β /Smad signaling in the nucleus by stabilizing Smad transcription factor complexes on the promoter region of TGF β regulated genes. There it recruits transcriptional co-repressors including NCoR and HDACs to form an inhibitory complex to repress TGF β /Smad dependent gene expression.

The bHLH transcription factor Scx has been shown to be induced by TGF β cytokines 1, 2, and 3 and R-Smad3^{20, 337, 345}. Scx has been implicated in promoting collagen production in numerous cell types including cardiac myofibroblasts²⁰. Moreover, Scx has been shown to work in a synergistic fashion with Smad proteins to promote collagen gene expression²⁰. R-Smad3 has also been implicated in transducing the TGF β signal from the cell membrane to the nucleus to positively regulate Scx gene expression²⁰. However, despite the convincing evidence for TGF β /Smad-induced Scx expression, no studies to date have evaluated the direct DNA:protein transcriptional mechanisms associated with TGF β ₁-dependent Smad signaling and Scx gene transcription.

Therefore, we sought to explore this proposed mechanism. We hypothesize that the hScxP is a direct target of Smad transcription factors that, upon TGF β ₁ stimulation, bind to the promoter region and positively regulate Scx gene transcription. To address our hypothesis, we aim to:

- i) Determine whether the hScxP is an indirect target of the TGF β inhibitory protein Ski through its interactions with Smad protein complexes to inhibit Scx gene transcription.
- ii) Determine whether Smad protein complexes directly interact with the hScxP and positively regulate Scx gene expression.

Methodology

Animal Ethics

All experimental protocols involving animal studies were reviewed and approved by the University of Manitoba's Animal Care Committee following the Canadian Council on Animal Care Standards.

***In silico* Analysis of the Scleraxis Promoter**

The mouse, rat, and human Scx gene sequences were obtained by searching the National Center for Biotechnology Information (NCBI) database using the key words "mouse Scleraxis", "rat Scleraxis", and "human Scleraxis". The FASTA sequences were downloaded into a word document. Using the FASTA gene sequence as the query, mouse, rat, and human Scx were localized to their appropriate chromosomal locations using NCBI Blast. The FASTA sequences for the chromosomal regions of interest were downloaded into a word document. The translational start site (ATG) was identified for each Scx gene, as was the 5' untranslated region (UTR), exons, introns, translational stop, and the 3' UTR. Identification of potential Smad binding element (SBE) sequences was determined by Blast searching all SBE sequence possibilities and highlighting them within the first 10,000 base pairs upstream of the translational start of the Scx gene. Other promoter elements were also identified including, but not limited

to NF- κ B binding sites (GGGRNNYYCC; where R is any purine, Y is any pyrimidine, and N is any nucleotide), E-Boxes (CANNTG), TATA, and CAAT-boxes.

To identify regions of potential significance, mouse, rat, and human promoters were aligned against each other (ClustalW, Expasy) to detect regions of high identity and similarity. Based on these alignments, the hScxP was split into 2 regions, the proximal (~1600 bp (1580 bp actual) 5' of the ATG; Appendix A, Figure A-1) and the distal promoters (a further 2000 bp upstream of the proximal promoter; Appendix A, Figure A-2). The location and size of the proximal region of the hScxP to be examined was chosen for evaluation as it contains numerous putative transcription factor binding motifs that may be indicative of a key regulatory region of the hScx gene.

Cloning of the Human Scleraxis Promoter

The hScxP was purchased from Genecopia and cloned into the *KpnI* and *HindIII* sites of a pGL4.10 [luc2] reporter vector (Promega) by the manufacturer. Upon arrival, bacteria was streaked out on Carbenicillin containing agar plates (100 μ g/mL) and incubated overnight at 37°C. Individual colonies were picked from the plate and overnight cultures grown in Luria broth (LB) containing Carbenicillin (100 μ g/mL). DNA was isolated using the Qiagen mini-prep kit and subjected to restriction enzyme analysis. 200 ng of vector DNA was digested in NEB buffer 2.1 with *KpnI* and *HindIII* (New England Biolabs) for 1 hour at 37°C in a water bath. 6x DNA loading buffer (30% glycerol (v/v), 0.25% bromophenol blue (w/v), 0.25% xylene cyanol FF (w/v)) was added to each reaction to a final concentration of 1x and separated on a 1.0% agarose gel at 120 volts for 1 hour. The gel was imaged using the Bio-Rad Gel Doc system and analyzed using Quantity One Software (BioRad). The presence of a single

band at ~1600 base pairs (bp) (1580 bp actual) would suggest the presence of the insert. The insert was confirmed by DNA sequencing analysis (TCAG, Toronto, Canada).

Luciferase Assay

1.0×10^6 NIH-3T3 (American Type Culture Collection (ATCC)) cells were plated in a T-75 (Corning) cell culture flask in Dulbecco's Modified Eagle's Medium (DMEM; Gibco)/high glucose supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin (Gibco), and 100 U/mL streptomycin (Gibco). Cells were allowed to proliferate until the culture flask was approximately 60% confluent. Cells were lifted using TrypLE (Gibco) reagent. TrypLE was neutralized with 2x volume 10% DMEM-FBS and cells pelleted by centrifugation at 2000 rpm for 5 minutes at room temperature. The supernatant was discarded and the cell pellet re-suspended in fresh 10% DMEM-FBS. Cells were counted using an automated cell counter (Moxi) by diluting the concentrated suspension 10x. The reading was taken twice and the average was used to determine cell numbers. Approximately 25,000 cells were added to each well of a 6-well dish based on the following equation:

$$\text{A) } \textit{Number of cells per mL} = (\textit{number of cells counted} * \textit{dilution})$$

$$\text{B) } \textit{mL of cells required} = \frac{25,000 \text{ cells}}{\textit{Number of cells per mL}}$$

Figure 11. Equation for Plating of NIH-3T3 Cells. **A)** Formula for calculating the number of cells in a given cell suspension, **B)** Formula for calculating the number of milliliters required to plate 25000 cells in a given cell culture dish.

Each well was filled to 2.0 mL using 10% DMEM-FBS. Cells were allowed to adhere and proliferate for 48 hours. Next, culture medium was removed, cells washed in 1x phosphate buffered saline (PBS), and 1.5 mL of serum/antibiotic free Opti-MEM I (Gibco) media was added to each well. For TGF β ₁ studies, each well was transfected with 1.5 μ L Lipofectamine 3000 (Invitrogen), 1.0 μ L P3000 (Invitrogen), 250 ng hScxP pGL4.10[luc2], 250 ng empty reporter vector, and 5.0 ng renilla vector DNA for 24 hours. The next day, Opti-MEM I media was removed, cells gently washed in 1x PBS, and 1.5 mL serum free DMEM supplemented with antibiotics was added to each well. Cells were treated with recombinant human TGF β ₁ (rhTGF β ₁; Cell Signaling) ranging in concentration from 0.5-10 ng/mL. Luciferase assays were performed 24 hours after TGF β ₁ treatment in triplicates using the Dual-Luciferase assay kit (Promega) and the GloMax luminometer (Promega). Individual samples were normalized to renilla expression and then to untreated control cells.

For studies including co-transfection of R-Smad expression vectors, Opti-MEM I media was added to each well as described above. NIH-3T3 cells were transfected with 1.5 μ L Lipofectamine 3000, 1.0 μ L P3000, 100 ng hScxP pGL4.10[luc2], 50-400 ng human Smad2-pcDNA3 or human Smad3-pCMV-Tag2B expression vectors, 100-350 ng empty pcDNA3 or pCMV-Tag2B (to equalize DNA mass), and 5.0 ng renilla. Twenty-four hours later, cells were harvested and read in triplicates as described by the manufacturer (Promega).

Isolation of Primary Cardiac Myofibroblasts

Primary cardiac fibroblasts were isolated from 150-250 gram Sprague-Dawley male rats *via* retrograde Langendorff perfusion as previously described^{22, 96}. Rats were

anesthetized with a cocktail of ketamine:xylazine *via* intraperitoneal (I.P.) injection. On loss of reflex, heparin (7.0 mg/kg) was administered through the femoral vein to prevent clotting. The chest was then opened, heart removed, and placed in a 100 mm cell culture dish containing DMEM_{F12} supplemented with 100 U/mL penicillin, 100 U/mL streptomycin, 1.2 g sodium bicarbonate, and 1.0 μ M ascorbic acid. Excess tissue was removed and the heart hung from the aorta. The heart was secured to the cannula using 3-0 braided silk suture (Deknatel). Once hung, the hearts were perfused with DMEM_{F12} for 5 minutes followed by a 6-minute perfusion with Spinner-Modified Minimum Essential Media (SMEM) to stop cardiac contraction. Following cardiac arrest, the hearts were perfused with 0.1% collagenase type II (298 U/mL; Worthington) for 20 minutes to digest the heart. After 20 minutes the hearts were removed from the cannula, placed in a 100 mm cell culture dish containing 0.05% collagenase type II (149 U/mL), and torn into small fragments using forceps. The heart was digested for an additional 15 minutes by incubation at 37°C in the cell culture dish. The collagenase reaction was then neutralized by the addition of 2x volume 10% DMEM_{F12}-FBS supplemented with 100 U/mL penicillin, 100 U/mL streptomycin, 1.2 g sodium bicarbonate, and 1.0 μ M ascorbic acid. The tissue/cell suspension was then transferred to a 50 mL conical and large heart fragments allowed to settle for 5 minutes. The supernatant was transferred to a new 50 mL conical and centrifuged at 2000 rpm for 5 minutes. The supernatant was removed and discarded. The cell pellet was re-suspended in 10% DMEM_{F12}-FBS and evenly distributed amongst 4x T-75 cell culture flasks and incubated for 2 hours at 37°C to allow fibroblasts to adhere. These cells are considered P0 primary cardiac fibroblasts. After 2 hours, the cells were washed twice

in 1x PBS, and 10 mL fresh 10% DMEM_{F12}-FBS was added to each flask, and the cells returned to the incubator. Twenty-four hours later the media was removed and the cells washed with 1x PBS. Fresh 10% DMEM_{F12}-FBS was added and the cells were incubated at 37°C in a humidified environment supplemented with 5.0% CO₂. Seventy-two hours later, cells were passaged to P1 cardiac myofibroblasts. Briefly, culture medium was removed, cells washed in 1x PBS, and incubated for 5 minutes with TrypLE (Gibco) to gently lift the cells from the T-75 cell culture flask. TrypLE was neutralized with 2x volume 10% DMEM_{F12}-FBS and cells pelleted by centrifugation at 2000 rpm for 5 minutes at room temperature. The supernatant was removed and the cell pellet re-suspended in 10% DMEM_{F12}-FBS. Cells were counted using an automated cell counter as described above. Cells were plated as appropriate for the given experiments.

Protein Isolation and Quantification

P0 primary cardiac fibroblasts were passaged into P1 cardiac myofibroblasts and plated into 100 mm cell culture dishes. 1.0×10^6 P1 myofibroblasts were infected with adenovirus containing either haemagglutinin (HA)-human Ski (Ad-HA-Ski) or LacZ (Ad-LacZ) at the time of plating with a multiplicity of infection (MOI) of 50. Twenty-four hours later cells were harvested. Briefly, culture medium was removed and the cells washed twice in 1x PBS. Cells were left in the second wash on ice until ready for cell lysis. For cell lysis, PBS was removed and cultures treated with 100 μ L radioimmunoprecipitation assay (RIPA) lysis buffer (150 mM NaCl, 1.0% nonidet P-40 (NP-40), 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris) containing protease (20 μ M leupeptin, 15 μ M pepstatin A, 0.80 μ M aprotinin, 1.04 mM

[4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride] (AEBSF), 40 μ M Bestatin, 1.4 mM E-64 (Sigma-Aldrich Corporation, St. Louis, MO)) and phosphatase (10 mM NaF, 1.0 mM Na_3VO_4 , and 1.0 mM EGTA) inhibitors. Cells were scraped from the plate in RIPA buffer using a sterile plastic scraper. The protein containing solution was transferred to a 1.5 mL microcentrifuge tube and left on ice for 60 minutes. The solution was then sonicated with three, 10 second pluses with a 5-minute incubation on ice between each pulse. After the final pulse, the solution was centrifuged at 16000 $\times g$ for 20 minutes at 4°C to pellet cellular debris. The supernatant was removed and placed in a fresh 1.5 mL microcentrifuge tube and stored at -20°C until ready for protein quantification and Western blot analysis.

Protein quantification was achieved using the bicinchoninic acid (BCA) assay. Protein samples were diluted 4x in RIPA buffer and 10 μ L of sample was added in triplicates to a 96-well cell culture dish. Bovine serum albumin (BSA) standards ranging from 0.0 mg/mL to 2.0 mg/mL were prepared in RIPA buffer and also added to the plate in triplicates to generate a standard curve. A 50:1 solution of bicinchoninic acid and copper (II) sulfate (ThermoFisher) was prepared and 200 μ L was added to each well. The plate was sealed with parafilm and incubated at 37°C for 30 minutes before being read at 560 nm on a plate reader (BioRAD iMark).

Western Blotting

All protein samples underwent 1-dimensional 8-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 150 volts for approximately 1.5 hours in a discontinuous system at room temperature. Each lane was loaded with 5-20 μ g of protein. Proteins were transferred to 0.45 μ m polyvinylidene fluoride (PVDF;

Bio-RAD) membrane at 300 milliAmps for 1.25 hours at 4°C on ice. Membranes were blocked in 5.0% skim milk dissolved in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1.0 hour at room temperature with constant shaking. The membranes were washed two times for 15 minutes each in TBS-T prior to the addition of primary antibodies. Primary polyclonal antibodies for Ski (Santa Cruz Biotech), Hemagglutinin (HA; Rockland), Scleraxis (generated in the laboratory of Dr. Mike Czubryt), p44/42, phospho-p44/42, c-Jun, phospho-c-Jun, p38, and phospho-p38 (Cell Signaling) were incubated overnight in either 3.0% skim milk TBS-T or 5.0% BSA TBS-T at a concentration of 1:1000 at 4°C with gentle shaking. Loading control was accomplished using β -Tubulin (1:5000; Abcam) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000; Cell Signaling) in 3.0% skim milk TBS-T and 5.0% BSA TBS-T respectively. Goat-anti-mouse or goat-anti-rabbit horseradish peroxidase (HRP)-linked IgG secondary antibodies (1:2000; Jackson Laboratories) were used to detect the primary antibodies and was accomplished using Pierce ECL Western blotting substrate (Life Technologies). Protein bands were visualized using CL-Xposure blue X-ray film (Life Technologies). Film images were digitized using the GS-800 densitometer (BioRad) and protein expression determined using Quantity One Software (Bio-Rad).

Generation of Expression Vectors

Scleraxis

The cDNA open reading frame (ORF) of human Scx (hScx) was purchased from Genecopia (Massachusetts, USA). Upon arrival, bacteria were streaked onto agar plates containing Kanamycin (100 μ g/mL) and were incubated overnight at 37°C. The next day individual clones were chosen and overnight cultures grown in LB containing

Kanamycin (100 µg/mL) at 37°C with constant mixing (300 rpm). Vector DNA was isolated using the Qiagen mini-prep kit and concentration determined by UV absorbance at 260 nm (GeneQuant III). Vector DNA containing hScx was then sent for sequencing to confirm the identity of the insert (TCAG, Toronto, Canada). Upon confirmation, hScx was subjected to PCR (Table 2) to add *EcoRI* and *XhoI* cloning sites for insertion into a pcDNA3 expression vector. The PCR product was stored at -20°C until ready for sub-cloning.

<u>Step</u>	<u>Event</u>
1	95°C for 5 minutes 1x
2	95°C 30 seconds
3	60°C 30 seconds
4	70°C 2 minutes
5	Step 2 - 4 40x
6	70°C for 4 minutes 1x
7	10°C Hold

Table 2. PCR Cycling for Cloning Human Scleraxis into pcDNA3.

10 µg empty pcDNA3 expression vector (Stratagene) was digested with *EcoRI* and *XhoI* in NEB buffer 2.1 for 3 hours at 37°C in a water bath. Following digestion, 1/10th volume of Antarctic phosphatase buffer and 2.0 U of Antarctic Phosphatase (New England Biolabs) were added to the reaction and incubated for an additional hour at 37°C in a water bath to dephosphorylate DNA ends. Following incubation, Antarctic

Phosphatase was heat inactivated by incubation at 70°C in a heat block for 5 minutes. Linearized vector was purified using the QiaQuik PCR purification kit and samples stored at -20°C until ready for use.

The hScx PCR product from above underwent digestion with *EcoRI* and *XhoI* as described above. DNA loading buffer (6x) was added to each sample to a final concentration of 1x and subjected to electrophoresis on a 1.0% agarose gel with SYBR safe (Invitrogen) at 120 volts for 1 hour. Bands were visualized under a long wave UV light and excised from the gel using a scalpel. DNA was extracted from the gel using DNA Extraction Columns (Millipore). DNA concentration was determined *via* UV absorbance.

Ligation of the purified hScx ORF into pcDNA3 was accomplished using the NEB quick ligation kit. A 3:1 molar ratio of insert to vector was used per ligation. Each reaction (vector, vector + ligase, vector + insert + ligase) was incubated at room temperature for 10 minutes and 5.0 µL of reaction used to transform AG1 (Agilent) or NEB-5α (New England Biolabs) competent cells as per manufacture's protocol. After a 30 minute incubation on ice, bacteria were heat shocked at 42°C in a water bath for 45 seconds to help transform with DNA. One milliliter of LB was added to each reaction and cells incubated at 37°C with constant mixing (300 rpm) for 1 hour. The bacterial solution was then spun at 16000 *xg* for 10 seconds to pellet cells. The supernatant was removed, bacteria re-suspended in 100 µL LB, and 75 µL cell suspension was spread and 25 µL streaked onto agar plates containing Carbenicillin (100 µg/mL). The plates were incubated overnight at 37°C and the next analyzed for efficiency.

Ten to twenty colonies from the vector + insert + ligase reaction were picked from the plate and overnight cultures grown in LB containing Carbenicillin (100 µg/mL) at 37°C in a shaking incubator at 300 rpm. DNA was isolated from bacteria using the Qiagen mini-prep kit. The concentration of each clone was determined and 200 ng DNA was subjected to double enzymatic digestion in NEB buffer 2.1 with *EcoRI* and *XhoI* for 1 hour at 37°C in a water bath. Samples were then subjected to 1.0% agarose gel electrophoresis with SYBR safe (Invitrogen) at 120 volts for 1 hour. Gels were imaged using a Bio-Rad Gel Doc imager. The presence of a single band at 600 bp confirmed the presence of hScx. Two clones were sent for sequencing (TCAG, Toronto, Canada) to confirm insert sequence.

Ski

The ORF of human Ski (hSki) was PCR'd from a pShuttle2 vector containing hSki and cloned into the *BamHI* and *XhoI* cloning sites of pcDNA3 as described above. Clones positive for the insertion of hSki were determined by restriction enzyme analysis and confirmed by DNA sequencing (TCAG, Toronto, Canada) as described above.

Cell Fractionation

Untreated P1 cardiac myofibroblasts and Cos7 cells were grown on 100 mm cell culture dishes in either 10% DMEM_{F12}-FBS or 10% DMEM-FBS respectively until they reached 70-80% confluency. Cytoplasmic and nuclear fractionation was performed using the NE-PER Cytoplasmic and Nuclear Extraction Kit (Pierce Scientific) according to manufacturer's directions. Samples were stored at -80°C until ready for protein quantification and Western Blot or EMSA analysis.

Electrophoretic Mobility Shift Assay (EMSA)

Biotinylated and non-labelled probes encompassing conserved SBE in the mouse/human/rat Scx promoter (Table 3) were synthesized commercially (Sigma). Duplexes from single-stranded probes were made by adding equal volumes of 5.0 μ M stocks of complementary oligonucleotides encompassing the appropriate SBE and incubated at 95°C for 5 minutes in a heat block. The heat was then turned off and probes allowed to gradually cool to room temperature. Gel shift assays were performed according to the Lightshift Chemiluminescent EMSA kit (Pierce Scientific) as per manufacturer's directions. Briefly, 500-1000 ng of nuclear lysate was incubated in binding buffer (10 mM Tris, 50 mM KCl, 1.0 mM dithiothreitol (DTT), 50 ng/ μ L sonicated salmon sperm DNA, pH 7.5) and 20 fmol biotinylated probe for 20 minutes at room temperature. For cold competitions, a 500-fold molar excess of unlabelled probe was included in the binding reactions for 20 minutes prior to the addition of biotinylated probe. For supershift reactions, 500-1500 ng of anti-Smad2 or anti-Smad3 antibodies (Cell Signaling) were incubated in binding buffer plus nuclear lysate overnight at 4°C. DNA:protein complexes were subjected to 6% native PAGE at 100 volts for 60 minutes and transferred to 0.45 μ m positively charged nylon membrane (Biodyne, ThermoFisher) at 380 mA for 30 minutes. DNA was cross-linked to the membrane in a UV cross-linking oven at 120 mJ for 60 seconds. DNA was visualized using ECL and CL-Xposure blue X-Ray film (Life Technologies).

<u>Probe</u>	<u>Sequence (5 – 3)</u>
Distal WT SBE Forward	AGGGCTCTGGGCAGCCAC <u>AGAC</u> AGGGATGACCTCATCACC
Distal WT SBE Reverse	GGTGATGAGGTCATCCCTGTCTGTGGCTGCCCCAGAGCCCT
Distal MT SBE Forward	CAGGGCTCTGGGCAGCCAC <u>GCATG</u> CGGGGATGACCTCATCACCCAC
Distal MT SBE Forward	GTGGGTGATGAGGTCAATCCCGCATGCTGGCTGCCAGAGCCCTG
Proximal WT SBE Forward	CACATGGGAGGCCAAAGAC <u>AGAC</u> AGGGTGCGGTCAGGCAGC
Proximal WT SBE Reverse	GCTGCCCTGACCGCACCCCTGTCTGTCTTTGCCCTCCCATGTG
Proximal MT SBE Forward	CACATGGGAGGCCAAAGAC <u>GCATG</u> CGGGTGCGGTCAGGCAGCAG
Proximal MT SBE Reverse	CTGCTGCCCTGACCGCACCCGCATGCTCTTTGCCCTCCCATGTG

Table 3. Electrophoretic Mobility Shift Assay Probes. Electrophoretic Mobility Shift Assay probe sequences for the distal and proximal SBEs in the hScxP. Bold and underlined sequences identify the position of the SBE. Reproduced with permission³⁴⁶.

Immunoprecipitation

1.0×10^6 P1 cardiac myofibroblasts were plated into a 100 mm cell culture dish and infected with either Ad-LacZ or Ad-HA-Ski at an MOI of 50 at the time of plating. Twenty-four hours later, cells were washed twice in 1x PBS and lysed in 1.0 mL of IP lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1.0 % NP-40, 1.0 mM EDTA pH 8.0, 5.0% glycerol) with gentle agitation for 5 minutes at room temperature. The plate was then scraped with a plastic cell scraper and the solution added to a 1.5 mL microcentrifuge tube. Samples were then centrifuged at $13000 \times g$ for 10 minutes at 4°C to remove cellular debris. The sample volume was then split in half and added to new microcentrifuge tubes, one for antibody pull down and the other as an IgG control. c-Jun (Cell Signaling; 20 μg) primary antibody or normal rabbit IgG (Cell Signaling; 20 μg) were added to the appropriate tube and incubated overnight at 4°C with rotation to form protein:antibody complexes. The next day 200 μL Protein A agarose beads (50% slurry; ThermoFisher) was added to each tube and incubated for 2 hours at room temperature with rotation. The beads were pelleted at $2500 \times g$ for 2 minutes, the supernatant removed, and the beads suspended in IP wash buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, pH 7.2). This process was repeated 3 times with IP wash buffer and once with DDW. After the final wash, the supernatant was removed and the beads suspended in 60 μL 3x IP loading buffer (187.5 mM Tris-HCl pH 6.8, 6% SDS (w/v), 30% glycerol, 0.03% Phenol Red, 125 mM DTT). Samples were briefly vortexed, and centrifuged at $14000 \times g$ for 1 minute. Samples were then incubated at 95°C for 5 minutes in a heat block. Following incubation, the samples were centrifuged at $14000 \times g$ for 1 minute and 50 μL of protein sample loaded directly onto a 10% SDS-PAGE

gel. Proteins were separated and detected as described above for standard Western blotting.

Chromatin Immunoprecipitation

1.0×10^6 P1 cardiac myofibroblasts were seeded onto a 100 mm cell culture dish and treated with Ad-LacZ, or Ad-HA-Ski at an MOI of 50 at the time of plating. Twenty-four hours later, 37% formaldehyde was added to the culture medium to a final concentration of 1.0% to cross-link DNA:protein complexes and was incubated at room temperature for 10 minutes with gentle shaking. The formaldehyde was neutralized with glycine at a final concentration of 0.25 M for 5 minutes with gentle shaking at room temperature. The culture media was then removed and cells washed twice in PBS containing protease inhibitors (Sigma) and 1.0 mM PMSF. After the final wash, 1.0 mL wash buffer was added to the plate and cells scraped from the plate and added to a 1.5 mL microcentrifuge tube. The cells were then pelleted by centrifugation at 2000 rpm for 5 minutes at 4°C. The supernatant was discarded and the pellet suspended in 200 μ L SDS lysis buffer (50 mM Tris-HCl pH 8.0; 10 mM EDTA, 1.0% SDS, 0.22 μ m syringe filtered) containing protease inhibitors and 1.0 mM PMSF. DNA was sheared by sonication with 3x 10 second pulses on level 4. The cells were incubated on ice for 5 minutes between each pulse. Following shearing, the samples were centrifuged at 16000 $\times g$ for 10 minutes at 4°C. The supernatant was transferred to a new microcentrifuge tube. A 10% aliquot of each sample was removed and placed in a new microcentrifuge tube and frozen to be used as an input control for PCR. The remaining sample was diluted 10-fold in ChIP IP dilution buffer (0.01% SDS, 1.1% Triton X-100, 167 mM NaCl, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.0, 0.22 μ m syringe filtered)

containing protease inhibitors. Each sample was then pre-cleared with 50 μ L of Protein A agarose beads (50% slurry; ThermoScientific) to reduce non-specific binding *via* rotation for 60 minutes at 4°C. Agarose beads were pelleted by centrifugation of 2000 rpm for 1 minute at room temperature. The supernatant was removed and each sample split in half and placed into a new 2.0 mL microcentrifuge tube. Antibody against Ski (1:50; Santa Cruz) or rabbit IgG (1:50; Santa Cruz) was added to the appropriate tube and incubated overnight at 4°C with constant, gentle rotation. The next day, 100 μ L of Protein A agarose was added to each tube and incubated at 4°C with constant rotation for an additional 60 minutes. Samples were then centrifuged at 2000 rpm for 1 minute at room temperature and the supernatant discarded. The agarose beads were washed 2x for 5 minutes each with constant rotation with 1) low salt buffer (0.1% SDS, 0.1% Triton X-100, 150 mM NaCl, 2.0 mM EDTA, 20 mM Tris-HCl pH 8.0, 0.22 μ m syringe filtered); 2) high salt buffer (0.1% SDS, 0.1% Triton X-100, 500 mM NaCl, 2.0 mM EDTA, 20 mM Tris-HCl pH 8.0, 0.22 μ m syringe filtered); 3) lithium salt buffer (0.5 M LiCl, 1.0% NP-40, 1.0% deoxycholic acid; 100 mM Tris-HCl pH 9.0, 0.22 μ m syringe filtered); 4) TE buffer (10 mM Tris-HCl pH 8.0, 1.0 mM EDTA, 0.22 μ m filtered). Agarose beads were pelleted by centrifugation at 2000 rpm for 1 minute at room temperature between each wash. After the final wash, 250 μ L elution buffer (1.0% SDS, 0.1M NaHCO₃) was added to each tube and incubated for 15 minutes at room temperature with rotation. The beads were pelleted and the supernatant added to a new 1.5 mL microcentrifuge tube. The elution step was repeated to give a final eluted volume of 500 μ L. DNA:protein complexes were reverse cross-linked in 0.3 M NaCl at 65°C overnight in a heat block. The next day, 5x Proteinase K buffer (50 mM Tris-

HCl pH 7.5, 25 mM EDTA, 1.25% SDS) was added to a final concentration of 1x and 60 µg/mL of Proteinase K added to each reaction and incubated at 45°C for 2 hours. An equal volume of phenol:chloroform:isoamylalcohol (Invitrogen) was added to each tube and mixed well by vortexing. Samples were centrifuged at 16000 *xg* for 5 minutes at room temperature. The top aqueous layer was removed and placed in a fresh 1.5 mL microcentrifuge tube. DNA was precipitated overnight with 2x volume 100% ethanol, 1/10th volume NaOAc, and 20 µg glycogen (Invitrogen) at -20°C. The next day, samples were centrifuged at 16000 *xg* at 4°C for 30 minutes to pellet DNA. The supernatant was removed and the pellet washed in 75% ethanol. DNA was pelleted again at 16000 *xg* for 10 minutes at room temperature. The supernatant was removed and discarded and the DNA pellet allowed to air dry for 10 minutes at room temperature. Each pellet was suspended in 50 µL of nuclease free water (Ambion) and the optical density at 280 nm determined to calculate DNA concentration. Samples were amplified *via* PCR using the primers listed in Table 4, separated on a 2% agarose gel, and visualized using a Gel Doc system (BioRad).

<u>Primer</u>	<u>Sequence (5' – 3')</u>
Rat Scx Distal Promoter Forward	GCCAGAGCTTTACCTTCACA
Rat Scx Distal Promoter Reverse	CAGGTTTGTCTCCCTGTGGT

Table 4. ChIP Primers.

Quantitative Real-Time PCR

1.0×10^6 P1 cardiac myofibroblasts were seeded onto a 100 mm cell culture dish and treated with Ad-LacZ, or Ad-HA-Ski at an MOI of 50 at the time of plating. Twenty-four hours later, the medium was removed and the cells were washed twice in 1x PBS and harvested in 1.0 mL Trizol Reagent (Life Technologies). Total RNA was isolated as per manufacturer's directions. Samples were treated with DNase I (NEB) to remove contaminating genomic DNA (gDNA) prior to cDNA synthesis. cDNA was synthesized using the BioRAD iScript Select cDNA synthesis kit using random primers as per the manufacturer's protocol. qRT-PCR was performed using SYBR select Master Mix for CFX (Life Technologies) as per the manufacturer's protocol on a BioRAD iQ5 or Mini-Opticon in triplicate using primers against: Scx, collagen 1 α 1, collagen 1 α 2, collagen 3 α 1, and GAPDH (Table 5). The cycling conditions were as outlined in Table 6. mRNA levels were calculated using the $2^{-\Delta\Delta C_t}$ method and normalized to GAPDH.

<u>Primer</u>	<u>Sequence (5'-3')</u>
Scleraxis Forward*	AACACGGCCTTCACTGCGCTG
Scleraxis Reverse*	CAGTAGCACGTTGCCCAGGTG
Collagen 1 α 1 Forward	TGCTCCTCTTAGGGGCCA
Collagen 1 α 1 Reverse	CGTCTCACCATTAGGGACCCT
Collagen 1 α 2 Forward	GTCCCCGAGGCAGAGAT
Collagen 1 α 2 Reverse	CCTTTGTCAGAATACTGAGCAGC
Collagen 3 α 1 Forward	GGTTTCTTCTCACCCTGCTTC
Collagen 3 α 1 Reverse	GGTTCTGGCTTCCAGACATC
GAPDH Forward	TGCACCACCAACTGCTTAGC
GAPDH Reverse	ACGGCCATCACGCCACAGC

Table 5. qPCR Primers. * Previously published by Bagchi and Czubryt²⁰.

<u>Step</u>	<u>Event</u>
1	50°C 5 minutes 1x
2	95°C 5 minutes 1x
3	95°C 15 seconds
4	61°C 30 seconds – Plate Read
5	3-4 40x
6	55°C → 95°C – Melt Curve
7	End

Table 6. qPCR Cycling Conditions.

Inhibition of MEK1/2

P1 cardiac myofibroblasts were seeded onto a 100 mm cell culture dish with 10% DMEM_{F12}-FBS growth media. Twenty-four hours later the cells were washed in 1x PBS and starved in serum-free DMEM_{F12} supplemented with antibiotics for 24 hours. The next day, cells were treated with 10 μ M U0126 (Cell Signaling) or vehicle (dimethyl sulfoxide (DMSO)) for 2 hours followed by 10 ng/mL rhTGF β ₁ for an additional 1 or 24 hours. Cells were harvested for Western Blot analysis as described above.

Rat Model of Myocardial Infarction

Rats were randomized to two groups: i) sham operated, where the animals underwent surgery but did not have ligation of the LAD-CA, and ii) ligated, where the LAD-CA was permanently occluded. The animals were then further randomized into sub-groups of 2-weeks, 4-weeks, and 8-weeks following surgery (Figure 12). Upon removal, the hearts were dissected into LV viable tissue (border zone to the infarct) and LV scar (the collagenous scar itself) in those animals that underwent ligation. The whole LV from sham operated animals was also collected as a control. Samples were flash frozen in liquid nitrogen and stored at -80°C until ready for processing (Figure 12).

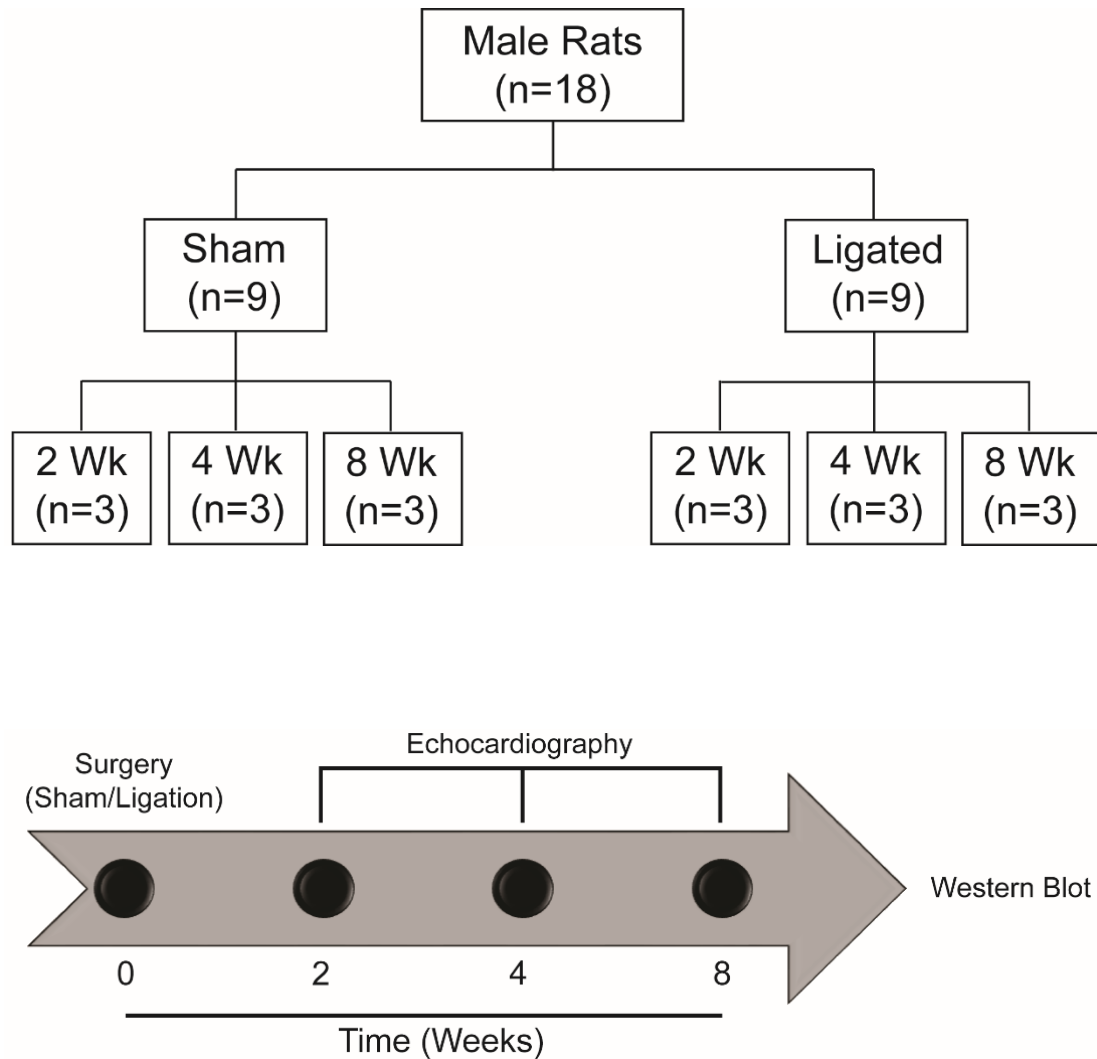


Figure 12. Rat MI Randomization Scheme and Surgery Timeline. Male rats were randomized into 2 groups – sham operated and LAD-CA ligation. Animals were further randomized to be euthanized at 2, 4, or 8-weeks post-MI and tissue collected for Western blot analysis.

Echocardiography

Non-invasive transthoracic echocardiography (TTE) was performed as previously described at 2, 4, and 8-weeks post-MI^{347, 348}. Animals were anesthetized with 3.0% isoflurane and maintained at 2.0% isoflurane. The hair from the chest was removed using an electric razor and the area cleaned with an alcohol wipe. Acoustic gel was applied to the chest and TTE was performed using a 5.0 MHz probe (Vivid 7,

GE Medical Systems, Milwaukee, MI, USA) in the parasternal short axis view at the level of the papillary muscles to acquire M-mode images. Processing of M-mode TTE images was accomplished offline using EchoPAC PC software (v.112, GE Medical Systems, Milwaukee, MI, USA).

Statistics

All data is expressed as mean $\pm$ standard error (SE). Comparisons between 2 groups was accomplished using the Students' *t*-test. Differences between multiple groups were compared using either a one-way ANOVA with a Student-Newman Keuls' *post-hoc* test or a two-way ANOVA with a Bonferroni *post-hoc* test. A *P*-value less than 0.05 ($P < 0.05$) was considered significant. All statistics were performed using Graphpad Prism 5 statistical analysis software.

Results

Ski is a Negative Regulator of TGF β ₁/Smad Signaling

The primary function of Ski is to repress TGF β signaling through disruption of Smad protein complexes at the promoter region of TGF β /Smad-dependent genes³²¹. It accomplishes this action through the recruitment of a transcriptional co-repressor complex that ultimately prevents gene expression²⁸⁰. We exposed P1 primary cardiac myofibroblasts to Ad-HA-Ski at an MOI of 50 for 24 hours. Ectopic expression of Ski resulted in an 8 to 10-fold increase ($*P < 0.001$; $n=4$) in Ski protein expression as compared to Ad-LacZ and untreated controls (Figure 13).

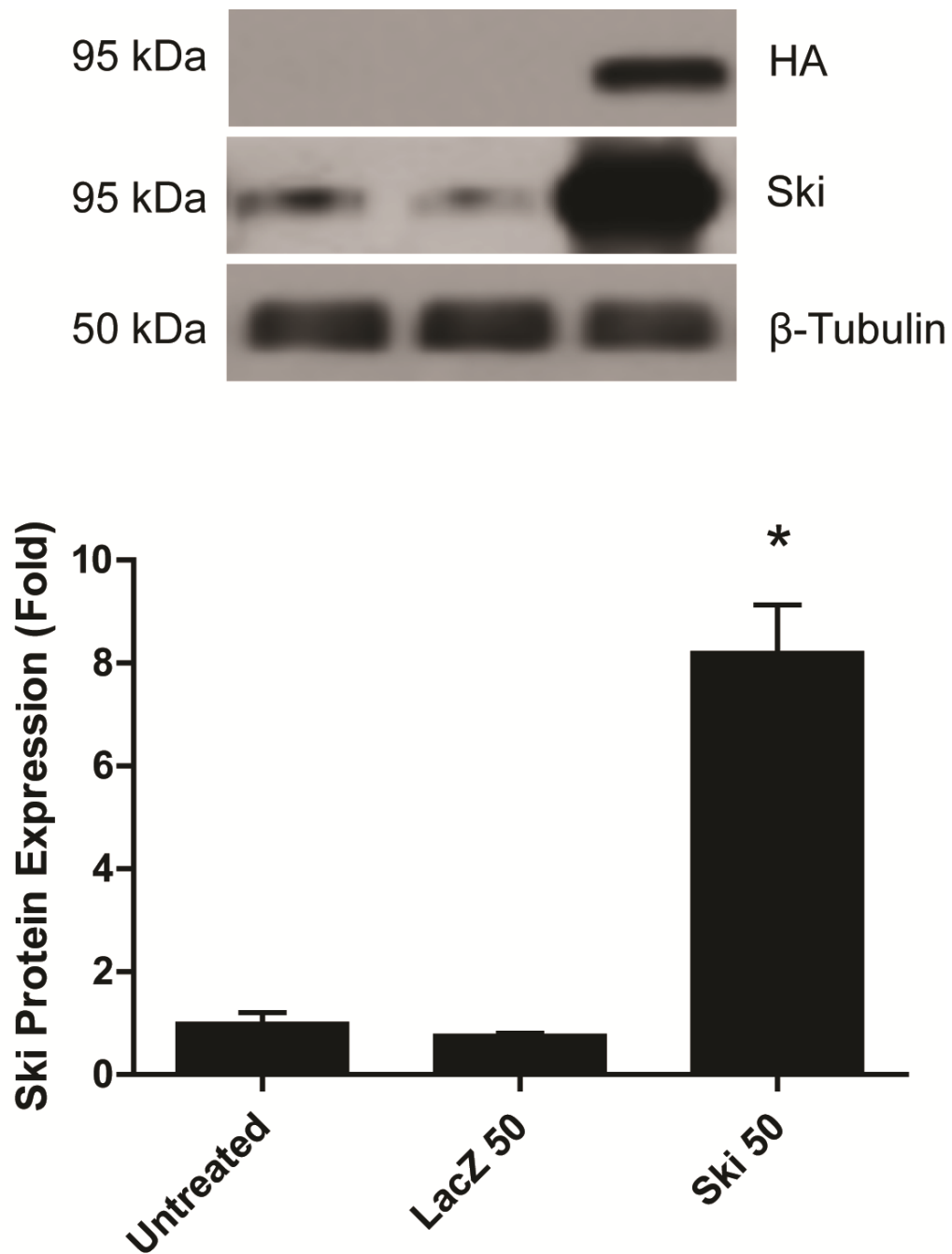


Figure 13. Overexpression of Ski in Primary P1 Cardiac Myofibroblasts. Using an adenoviral approach, we overexpressed Ski in P1 primary cardiac myofibroblasts for 24 hours and obtained an 8 to 10-fold increase in Ski protein levels as compared to Ad-LacZ and untreated controls. β -Tubulin was used as a loading control and HA used to demonstrate adenoviral derived Ski (* $P < 0.001$; $n=4$). Reproduced with permission³⁴⁶.

To determine what effect, if any, increased Ski expression had on known TGF β ₁/Smad-dependent gene expression in our model system, we isolated RNA from P1 myofibroblasts and assayed for the relative abundance of mRNA for fibrillar collagens type I and type III. As predicted, we found that overexpression of Ski significantly reduced the expression of fibrillar collagens 1 α 1 (**P* < 0.05; n=3), 1 α 2 (**P* < 0.05; n=3), and 3 α 1 ([†]*P* < 0.01; n=3) by approximately 50% as compared to Ad-LacZ and non-transfected controls (Figure 14). Thus, adenovirally delivered, ectopic expression of Ski in our system is sufficient to repress known downstream targets³⁴⁹⁻³⁵² of TGF β ₁/Smad-dependent signaling.

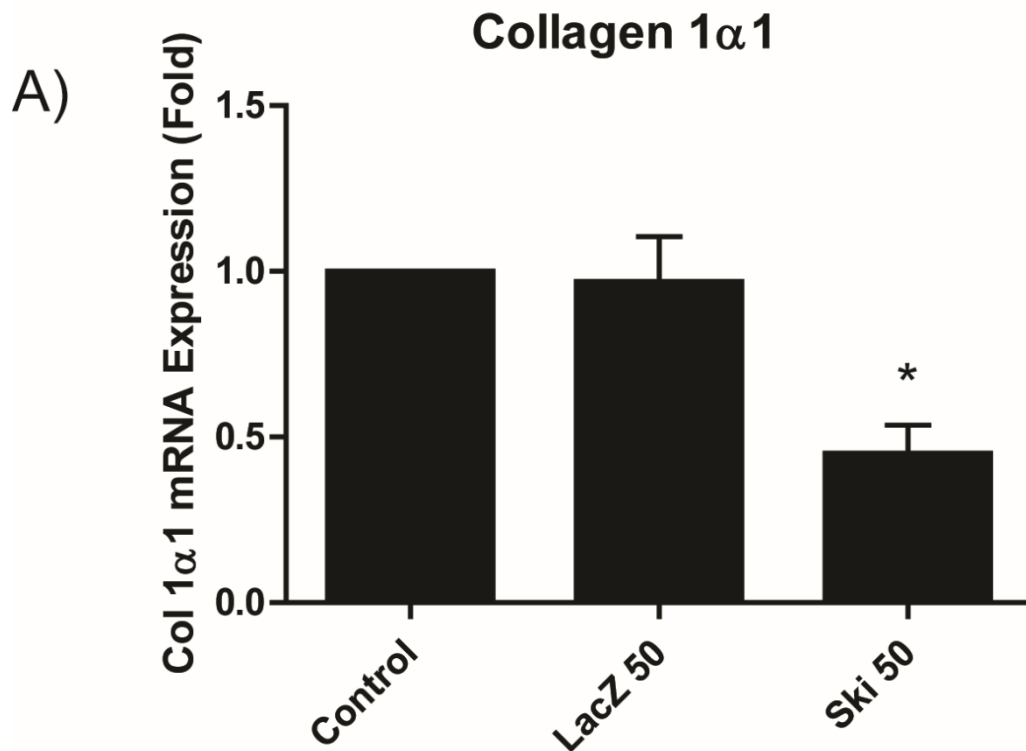


Figure 14. mRNA Expression of Fibrillar Collagens in Ski Overexpressing P1 Myofibroblasts.

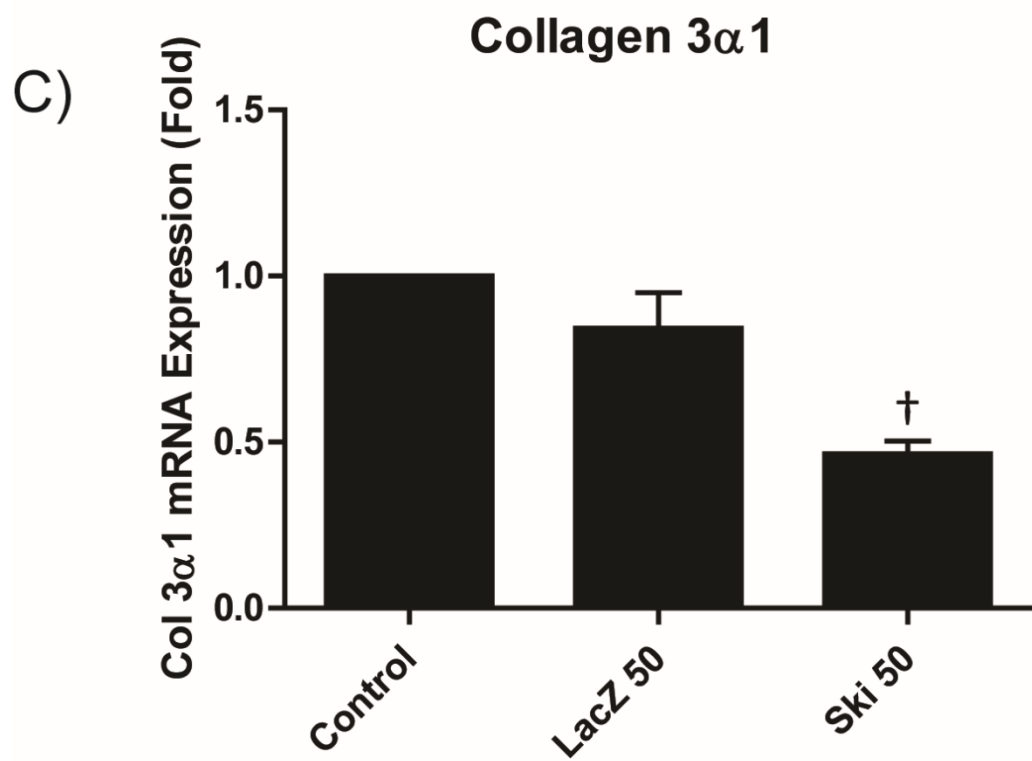
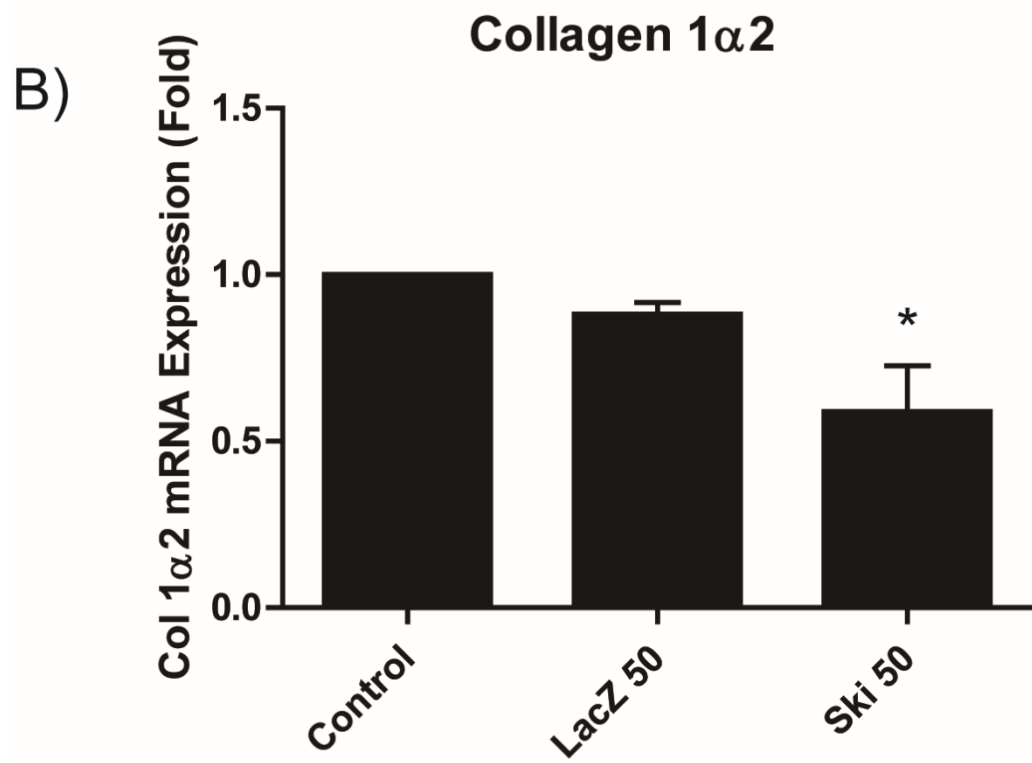


Figure 14. mRNA Expression of Fibrillar Collagens in Ski Overexpressing P1 Myofibroblasts. mRNA was isolated from P1 myofibroblasts transfected with Ad-HA-Ski 50 or Ad-LacZ 50 and the relative abundance of pro-fibrotic collagen expression was evaluated. There was approximately a 50% reduction in collagen 1 α 1 (**P* < 0.05; n=3), 1 α 2 (**P* < 0.05; n=3), and 3 α 1 ([†]*P* < 0.01; n=3) gene expression in those cells treated with Ad-HA-Ski for 24 hours.

Ski Represses Scleraxis Expression

Scleraxis is a pro-fibrotic agent that has been shown to regulate collagen gene expression in a synergistic manner with Smad proteins^{20, 210, 334}. Moreover, expression of Scx itself has been shown to be regulated by TGF β and Smad proteins^{20, 337, 345}. Thus, we wanted to know whether Ski, a potent inhibitor of TGF β /Smad signaling, was capable of repressing Scx mRNA and protein expression. Overexpression of Ski resulted in a 50-60% reduction in Scx mRNA (**P* < 0.01; n=4) and an 80-90% reduction in Scx protein (**P* < 0.05; n=3) levels as compared to Ad-LacZ and untransfected controls (Figure 15).

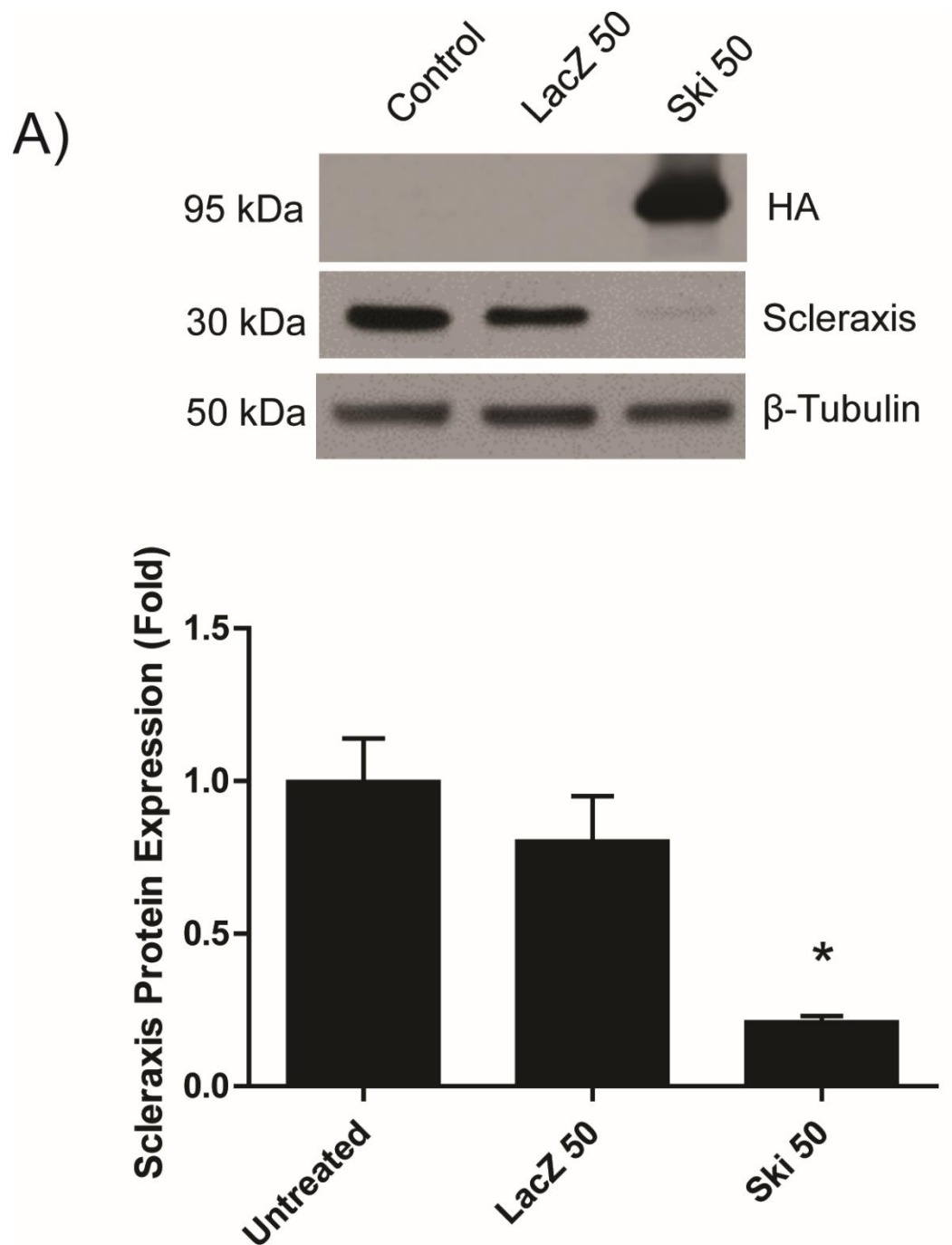


Figure 15. Scleraxis Protein and mRNA Expression in Ski Overexpressing Myofibroblasts.

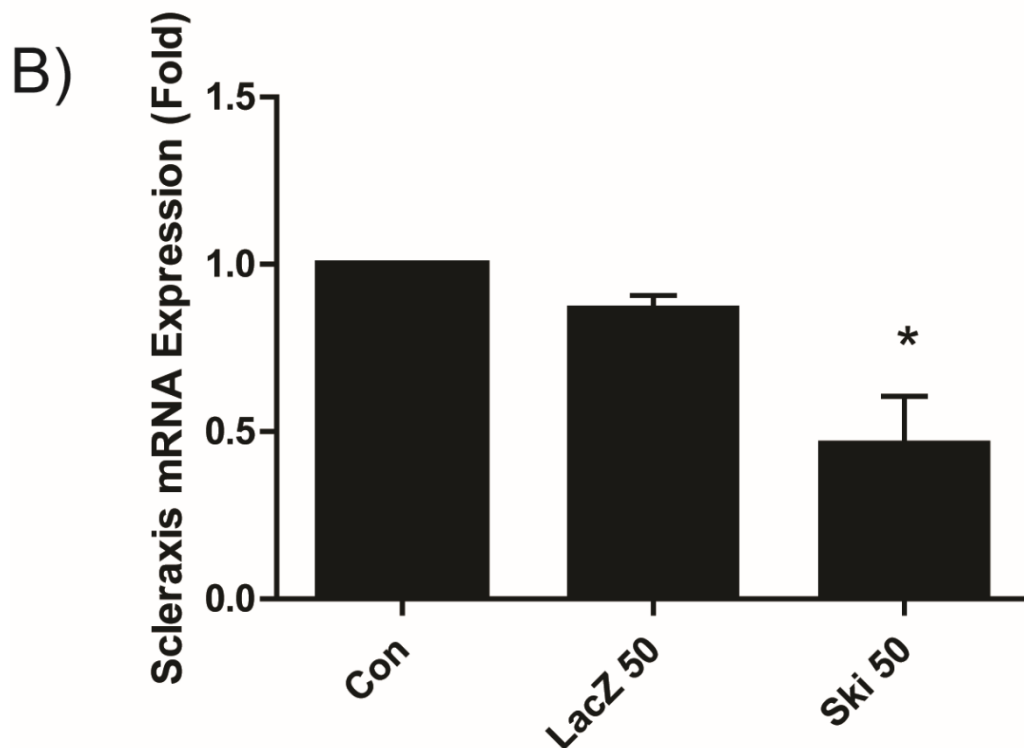


Figure 15. Scleraxis Protein and mRNA Expression in Ski Overexpressing Myofibroblasts. Overexpression of Ski in P1 myofibroblasts for 24 hours was able to significantly repress Scx **A)** protein ($*P < 0.05$; $n=3$) and **B)** mRNA ($*P < 0.01$; $n=4$) levels as compared to Ad-LacZ and untransfected controls. Reproduced with permission³⁴⁶.

***In silico* Analysis of the Scleraxis Promoter**

Previous studies indicate that Scx protein levels can be induced by TGF β and Smad proteins^{20, 337, 345}. However, no one to date has evaluated the direct DNA:protein transcriptional mechanisms that underpin this relationship. Thus, we set forth to determine whether there are any potential regulatory elements (SBE) for which Smad proteins may be capable of interacting within the Scx promoter that might suggest a direct role for Smad proteins in promoting Scx expression. *In silico* analysis of the human, rat, and mouse promoter regions (~ 10000 bp 5' to the ATG start codon)

revealed the presence of several putative SBE with the consensus sequence *CAGACA*. We performed a Clustal W alignment of this region and found that the 1580 bp most proximal to the ATG codon contained 2 highly conserved SBE motifs that may be functionally significant sites for Smad protein complexes to bind (Figure 16) in addition to the presence of several other putative transcription factor binding motifs. Clustering of putative transcription factor binding sites may suggest a regulatory region of the hScxP that may be important in Scx expression. Furthermore, the 5' and 3' regions flanking these motifs show high similarity and identity amongst the various species (Figure 16; starred (*) nucleotides) which may suggest that these play a role in protein complex docking to SBE sites.

Putative SBEs:

Proximal:

```
Mm: AGTGCACATGGAAGGAGAGT CAGACA GGGTGTGGCCAGGCACTCAG -445
Hs: GCGCACATGGGAGGCAAAGA CAGACA GGGTGC GGTCAGGCAGCAGG -490
Rn: AGTGCACATGGAAGGAGAGT CAGACA GGGTGTGGCCAGGCACTCAG -444
      *** * * * * ***** ** ***** *
```

Distal:

```
Mm: GCCAAGGCTCTGGGCAGCCA CAGACA GGGATGACCTCATCGCCTAT -1260
Hs: GCCAGGGCTCTGGGCAGCCA CAGACA GGGATGACCTCATCACCCAC -1211
Rn: GCCAAGGCTCTGGGCAGCCA CAGACA GGGATGACCTCATCGCCTAT -1297
      **** ***** ** *
```

Figure 16. Conserved SBE of the Mouse, Human, and Rat Scleraxis Promoter. Clustal W alignment of the mouse, human, and rat promoter regions that contain highly conserved SBE. The putative SBE is highlighted in purple. The star (*) beneath the alignment marks bp that are conserved amongst all 3 species. Reproduced with permission³⁴⁶.

TGF β ₁ Promotes Scleraxis Expression

To determine whether TGF β ₁ is capable of inducing expression of Scx protein in our model system, we treated P1 cardiac myofibroblasts with rhTGF β ₁ cytokine (10 ng/mL) for 24 hours followed by Western blot analysis. In line with previous reports^{20, 337, 345}, we found that TGF β ₁ induced a 2 to 3-fold increase (**P* < 0.01; n=4) in Scx protein levels as compared to untreated controls (Figure 17).

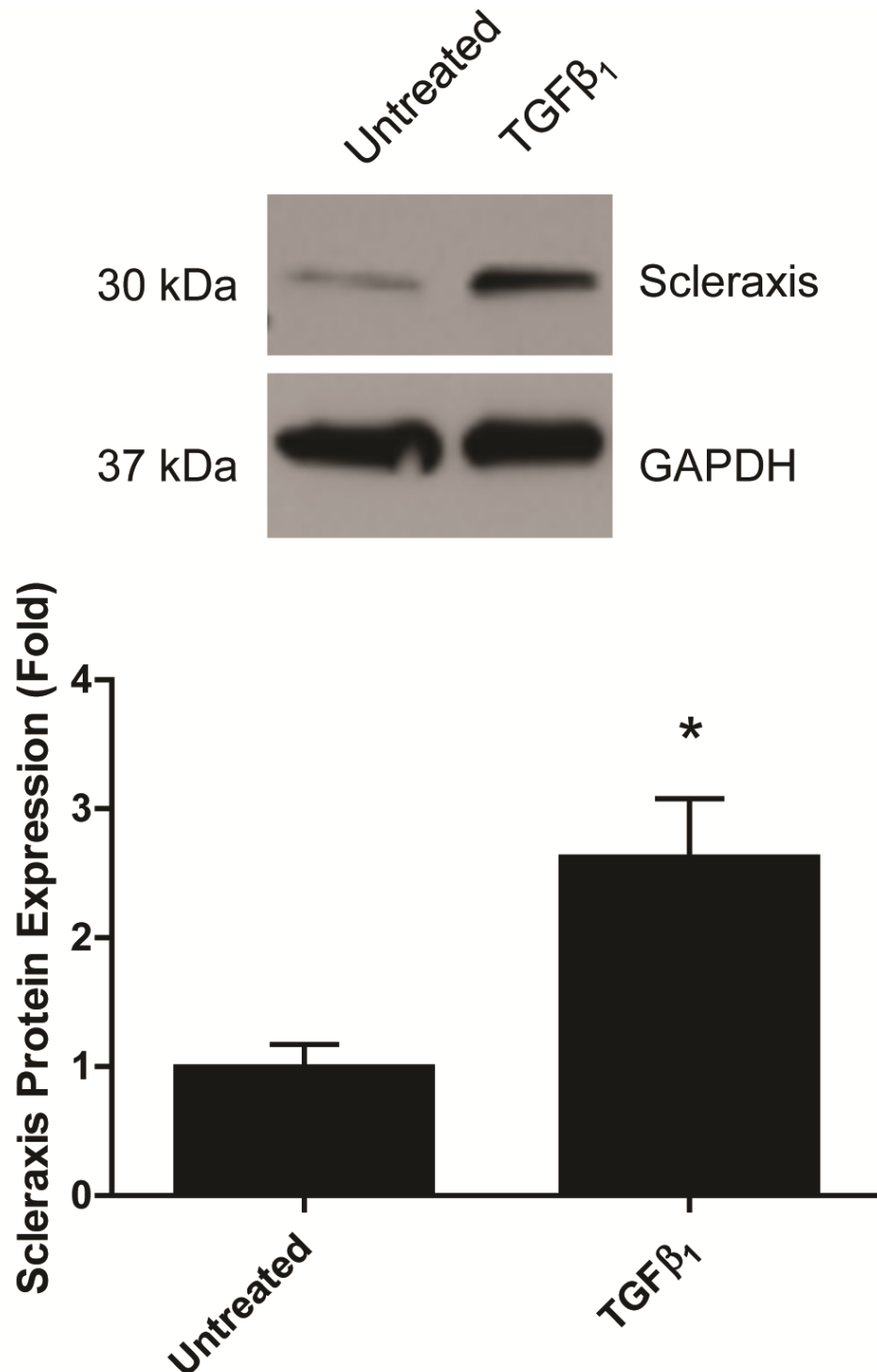


Figure 17. TGFβ₁ Stimulation of Scleraxis. P1 cardiac myofibroblasts were seeded into 100 mm cell culture dishes and allowed to adhere to the dish. Twenty-four hours later the cells were serum starved for an additional 24 hours. Cells were then treated with TGFβ₁ (10 ng/mL) for 24 hours and harvested for Western blot analysis. There was a 2 to 3-fold increase in Scx protein levels in those cells treated with TGFβ₁ as compared to untreated controls (**P* < 0.01; n=4). Reproduced with permission³⁴⁶.

We next wanted to determine the functional significance of the SBE motifs that we have identified. Approximately 1600 bp (1580 bp) of the hScxP that contained the sites of interest was purchased from Genecopia and cloned into a pGL4.10 [luc2] reporter vector (hScxP pGL4.10 [luc2]). As primary cardiac myofibroblasts are difficult to transfect with plasmid DNA, we transfected NIH-3T3 cells with hScxP pGL4.10 [luc2] using Lipofectamine 3000 for 24 hours. The next day the DNA:lipid complexes were removed and the cells treated with rhTGF β_1 (0-10 ng/mL) for 24 hours. We found that with increasing doses of rhTGF β_1 there was a dose-dependent increase in luciferase expression up to 5.0 ng/mL (Figure 18; ** $P < 0.01$; *** $P < 0.001$; n=6). This result suggests that the 2 identified putative SBE may be functionally important in regulating Scx transcription.

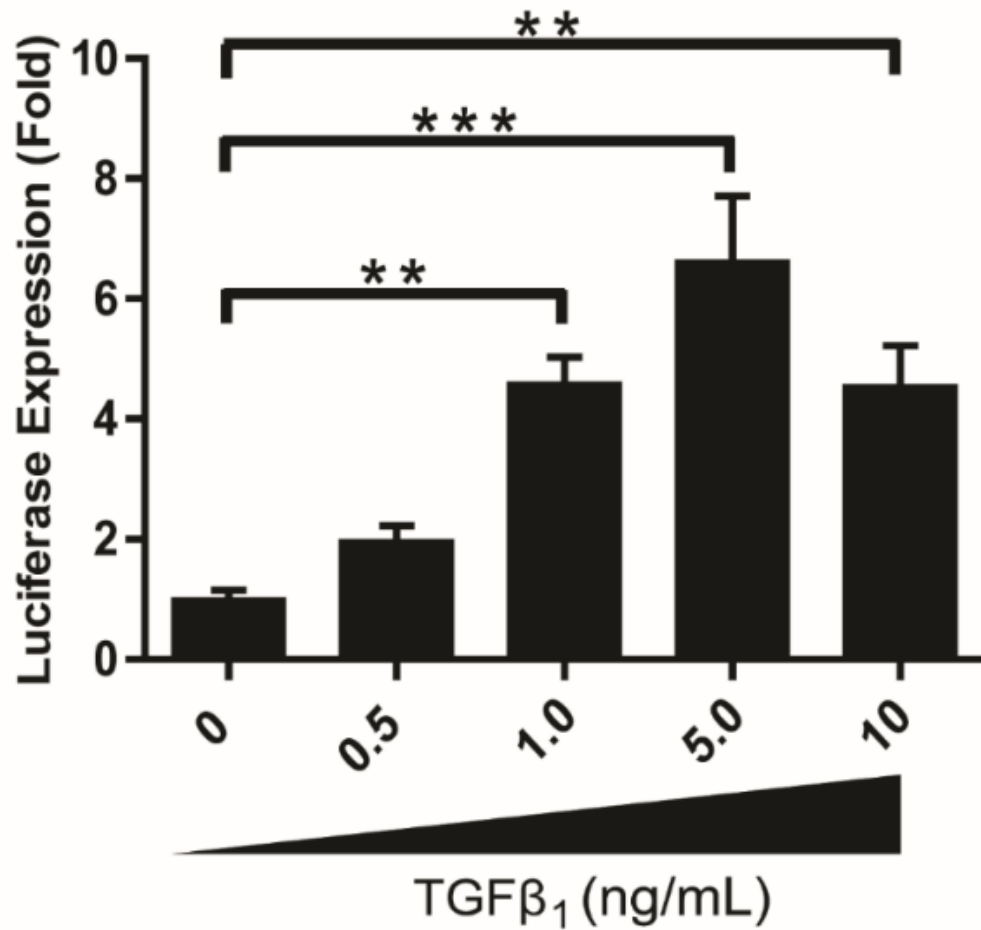


Figure 18. Activation of the Human Scleraxis Promoter by TGFβ₁. NIH-3T3 cells were transfected with 500 ng of our hScxP pGL4.10 [luc2] reporter construct and treated with increasing doses of TGFβ₁. We observed a dose-dependent increase in luciferase reporter with TGFβ₁ treatment that demonstrated a maximal signal with 5.0 ng/mL TGFβ₁ (***P* < 0.01; ****P* < 0.001; n=6). Reproduced with permission³⁴⁶.

TGFβ₁ Promotes Scleraxis Expression in a Smad-Independent Manner

Although we demonstrated that rhTGFβ₁ is capable of activating our isolated region of the hScxP, we still do not know whether Smad proteins are the major transducers of the TGFβ₁ signal from the cell membrane to the promoter region of Scx. Thus, we extended our luciferase studies and evaluated the effects of human Smad2 (hSmad2) and human Smad3 (hSmad3) overexpression using pcDNA3 and pCMV-

Tag2B expression vectors in combination with our hScxP pGL4.10[luc2] reporter. Neither transfection with hSmad2 nor hSmad3 demonstrated any significant difference in hScxP promoter activation as compared to co-transfection with empty expression vector controls (Figure 19A and B; $P > 0.05$; $n=4$).

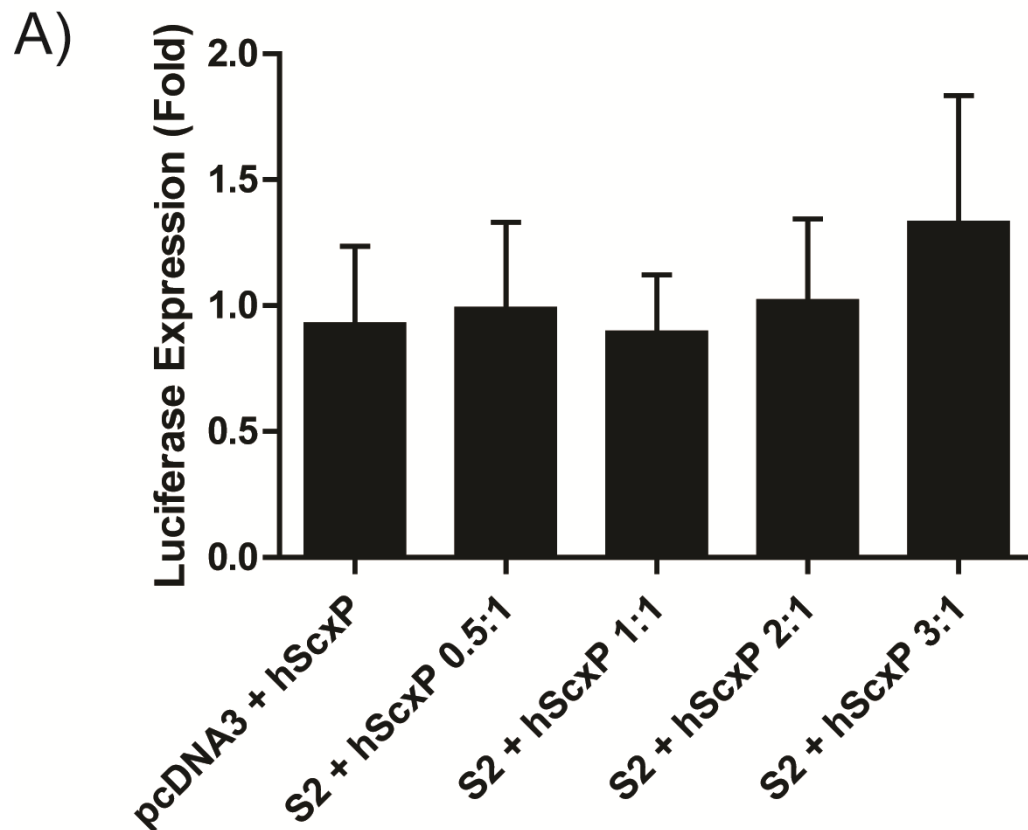


Figure 19. hSmad2/3 Luciferase of the Human Scleraxis Promoter.

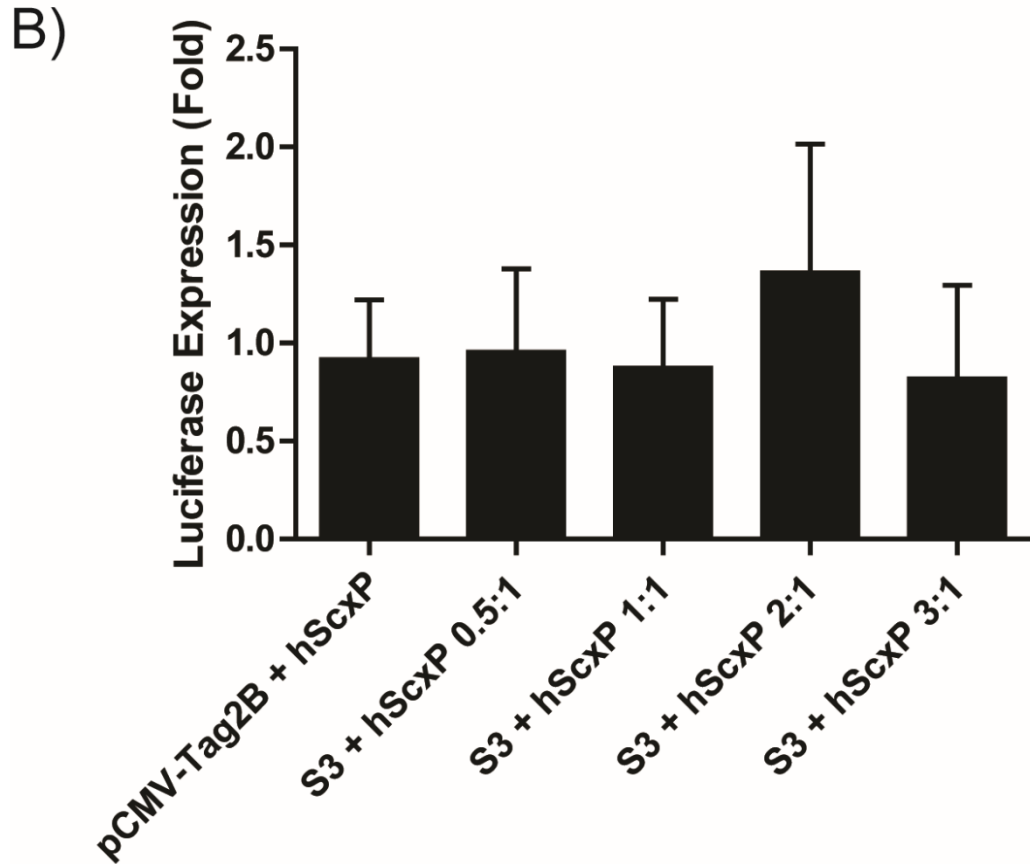
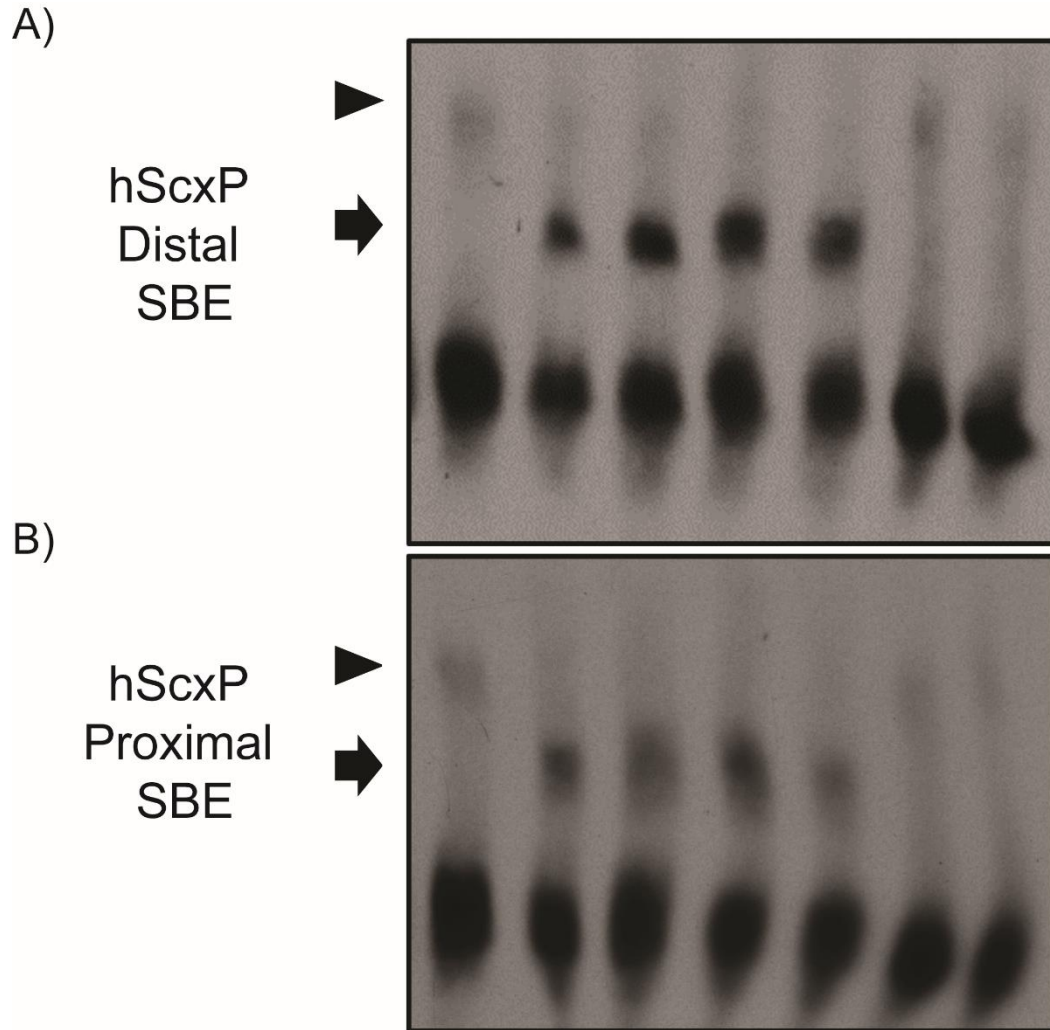


Figure 19. hSmad2/3 Luciferase Assay of the Human Scleraxis Promoter. Co-transfection of NIH-3T3 cells with hSmad2 and hSmad3 expression vectors with our hScxP reporter construct. Neither **A)** hSmad2 ($P > 0.05$; $n=4$) nor **B)** hSmad3 ($P > 0.05$; $n=4$) could promote luciferase expression of our reporter construct regardless of the amount of expression vector that was transfected. S2 – Human Smad2; S3 – Human Smad3. Reproduced with permission³⁴⁶.

A second question that we addressed was whether Smad proteins could bind to the hScxP but could not activate it. To evaluate this relationship, we conducted electrophoretic mobility gel shift assays (EMSA) using the nuclear extract from Cos7 (Appendix A, Figure A-2) and primary P1 cardiac myofibroblasts (Figure 20). Biotinylated probes encompassing both conserved putative SBE were synthesized. Protein lysates were incubated overnight with antibodies against Smad2 and Smad3 to form DNA:protein complexes to show a supershift reaction, indicative of DNA:protein

binding. Unlabelled and mutant SBE probes were synthesized as negative controls. As seen in Figure 20, incubation of nuclear cell lysate from P1 myofibroblasts resulted in a shift in the labelled probe (Figure 20 arrow; n=3), however, pre-incubation with Smad2 and Smad3 antibodies (for supershift reactions) demonstrated no further shift of the labelled probe. Furthermore, although our unlabelled probe (in 500 molar excess) prevented shifting of the biotinylated probe, our mutant probes in which we disrupted the putative SBE motif (Table 3) also prevented probe shifting (Figure 20; n=3). Taken together these results indicate that R-Smad proteins may not interact (or at least not directly interact with) with this region of the hScxP.



Input		+	+	+	+	+	+
Anti-Smad 2			+				
Anti-Smad 3				+			
Normal Rabbit IgG					+		
500x Cold						+	
500x Mutant Cold							+
Probe	+	+	+	+	+	+	+

Figure 20. Electrophoretic Mobility Gel Shift Assay of the Distal and Proximal SBE of the Human Scleraxis Promoter. Nuclear lysates from P1 cardiac myofibroblasts were incubated with biotinylated probes encompassing the 2 putative SBEs within the hScxP. To confirm DNA:protein interactions, reactions were incubated overnight with antibodies against Smad2, Smad3, or rabbit IgG control.

Incubation with just the biotinylated probe and nuclear lysate demonstrate a clear interaction with both the **A)** distal, and **B)** proximal SBEs found within the hScxP. However, co-incubation with Smad antibodies was unable to further demonstrate specific R-Smad protein binding to the probes. Furthermore, unlabelled probe that has had the SBE motif mutated was still capable of outcompeting the WT biotinylated probe. Figure is a representative of 3 individual experiments. Re-produced with permission³⁴⁶. Triangles indicate non-specific binding; Arrows indicate DNA:protein bound complexes.

Scleraxis Expression is Modulated by p44/42 MAPK Signaling Pathway

We observed that TGF β ₁ promotes Scx expression, but that this event did not occur directly *via* R-Smad proteins in our isolated region of the hScxP, *ergo* we explored whether a TGF β ₁-mediated, Smad-independent pathway might be involved. As the Erk1/2-MAPK signaling pathway lies downstream of T β Rs and has been shown to be activated in response to TGF β ₁ stimulation^{162, 219, 240} we determined whether this pathway plays a role in Scx gene regulation. We serum-starved P1 myofibroblasts for 24 hours to normalize culture conditions and remove cytokines from the medium that are present in the FBS. We then pre-treated primary cardiac myofibroblasts with either the MEK1/2 inhibitor U0126 (10 μ M) or DMSO as a vehicle control. Three hours later we stimulated these cells with 10 ng/mL rhTGF β ₁ for an additional 21 hours in the presence of the vehicle or U0126. After 24 hours of treatment we found that rhTGF β ₁ stimulation induced a 2-fold increase (* P < 0.05; n=5) in Scx protein expression in the vehicle control as compared to untreated controls (Figure 21A). Pre-treatment with U0126 modestly, but not significantly (P > 0.05; n=5) blunted the effect of TGF β ₁ stimulation on Scx protein expression (Figure 21A).

We next wanted to determine what degree of p44/42 inhibition we were able to acquire using U0126 over the course of 24 hours. As seen in Figure 21B we were able

to obtain a 70-80% reduction ($*P < 0.05$; $n=5$) in phosphorylation of p44/42 (phospho-p44/42) as compared to vehicle and untreated controls despite the presence of rhTGF β_1 . However, we did not see any change in phospho-p44/42 in the DMSO + TGF β_1 treatment group as compared to untreated controls. To determine whether we missed the flux of rhTGF β_1 down the p44/42 pathway we repeated our experiment but this time harvesting cells after 1 hour of rhTGF β_1 stimulation. After 1 hour of rhTGF β_1 stimulation, we saw nearly a 1.5-fold increase ($*P < 0.05$; $n=5$) in phospho-p44/42 levels in the TGF β_1 + DMSO treatment group as compared to untreated controls. In those cells that were pretreated with U0126 we saw no evidence of p44/42 phosphorylation despite the presence of rhTGF β_1 (Figure 21D; $^{\dagger}P < 0.01$ vs. TGF β_1 + DMSO; $^{\ddagger}P < 0.001$ vs. untreated; $n=5$). Furthermore, after just 1 hour of rhTGF β_1 stimulation with or without the presence of U0126, we saw no significant difference in the protein levels of Scx (Figure 21C; $P > 0.05$).

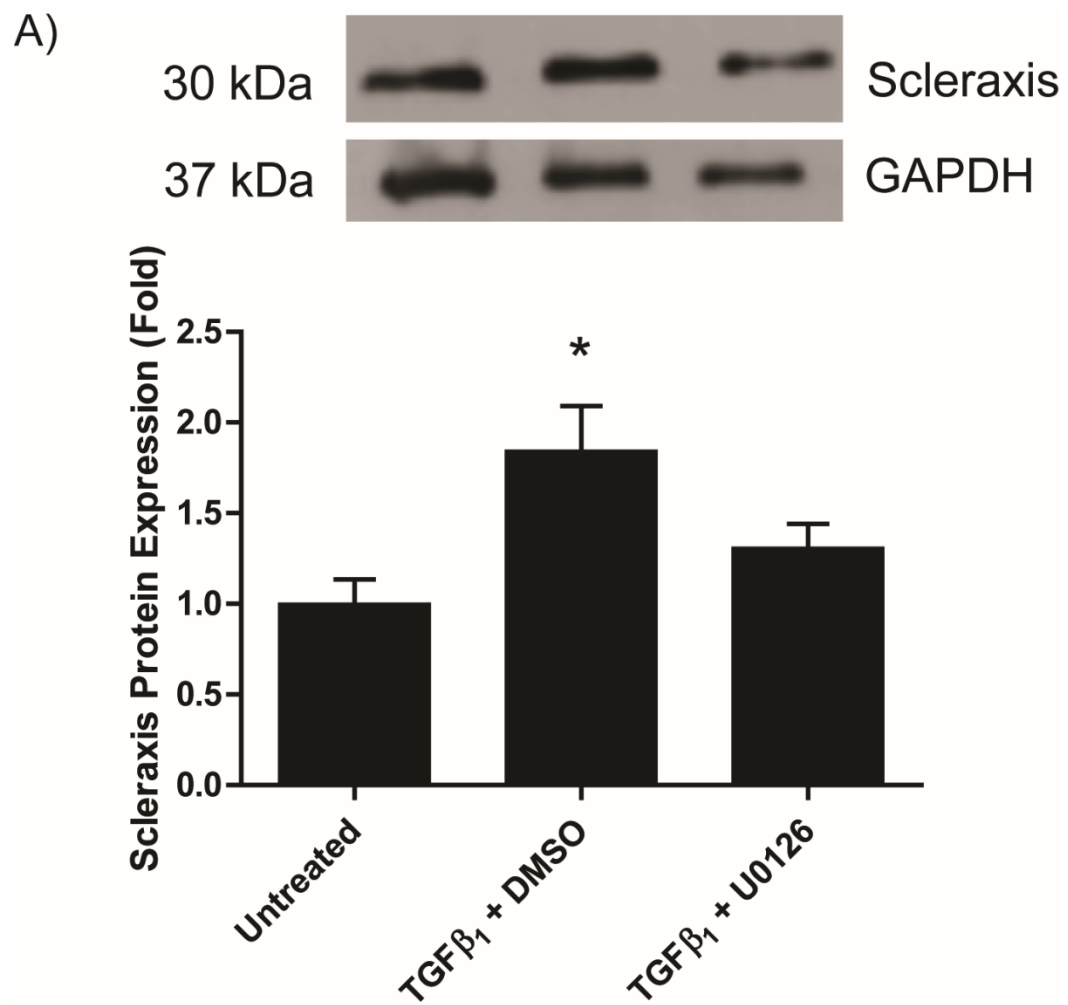


Figure 21. Temporal Phosphorylation of p44/42 by TGFβ₁ and its Effect on Scleraxis Protein Expression

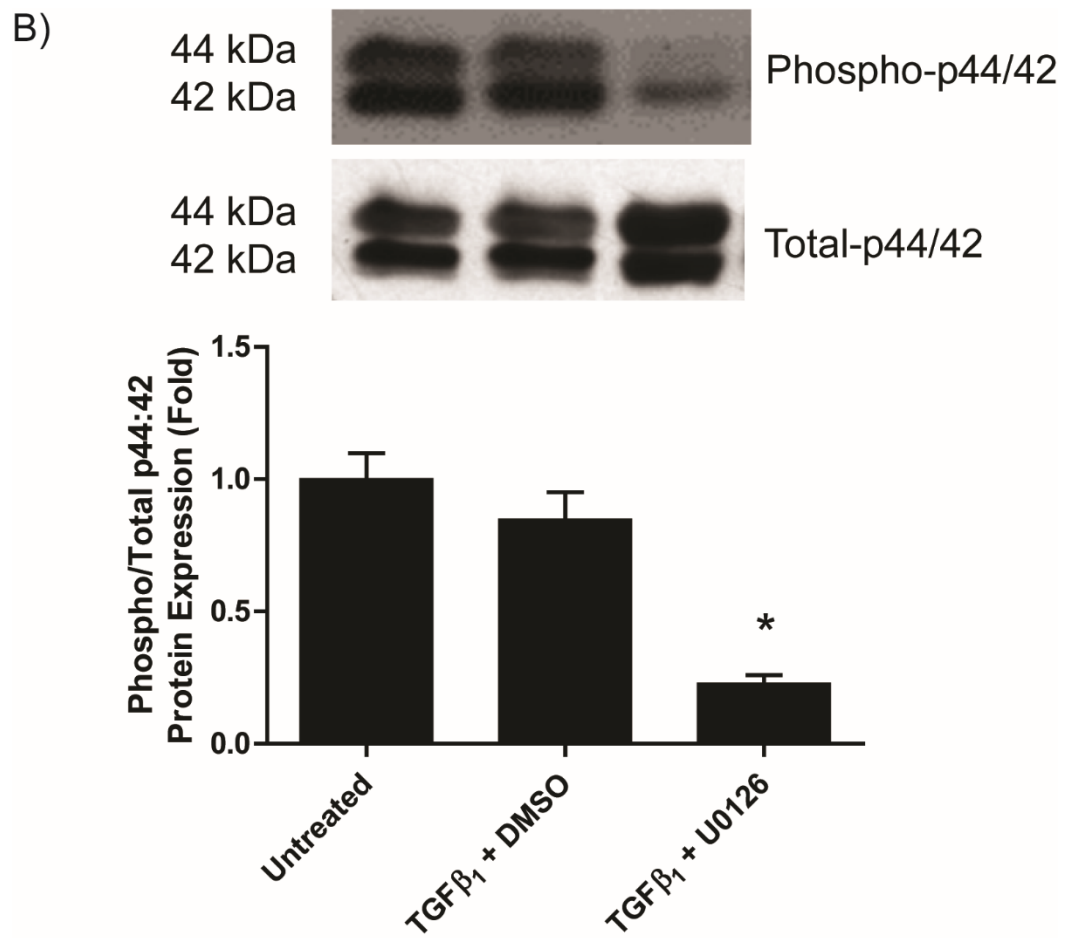


Figure 21. Temporal Phosphorylation of p44/42 by TGFβ₁ and its Effect on Scleraxis Protein Expression

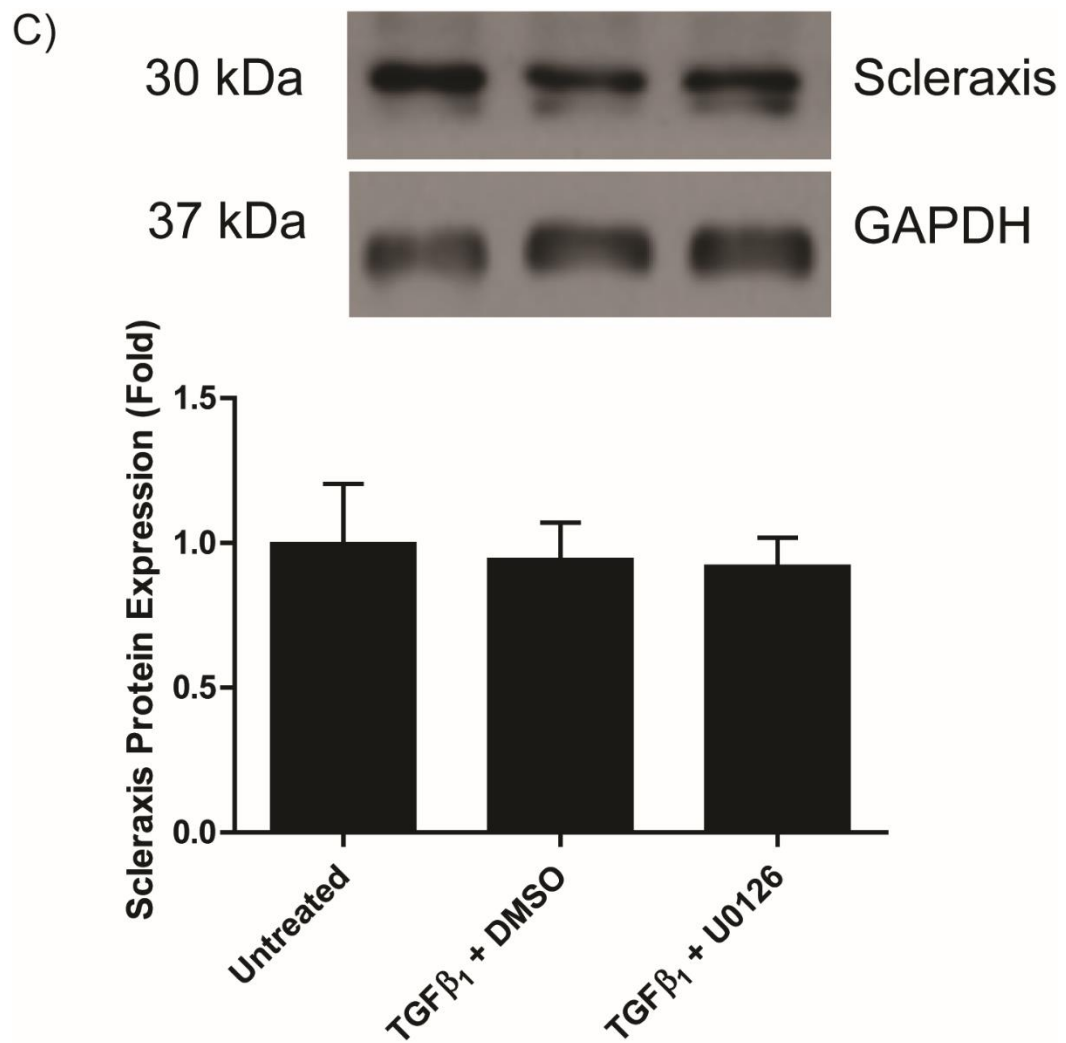


Figure 21. Temporal Phosphorylation of p44/42 by TGF β_1 and its Effect on Scleraxis Protein Expression

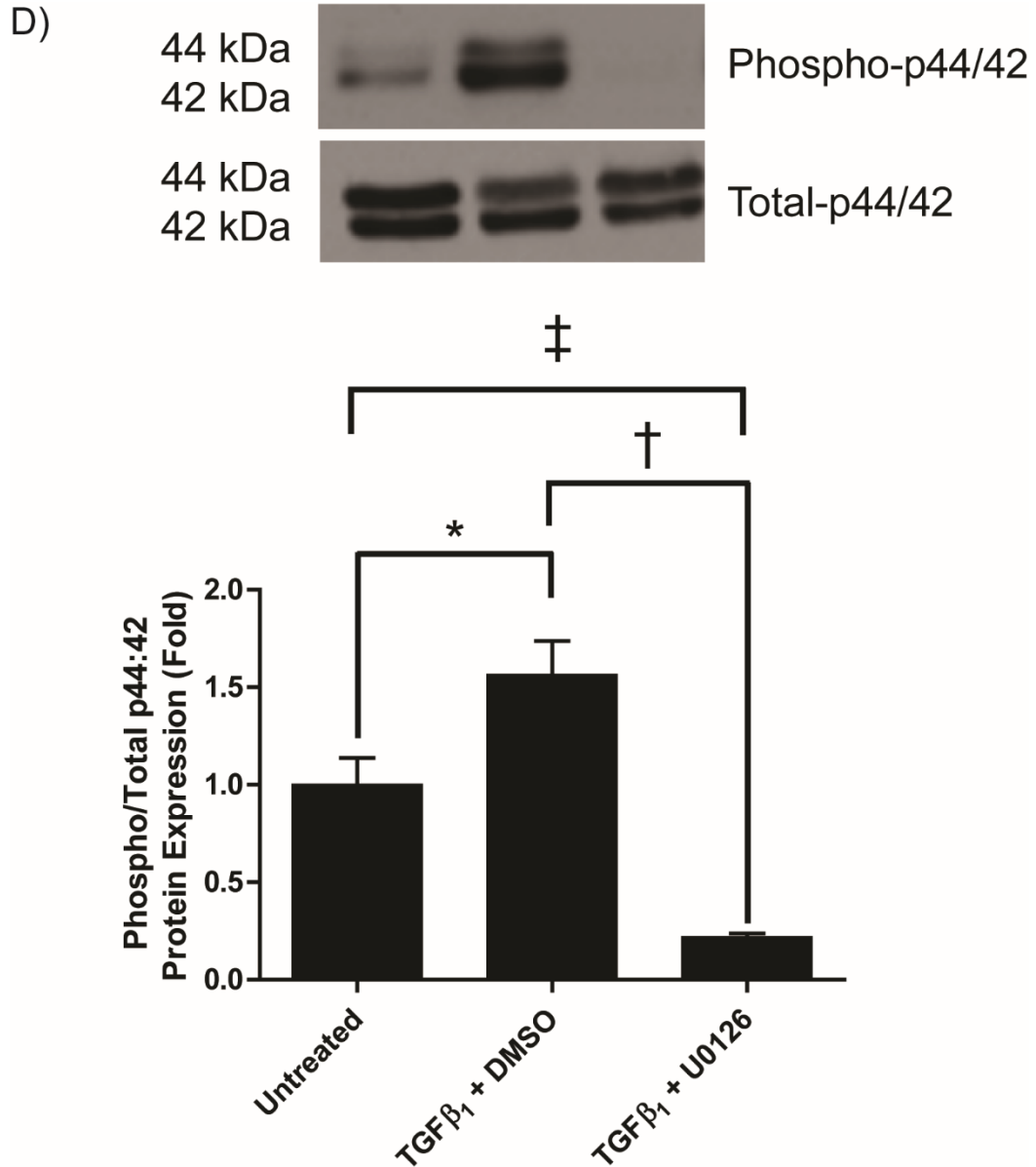


Figure 21. Temporal Phosphorylation of p44/42 by TGFβ₁ and its Effect on Scleraxis Protein Expression. **A)** Twenty-four hours of TGFβ₁ stimulation resulted in a 2-fold increase in Scx protein levels (**P* < 0.05 vs. untreated; n=5). Co-treatment with U0126 (10 μM) modestly counteracted TGFβ₁-mediated induction of Scx. **B)** U0126 significantly (**P* < 0.05 vs. untreated and TGFβ₁ + DMSO; n=5) repressed phosphorylation of p44/42 after 24 hours. **C)** After 1 hour of TGFβ₁ stimulation, there was no difference in Scx protein levels amongst any of the treatment groups. **D)** One hour following TGFβ₁ stimulation there was a significant (**P* < 0.05 vs. untreated; n=5) increase in phosphorylation of p44/42 compared to untreated controls. Co-treatment with U0126 significantly repressed p44/42 phosphorylation (†*P* < 0.01 vs. TGFβ₁ + DMSO; ‡*P* < 0.001 vs. untreated; n=5). Reproduced with permission³⁴⁶.

Finally, we wanted to understand what, if any, effect U0126 had on the basal expression of Scx protein in the absence of rhTGF β ₁. Pre-treatment with either U0126 or vehicle had no effect on Scx protein level as compared to untreated controls after 24 hours (Figure 22; $P > 0.05$; $n=4$). Taken together these results suggest that when there is an abundance of TGF β ₁ cytokine to promote TGF β signaling, Scx expression may be induced *via* a Smad-independent, p44/42-MAPK signaling pathway.

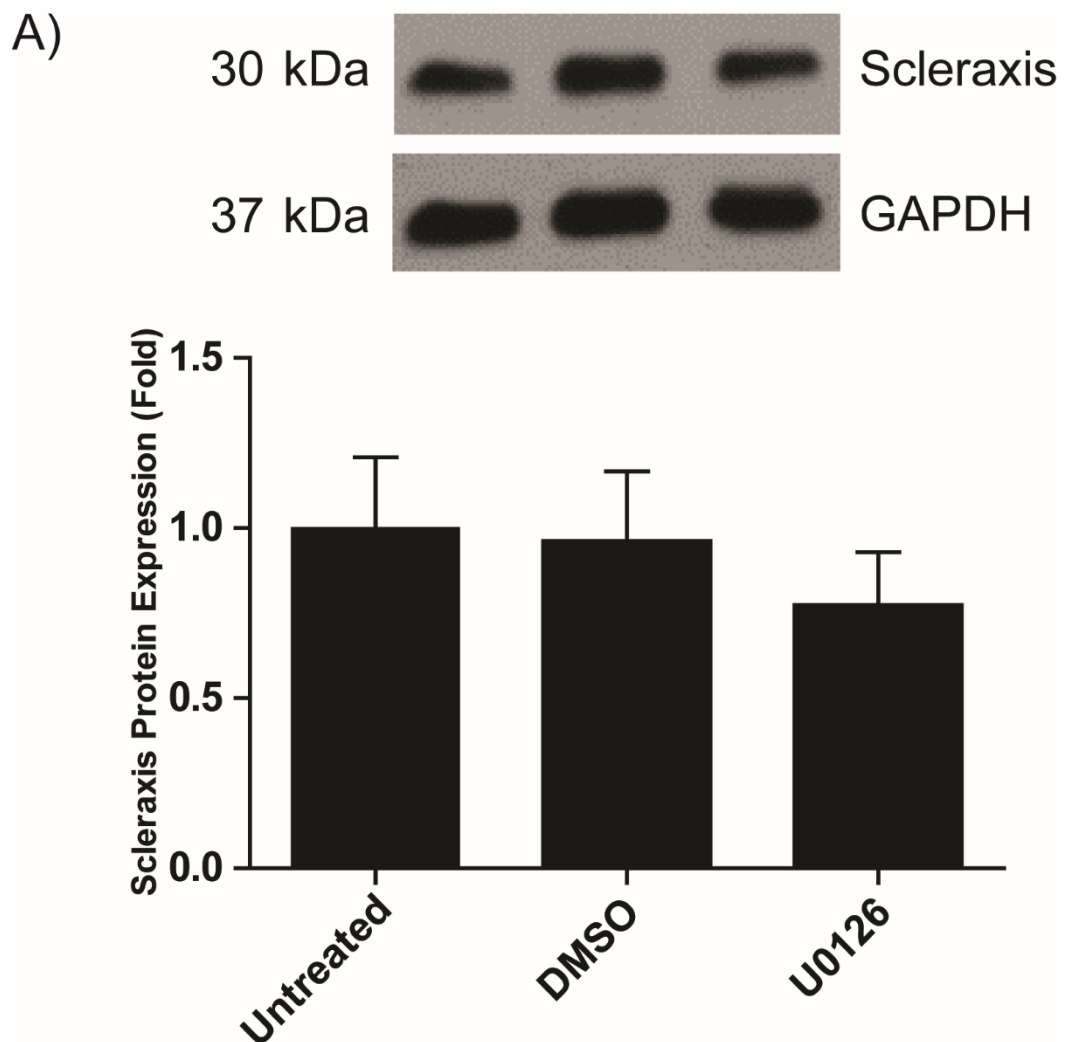


Figure 22. U0126 Treatment of P1 Cardiac Myofibroblasts and Scleraxis Expression.

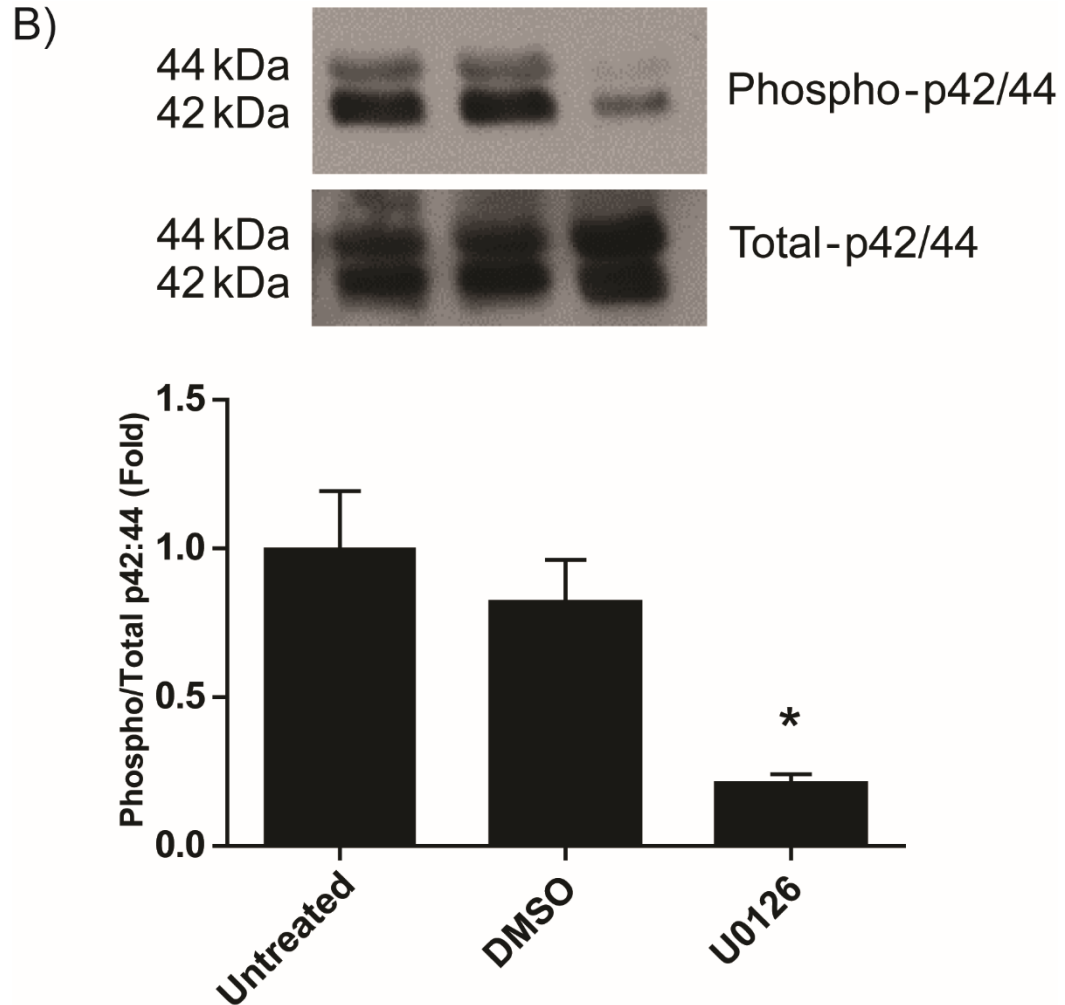


Figure 22. U0126 Treatment of P1 Cardiac Myofibroblasts and Scleraxis Expression. A) After 24 hours of incubation, U0126 treatment alone has no effect on the endogenous levels of Scx ($P > 0.05$; $n=4$). B) U0126 significantly ($*P < 0.05$; $n=4$) prevented phosphorylation of p44/42 for 24 hours. Re-produced with permission³⁴⁶.

Ski Does Not Affect Phosphorylation of MAPK Protein Kinases

Ski has been well documented as a potent inhibitor of TGF β /Smad signaling^{22, 187, 321}. However, as Scx does not appear to be transcriptionally regulated through Smad proteins in our hScxP reporter construct and we previously demonstrated that Ski could repress Scx expression, we undertook studies to determine whether or not Ski is capable of interfering with MAPK signaling pathways. As seen in Figure 23, ectopic expression

of Ski had no effect on the phosphorylation of either p44/42 or p38 ($P > 0.05$; $n=4$) as compared to Ad-LacZ and untreated controls.

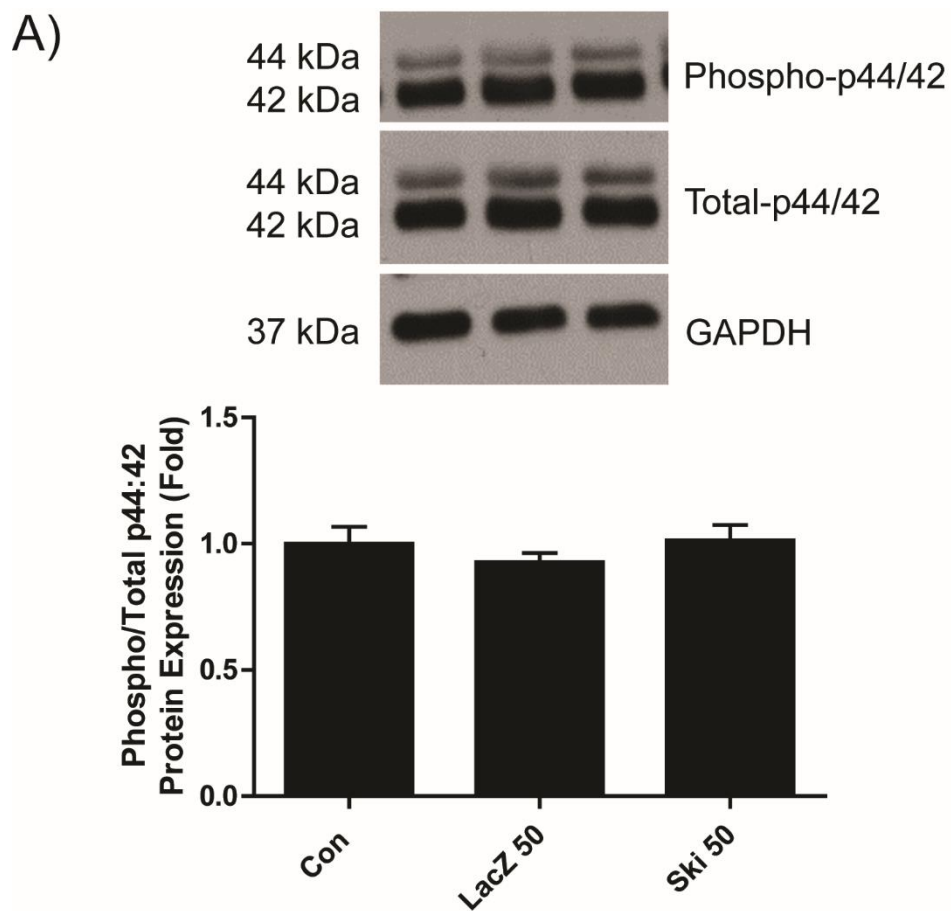


Figure 23. Phosphorylation of MAPK Proteins Following Ski Expression.

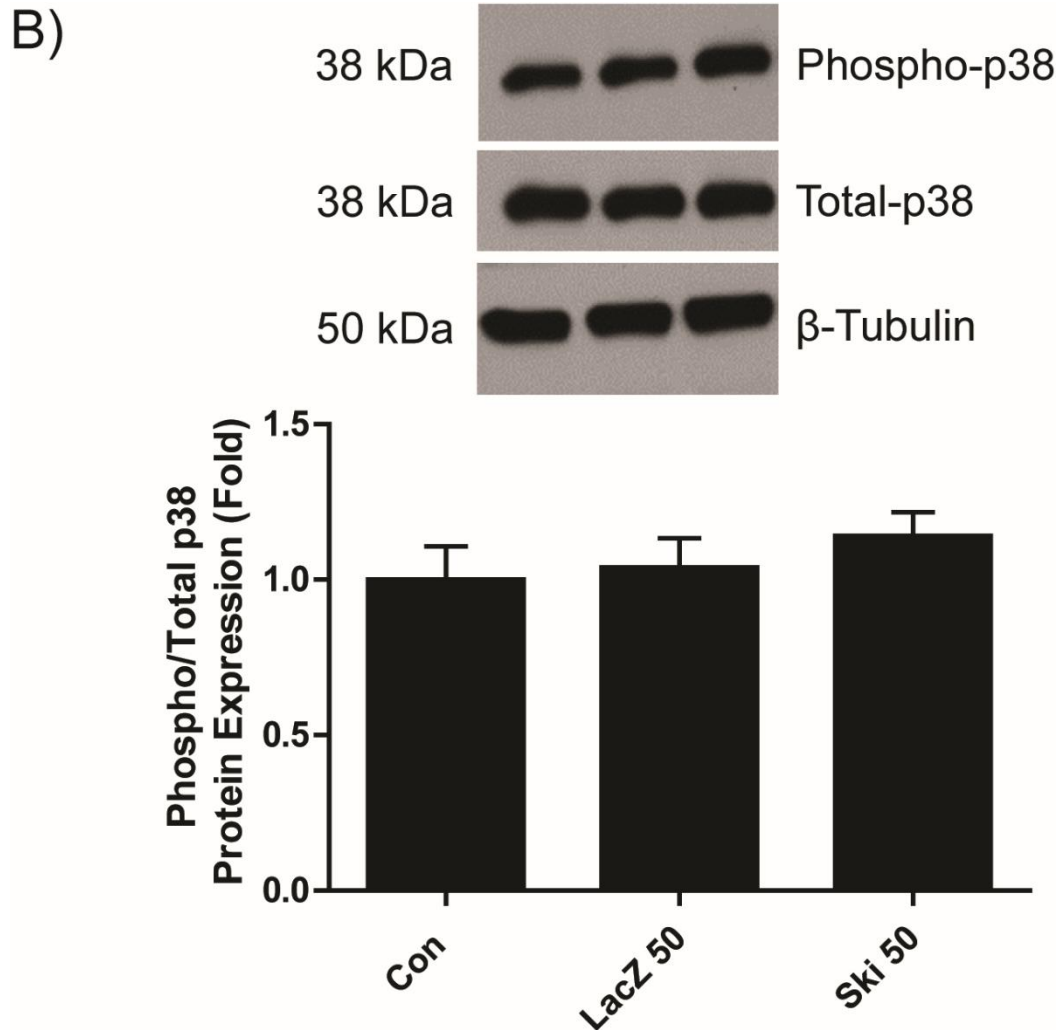


Figure 23. Phosphorylation of MAPK Proteins Following Ski Expression. Overexpression of Ski for 24 hours had no effect on the phosphorylation levels of either **A)** p44/42, or **B)** p38 ($P > 0.05$; $n=4$).

Ski Interacts with c-Jun to Repress MAPK Signaling

As Ski is primarily a nuclear protein where it exerts its effects on Smad transcription factors^{187, 321} it was not surprising that Ski had no significant effect on the phosphorylation of either p44/42 or p38-MAPK kinases. However, Ski has previously been shown to form a protein:protein complex with the downstream transcription factor c-Jun³⁵³. Thus, we chose to explore whether Ski was capable of forming an inhibitory

complex with c-Jun in our primary cell culture model system. We performed immunoprecipitation studies and used a c-Jun antibody to pull down complexes in LacZ and Ski overexpressing P1 myofibroblasts. In those cells which overexpressed Ski, we were able co-precipitate Ski with c-Jun whereas we saw no evidence of Ski in our pulldown lysates when cells were treated with LacZ (Figure 24A). Whole cell lysates (WCL) from P1 myofibroblasts were used as internal controls to confirm the presence of both Ski and c-Jun (Figure 24A).

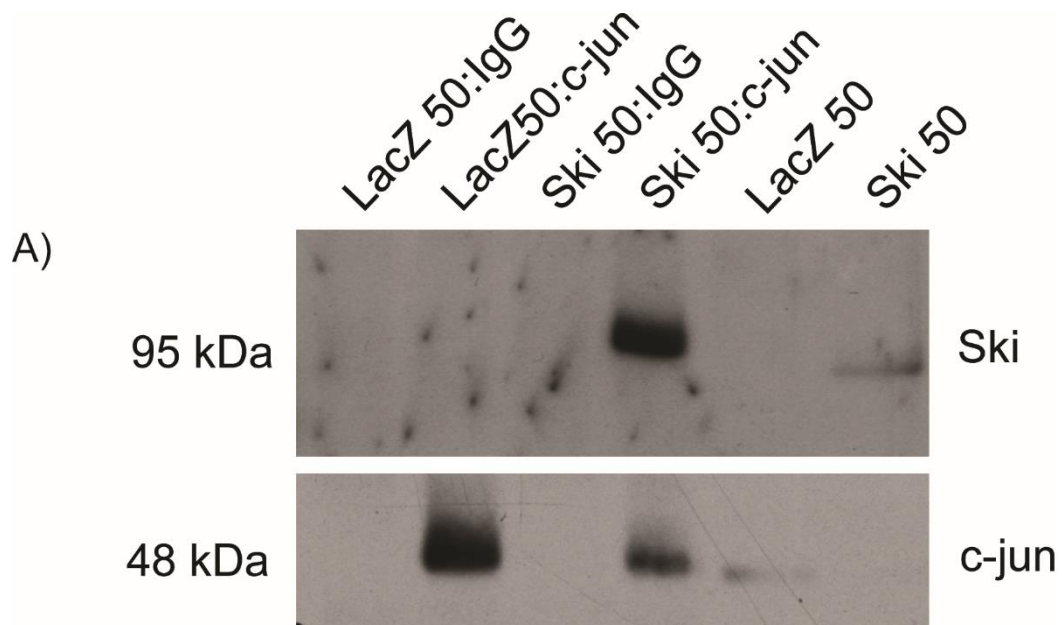


Figure 24. Interactions between Ski, c-Jun, and the Scleraxis Promoter.

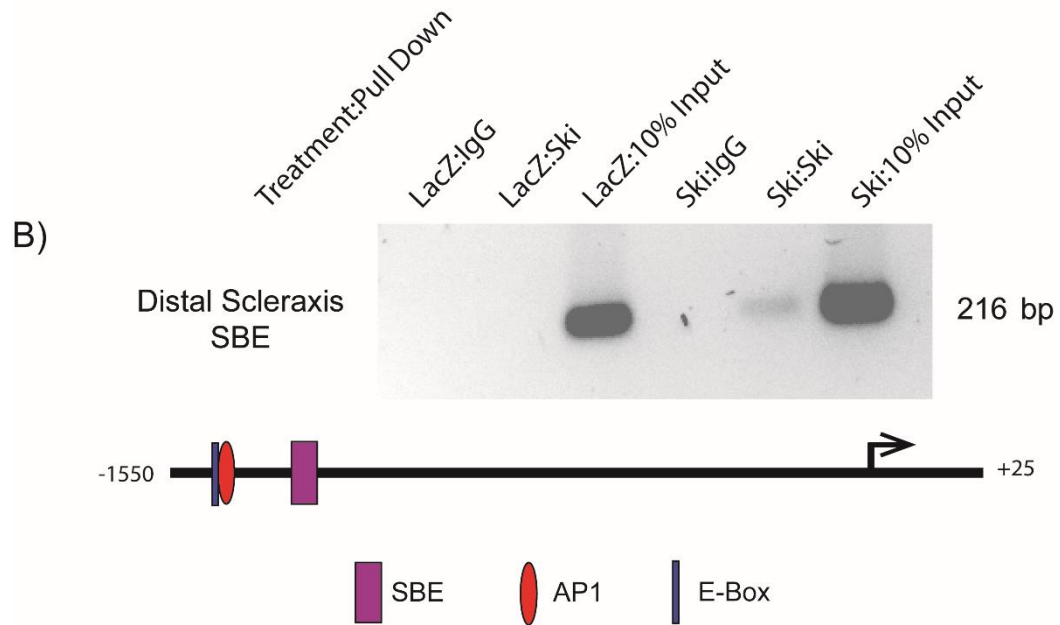


Figure 24. Interactions between Ski, c-Jun, and the Scleraxis Promoter. A) Immunoprecipitation studies demonstrated an interaction between c-Jun and Ski in Ad-HA-Ski treated P1 cardiac myofibroblasts. Antibody against c-Jun was able to pull down Ski with c-Jun, demonstrating an interaction between these proteins. Reproduced with permission³⁴⁶. **B)** Chromatin immunoprecipitation studies between Ski and the rat Scx promoter. Adenoviral overexpression of Ski in P1 rat cardiac myofibroblasts led to an association of Ski with the Scx promoter as indicated by PCR analysis. Approximate locations of the potential activating protein (AP1) binding site in relation to the SBE is highlighted in the graphical representation. Images are a representative of 3 individual experiments.

We saw a diminished signal for c-Jun in those cells that overexpressed Ski as compared to LacZ treated controls. This may be the result of antibody masking during the IP procedure which reduced the amount of c-Jun that could be co-precipitated, or this may be due to an actual reduction in c-Jun protein expression as c-Jun is part of the AP-1 transcription factor complex that has been shown to positively regulate its own expression. However, this relationship is speculative and requires further study.

To explore Ski's ability to interact with the Scx promoter we performed ChIP studies in P1 cardiac myofibroblasts overexpressing Ski (Figure 24B). Using a Ski

antibody to pull down DNA:protein complexes we were able to isolate a portion of the distal rat Scx promoter that corresponded to the distally conserved SBE. The dataset presented earlier in this thesis indicate however, that Smad proteins do not play a role in the regulation of Scx. Upon closer analysis of the promoter sequence, we identified an AP1 binding site that lies within 200 bp (192 bp) of the SBE that may be important in regulating Scx (Figure 24B).

Myocardial Function in the Post-MI Heart

To provide a clinically relevant aspect to our *in vitro* studies, we undertook an *in vivo* study of experimental heart failure where we induced a MI in rats by complete ligation of the LAD-CA and followed their cardiac function for up to 8 weeks post-MI. Using non-invasive TTE, we imaged the heart in the parasternal short axis view to obtain M-mode images. We observed that the movement in the anterior wall of infarcted animals was significantly blunted as compared to that of sham operated animals (Figure 25).

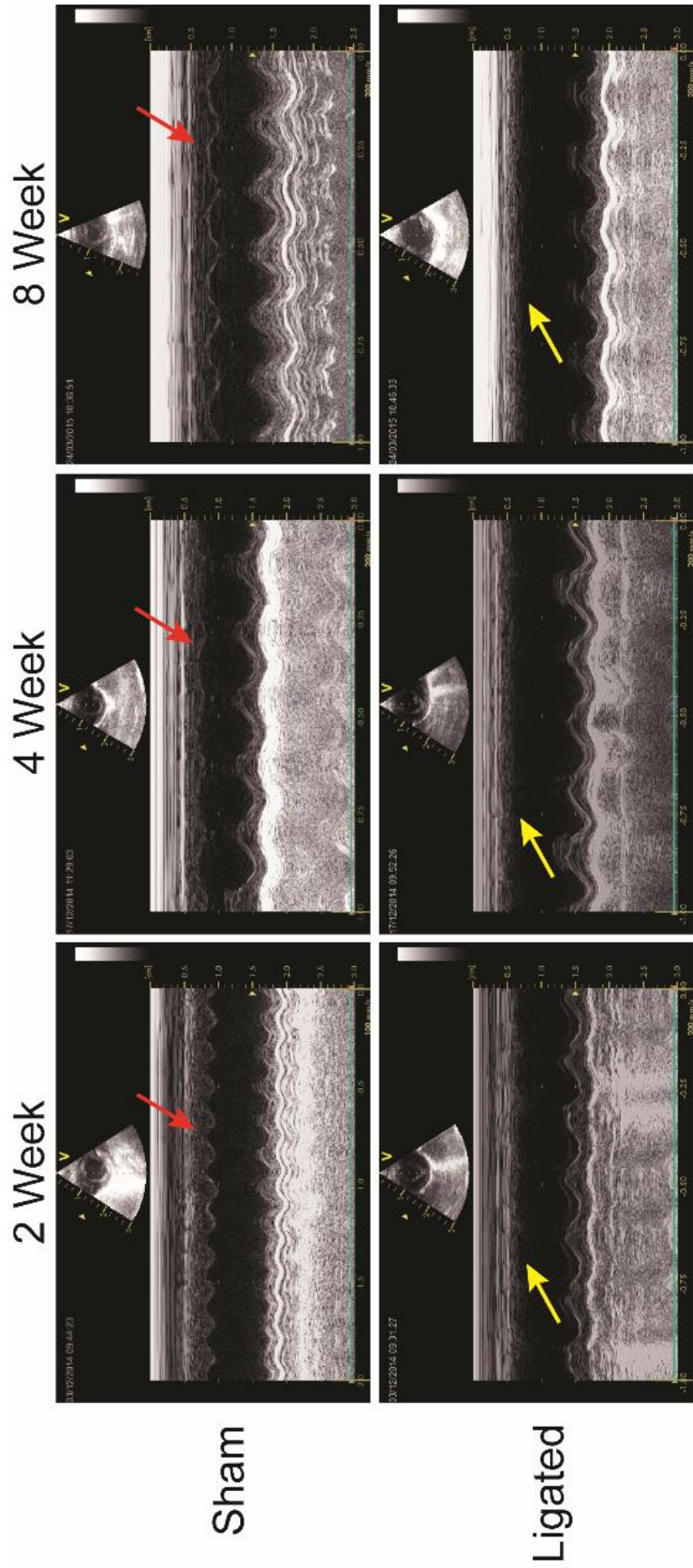


Figure 25. Echocardiographic M-Mode Images of the Sham Operated and Ligated Left Ventricle. Transthoracic echocardiography (TTE) was performed on animals 2, 4, and 8-weeks post-MI. Representative M-mode images of sham and ligated animals are depicted above. Note the relative akinetic motion of the anterior wall where the left anterior coronary artery (LAD-CA) was tied off to induce a MI. Images are representative of 4 individual animals. Red arrows indicate movement of the anterior wall in sham operated animals. Yellow arrows point towards the akinetic motion of the anterior wall following LAD-CA.

We also found that there was a significant ($*P < 0.001$; $n=4$) reduction in LV fractional shortening in ligated animals as compared to sham. There was no difference in the reduction in fractional shortening over time in ligated animals (Figure 26).

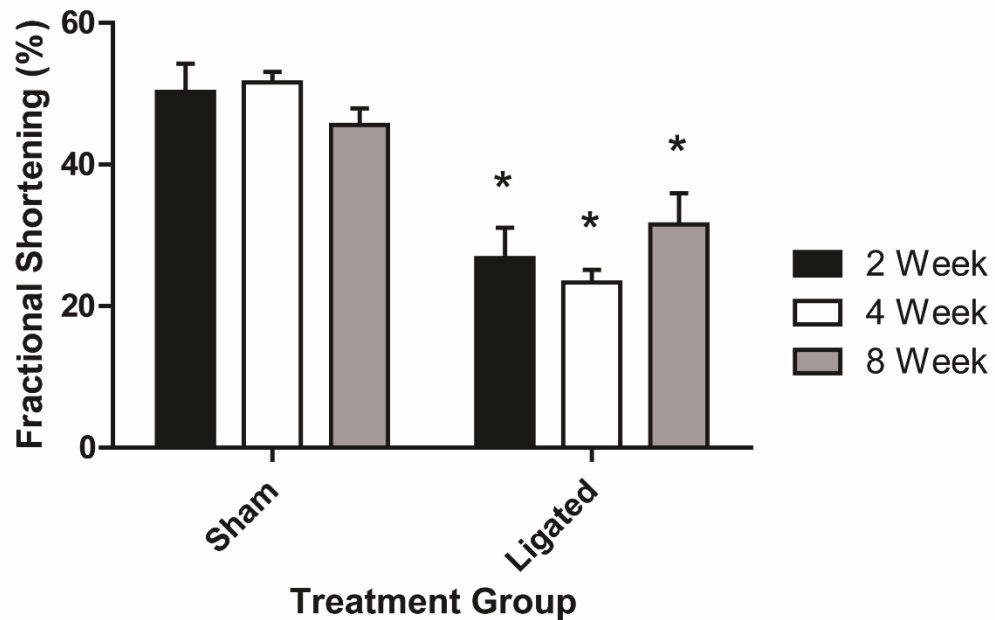


Figure 26. Cardiac Function in the Post-MI Heart. We performed transthoracic echocardiography on rats 2, 4, and 8-weeks following surgery. We observed a significant ($*P < 0.001$ vs. sham operated animals; $n = 4$) reduction in fractional shortening following coronary ligation. There was no significant difference in the reduction in fractional shortening over time.

MAPK Signaling is Elevated in the Post-MI Heart

In order to translate our *in vitro* findings, we used our rat MI model to explore the expression profile of MAPK proteins in the post-MI heart out to 8-weeks following surgery. We found a significant, time-dependent increase in the phosphorylation of p38 in those rats that underwent LAD-CA ligation as compared to sham operated controls

(Figure 27A; $^{\dagger}P < 0.001$ vs. 8-week Sham LV and CL viable; $^{*}P < 0.001$ vs. 2-week scar; $^{\dagger}P < 0.01$ vs. 4-week scar; $n=3$). We noted a trend in increasing phosphorylation of p44/42 but this did not reach significance (Figure 27B; $P > 0.05$; $n=3$). Furthermore, we found that there was a significant increase in phospho ($^{*}P < 0.001$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.05$ vs. sham LV; $^{\&}P < 0.01$ vs. CL viable) and total ($^{\dagger}P < 0.01$, $^{\ddagger}P < 0.05$ vs. sham LV; $^{\#}P < 0.05$ vs. CL viable) c-Jun expression only in those hearts that underwent ligation (Figure 27C, D, and E), and its levels were higher in the infarct scar as compared to the viable boarder zone tissue (Figure 27C, D, and E). As c-Jun has been shown to be a positive regulator of itself^{354, 355} and thus increased phosphorylation (active protein) would lead to an increase in total protein, we presented this data as phospho c-Jun/GAPDH and total c-Jun/GAPDH to avoid masking of the c-Jun effect that would be seen with phospho/total c-Jun. This effect is seen in Figure 27F.

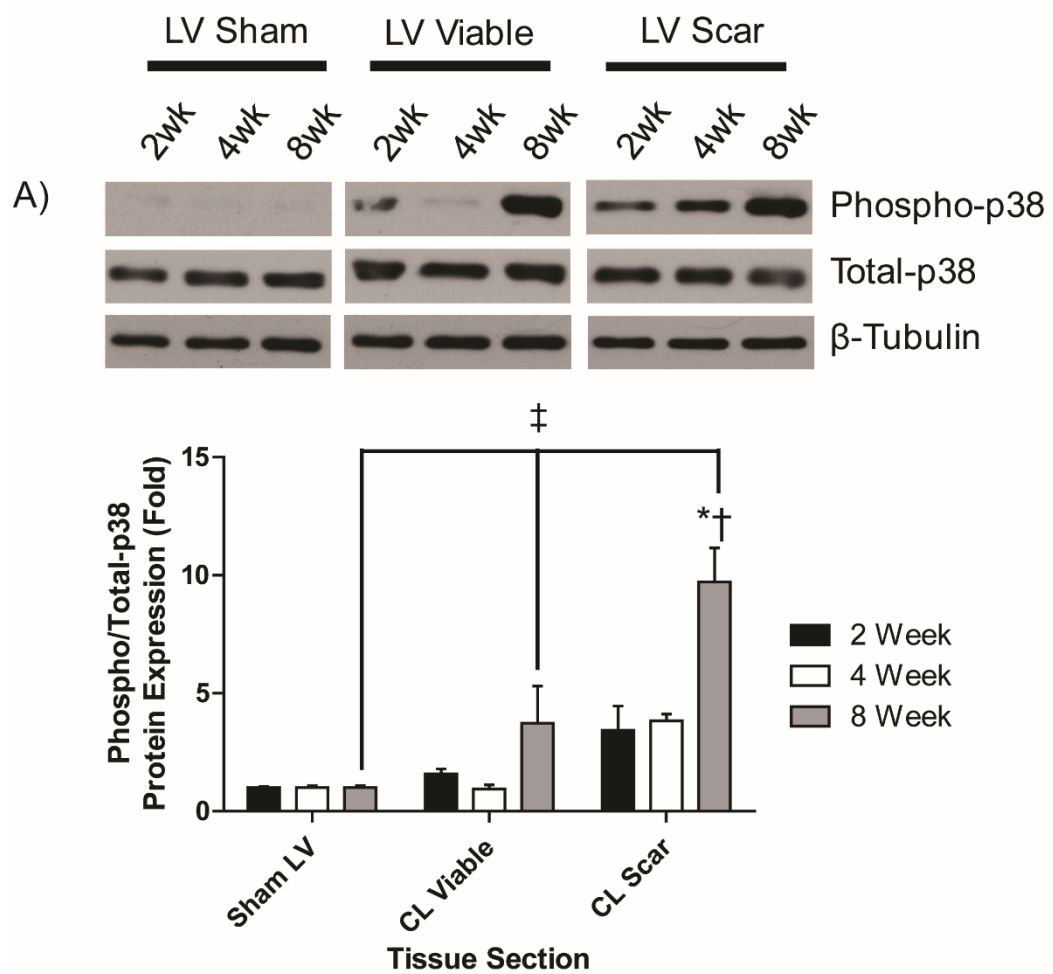


Figure 27. *In vivo* Expression and Phosphorylation of MAPK Proteins Post-MI.

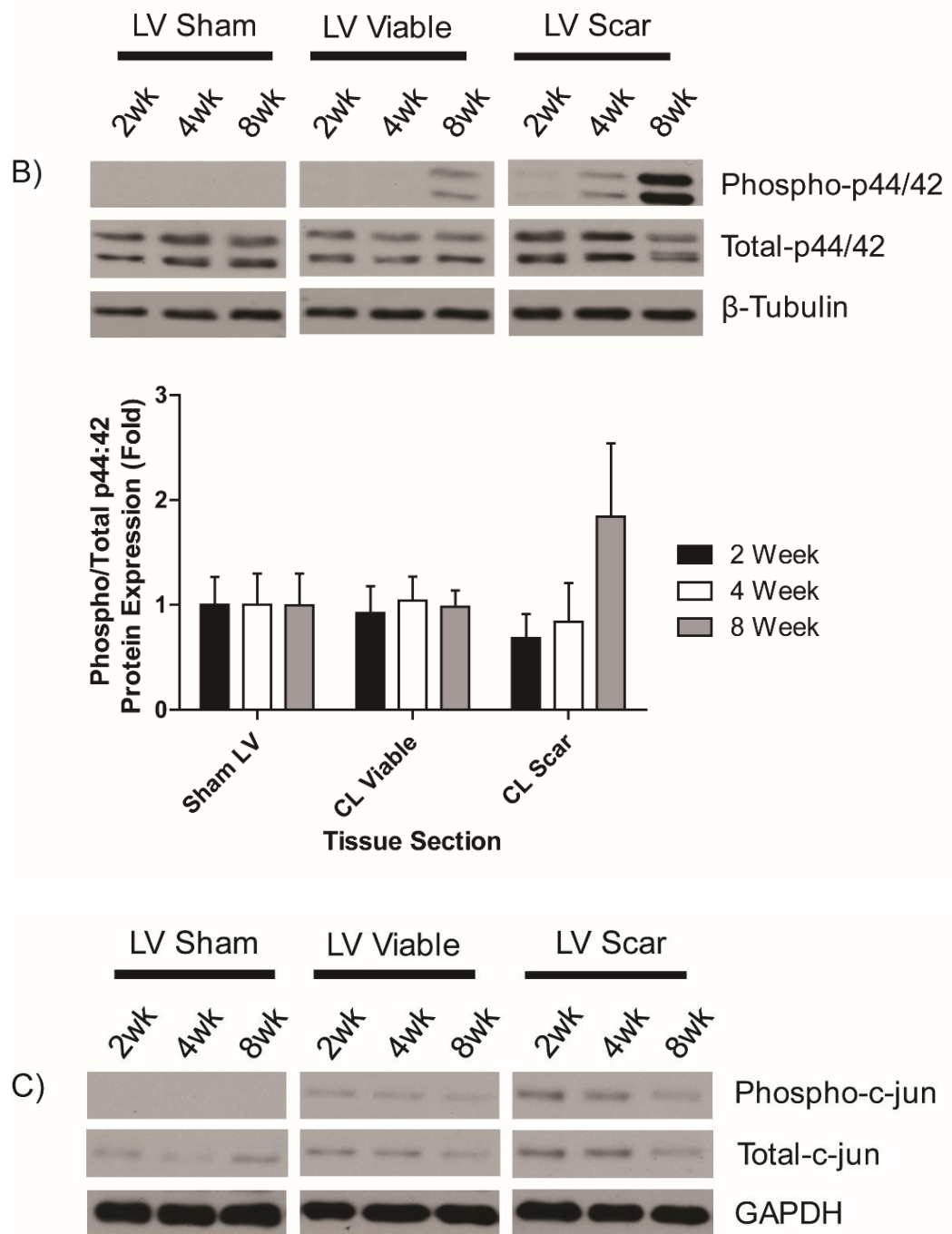


Figure 27. *In vivo* Expression and Phosphorylation of MAPK Proteins Post-MI.

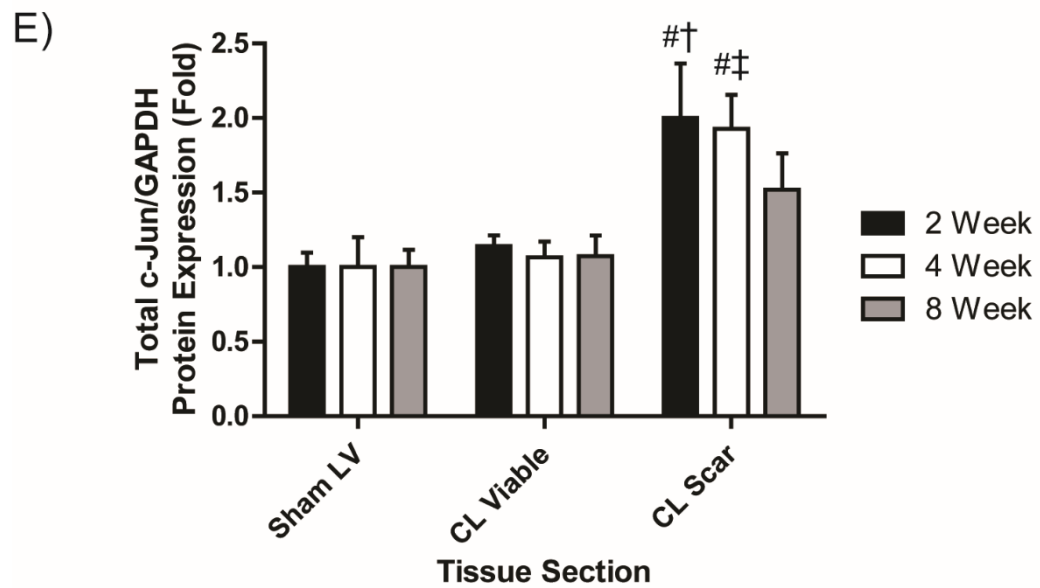
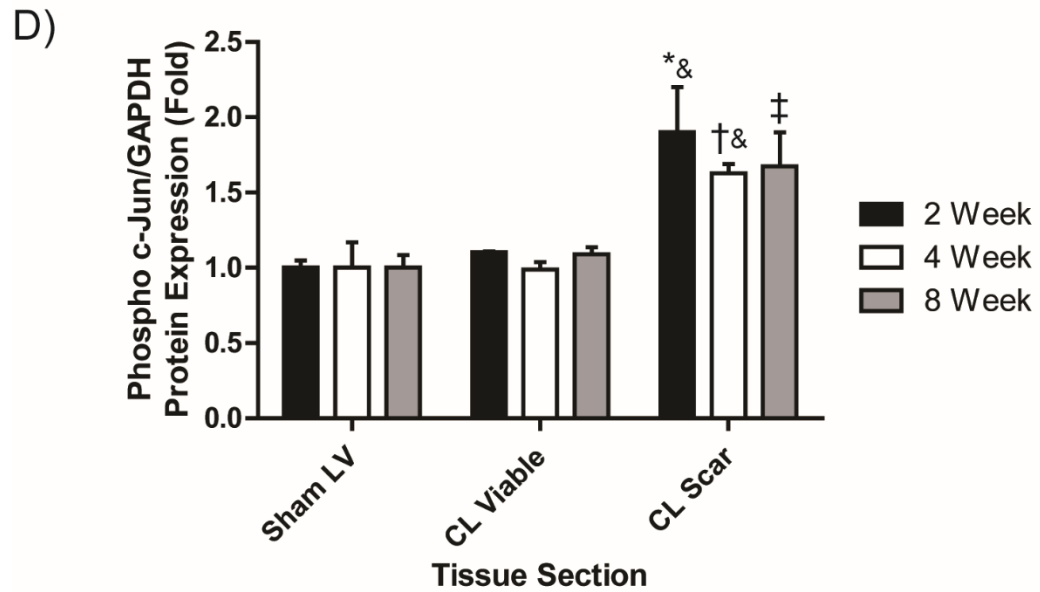


Figure 27. *In vivo* Expression and Phosphorylation of MAPK Proteins Post-MI.

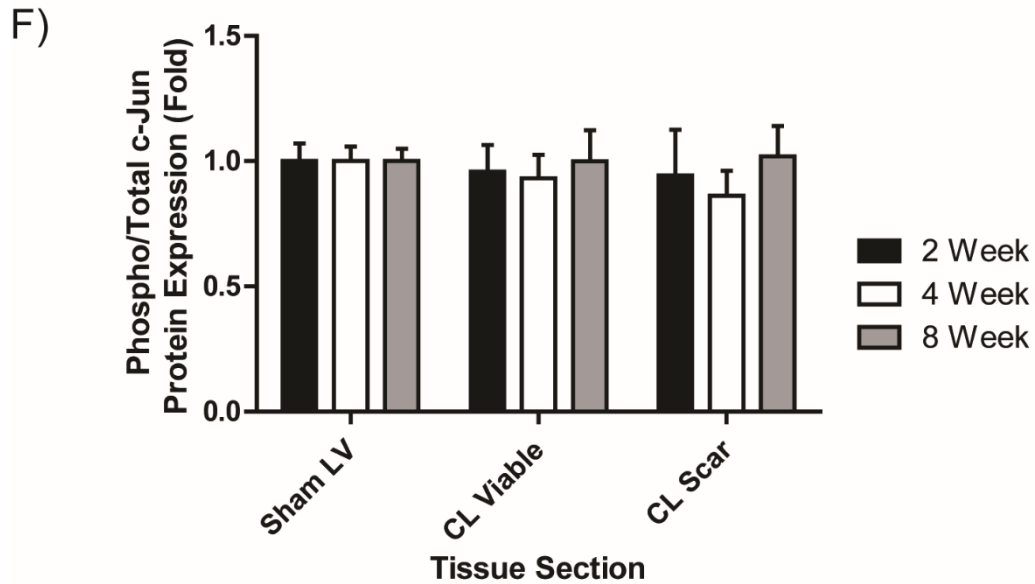


Figure 27. *In vivo* Expression and Phosphorylation of MAPK Proteins Post-MI. Western blot analysis of MAPK proteins in the 2, 4, and 8-week post-MI heart. **A)** There was a significant increase in phospho-p38 levels at 8-weeks post-MI in the scar tissue as compared to 2 and 4-week time points ($^{\dagger}P < 0.001$ vs. 8-week Sham LV and CL viable; $^*P < 0.001$ vs. 2-week scar; $^{\dagger}P < 0.01$ vs. 4-week scar; $n=3$). **B)** There was no significant difference in phospho-p44/42 ($P < 0.05$). There was a significant increase in both **C, D)** phospho ($^*P < 0.001$, $^{\dagger}P < 0.01$, $^{\dagger}P < 0.05$ vs. sham LV; $^{\&}P < 0.01$ vs. CL viable) and **C, E)** total ($^{\dagger}P < 0.01$, $^{\dagger}P < 0.05$ vs. sham LV; $^{\#}P < 0.05$ vs. CL viable) c-Jun levels in the infarct scar at all time points when compared to GAPDH. **F)** There was no difference in phospho/total c-Jun levels ($P > 0.05$; $n=3$).

Discussion

The current study provides data for the proposal of a novel pathway for Scx expression through a TGF β /Smad-independent mechanism. rhTGF β ₁ stimulation of primary cardiac myofibroblasts produced a robust (2-fold) increase in Scx protein expression as compared to untreated cells. Overexpression of the TGF β /Smad inhibitor Ski was able to significantly repress Scx mRNA and protein expression indicating a role for TGF β /Smad-dependent signaling in the regulation of Scx. We then obtained a portion of the hScxP and performed luciferase reporter gene assays using NIH-3T3

cells. Corroborating our studies in primary myofibroblasts, rhTGF β ₁ stimulation of the hScxP resulted in a robust, dose-dependent increase in luciferase reporter activity up to 5.0 ng/mL of rhTGF β ₁. However, we could not recapitulate this finding with the co-expression of hSmad2 or hSmad3 with hScxP in our reporter construct, suggesting that our region of the hScxP may not be regulated by Smad proteins, rather Scx transcription may be modulated in a TGF β /Smad-independent manner. As Scx has been previously suggested to be regulated by p44/42 MAPK signaling³³⁷, we explored the effects of the MEK1/2 inhibitor U0126 on Scx expression. rhTGF β ₁ stimulation of P1 myofibroblasts resulted in a rapid (1 hour) induction of p44/42 phosphorylation, demonstrating the ability of TGF β ₁ to signal in a Smad-independent manner in our model system. Co-treatment of P1 myofibroblasts with U0126 and rhTGF β ₁ blunted the increase in Scx protein levels that were seen with rhTGF β ₁ treatment alone. As Ski is primarily known for its role in regulating TGF β /Smad signaling, and we found that Scx appears not to be significantly modulated by Smad signaling in our reporter construct, we explored a role for Ski in repressing Scx expression in a Smad independent manner. We were able to co-precipitate Ski with the transcription factor c-Jun, suggesting a protein:protein interaction between them. Furthermore, using ChIP we were able to isolate a portion of the rat Scx promoter in Ski overexpressing cells, indicating that Ski may interact with other transcription factors, such as AP1, at the promoter region of Scx to regulate gene expression. Providing a translational element to our studies, we induced an MI in rat hearts and followed the animals for 8-weeks. We found that there was increased phosphorylation of the MAPKs p38 and p44/42 as early as 2-weeks post-MI in the infarct scar. Furthermore, we found that there was

increased kinase phosphorylation in LV viable tissue (the border zone immediately surrounding the scarred region) by 2-weeks and 8-weeks post-MI for p38 and p44/42 respectively.

We are not the first group to demonstrate a role for induction of Scx expression by TGF β ₁. Several other groups have identified a role for each TGF β isoform (1, 2, and 3) in promoting Scx expression^{20, 337, 345}. A 2013 study by Barsby and Guest showed that Scx expression correlated with increased TGF β ₁₋₃ in the tendons of injured horses *in vivo*³⁴⁵. Using an *in vitro* model, they allowed undifferentiated equine embryonic stem cells (ESC) to spontaneously differentiate in the presence of TGF β ₁₋₃. They found that in the presence of TGF β ₁₋₃, there was a significant upregulation of Scx staining in the differentiated ESCs. Furthermore, they noted that while all 3 isoforms of TGF β were capable of promoting Scx expression, TGF β ₃ provided the greatest effect. A 2009 study by Espira *et al.* analyzed the effects of cell passaging and TGF β ₁ on Scx production in primary cardiac fibroblasts and myofibroblasts²¹⁰. With each passage they found that Scx mRNA increased reaching a 4-fold increase within 2 passages as compared to unpassaged controls. Stimulating P2 myofibroblasts with 10 ng/mL of TGF β ₁ resulted in a further increase in Scx mRNA and protein expression over untreated controls²¹⁰. A third study to demonstrate a role for TGF β -mediated Scx expression used primary cell cultures and cell lines to describe a role for TGF β ₂. Using a porcine model, they isolated myofibroblast-like interstitial cells from heart valves and treated them with 200 pg/mL TGF β ₂ for 48 hours. Similar to our current study and those previously published, they found that TGF β ₂ stimulation significantly increased Scx protein expression as compared to vehicle controls. This group recapitulated their

findings using NIH-3T3, C3H10T_{1/2}, and avian valve precursor cultures. The results presented in our study are in line with those previously published and clearly demonstrate a role for TGF β -mediated Scx expression.

Despite the overwhelming evidence for TGF β -mediated Scx expression, no study to date has explored the direct DNA:protein transcriptional mechanisms that underpin this relationship. Therefore, we obtained approximately 1600 bp (1580 bp actual) of the hScxP and explored the relationship between TGF β ₁, R-Smads, and Scx using luciferase reporter assays. We chose to explore the effects of TGF β ₁ over TGF β ₂ and TGF β ₃ as TGF β ₁ has been described as one of the driving forces behind myocardial remodelling in heart disease. Although we found that TGF β ₁ could promote luciferase expression from the hScxP, overexpression of R-Smad proteins had no effect on our isolated region of the hScxP as indicated by luciferase expression. Our results here differ from those published by Bagchi and Czubryt who demonstrated that adenoviral delivery of R-Smad3 to primary rat cardiac myofibroblasts could significantly increase Scx protein expression²⁰. Furthermore, they showed that adenoviral delivery of I-Smad7 reduced Scx protein levels by as much as 40%²⁰. As R-Smad3 is a downstream effector of the TGF β ₁ signal, and I-Smad7, an endogenous inhibitor of this signal, they proposed that Smad proteins lie downstream of TGF β ₁ and that they directly regulate Scx gene expression²⁰. There are several possibilities for the differences that we observed in our study compared to that of Bagchi and Czubryt. First, it is possible that we did not capture the functionally significant SBE for Scx in our luciferase promoter construct as we only isolated the portion that we considered to be the important regulatory region of Scx gene expression due to the abundance and clustering of

putative transcription factor binding sites as well as the high identity of the selected promoter region across mouse, human, and rat species. Second, it is possible that Smad proteins play a role in Scx expression through enhancer elements located elsewhere in the genome. Finally, it is possible that Smads do not directly regulate Scx gene expression at all, but rather act as an intermediate step towards the induction of a yet to be identified transcription factor that does directly bind to and activate the promoter of Scx.

As we did not find any functional significance for Smad proteins in the regulation of Scx, we consulted the literature to identify other regions and proteins of interest. We found but one article that described a role for MAPK signaling in mediating Scx expression in heart valves³³⁷. Thus, based on the data presented we sought to determine if p44/42 (Erk1/2) signaling played a role in Scx regulation. We utilized U1026 as a known MEK1/2 inhibitor with and without rhTGF β ₁ stimulation of primary cardiac myofibroblasts. As previously described, rhTGF β ₁ treatment increased Scx protein levels whereas co-treatment with U0126 and rhTGF β ₁ modestly, but not significantly, reduced Scx protein levels as compared to rhTGF β ₁ and vehicle treated controls. Furthermore, we found that rhTGF β ₁ stimulation resulted in rapid (within 1 hour) phosphorylation of p44/42, demonstrating a potential role for a TGF β ₁/Smad-independent signaling pathway in the regulation of Scx. Our results contrast those previously reported by Barnette *et al.*³³⁷. Using a porcine model of valvular interstitial cells they demonstrated that p44/42 MAPK signaling represses Scx expression as opposed to increases its expression as we have shown in the current dataset. In contrast to our results where we saw increased phosphorylation of p44/42 following TGF β ₁

stimulation, Barnette and colleagues saw no increase in p44/42 phosphorylation after TGF β ₂ treatment. Moreover, when they overexpressed MEK1 (an upstream kinase of p44/42) prior to TGF β ₂ stimulation, they observed a significant reduction in Scx expression as compared to TGF β ₂ stimulated cells alone. Although both studies agree on the ability of TGF β to promote Scx expression, the mechanisms proposed by our group and that of Barnette³³⁷ are fundamentally different. First, we utilized primary cardiac myofibroblasts from adult male rat hearts. These cells have been identified to play an important role in myocardial remodelling in the wounded heart. In those studies conducted by Barnette *et al.* they used an embryonic mesenchymal cell type that mimics the primary cell type. Furthermore, they used avian precursor cells to provide proof-of-principle to those studies conducted in their cell line model with C3H10T_{1/2}. The use of cell lines and embryonic cells in their study may distort the true mechanism for Scx regulation by these pathways as these cell types are immortal and not fully differentiated. Differences in developmental stage may account for the conflicting results between our 2 studies. Second, Barnette *et al.* used TGF β ₂, whereas we utilized TGF β ₁. Although these cytokines are highly similar, it may be that they elicit a different response from the T β R's with respect to Smad-independent pathways. Finally, we used 10 ng/mL rhTGF β ₁ (~400 nM) in serum starved cells, whereas Barnette's group used 200 pM TGF β ₂ in complete growth medium for 2 days. As normal growth media contains a plethora of cytokines and growth factors to maintain cell growth and viability, it would be difficult to elucidate the true role of TGF β ₂ on Scx expression. The presence of multiple growth factors and cytokines would provide high background and may alter the effect of TGF β ₂.

Historically, Ski is recognized as a potent inhibitor of TGF β /Smad signaling. However, as Scx appears not to be regulated through such a mechanism we set out to define a role for Ski in our model. As Ski is not a transcription factor itself, but rather more of a scaffolding protein that binds to transcription factors we decided to look at transcription factors that lie downstream of the MAPK signaling pathways to see if Ski could interact with them to prevent Scx gene expression. A previous study published by Pessah *et al.* demonstrated that c-Jun directly interacts with Ski and that upon TGF β stimulation, this complex dissociates and de-represses c-Jun³⁵³. Using an AP-1 Lux luciferase reporter, they co-transfected HepG2 cells with c-Jun and Ski. They found that increasing amounts of Ski repressed c-Jun's ability to transactivate the AP-1 Lux reporter. Co-immunoprecipitation studies demonstrated a clear protein:protein interaction between c-Jun and Ski. These results are in agreement with our current study. We found that with adenoviral overexpression of Ski we could co-precipitate Ski and c-Jun. This data, although preliminary, provides an alternative method for Ski-mediated repression of Scx through a Smad-independent mechanism. Upon closer analysis, we identified a putative AP1/CREB binding site within our isolated hScxP. We showed that Ski was part of a DNA:protein complex within the rat Scx promoter as we were able to pull down a portion of the rat Scx promoter using the ChIP technique. The region isolated correlated with the location that contained the identified AP1 transcription factor binding site. This site may play a role in TGF/Smad-independent p44/42-MAPK regulation of Scx.

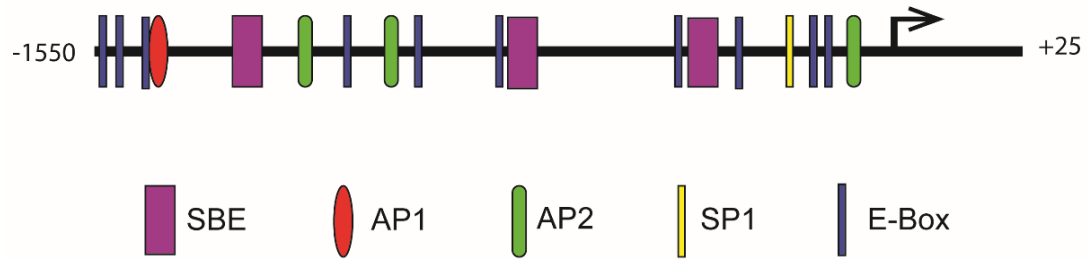


Figure 28. Graphic Depiction of the Human Scleraxis Promoter and Putative Transcription Factor Binding Sites. Reproduced with permission³⁴⁶.

The combination of our *in vitro* work and the use of a whole animal model allowed us to approach the overall hypothesis with a more clinically relevant perspective. Thus, we permanently ligated the LAD-CA to produce a large MI to mimic the clinical setting. Animals were euthanized at either 2, 4, or 8-weeks post-MI, and myocardial tissue collected for Western blot analysis. Corroborating our *in vitro* studies with $TGF\beta_1$, a well-known mediator of the chronic wound healing response, we observed that as early as 2-weeks after MI, there was induction of both the p38 and p44/42 MAPK signaling pathways as identified by kinase phosphorylation within the infarct scar (Figure 27A and B). Furthermore, we noted phosphorylation of the transcription factor c-Jun in the border zone within 2-weeks following LAD-CA ligation that was sustained out to the 8-week time point. This trend continued in the infarct scar where there was a further increase in phospho-c-Jun levels as compared to border zone tissue and sham operated controls (Figure 27C and D). Our data is supported by numerous other reports³⁵⁶⁻³⁵⁹. In a mouse model of MI, Yeh *et al.* noted a spatial and temporal expression pattern for phospho-p38 and phospho-p44/42 in the post-MI heart. Much akin to our current study, Yeh *et al.* found significant

phosphorylation of p38 in areas remote to the infarct scar that progressively increased out to 12-weeks, a further 4-weeks past the latest time point in our study³⁵⁶. Furthermore, they observed a rapid and robust increase in phospho-p38 levels in the infarct scar itself as early as 1-week post-MI, which stabilized at the 12-week time point³⁵⁶. Analogous to our study, Yeh *et al.* also demonstrated a spatial and temporal distribution of phospho-p44/42. Compared to the results obtained for phospho-p38, phospho-p44/42 levels rose significantly at later time points that are typically associated with extensive fibrosis and non-myocyte proliferation (i.e. increased numbers of resident myofibroblasts)¹²³. The results of our study recapitulate and mirror those of Yeh *et al.* with respect to both phospho-p38 and phospho-p44/42 spatial and temporal activation patterns in the post-MI heart. The role of p38 in the post-MI heart is further highlighted by a study by See *et al.* who subjected rats to complete ligation of the LAD-CA and treated them with the p38 inhibitor RWJ³⁵⁹. In a more clinically relevant evaluation of the post-MI heart, those animals pretreated with RWJ had a significantly smaller infarct size (2.6% vs. 17.1% for the vehicle controls) over a 4-week period³⁵⁹. This reduction in scar expansion also correlated with improved myocardial dimensions and functional capacity as determined by 2-dimensional transthoracic echocardiography³⁵⁹. Collagen deposition was also significantly blunted by RWJ pre-treatment. Taken together, these results complement and extend those results presented here in our dataset, as well as that provided by Yeh *et al.* It is clear that MAPK signaling plays a prominent role in the post-MI heart and the chronic, maladaptive wound healing process.

Conclusions and Future Directions

Taking our *in vitro* and *in vivo* data together, we propose a new mechanism for the regulation of Scx in a Smad-independent manner (Figure 29). We believe that TGF β ₁ promotes the phosphorylation of p44/42 *via* T β R's which in turn promotes the expression of Scx, possibly through the c-Jun and the AP-1 transcription factor complex. Furthermore, we believe that Ski plays a significant role in mediating Scx expression and that it may exert its effects through a c-Jun dependent mechanism rather than its traditional interactions with Smad transcription factor complexes. Based on the studies presented here, we believe that Smad proteins are not directly involved in Scx regulation, however their role as an indirect mediator is still open for interpretation and requires further study.

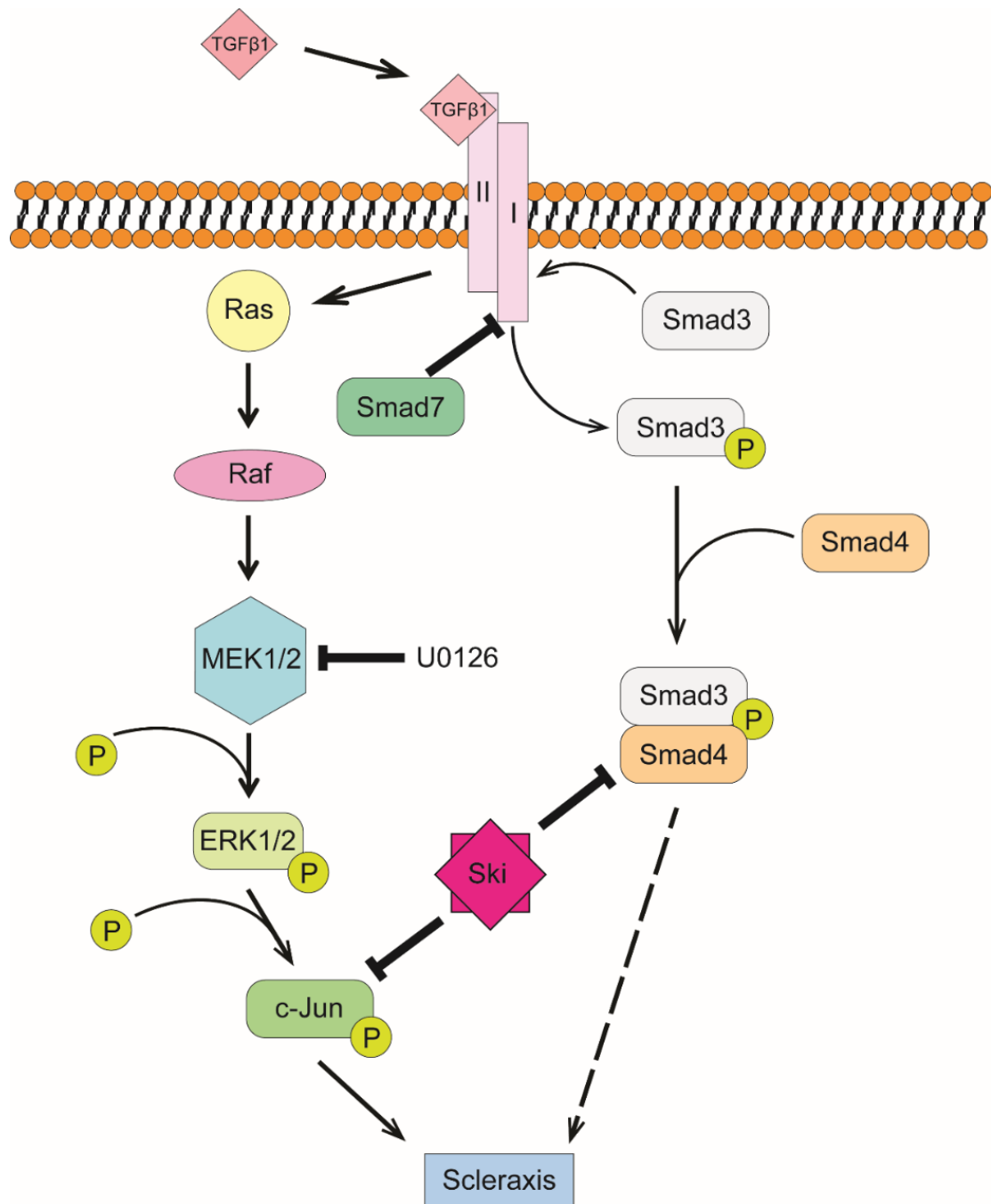


Figure 29. Proposed Mechanism for the Regulation of Scx by p44/42 (Erk1/2) and Ski. TGFβ₁ is a powerful inducer of Scx expression. We have demonstrated that TGFβ₁ may exert its effects on Scx expression through a TGFβ₁/Smad-independent mechanism that includes the p44/42-MAPK signaling pathway in combination with c-Jun activation in cardiac myofibroblasts. As U0126 could not completely counteract the effect of TGFβ₁ on Scx protein expression, it is plausible that Smad proteins may still be involved in regulating Scx through an intermediate mechanism that has yet to be described. We have shown Ski to be a potent inhibitor of Scx signaling and we

propose that Ski exerts its effects through inhibition of p44/42 in addition to its role in preventing Smad mediated gene regulation. Reproduced with permission³⁴⁶.

Further work needs to be done to tease out the precise mechanism by which TGF β ₁ promotes p44/42 phosphorylation. A detailed and exhaustive study needs to explore whether the T β R's are capable of promoting p44/42 phosphorylation through the previously identified Ras/Raf/MEK pathway. This will provide the missing link between p44/42 and the T β R's in this model system and will identify the true role of TGF β /Smad-independent pathways in regulation of Scx gene expression. Moreover, an extensive study is required to explore the effects of the other recognized MAPK signaling pathways that have been shown to be modulated through TGF β signaling. It may be that there is extensive cross talk and input amongst these pathways such that not just one but all play a small part in regulating Scx gene expression.

Additional work to explore the mechanisms associated with Ski mediated transcriptional repression of Scx is required. While it is clear from our current studies that Ski is a potent inhibitor of Scx gene and protein expression, the inability of Smad proteins to positively regulate Scx bring into question how Ski can promote such a dominant effect. We did however provide some insight into this mystery. We were able to co-precipitate Ski with c-Jun when Ski was overexpressed as previously reported by Pessah *et al.*³⁵³, suggesting a little defined role for Ski outside of TGF β /Smad signaling. However, whether c-Jun is a potent transcriptional regulator of Scx gene expression is currently unknown and warrants further investigation.

Finally, further work is needed to explore the spatial, temporal, and sub-cellular distribution of Ski and Scx in the post MI heart. A previous study conducted in our

laboratory by Cunningham *et al.* demonstrated that Ski protein levels rise in the cytosolic fraction of protein isolated from rat hearts that have undergone LAD-CA ligation²². The precise mechanism for the accumulation of Ski within the cytosolic fraction post-MI is currently unknown and merits further evaluation. Even less is known with regards to the sub-cellular distribution of Scx in the post-MI setting as well as its spatial and temporal compartmentalization within the infarct and border zone. A clearer understanding into the distribution and expression patterns of both Ski and Scx in the post-MI setting is needed to gain better insight into the molecular mechanisms that underpin the chronic, maladaptive wound healing response and may lead to therapeutic remedies to prevent the onset of heart failure in the post-MI patient population.

Chapter 4: Transcriptional Regulation of Ski

Rationale, Hypothesis, and Objectives

Originally identified as the transforming agent of the Sloan-Kettering retroviruses in avian fibroblasts, Ski is now better known for its role as a potent endogenous antagonist of TGF β signaling. Functionally, Ski forms a transcriptional inhibitory complex that has been shown to include NCoR and HDAC proteins³²⁰. Furthermore, site-directed mutagenesis of the NCoR binding domain within Ski, significantly represses the transcriptional repression capability of Ski. Despite widespread knowledge of Ski and its role in TGF β signaling, relatively little is known with respect to the transcriptional mechanisms that govern the expression of Ski itself. As TGF β is a prominent player in numerous pathologies and Ski is a natural inhibitor of TGF β /Smad-dependent signaling, it would be beneficial to better understand the mechanisms regulating Ski expression.

A 2012 study by Li *et al.* found that Ski was directly regulated by PPAR δ and that the rat Ski promoter contained a functional PPRE²⁹¹. Treatment with PPAR δ resulted in an increase in Ski expression that could be significantly blunted by an irreversible inhibitor against PPAR δ ²⁹¹. Outside of this single study that shows a mechanism for regulating Ski transcription, nothing else is known with regards to other positive or negative regulators of Ski expression. As Ski is an anti-fibrotic protein that is a potent inhibitor of Scx expression we set out to explore the reverse relationship. We hypothesize that Scx is a potent inhibitor of Ski expression, and that it binds directly to the promoter region of Ski to repress Ski transcription. To explore our hypothesis, we will:

- 1) Determine whether the human Ski proximal promoter (hSkiPP) is a direct target of the pro-fibrotic transcription factor Scx through direct interaction with E-box binding motifs found within the hSkiPP to inhibit Ski transcription.
- 2) Determine whether ectopic overexpression of Scx negatively regulates Ski mRNA and protein expression in primary cardiac myofibroblasts, thus promoting a net pro-fibrotic environment.

Methodology

Animal Ethics

All experimental protocols involving animal studies were reviewed and approved by the University of Manitoba's Animal Care Committee following the Canadian Council on Animal Care Standards.

***In silico* Analysis of the Ski Promoter**

The mouse, rat, and human Ski gene sequences were obtained by searching the NCBI database using the key words "mouse Ski", "rat Ski", and "human Ski". The FASTA sequences were downloaded into a Word document. Using the FASTA gene sequence as the query, mouse, rat, and human Ski were localized to their appropriate chromosomal locations using NCBI Blast. The FASTA sequences for the chromosomal regions of interest were downloaded into a Word document. The translational start site (ATG) was identified for each species, as were exons, introns, and the translational stop. Identification of potential E-box binding motifs were determined by Blast searching all iterations of the E-box consensus sequence (CANNTG) and highlighting them within the first 10,000 base pairs upstream of the translational start of the Ski gene. Other promoter elements were also identified including, but not limited to NF-

κB binding sites (GGGRNNYYCC; where R is any purine, Y is any pyrimidine, and N is any nucleotide), and CAAT-boxes.

To identify regions of potential significance, mouse, rat, and human Ski promoters were aligned to one another (ClustalW, Expasy) to detect regions of high identity and similarity. Based on these alignments, the hSkiPP was split into 2 regions, the proximal (~2000 bp 5' of the ATG; Figure A-1) and the distal promoters (a further ~2000 bp upstream of the proximal promoter; Figure A-2).

Isolation of BAC DNA

Two bacterial artificial chromosomes (BAC; RP11-659D23 and RP11-719N13) containing the region of chromosome 1 containing human Ski were purchased from Invitrogen (Cat #: RPC111.C). Upon arrival, 20 µL of the bacterial BAC stab was streaked out on an agar plate containing chloramphenicol (100 µg/mL) and incubated over night at 37°C in a dark, humidified environment. The next day individual clones were picked from the plate and placed into a 50 mL starter culture of LB containing chloramphenicol (100 µg/mL) for 2 hours. The 50 mL starter culture was used to inoculate 1000 mL of LB in a 2.0 L Erlenmeyer flask containing chloramphenicol (100 µg/mL). The culture was incubated overnight at 37°C with constant agitation at 300 rpm. Isolation of the BAC DNA from the bacteria was conducted using a Qiagen Large Construct Kit (Cat #12462) as per the manufacture's directions.

The DNA pellet was re-suspended in nuclease free water and its concentration determined *via* its 260 nm spectrophotometry reading using the GeneQuant III.

Cloning of the 1.8 kb Human Ski Promoter

Primers were designed to amplify approximately ~2000 bp (1816 bp actual) of the human Ski proximal promoter (hSkiPP) from the BAC and were commercially synthesized (Sigma-Aldrich; Table 7).

<u>Probe</u>	<u>Sequence (5' – 3')</u>
Human Ski Proximal Promoter Forward	CAGTCACTCGAGGTCACGGGGCCGAAGTCCTGATAAG
Human Ski Proximal Promoter Reverse	CAGTCAAAGCTTGTCACCTGGCGCTCCTTCTGTAG

Table 7. Primer Sequences for the Synthesis of the Human Ski Proximal Promoter.

Due to the high GC content of the hSkiPP, an enhancer cocktail (Table 8) was used to reduce the amount of non-specific binding and secondary structure that may occur during PCR cycling. The PCR reaction was set up as listed in Table 9 and run on a MJ Thermocycler:

<u>Solution</u>	<u>Stock Concentration</u>	<u>Final Concentration</u>
Nuclease Free Water	-	To 50 μ L
Deep Vent Buffer	10x	1x
DMSO	-	5.0%
Betaine	5.0 M	1.3 M
Forward Primer	5.0 μ M	300 nM
Reverse Primer	5.0 μ M	300 nM
dNTP	2.5 mM	200 μ M
DNA	-	-
7-deaza-dGTP	5.0 mM	50 μ M
Deep Vent	2.0 U/ μ L	2.0 U

Table 8. PCR Reaction Composition for the Synthesis of the Human Ski Proximal Promoter.

<u>Step</u>	<u>Event</u>
1	95°C for 5 minutes 1x
2	95°C 30 seconds
3	60°C 30 seconds
4	70°C 2 minutes
5	Step 2-4 40x
6	70°C for 4 minutes 1x
7	10°C Hold

Table 9. Thermocycling Profile for Amplification of the Human Ski Proximal Promoter from a Bacterial Artificial Chromosome.

Following PCR, 10 μ L 6x loading buffer was added to each reaction. Fifteen microliters of each reaction was loaded onto a 1.0% agarose gel and subjected to electrophoresis at 120 volts for 60 minutes at room temperature. Bands were visualized using a Bio-Rad Gel Doc system.

The remainder of the PCR reactions that produced a band of interest were subjected to electrophoresis on a 1.0% agarose gel at 120 volts for 60 minutes at room temperature and the band visualized using a UV long wave light table and cut from the gel using a scalpel. Gel pieces containing DNA of interest were placed into a DNA spin column (Millipore) and subjected to centrifugation at 5,000 xg for 10 minutes at room temperature. Flow through from the column was purified using the QiaQuik system (Qiagen) as per the manufacture's directions. DNA concentration was determined by UV spectrophotometry using the Gene Quant III system. 98 μ L nuclease free water was used as a blank for the spectrophotometer. 2.0 μ L concentrated DNA solution was

added to the blank and gently mixed to give a 50x dilution. The sample was read and absorbances at 230 nm, 260 nm, and 280 nm was recorded. Ratios of 260 nm/280 nm and 260 nm/230 nm were also reported to identify any possible contamination. DNA concentrations were determined (Figure 30) based on the equation below:

$$DNA\ Concentration\ \left(\frac{\mu g}{\mu L}\right) = \frac{A_{260} * 50 * 0.1}{2}$$

Figure 30. Formula for Calculating DNA Concentration.

To create a cleaner, more specific product, the DNA of interest was used as template to conduct a nested PCR reaction (Tables 10 and 11). DNA was gel purified using spin columns (Millipore) and cleaned using the QiaQuick system (Qiagen) as described above.

<u>Solution</u>	<u>Stock Concentration</u>	<u>Final Concentration</u>
Nuclease Free Water	-	To 50 μ L
Deep Vent Buffer	10x	1x
DMSO	-	5.0%
Betaine	5.0 M	1.3 M
Forward Primer	5.0 μ M	300 nM
Reverse Primer	5.0 μ M	300 nM
dNTP	2.5 mM	200 μ M
DNA		25 ng
7-deaza-dGTP	5.0 mM	50 μ M
Deep Vent	2.0 U/ μ L	2.0 U

Table 10. Nested PCR Reaction.

<u>Step</u>	<u>Event</u>
1	95°C 5 minutes 1x
2	95°C 30 seconds
3	60°C 30 seconds
4	70°C 2 minutes
5	Step 2 - 4 40x
6	70°C 4 minutes 1x
7	10°C Hold

Table 11. Nested PCR Cycling Conditions.

2.0 μL of nested PCR DNA fragment and 1.0 μg pGL4.10 [luc2] vector were subjected to a restriction enzyme digest with *HindIII* and *XhoI* to remove the ends from the DNA and to create a splice site within the vector. The reaction was incubated for 60 minutes at 37°C in a temperature controlled water bath. The PCR fragment containing the hSkiPP was ligated into the pGL4.10 [luc2] vector using a Quick Ligation Kit (New England Biolabs) as per the manufacture's directions. The reaction was incubated at room temperature for 5 minutes to allow for maximal vector/fragment ligations. Immediately following ligation, the DNA product was transformed into AG1 (Agilent) or NEB-5 α (New England Biolabs) competent bacteria. 5.0 μL of the ligation reaction was added to 50 μL of competent bacteria and incubated on ice for 30 minutes. Transformation of bacteria was accomplished by heat shocking bacteria in a 42°C water bath for exactly 30 seconds as per the manufacture's protocol. The solution was placed back on ice for an additional 5 minutes. Following incubation, 950 μL LB containing no antibiotics was added to the reaction and incubated at 37°C for 60 minutes with constant agitation at 300 rpm. After 60 minutes, bacteria were pelleted by centrifugation at 18,000 $\times g$ for 30 seconds. The LB supernatant was removed and the bacterial pellet re-suspended in 100 μL fresh LB. From this, 25 μL of concentrated bacteria were streaked and 75 μL spread onto Carbenicillin (100 $\mu\text{g}/\text{mL}$) containing agar plates and incubated overnight at 37°C. The next day, colonies of interest were identified and picked from the plate, and incubated in LB containing Carbenicillin (100 $\mu\text{g}/\text{mL}$) overnight. Cultures were incubated at 37°C with constant agitation at 300 rpm for at least 18 hours. The next day DNA, was isolated from the bacteria cultures using a Miniprep kit (Qiagen) as per the manufacture's directions. To determine which of the

clones contained the insert of interest, we performed a restriction digest in a water bath at 37°C for 60 minutes.

Following digestions, 3.0 µL 6x loading buffer was added to each reaction and 15 µL of each reaction volume was subjected to electrophoresis on a 1.0% agarose gel at 120 volts for 60 minutes. Bands were visualized using a Bio-Rad Gel Doc system with a 2.5 second exposure and analyzed using Quantity One Software (BioRad). Clones that were positive for the insert of interest were sent for sequencing to confirm that they contained the sequence of interest (TCAG, Toronto, Ontario, Canada).

Isolation of Primary Cardiac Myofibroblasts

P1 cardiac myofibroblasts were isolated as described in Chapter 3 (page 66-68) of this thesis.

Cell Culture

P1 primary rat cardiac myofibroblasts and Cos7 cells were maintained in a standard cell culture incubator at 37°C with 5.0% CO₂. P1 myofibroblasts were maintained in a 10% DMEM_{F12}-FBS solution supplemented with 100 U/mL penicillin, 100 U/mL streptomycin, 14 mM sodium bicarbonate, and 1.0 µM ascorbic acid. Cos7 cells were kept in a 10% DMEM-FBS solution containing 100 U/mL penicillin, and 100 U/mL streptomycin.

Luciferase Assay

1.0×10^5 Cos7 cells were seeded into each well of a 6-well cell culture dish and allowed to adhere and proliferate overnight. The next day the cells were washed twice in 1x PBS and 1.5 mL of Opti-MEM cell culture medium (Gibco) was added to each well. Cells were then co-transfected with 500 ng of hSkiPP pGL4.10 [luc2] reporter

vector and 500 ng of one of hScx-pcDNA3, p65-pCMV4, or empty expression vector (pcDNA3 or pCMV-Tag2B) using Lipofectamine 3000 (Invitrogen). Twenty-four hours later, cells were harvested and analyzed using a Dual-Luciferase assay kit (Promega) and the GloMax luminometer (Promega) according to the manufacture's directions. Individual samples were normalized to renilla expression.

For cells that were treated with recombinant human TNF α (rhTNF α ; Cell Signaling), 1.0 μ g hSkiPP pGL4.10 [luc2] was transfected into Cos7 cells using Lipofectamine 3000. Twenty-four hours after transfection, DNA:lipid complexes were removed and the media replaced. Cells were then treated with increasing concentrations of rhTNF α (1.0, 10, 100 ng/mL) or DMSO as a vehicle control. Twenty-four hours later, the cells were harvested for luciferase activity as previously described.

Protein Isolation and Western Blot Analysis

Whole cell protein lysate from P1 myofibroblasts and Cos7 cells was isolated, quantified, and analyzed as described in Chapter 3 (page 68-70) of this thesis. Primary antibody against p65 (1:1000; Cell Signaling) was used in a 5.0% BSA TBS-T solution overnight with constant shaking at 4°C. Secondary goat-anti-rabbit (1:20000; Jackson Labs) was diluted in a 3.0% skim milk TBS-T solution and incubated for 60 minutes at room temperature to detect the primary antibody. Blots were developed with Pierce ECL Western blotting substrate (Life Technologies). Protein bands were visualized using CL-Xposure blue X-ray film (Life Technologies). Images were acquired using the Bio-Rad densitometer GS-800 and protein expression determined using Quantity One Software (Bio-Rad).

Quantitative Real-Time PCR

1.0×10^6 P1 cardiac myofibroblasts were seeded onto a 100 mm cell culture dish and treated with Ad-LacZ, or HA-mouse Scx (Ad-HA-mScx) at an MOI of 50 at the time of plating. Twenty-four hours later the medium was removed and cells washed twice in 1x PBS and harvested in 1.0 mL Trizol Reagent (Life Technologies). Total RNA was isolated as per manufacturer's directions. Samples were treated with DNase I (NEB) to remove contaminating genomic DNA prior to cDNA synthesis as per the manufacturer's protocol. cDNA was synthesized using the BioRad iScript Select cDNA synthesis kit using random primers as per the manufacturer's protocol. qRT-PCR was performed using SYBR select Master Mix for CFX (Life Technologies) as per the manufacturer's protocol on a BioRad iQ5 in triplicate. The cycling conditions were as outlined in Table 12 and primers detailed in Table 13. Ski and Scx mRNA levels were calculated using the $2^{-\Delta\Delta C_t}$ method and normalized to GAPDH.

<u>Step</u>	<u>Event</u>
1	50°C 5 minutes 1x
2	95°C 5 minutes 1x
3	95°C 15 seconds
4	61°C 30 seconds – Plate Read
5	3-4 40x
6	55°C → 95°C – Melt Curve
7	End

Table 12. Real-time qPCR Cycling Conditions.

<u>Primer</u>	<u>Sequence (5'-3')</u>
Rat Ski Forward	CAGCATGCAGAGGCTGACC
Rat Ski Reverse	CCAGCTCAGCGTTGCTCTC
Scleraxis Forward*	AACACGGCCTTCACTGCGCTG
Scleraxis Reverse*	CAGTAGCACGTTGCCCAGGTG
GAPDH Forward	TGCACCACCAACTGCTTAGC
GAPDH Reverse	ACGGCCATCACGCCACAGC

Table 13. Real-time qPCR Primer Sequences. *As previously published by Bagchi and Czubyrt²⁰.

Statistics

All data is expressed as mean $\pm$ SE. Comparisons between 2 groups was accomplished using a Students *t*-test. Differences between multiple groups was compared using one-way ANOVA with a Student-Newman-Keuls *post hoc* test. A *P*-value less than 0.05 ($P < 0.05$) was considered significant. All statistics were performed using Graphpad Prism 5 statistical analysis software.

Results

In silico Analysis of the Ski Promoter

Like Scx, relatively little is known with regards to the transcriptional regulation of Ski. There is but one study that has shown Ski to be negatively regulated by PPAR δ ²⁹¹. Thus, we set out to determine whether the Ski promoter region contained any sites that may serve as putative transcription factor binding sites. We found that the human and mouse regions 5' to the ATG start codon were fairly well conserved,

however the rat sequence significantly diverged from that of human and mouse (Appendix C, Figure C-1 and C-2). Using the mouse and human promoter regions, we performed a Clustal W alignment and were able to identify several putative binding sites for transcription factors including bHLH proteins (E-box), and NF- κ B (Appendix C, Figure C-1 and C-2).

Scleraxis Does Not Regulate Transcription of Ski

In Chapter 3, we demonstrated that Scx is regulated at the transcriptional level by Ski not through its ability to inhibit TGF β /Smad signaling, but through Ski's ability to inhibit MAPK signaling through the repression of c-Jun. We next chose to explore the reverse relationship between Ski and Scx. We asked whether the Ski promoter is a direct target of the bHLH transcription factor Scx. To explore this relationship, we PCR cloned 1.8 kb of the hSkiPP into a pGL4.10 [luc2] reporter vector (hSkiPP pGL4.10 [luc2]) and conducted luciferase assays with overexpression of hScx. This region was chosen due to the presence of several potential putative transcription factor binding motifs for Scx (e.g.s E-boxes) as well as sites for NF- κ B. We found that there was no significant difference ($P > 0.05$; n=6) in the levels of luciferase expression between hScx and empty vector transfected cells (Figure 31).

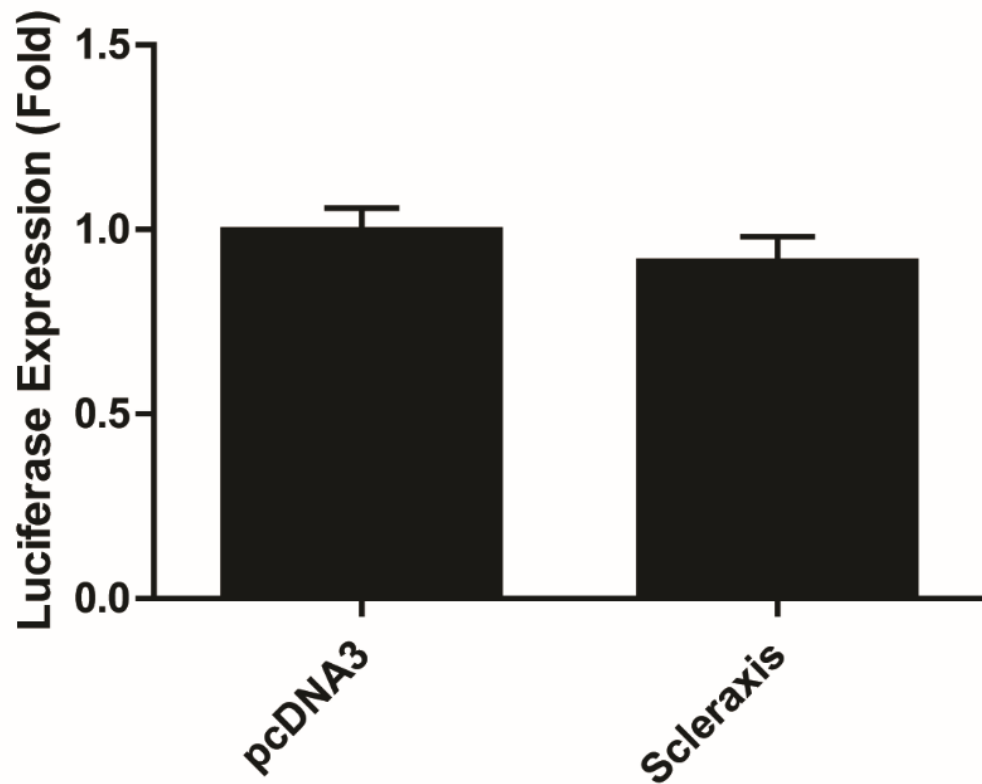


Figure 31. Scleraxis Regulation of the Human Ski Proximal Promoter. Co-transfection of Cos7 cells with hSkiPP and hScx yielded no significant change in luciferase reporter activity as compared to empty expression vector control. ($P > 0.05$; $n=6$).

To confirm our results in a more relevant model system, we infected primary P1 cardiac myofibroblasts with Ad-HA-mScx or Ad-LacZ at an MOI of 50. We obtained a 20-fold increase ($*P < 0.001$; $n=3$) in Scx mRNA expression with our adenoviral construct (Figure 32A). In agreement with our luciferase results, overexpression of Scx had no effect on Ski mRNA (Figure 32B) or protein (Figure 32C) levels in rat P1 cardiac myofibroblasts. These results taken together suggest that Ski gene expression may not be regulated by Scx.

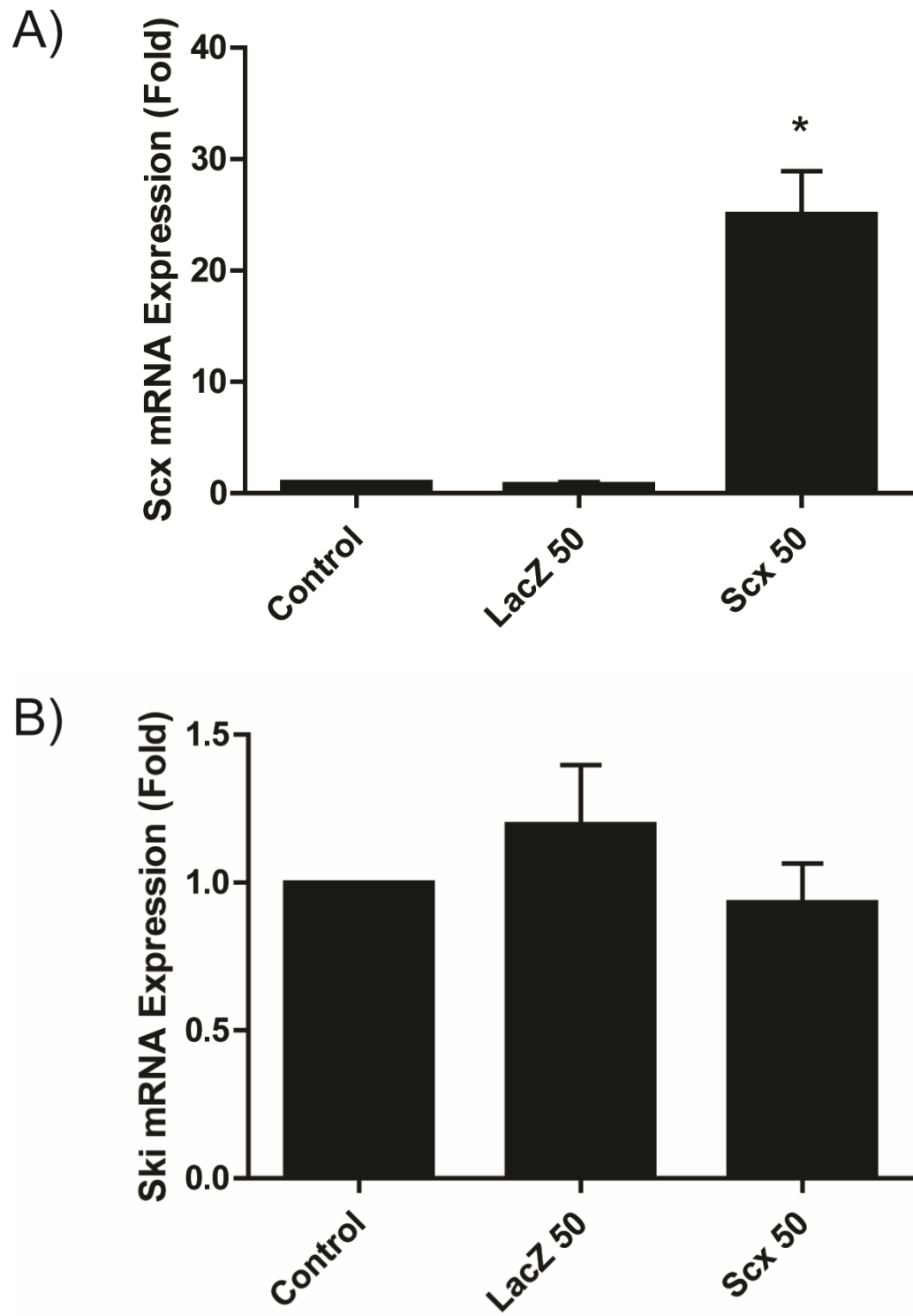


Figure 32. Scleraxis Overexpression in Primary Cardiac Myofibroblasts and Ski Expression.

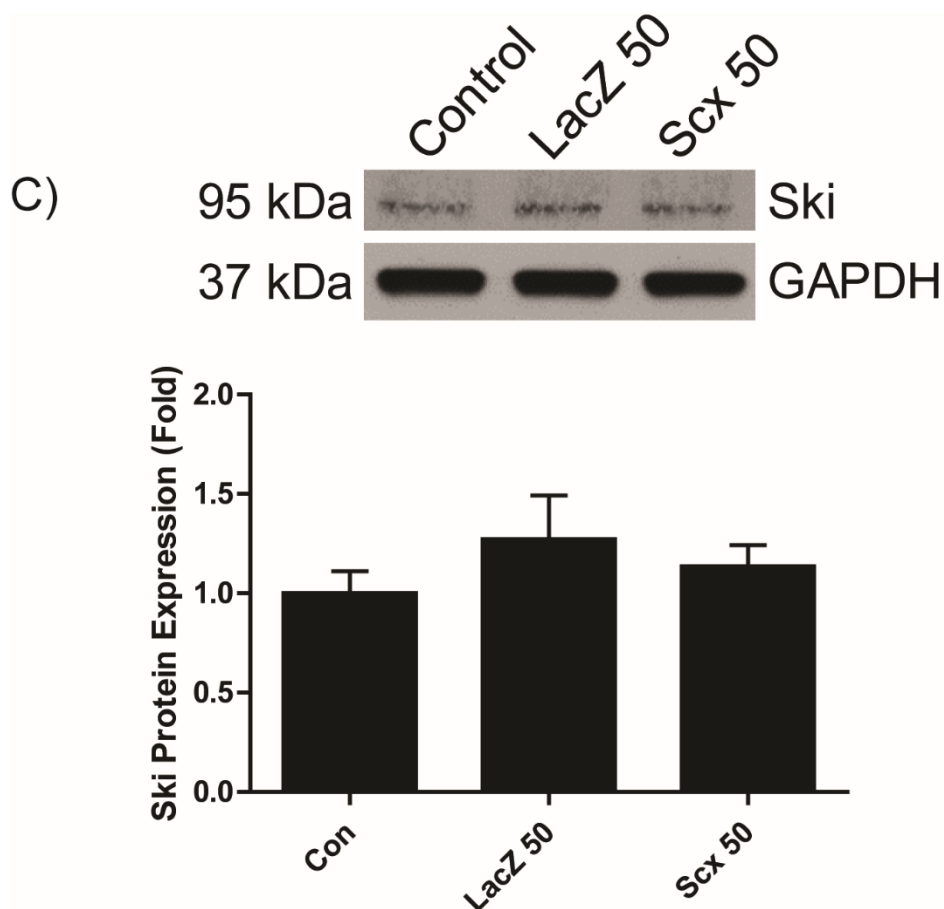


Figure 32. Scleraxis Overexpression in Primary Cardiac Myofibroblasts and Ski Expression. A) Ad-HA-mScx treatment of P1 cardiac myofibroblasts yielded a 20-fold increase in Scx mRNA levels as compared to Ad-LacZ and untreated controls ($*P < 0.001$; $n=3$). Ad-HA-mScx had no effect on Ski B) mRNA ($P > 0.05$; $n=3$), or C) protein ($P > 0.05$; $n=4$) levels in primary cardiac myofibroblasts.

p65-NF- κ B is a Negative Regulator of Ski Gene Expression

As Scx appears not to be a critical regulator of Ski gene expression, we sought to determine whether any of the other identified putative transcription factor binding sites were able to regulate Ski gene expression. From our Clustal W alignments of the human and mouse Ski promoters we identified a well conserved NF- κ B binding site that may play a role in regulating Ski. Using Cos7 cells we conducted luciferase assays using a p65-pCMV4 expression vector for 24 hours. Unlike Scx, overexpression of p65

resulted in a 50% reduction ($*P < 0.01$; $n=6$) in luciferase expression as compared to the empty vector control (Figure 33A). Western blot analysis of WCL demonstrated that we were able to obtain a 3-fold ($*P < 0.001$; $n=4$) increase in p65 protein expression over empty vector controls (Figure 33B).

We next determined whether $\text{TNF}\alpha$, a well known inducer of p65, could promote a response similar to that of p65 in our luciferase reporter assay. We treated Cos7 cells that had been transfected with our hSkiPP luciferase reporter with increasing concentrations of rh $\text{TNF}\alpha$ (1.0 ng/mL, 10 ng/mL, and 100 ng/mL) or vehicle (DMSO) control. We observed a 50-60% reduction in luciferase reporter construct with as little as 1.0 ng/mL $\text{TNF}\alpha$ as compared to our vehicle treated control (Figure 33C; $*P < 0.05$; $n=4$). Furthermore, although we performed a 10-fold dose response experiment, we found that there was no further reduction in luciferase expression with either 10 ng/mL or 100 ng/mL compared to 1.0 ng/mL. This suggests that although $\text{TNF}\alpha$ appears to be capable of repressing Ski expression, there is a limit to its potency as a 100-fold increase in $\text{TNF}\alpha$ could not further affect Ski luciferase expression.

To address Ski expression in response to p65 and $\text{TNF}\alpha$, we performed qPCR analysis on lysates isolated from Cos7 cells 24 hours after treatment. Both p65 and $\text{TNF}\alpha$ (10 ng/mL) treatment regimes resulted in a 50% reduction in Ski mRNA levels as compared to empty vector (Figure 33D; $*P < 0.05$; $n=3$) and vehicle treated (Figure 33E; $\#P < 0.001$; $n=3$) controls respectively.

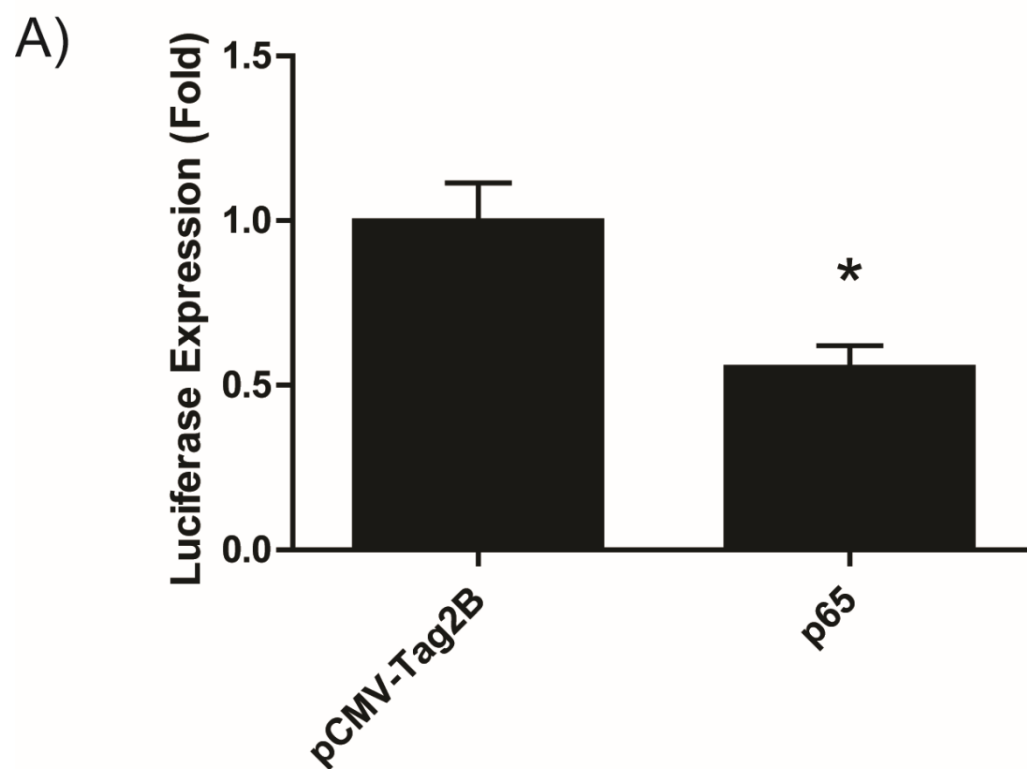


Figure 33. p65/TNF α -Mediated Regulation of the Human Ski Proximal Promoter.

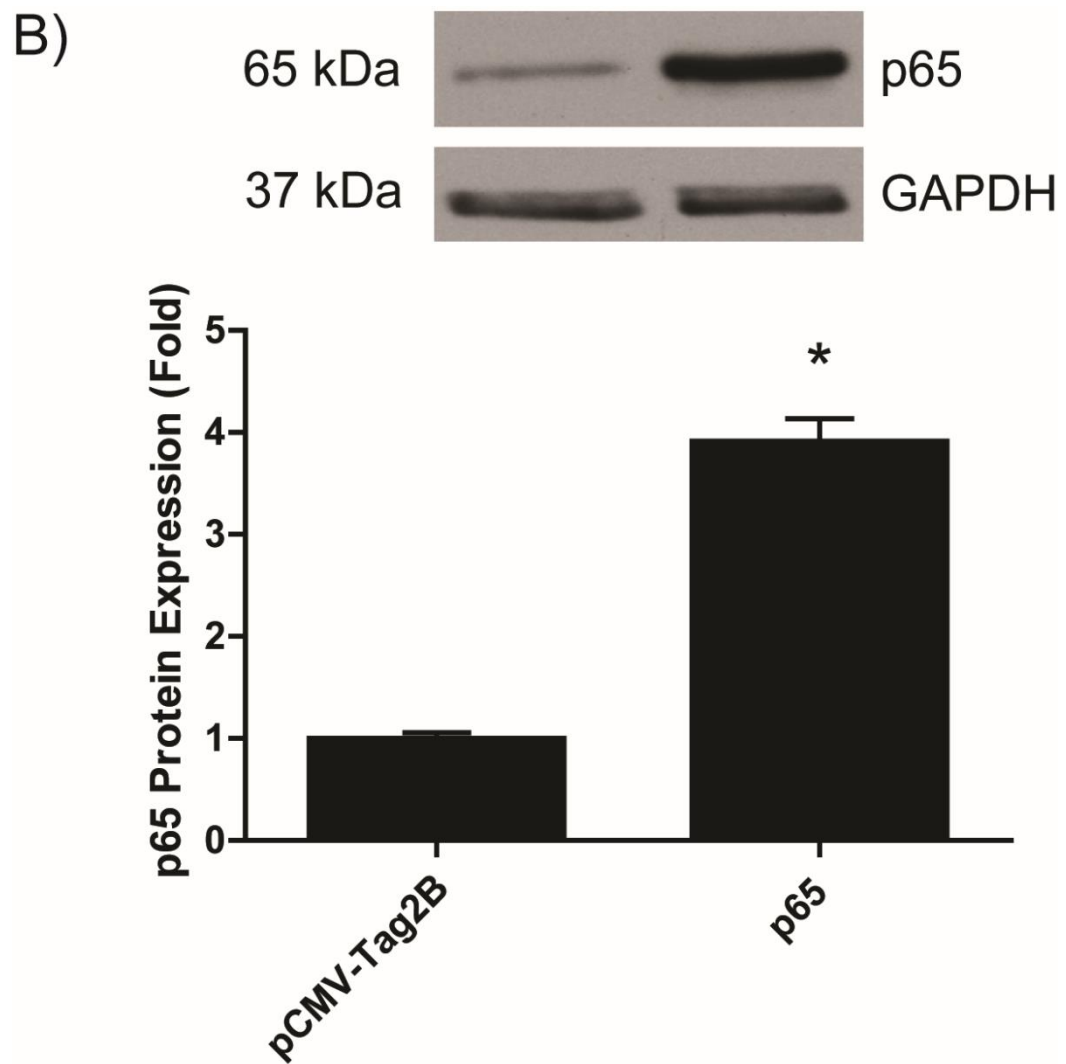


Figure 33. p65/TNF α -Mediated Regulation of the Human Ski Proximal Promoter.

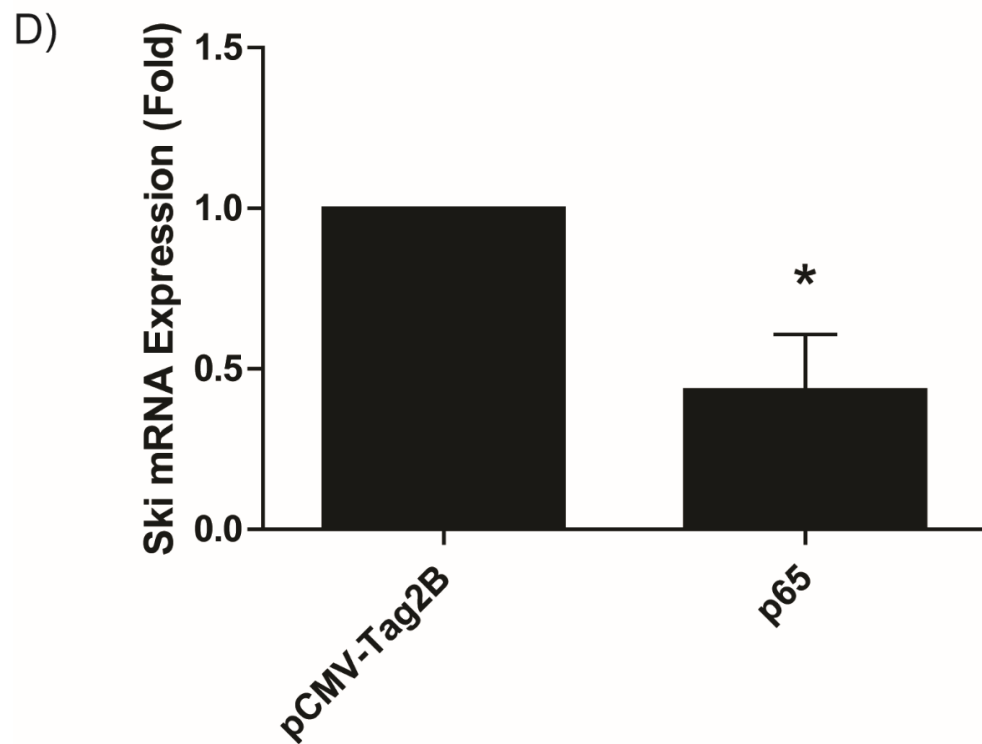
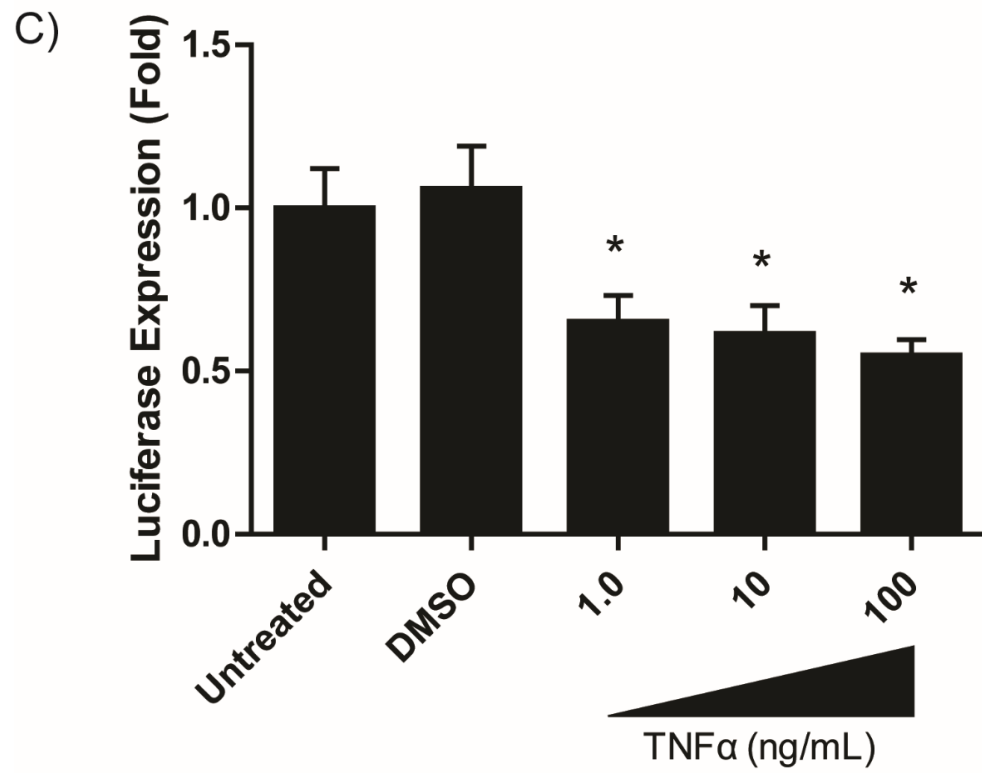


Figure 33. p65/TNF α -Mediated Regulation of the Human Ski Proximal Promoter.

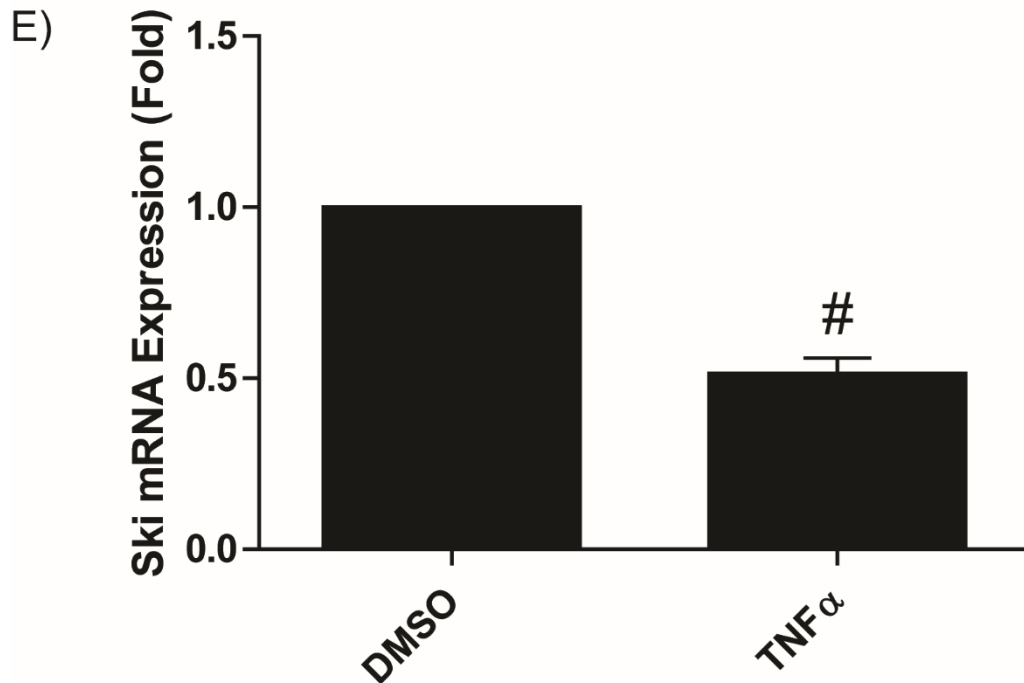


Figure 33. p65/TNF α -Mediated Regulation of the Human Ski Proximal Promoter.

A) Co-transfection of Cos7 cells with hSkiPP and p65 resulted in a 50% reduction in luciferase reporter activity ($*P < 0.01$; $n=6$). **B)** Co-transfection of Cos7 with hSkiPP and p65 yielded a 3-fold increase in p65 protein expression compared to empty expression vector control ($*P < 0.001$; $n=4$). **C)** Treatment of Cos7 cells transfected with hSkiPP alone for 24 hours demonstrated a 40% reduction with as little as 1.0 ng/mL of TNF α in luciferase expression as compared to untreated and DMSO treated controls ($*P < 0.05$; $n=4$). There was no significant difference in luciferase expression with increasing doses of TNF α . **D)** Transfection of Cos7 cells with p65 for 24 hours resulted in a 50% reduction in Ski mRNA levels as compared to empty vector controls ($*P < 0.05$; $n=3$). **E)** TNF α treatment (10 ng/mL) of Cos7 cells for 24 hours resulted in a significant (50%) reduction in Ski mRNA levels as compared to vehicle control ($^{\#}P < 0.001$; $n=3$).

Discussion

In the current study we have provided insight into the molecular mechanisms that govern the transcriptional regulation of Ski. Specifically, we have demonstrated that the bHLH transcription factor Scx has no discernible effect on Ski transcription.

Co-transfection of Cos7 cells with Scx and our hSkiPP reporter construct did not result in elevated luciferase expression suggesting that Scx does not mediate Ski transcription. As we cloned only 1.8 kb of the hSkiPP, and hence may have missed a true functional E-box for Scx, we conducted follow-up studies in primary P1 rat cardiac myofibroblasts using an adenoviral construct to overexpress Scx. Twenty-four hours of Scx expression did not alter Ski transcript (mRNA) or protein levels, thus corroborating our luciferase reporter assays. If our promoter reporter construct had excluded a functionally significant binding domain for Scx, our adenoviral studies would have conflicted those of our luciferase experiments and suggest that we need to look further upstream of the promoter region we had isolated.

Scx is a bHLH transcription factor that can form homo- and hetero-dimers with itself and other bHLH proteins such as E12 or E47^{334, 360-362}. It is possible that in our model system, overexpression of Scx alone may not be sufficient to promote changes in Ski expression. Consultation of the literature indicates that this notion is not unreasonable, as a previous study conducted by Lejard *et al.* demonstrated that Scx/E47 heterodimers formed a functional complex that bound to the TSE2 site within the collagen 1 α 1 minimal promoter that contained an E-box binding domain whereas Scx/E12, Scx/Scx, E12/E12, and E47/E47 hetero- and homo-dimers were unable to transactivate the promoter³³⁴. Further support for this view is provided by data from a study conducted in 2000 by Carlberg *et al.* showing that Scx formed a heteromeric complex with E47 to significantly upregulate the muscle creatine kinase (MCK) promoter³⁶⁰. Neither Scx nor E47 on its own could promote a response as robust as Scx/E47 could when co-expressed. Scleraxis/E12 hetero-dimer complexes have also

been shown to play a role in the regulation of the MCK promoter. Cserjesi *et al.* found that the Scx/E12 complex can bind to the MCK enhancer element³⁶¹. Furthermore, they found that Scx/E12 could transactivate their chloramphenicol acetyl transferase (CAT)-reporter construct indicating a functional role for this complex in the regulation of MCK³⁶¹. Scleraxis/E47 complexes have also been demonstrated to regulate the expression of Sox-9 cooperatively with p300³⁴³.

As Scx appears not to be a major regulator of Ski expression as hypothesized, we set out to examine the potential importance of other transcription factors that were identified to contain a DNA binding motif within the hSkiPP. Careful analysis of the hSkiPP DNA sequence demonstrated the presence of a single NF- κ B binding domain that was well conserved between mice and humans. Thus, we explored the effects of p65 (a subunit of NF- κ B) and TNF α (an upstream cytokine to NF- κ B activation) on hSkiPP expression. Co-transfection of p65 and our hSkiPP revealed a 50% reduction in luciferase expression as compared to untreated controls. Our studies using a 10-fold dilution of TNF α demonstrated the same result to that seen with p65 over expression. However, there appears to be a maximal effect for TNF α on repressing Ski expression as there was no significant difference between those cells treated with 1.0 ng/mL compared to those treated with 10 or 100 ng/mL. To evaluate whether TNF α exerted an effect on Ski transcript levels, we measured Ski mRNA levels following p65 and TNF α treatment. In both instances, we found a significant 50% reduction in Ski mRNA levels as compared to empty vector and vehicle treated controls after 24 hours, further corroborating our luciferase studies. Due to the lack of an antibody to recognize Ski protein levels within Cos7 cells (monkey kidney fibroblasts), we were unable to

measure the effect that p65 and TNF α have on Ski protein. Nevertheless, we have preliminary data that indicates that p65 is a component of a transcription factor complex that binds to a κ B motif within the hSkiPP as demonstrated by ChIP (Appendix D, Figure D-1). This preliminary result suggests a DNA:protein interaction between p65 and the hSkiPP that may be important in the transcriptional regulation of Ski.

Our current study is the first to demonstrate a role for TNF α /p65 regulation of Ski. We are not, however, the first group to propose a role for p65 and NF- κ B in the pathogenesis of fibrosis and heart failure. In a mouse MI model, Hamid *et al.* found that there was a sustained and myocyte-localized activation of p65 that correlated with increased fibrotic remodelling of the LV, increased apoptosis, and decreased animal survival²⁶³. Using a myocyte specific transgenic model of chronic p65 activation, they found that p65 provoked greater tissue remodelling and reduced cardiac function as compared to WT controls²⁶³. Recent studies have shown that integrin-linked kinase (ILK) can activate NF- κ B and deletion of ILK results in spontaneous heart failure^{363, 364}. A 2014 study by Thakur *et al.* looked at the effects of ILK in neonatal cardiac fibroblasts³⁶⁵. They found that ILK could promote collagen gene expression and that overexpression of I κ B α (an endogenous inhibitor of NF- κ B signaling) could significantly repress collagen gene expression. Furthermore, knockdown of ILK significantly repressed angiotensin II mediated collagen production³⁶⁵. Ultimately, they demonstrated that ILK promoted the activation of NF- κ B and translocation of p65 to the nucleus to promote a pro-fibrotic gene program³⁶⁵. Based on our current dataset and those previously published, it is reasonable to postulate that p65 NF- κ B may play a significant role in the fibrotic remodelling process in cardiac myofibroblasts. The

precise importance of p65-mediated repression of Ski in the cardiac myofibroblasts is only now beginning to be brought to light and merits further evaluation.

We are not the first group to define a role for a transcriptional regulator of Ski. Li *et al.* 2012 defined a role for PPAR δ in the activation of Ski gene expression²⁹¹. They clearly demonstrated that the synthetic PPAR δ ligand GW501516 could promote Ski expression at both the mRNA and protein levels²⁹¹. Furthermore, they showed that GW501516 could promote Ski gene expression *via* their luciferase constructs containing the rat Ski promoter. This study provided support and further evidence to previous work published by Teunissen *et al.* who demonstrated that PPAR δ inhibited cardiac fibroblast proliferation and phenoconversion into a cardiac myofibroblast³⁶⁶. These studies and our current dataset provide insight into the molecular mechanisms that govern Ski expression and the phenotype of cardiac fibroblasts and myofibroblasts.

Conclusions and Future Directions

For the first time, we have demonstrated a role for p65 and TNF α in the transcriptional repression of Ski. We believe that p65 NF- κ B expression may play a significant role in repressing Ski following an MI, thus promoting the formation of a pro-fibrotic environment. However, more work needs to be done to further define the role of p65 and TNF α in the transcriptional regulation of Ski. Specifically, studies aimed at elucidating direct DNA:protein interactions between p65 and the promoter region of Ski need to be done to show the functional significance of p65. Moreover, translation of these findings from cell lines into a primary fibroblast and myofibroblast cell culture model would provide further strength to the relationship between p65 and Ski expression. The lack of an antibody able to detect endogenous Ski protein levels in

lysates isolated from Cos7 cells is another limitation to our current study. Translating our findings into a cell model in which our Ski antibody does detect endogenous Ski protein levels would not only strengthen our current dataset, but also provide evidence towards the conserved nature of Ski protein and its regulation by TNF α . Finally, understanding the spatial, temporal, and sub-cellular localization/expression pattern of these proteins using an *in vivo* animal MI model would lead to a more thorough understanding of the mechanisms associated with the regulation of anti-fibrotic genes in the post-MI heart and may lead to better therapeutic strategies to combat maladaptive cardiac wound healing.

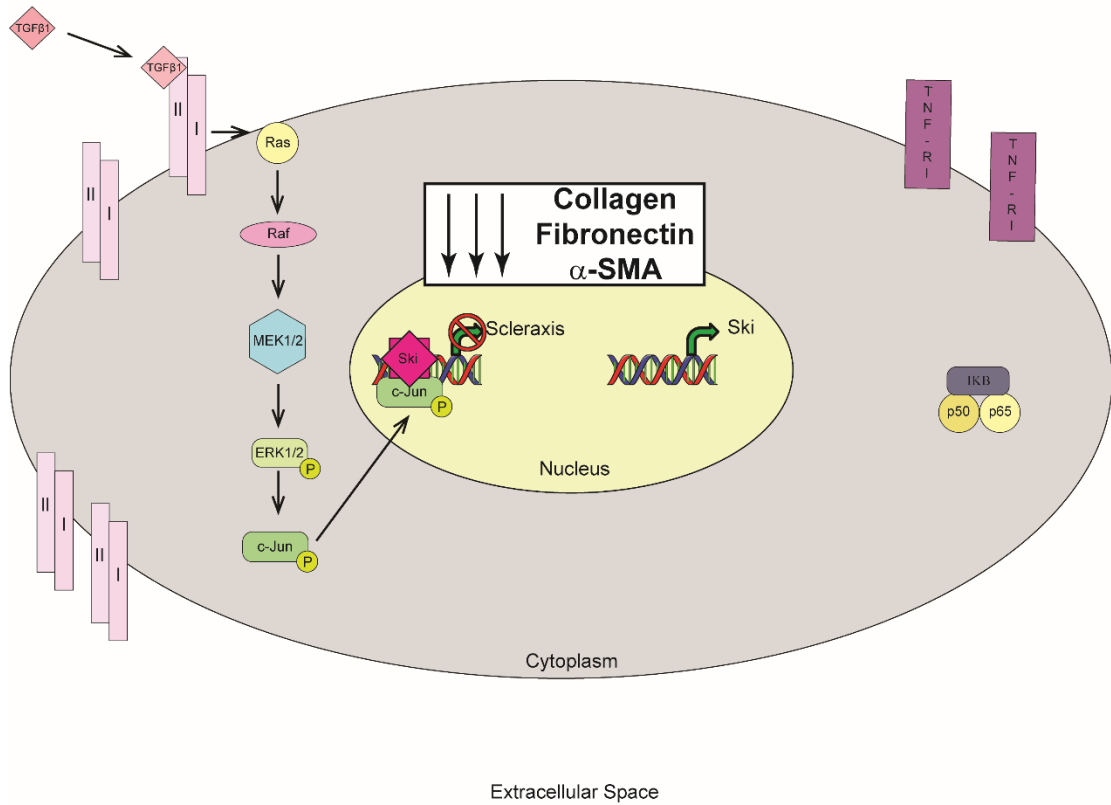
In the current study, we also provided evidence against the transcription factor Scx as being a novel negative regulator of Ski. We could not detect any difference in Ski mRNA or protein expression with Scx overexpression in our model systems. As discussed above this may not represent the true role of Scx in the regulation of Ski as many models have shown Scx to be unable to promote gene expression as a homo-dimer. Scleraxis required dimerization to other bHLH proteins to form a transcriptional hetero-dimer complex to alter gene expression. Thus, further studies need to be undertaken with Scx and other bHLH proteins to better understand their role, if any, in the transcriptional profile of Ski. Co-transfection of cells with both Scx and E12 or E47 in all combinations would provide further evidence for or against bHLH proteins in the regulation of Ski. Although Scx does not appear to be a regulator of Ski expression, it may still have an effect on the sub-cellular localization of Ski. A study previously published by our laboratory demonstrated an accumulation of Ski within the cytoplasm post-MI. As both Ski and Scx are nuclear proteins, it may be possible that Scx regulates

some process that marks Ski for nuclear export, thus freeing itself and other pro-fibrotic transcription factors, such as Smads, to promote pro-fibrotic gene expression.

Chapter 5: Overall Discussion, Conclusion, and Future Directions

Increased deposition of fibrillar collagens by myofibroblasts in the injured heart is considered a primary contributor to cardiac dysfunction and heart failure. Of the many molecular mechanisms that have been described to promote the myofibroblast phenotype and production of fibrillar collagens and matrix proteins, the TGF β pathway has been implicated as the primary contributor to the pro-fibrotic response. Data from previous studies indicate that TGF β and Smad proteins are potent inducers of Scx however these studies neglected to evaluate the direct DNA:protein transcriptional mechanisms of this relationship^{20, 334, 337, 345}. Our current data indicate that although TGF β is indeed a potent inducer of Scx, it does so through a Smad-independent signaling pathway. This result is not overly surprising due to the large amount of cross-talk between TGF β and other signaling pathways. However, the interplay between these vast number of molecular signaling pathways makes it difficult to suggest that any one of these Smad-independent pathways is the only contributor to Scx expression. It is likely that several of these pathways are intertwined such that in the setting of cardiac injury, Scx will be expressed to promote the wound healing response no matter what. This is ultimately a limitation of our study as we did not evaluate the effects of the 4 other well-defined TGF β /Smad-independent signaling pathways that could be elicited by TGF β and thus constrain the current study. Although we began to define the role for Smad proteins and p44/42 MAPK signaling in the cardiac myofibroblasts, a complete block of Scx expression following TGF β ₁ stimulation using the MEK1/2 inhibitor U0126 was not observed. Thus, it is likely that there is at least one other Smad-independent pathway that may be playing a role in regulation of Scx.

Quiescent Fibroblast State



Activated Myofibroblast State

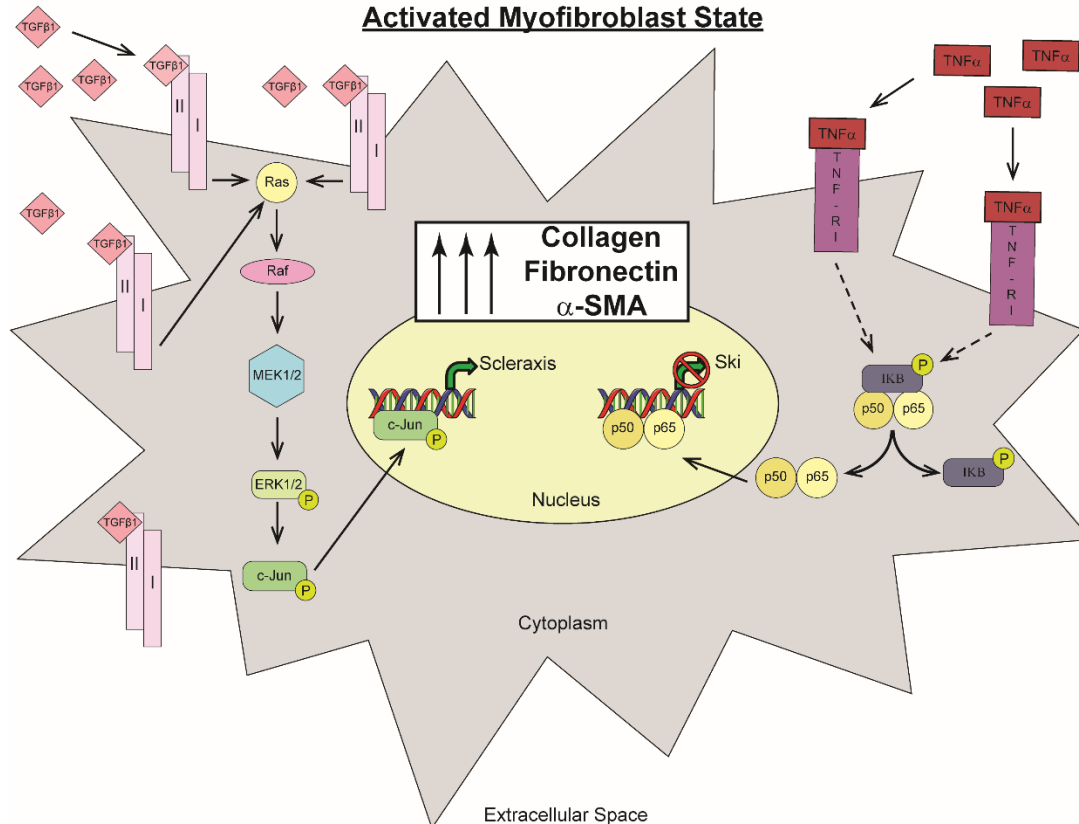


Figure 34. Schematic Summary for the Transcriptional Regulation of Ski and Scleraxis in the Primary Cardiac Myofibroblast and its Proposed Role in Fibrogenesis. In the quiescent state there is a small amount of free TGF β ₁ present to maintain the slow turnover of the matrix proteins. Ski is present and acts as a nuclear transcriptional repressor of pro-fibrotic signals, such as Scx, by inhibiting TGF β ₁-Smad independent c-Jun mediated transcriptional activation of Scx resulting in a net negative regulation and minimized deposition of ECM in the healthy heart. During periods of injury, such as following a large MI, Ski transcription is turned off by TNF α -p65 signaling. Repression of Ski protein and the up-regulation of TGF β ₁-Smad independent signaling allows for the derepression and transcription of the pro-fibrotic regulator Scx. Increase Scx protein in the cell can then act in a synergistic manner with Smad proteins to promote collagen and matrix synthesis.

Ski has been well defined as a potent, endogenous inhibitor of TGF β /Smad signaling. It has been shown to play a role in both the cancer and chronic wound healing settings through its interactions with Smad proteins. Our laboratory has previously defined a role for Ski expression in repressing the cardiac myofibroblast phenotype. Although a role for Ski was defined in the setting of the myofibroblast, little is known with regard to the mechanisms that regulate Ski gene expression. It has been previously published that although SnoN, a member of the Ski-Sno Superfamily of TGF β inhibitors, expression is transcriptionally regulated following TGF β stimulation, Ski does not share this feature. Following TGF β stimulation, SnoN is transcriptionally repressed by a negative feedback loop mechanism^{296, 367}. The promoter region of SnoN was identified to contain critical SBE motifs that played a central role in its regulation following TGF β ³⁶⁷ stimulation. However, Ski does not contain such motifs. One recent study has demonstrated that the promoter region of rat Ski contains a functional PPRE for PPAR δ that promotes Ski expression²⁹¹ and PPAR δ has additionally been shown to repress the cardiac myofibroblast phenotype³⁶⁶. Here we have defined a role for TNF α and p65 in the negative regulation of Ski expression. Following an MI, there is a

massive influx of inflammatory cytokines (including $\text{TNF}\alpha$) that initiate and propagate the wound healing response. As Ski is a negative regulator of collagen production it is logical to postulate that Ski might be repressed in the inflammatory setting thus paving the way for wound closure. Our studies into the transcriptional regulation of Ski do have several limitations. Although we defined a role for p65 in inhibiting Ski gene expression, we did not demonstrate DNA:protein interactions that would show a direct relationship between p65 and Ski expression. Additionally, the lack of an adequate antibody to detect endogenous Ski protein levels limits the scope of our findings. Furthermore, our studies in the regulation of Ski are confined to Cos7 cells. Whether $\text{TNF}\alpha$ and/or p65 have any significant role in regulating Ski expression in primary cardiac myofibroblasts merits further investigation.

Based on our studies, we conclude that:

- 1) Ski is central negative regulator of Scx gene expression;
- 2) $\text{TGF}\beta_1$ stimulation of Scx occurs through a Smad-independent pathway that includes p44/42 and c-Jun in primary cardiac myofibroblasts; and
- 3) $\text{TNF}\alpha$ and p65 are powerful repressors of Ski gene expression.

Further studies into the regulation of these two critical endogenous factors, which themselves serve as regulators of the fibrotic response, may lead to the development of novel therapeutic strategies that target one of, or both, proteins to alleviate chronic fibrosis in the post-MI setting.

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Appendices

Appendix A

Clustal W of the Mouse, Human, and Rat Scleraxis Proximal Promoter

MouseScxProm	-----GTCACAGGGTGGATCATGATGTCACCGCTGAAGAAGTCCACAGCTGGCTATG
HumanScxProm	-GCGGTTGGTCACCGGGTGGATCATGACGTCCCCGCTGAAGAAGTCGACAGCCGGCTGGG
RatScxProm	AGCGGTTGGTCACCGGGTGGATCATGACGTCCCCGCTGAAGAAGTCGACAGCCGGCTGGG

MouseScxProm	GAGAGAGGCAAAAGATGT-TAGGTTGGGGGGGCGAGGGCGTTCATGGA--CTTCAGA----
HumanScxProm	GGAGAAGATGTGGGCGTGTGGGCCAGAGTGGCTGAGGCCCTGCTGGTGCCTCCAGGACCC
RatScxProm	GGAGAAGATGTGGGCGTGTGGGCCAGAGTGGCTGAGGCCCTGCTGGTGCCTCCAGGACCC
	* ** * * * * * * * * *
MouseScxProm	--CTGT--AGGGCCAACCGTTGTTGCCTACCTCATAGGGATTGAAGCCTGAATCTCCG
HumanScxProm	CCGCCGCCAGGGGCCAGCCCTGCCACCTACCTCATAGGGGTTGAAGCCACATCCCCA
RatScxProm	CCGCCGCCAGGGGCCAGCCCTGCCACCTACCTCATAGGGGTTGAAGCCACATCCCCA
	* * ***** * * ***** ***** * *
MouseScxProm	AACTGGCCTCTCTGAAGCCGGTGTACCAGGGCAACCTGCTCATCAGTTAGCCGCAGATCA
HumanScxProm	AACTGGCCACTCTGCAGCCGCCGCCAGGGCCACCTGCTCATCCGTCACTCTCAGGTCC
RatScxProm	AACTGGCCACTCTGCAGCCGCCGCCAGGGCCACCTGCTCATCCGTCACTCTCAGGTCC
	***** ***** * ***** ***** * * * *
MouseScxProm	CGCCCTGTCATCTTGTCTTGCACAGTGCGCCTGAGGGAAAGCCAGTCAGG-----GC
HumanScxProm	CGCCCTGTCATCGGGTCCTGCACGGTGCGCCTG-GAGACCGCCAGTCAGACCCGCCAGC
RatScxProm	CGCCCTGTCATCGGGTCCTGCACGGTGCGCCTG-GAGACCGCCAGTCAGACCCGCCAGC
	***** * * * * * * * * * *
MouseScxProm	CATACTCCAGGCTTGAGG-----GACCACAGGTCCCACCCAGGTCCAACCTACCA
HumanScxProm	CCTGCCCCACCCCTCGGGCCACCCCGGCTGCTGGCCCCACCCACCCAGCC--CTCACCA
RatScxProm	CCTGCCCCACCCCTCGGGCCACCCCGGCTGCTGGCCCCACCCACCCAGCC--CTCACCA
	* * * * * * * * * * *
MouseScxProm	GAAATCTGGGTCATCCATTTTGTCCAGAACTGGTCGAGCTCATCTCGTGTCCGAGGGG
HumanScxProm	GTAGTCAGGATCGTCCATCTTGTCCAGGAAGTGGTCCAGCTCATCCGGGTCCGAGGGG
RatScxProm	GTAGTCAGGATCGTCCATCTTGTCCAGGAAGTGGTCCAGCTCATCCGGGTCCGAGGGG
	* * * * * * * * * * *
MouseScxProm	CTTGTAGATACGTTTGCCATCCAGGTCATAACCCACATGTGGGAATTCATCGTACCATGC
HumanScxProm	CTTGTAGATGCGCCTGCCATCCAGGTCGTAGCCACGTGGGGGAAGTCATCGTACCACTC
RatScxProm	CTTGTAGATGCGCCTGCCATCCAGGTCGTAGCCACGTGGGGGAAGTCATCGTACCACTC
	***** * ***** * * * * * *
MouseScxProm	CAGGGGCACATTGCCACAGTGTTCCGAATGTCCTGTAGC--ATAGTCACCACGTCAGCA
HumanScxProm	CAAGGGCACGTTGCCACCGTGTTCCGGATGTCCTGCAGCCGGGGGCCACCATGTCGGCA
RatScxProm	CAAGGGCACGTTGCCACCGTGTTCCGGATGTCCTGCAGCCGGGGGCCACCATGTCGGCA
	** ***** ***** ***** * * * *
MouseScxProm	CCTGTCCCTGCCATGCTTGATCCT-CTTCAAACCACCCAAGG--GCAGAAGAGCCACT--
HumanScxProm	TCTGATCCTACAAGGCCACCCCGCTTCGGGCCGCCAGGGGAGCAGGTGAGGGGGTTG
RatScxProm	TCTGATCCTACAAGGCCACCCCGCTTCGGGCCGCCAGGGGAGCAGGTGAGGGGGTTG
	** * * * * * * * * * *
MouseScxProm	-----CAAGATCAGCCTCAAGTGCCAGAACCAAGTGACCCAAAATCCGCCCT
HumanScxProm	CAGGGGGAGTGCAGGCCAGCCTCAAGTGCCCTGAGGTCACTGGCCCTGACCTACGCCTG
RatScxProm	CAGGGGGAGTGCAGGCCAGCCTCAAGTGCCCTGAGGTCACTGGCCCTGACCTACGCCTG
	* * ***** * * * * * *
MouseScxProm	AGCCTTGGCCCCGATGTCTGACGTGGCGCGGCAGGGGTGCTATGCCCTCCCCAAGCCC
HumanScxProm	TTCCCCAGCCCTGGGGTCTGACGTGGCATGGCGGCCACGCTGC-CCCCACCCCC--GCC
RatScxProm	TTCCCCAGCCCTGGGGTCTGACGTGGCATGGCGGCCACGCTGC-CCCCACCCCC--GCC
	* * * * * * * * * * *

MouseScxProm	TCGGAGGCGCCTGAGCTTTACCTTCACATTTTCCACTGAAGTGATGCCAACCCCTAGC-CT
HumanScxProm	TCGGAGGCGCCGGAGCTGCACCTTCACATTTTCCACTGAAGTGGTGCCAACCCAGAGCT
RatScxProm	TCGGAGGCGCCGGAGCTGCACCTTCACATTTTCCACTGAAGTGGTGCCAACCCAGAGCT

MouseScxProm	CCCCCTCACTGGATTCTTGCCCAAGGCTCTGGGCAGCCA
HumanScxProm	CCCCCTCACTGGATTCTTGCCCAAGGCTCTGGGCAGCCA
RatScxProm	CCCCCTCACTGGATTCTTGCCCAAGGCTCTGGGCAGCCA

MouseScxProm	CGCCTATGGGCTCCCTCCCTGGCAAAGCCTTAGGTACAAGTGGGTAGTTTACAAGGCA
HumanScxProm	CACCCACGGGCTCCCTCCCTGGCAGGGGCTTAGGCAGAAGTGGGCAGCT-ATAAGACG
RatScxProm	CACCCACGGGCTCCCTCCCTGGCAGGGGCTTAGGCAGAAGTGGGCAGCT-ATAAGACG
	* * *
MouseScxProm	GGAACCATGGGAAGACCCCA-----CCCCAACCTCAGGGAGAC--AAACCTGTTCTGCTC
HumanScxProm	GGGACTCGGGGAAGACCCCAACTGACCCATTCCACAGAGAGGCAGAAGCCAAGACCTCTC
RatScxProm	GGGACTCGGGGAAGACCCCAACTGACCCATTCCACAGAGAGGCAGAAGCCAAGACCTCTC
	** * *
MouseScxProm	GGCAGGTGGTCAAGGACCCCTTCCAGCACCCTGATCCTC--CCCCTCCCATTTCAGTG
HumanScxProm	TGTGGG-----GGACCCCT-----CACCGTCTGCCCTCGGCCCTCCC-----CA
RatScxProm	TGTGGG-----GGACCCCT-----CACCGTCTGCCCTCGGCCCTCCC-----CA
	* * *
MouseScxProm	GGCAGAGCAGCCACATTCTGGACACACATTCCTGGTTTTCAGCCCTGCCAGCCACCACC
HumanScxProm	GGCAGAACAGCCACGTTCTGGACGTACATTCTGGTTTCAGCCCTGCCAGCCGCCACC
RatScxProm	GGCAGAACAGCCACGTTCTGGACGTACATTCTGGTTTCAGCCCTGCCAGCCGCCACC

MouseScxProm	ACCCTCACTCCTCCCAGCCTGGGGACTGCTGCCCAGGCGGCCACTTCTGCAACAGTAGC
HumanScxProm	---CTCGCTCCTTCCAGCCTGGGGACCGCTGCCAGGAGGCTGCTTCTGCAACAGTAGC
RatScxProm	---CTCGCTCCTTCCAGCCTGGGGACCGCTGCCAGGAGGCTGCTTCTGCAACAGTAGC
	** * *
MouseScxProm	CACAGGGAGGAGGGGCC
HumanScxProm	CACAGGGAGG--GGCCAGGCAAGACAGAGCACAGCTGGGAGCCTGCCGGGCCCTGC
RatScxProm	CACAGGGAGG--GGCCAGGCAAGACAGAGCACAGCTGGGAGCCTGCCGGGCCCTGC
	** * *
MouseScxProm	AGCA---AGACCCCTTGGGCTCGTCCCCTGACCTGTCCACACCAGCCAGAAATGC
HumanScxProm	TGCAGGAGGGACCCCTTGGGCCCTGCCCC-CTGC---TG-----AGGGT
RatScxProm	TGCAGGAGGGACCCCTTGGGCCCTGCCCC-CTGC---TG-----AGGGT
	** * *
MouseScxProm	CCCTGCTCTGTGTACGCTCTCCACC---TTGAGGGCCACTTCCGAAGTGGAGGGAT
HumanScxProm	CCTTCCCTCTC-----CTCCCATGGAGATAGGGGGCCATCCCCAAATTGGATA-AT
RatScxProm	CCTTCCCTCTC-----CTCCCATGGAGATAGGGGGCCATCCCCAAATTGGATA-AT
	** * *
MouseScxProm	GGAGAGGTGCCGATCTTAGCTCTTATCCACGTGTGCACTGGCTTTGAGAAGCCCATGAA
HumanScxProm	GGGG-----TATGTGTGTGCT--TCCTGAGGGGCTGGTGGC
RatScxProm	GGGG-----TATGTGTGTGCT--TCCTGAGGGGCTGGTGGC
	** * *
MouseScxProm	GCATACAGCAAGGATGGGGGTGGGGCCCCCAAGCAGGATGGGTCAAAGGGGTAAAGATCTG
HumanScxProm	CC-TGGAG-AGGAACGGGGGAAGGAGGAAGGGGCAAGG-AGACCGACGGGTGAGAGGTC
RatScxProm	CC-TGGAG-AGGAACGGGGGAAGGAGGAAGGGGCAAGG-AGACCGACGGGTGAGAGGTC
	* * *
MouseScxProm	GCACAAGGCACAGGCCATGGAGTCAAGTCATGCCAACACACACCCATTCTAGACTG
HumanScxProm	-CACACAGCACAAGAACAGGGGACATGGCCAGCTAGCAGAAGGCACAG--TCTG--TG
RatScxProm	-CACACAGCACAAGAACAGGGGACATGGCCAGCTAGCAGAAGGCACAG--TCTG--TG

MouseScxProm	CTACTGCTGGAGTCACCCCATG-AGTGCACACACACACACTCTCTCTCTCTCTCT
HumanScxProm	CCAAGCCTGGAG--AGGCAGGTGCAGGA
RatScxProm	CCAAGCCTGGAG--AGGCAGGTGCAGGA
	* * *

MouseScxProm	CTCTCTCACATACAG
HumanScxProm	CT-----AAATACTCGCCTGTGCCCGCCCC-----TGACACAG
RatScxProm	CT-----AAATACTCGCCTGTGCCCGCCCC-----TGACACAG
	*** * * * * * * * * * *****
MouseScxProm	GCCTGGCACAAA-AGACCCAAGTGACATGGAAGG-AGAGT CAGAC AGGGTGTGGCCAGG
HumanScxProm	GCCTGGCACAGGCAGGCGCTGGCGCACATGGGAGGCAAAGAC CAGAC AGGGTGCGGTCAGG
RatScxProm	GCCTGGCACAGGCAGGCGCTGGCGCACATGGGAGGCAAAGAC CAGAC AGGGTGCGGTCAGG
	***** * * * * ***** * * * * *****
MouseScxProm	CACTCAGGGCCAGGGCAGCTGGGTGAACCTAGGCATGCCAGGAGAGAAAGCCTCTTGATT
HumanScxProm	CAG-CAGGGCCAGGGCGGCTGGGTAACTCAGTTGTGCCACGGGAGAAAACGGGGTGGT-
RatScxProm	CAG-CAGGGCCAGGGCGGCTGGGTAACTCAGTTGTGCCACGGGAGAAAACGGGGTGGT-
	** ***** ***** ***** ***** * ***** * **
MouseScxProm	CTTGATTTATTCTCTTCTGCCCTGTAGAATGGGTGGGATGGGCACCATTGTCTTGGGGCA
HumanScxProm	---GGGTTCTCCCTCT-CCCG--GGACGGG-----GGGACTGCAGTTTGGGGCC
RatScxProm	---GGGTTCTCCCTCT-CCCG--GGACGGG-----GGGACTGCAGTTTGGGGCC
	* * * * * * * * * * * ***** * * *****
MouseScxProm	CT-AGTAG---CACCCAGAAACG--CTCCAA-----CCAGAAA-----G
HumanScxProm	CTGAGTAACACAGCCAGAACGACCTCCAGTTCCTCCGCATCCCCAGAGACGGAACG
RatScxProm	CTGAGTAACACAGCCAGAACGACCTCCAGTTCCTCCGCATCCCCAGAGACGGAACG
	** ***** * ***** * * ***** ***** *
MouseScxProm	ATGCTC--GGGG-----GCCCTCT-----AGGCACGGCTATTAAAGGG
HumanScxProm	ATGCCCCCAAAGACCAGCCCCGCCCGCCCCACCCCGCCAAAGCGTGGCCAC--AGAAGG
RatScxProm	ATGCCCCCAAAGACCAGCCCCGCCCGCCCCACCCCGCCAAAGCGTGGCCAC--AGAAGG
	**** * * ***** * * * * * *
MouseScxProm	AAAAGGTGCACGCACGAGCTCTGAGTAACT-CCTCCAGGCCAGAAAGGGAGGCGGAGGC
HumanScxProm	CCGAGGGACGCG-GCGGGCGCTGCTCGAGGAGCCTCCGGGCTG--AGAGGGCGGGG--
RatScxProm	CCGAGGGACGCG-GCGGGCGCTGCTCGAGGAGCCTCCGGGCTG--AGAGGGCGGGG--
	* * * * * * * ***** * * ***** *
MouseScxProm	CAGGCGCGGGGAGGAGCTGA---CTGCTATAAAGGCACTGCTCGGCTGGCGGCCAC
HumanScxProm	CGTGCGCGGGGAGGGGCCGGGACCCGCTATAAAGGCGCAGCTCGG-----GGCCCCGC
RatScxProm	CGTGCGCGGGGAGGGGCCGGGACCCGCTATAAAGGCGCAGCTCGG-----GGCCCCGC
	* ***** * * ***** * ***** *****
MouseScxProm	TCCAGTCCGAA--CACATGTGCCCGCTCACCCGGCAGCCGCCACCGCGGGGCGCAGCG
HumanScxProm	TCCGGCCCCGGACGCACATGTGCGCGCGACGCCCGGCAGCTGCCACCGCGGGGCGCAGCC
RatScxProm	TCCGGCCCCGGACGCACATGTGCGCGCGACGCCCGGCAGCTGCCACCGCGGGGCGCAGCC
	*** * * ***** * * ***** *****
MouseScxProm	GAGACCCC-----TGGCCCG-GCGGCTGTGGGGACCTAAAGAGCGGCA
HumanScxProm	GAGACCCCGCGCCTCGCCCCGGCGGCGCGAGGCCGCGGCGGCCGAGGAGCGGCA
RatScxProm	GAGACCCCGCGCCTCGCCCCGGCGGCGCGAGGCCGCGGCGGCCGAGGAGCGGCA
	***** ***** * * * * * *****
MouseScxProm	TGAGCAGCGCACGGTGGAGCTGACGCCGCGCCCCCTGCCCGGCC ATG
HumanScxProm	TGAGCAGCGCGACAGAGCTGACGCCGCGCCCC--GCCGGCC ATG
RatScxProm	TGAGCAGCGCGACAGAGCTGACGCCGCGCCCC--GCCGGCC ATG
	***** * ***** ***** * * *****

Figure A-1. Clustal W of the Mouse, Human, and Rat Scleraxis Proximal Promoter. Alignment of the mouse, human, and rat Scleraxis proximal promoter regions was conducted using Clustal W software from ExPASy. The star (*) beneath the alignment marks bp that are conserved between both species. Green highlighted text marks the beginning of the ORF for each species. Pink highlighted sequences indicated the locations of potential SBE.

Clustal W of the Mouse, Human, and Rat Scleraxis Distal Promoter.

```

MouseScxPromDistal  ----AGAGTG  GGTATCGGGG  GTGGAAGGCC  ACAGCCCGCA  AGGCCTTCTT
HumanScxPromDistal  -----CA  GGC-TCGG--  GCTGAAGG--  -----
RatScxPromDistal    GCAAAGAGTG  GGTATCGGGG  GTGGAAGGCC  ACAGCCCGCA  AGGCCTTCTT
Clustal Consensus    **  ****  *  *****

MouseScxPromDistal  GTGGTGCCTA  TGGACAGGGA  GTGTGTTTAC  AGTGGGCTCC  AGGTGAAGGG
HumanScxPromDistal  -----  -----GA  G-----  ---GGGCTCT  -GCTG-----
RatScxPromDistal    GTGGTGCCTG  TGGA---AGA  GGGTGTATTAT  AGTGGGCTCC  AGGTGAAGGA
                      **  *  *****  *  **

MouseScxPromDistal  GCCGTCCAC  CTACCTCACA  --ACCCACCT  -ACCTCAGCA  CTTGTATATGG
HumanScxPromDistal  GCCGCCT---  CCACCCCA  --GCTTTCCT  CACCTCAGCA  TCCTGTATGG
RatScxPromDistal    GCCCTTCTAC  CTACCTCACA  CAACCCACCT  TACCTCAGCA  CTTGTATATGG
                      ***  *  ***  *  **  *  ***  *****  *****

MouseScxPromDistal  CTTGGTGGAA  AGATCCAGGT  CAAACCACAC  CAGTTTGCTG  TCATAGCTGC
HumanScxPromDistal  CTTGGTGGAA  AGATCCAGGT  CAAACCACAC  CAGCTTGCTA  TCGTAGCTCC
RatScxPromDistal    CTTGGTGGAA  AGATCCAGGT  CAAACCACAC  CAGTTTGCTG  TCATAGCTGC
                      *****  *****  *****  ***  *****  **  *****  *

MouseScxPromDistal  CACAGATGAT  GTTGTACCT  AGGGCCAGAA  GCAGCG----  -----
HumanScxPromDistal  CACAGATGAC  GTTGTACCT  AGGGCCAAAG  GCTGTGATGC  AGGTGTTCCC
RatScxPromDistal    CACAGATGAT  GTTGTACCT  AGAGCCAGAC  ACAGTG----  -----
                      *****  *****  **  *****  *  *  *  *

MouseScxPromDistal  -----CCT  GTAA-----  ---TTCTCAC  -GAGATGCAC  A-----
HumanScxPromDistal  GCTCTCACCT  GCAGGGTGCA  CTGCCCTGCC  CGTCACCTAC  ACCCCTCACC
RatScxPromDistal    -----TCT  GTAA-----  ---TTCTCCC  TAAGACCTAC  G-----
                      **  *  *  **  *  *  *  *

MouseScxPromDistal  ----GGGTTC  ACT-CCCCTA  CCC-----  TGATGTGGAG  CATCCAT-GC
HumanScxPromDistal  TGCAGGGTGC  ACTGCCCTA  CCCCCACCC  TCACCTGCAG  GGTACACCGC
RatScxPromDistal    ----GGGCTC  ACT-CCCCTG  CCC-----  TGACTTGGAG  AATACAT-GT
                      ***  *  ***  *****  ***  *  *  *  *  *  *

MouseScxPromDistal  TCCCC-----  -----  -----  -----  ----TCACCT
HumanScxPromDistal  CCCCCCCCC  ACCCTCACC  TGCAGGGTGC  ACCGCCCCCA  CCCCTCACC
RatScxPromDistal    TCCC-----  -----  -----  -----  ----TCACCT
                      ***  *****

MouseScxPromDistal  GCTGGATGTA  CAGCCATGCT  GGACACCCAC  TTGCAGTTGG  GCATCAGCTT
HumanScxPromDistal  GCAGGGTGCA  CCGCCAGGCT  GGACACCCAC  TTGCAGTTGG  GCATCAGCTT
RatScxPromDistal    GCTGGATGCA  CAGCCATGCT  AGACACCCAC  TTGCAGTTAG  GCATCAGCTT
                      **  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

MouseScxPromDistal  CTTGGTTAGC  TCCTGGCGCA  GCAGGTGGTA  AATGCGGATG  CTGCGCTGGG
HumanScxPromDistal  CTTGGTGAGC  TCCTGGCGCA  GCAGGTGGTA  GAGGCGGACG  CTGCGCTGGG
RatScxPromDistal    CTTCGTAAGC  TCCTGGCGTA  GCAGGTGGTA  AATGCGGATG  CTGCGCTGGG
                      ***  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

MouseScxPromDistal  AGGCCACGAG  CAGGAAGGGC  CGGGAGGGAT  GGAAGGCCAC  AACTGCACC
HumanScxPromDistal  ACGCCACCAA  CAGGAAGGGC  CGGGCAGGGT  GGAAGGCCAC  TCGCTGCACC
RatScxPromDistal    AGGCCACAAG  CAGGAAGGGC  CGCGTGGGAT  GGAAGGCCAC  AACTGCACC
                      *  *****  *  *****  *  *  *  *  *  *  *  *  *  *

MouseScxPromDistal  TGCCCGTGGC  TGCGGCGGAA  CGGGCTCTGG  CTGCGCCTCC  TGCTCACCTG
HumanScxPromDistal  TGTCCGTGGC  TGCGGCGGAA  CGGACTCTGG  CTGCGGCGAC  GGCTCAGCTG
RatScxPromDistal    TGTCCGTGGC  TGCGGCGGAA  TGGGCTCTGG  CTGCGCCTTC  TGCTCACCTG
                      **  *****  *  *****  *  *  *  *  *  *  *

MouseScxPromDistal  GTGAAGCAGC  ACTTGCGTGT  GCTCTTGACT  AGACAGCACC  ACCGCCAAAT
HumanScxPromDistal  GTGAATCAGC  ACCTGGGTGT  GGCTTGGGT  GGCCAGCACC  ACGGCCAGGT
RatScxPromDistal    GTGGATCAGC  ACTTGAGTGT  GCCCTTGGCT  GGACAGCACC  ACAGCCAGGT
                      ***  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

```

MouseScxPromDistal	AGTCCCCTCG	CCCATGCCAG	GTCACCTGTG	TCAGTGGCTA	GAGAAGAGCA
HumanScxPromDistal	AGTCCCCACG	CCCGTGCCAG	GTCACCTGCG	TCAGTGGCTG	CAGGAGAGCA
RatScxPromDistal	AGTCCCCTCG	CCCATGCCAG	GTCACCTGTG	TCAGTGGCTA	GAGAAGGGCA
	***** *	*** *****	***** *	***** *	** ** *
MouseScxPromDistal	GTGCTGGCTG	ATTGTCTGAC	CAGGTCTTCG	CGCGTCGCC-	CTCCCCGTCC
HumanScxPromDistal	AGGCTGGCTG	AGTGGCTGAG	CCGGCCCCCT	CCCGCTGCAG	CCTCACCCCC
RatScxPromDistal	ATGCTGGCTG	ATCGTCTGAC	CAGGTCTT-G	CAAGTCCCCG	CCCCCTTCC
	***** *	* * ****	* ** *	* * *	* * * **
MouseScxPromDistal	CA-----TC	ACATACTTTG	CTGTGGCAGA	TGCGTAGGCG	CAGACCCCTC
HumanScxPromDistal	AAGGCGCCCC	ACGTACCTTC	CCGTGGCAGA	TGCGCAGCCG	CAGGCCCACT
RatScxPromDistal	CA-----TC	ACATACTTTA	CTGTGGCAGA	TACGTAGTCG	AAGGCCCTC
	*	* ** ** *	* *****	* ** ** *	** ** *
MouseScxPromDistal	TGGTGCTCTT	CCTCCGAGAC	CTCTAGCCAC	CGGGCAGGCT	GCAGAGCTGG
HumanScxPromDistal	TGGCGTCCT	CCTCTGAGGC	CTCCAGCCAG	CGGGCCGGCT	GCAAGGGGGG
RatScxPromDistal	TGGCGTCTT	CCTCTGAGAC	CTCCAGCCAG	TGGGCAGGCT	GCAAAGTTGG
	*** * ** *	***** ** *	*** *****	***** *****	*** * ** *
MouseScxPromDistal	CTCCTCAGGT	GGAGTGAAGG	CTTCCAGCAG	CTGGTCTGTG	CTGCCACCA
HumanScxPromDistal	CTCCTCAGGC	GGGACGAAGG	CGCTCAACAG	CTGATCTGTG	CTGCCCGCCA
RatScxPromDistal	CTCTTCAGGG	GGAGTGAAGG	TTTCCAGCAG	CTGGTCTGTG	CTGCCACCA
	*** *****	** *****	*** ** *	*** *****	***** ** *
MouseScxPromDistal	GCAGCCGGTC	CCCCAGGGCT	GGGTTCAGCA	GCAACACGGC	ATCATCCCTA
HumanScxPromDistal	CCAGCCGGTC	CCCCAGAGCT	GGGTTCAGCA	GCAGCACCGA	GTCTCCTCTG
RatScxPromDistal	GCAGCCGGTC	CCCCAGGGCT	GGGTTCAGCA	GCACCACGGC	ATCATCCCTA
	***** *	***** ** *	***** *****	*** ** *	** *****
MouseScxPromDistal	CCAGCCGGGG	CAGACAGAA	GCCCCCTCCTC	AGCATCAGCC	CCACTGGCCT
HumanScxPromDistal	TGAGCCAGGC	CAGACAGCG	-----CCCCTA	AGCACA----	-----
RatScxPromDistal	CAAGCCAGGG	CAGACAGCT	-CCCCCTCCTC	AGCATAGTCC	CCACTTTCTC
	**** **	***** *	* ** *	****	
MouseScxPromDistal	GTGCCCCCTC	CAGC--CCTG	ACCCCCAGGG	CCCTACATC	ACATGGCAGC
HumanScxPromDistal	GTGCCACCCC	TGGCTCCCCA	CCCCCAGGG	-CCTCCACTT	ACACGGCTGC
RatScxPromDistal	GTGCCCCCTC	CAGC--CCTA	ACCCCCGGGG	TCCTCAATC	ACATGGCAGC
	***** * **	** ** *	***** ** *	*** ** *	*** ** ** *
MouseScxPromDistal	AGCTACCAGG	CATATGGTAG	GATTGGGGTT	CCAGGCGATG	CTCCGCACCA
HumanScxPromDistal	AGCCACCAGG	CAGACAGCG	GGCTGGGGTT	CCAGGCCACA	CTCTTCACCA
RatScxPromDistal	GGCTACTAAG	CATATGGTGG	GATTGGGGTT	CCAAGCAATG	CTCCGTATCA
	* ** * *	** * * *	* *****	*** ** *	*** * ** *
MouseScxPromDistal	CACCTCCAAC	ATGTACAGTC	TTCATACAGC	GGGCAGTGGC	CACCTCCCAG
HumanScxPromDistal	CGCCCCCAC	GGGAACAGTC	CTCACACAGC	GGGCAGTGGC	CACCTCCCAG
RatScxPromDistal	CACCTCCAAC	ACGCACCGTC	TTCATGCAGC	GGGCAGTGGC	ACCTCCCAG
	* ** * ** *	* ** ** *	*** *****	***** *****	***** *****
MouseScxPromDistal	AGCTTCAACG	TGCCATCGTC	TGAACCTGGG	CATAGCAG-G	GACGCTCTCA
HumanScxPromDistal	AGCCGCAGGG	AGCCGTCGTC	AGAGCCTGGA	TGCGGCAGAG	ACAGCTCTCA
RatScxPromDistal	AGCCTCAATG	TGCCGTCGTC	TGAACCTGGA	TATAGCAGTG	AAGGCTCTTA
	*** ** *	*** *****	** *****	**** *	***** *
MouseScxPromDistal	GCACCTGAGC	TGCTCACCCA	GAGTCCCTGT	CTCCTAGCTC	AGGGCAGCTC
HumanScxPromDistal	ACACCTGGCC	ATCCCA----	-----CCTGC	CCCCCAGCTC	AAGGCTGCCC
RatScxPromDistal	GCACCTGAGC	CTCTTAGCCA	G-GTCCCTGT	CTCCAGCTC	AGGGCAGCTC
	***** *	* *	****	* ** *****	* ** ** *
MouseScxPromDistal	CCACCTCAGG	CCCACCTGAA	GCTAGCCACT	GGCCCCCTGG	CGAGACACTG
HumanScxPromDistal	CCAGCTCGGA	CCCACCTGAA	ACCAGCCACT	GGCCCCCAGG	AGAGACACTG
RatScxPromDistal	CCACCTCAGG	CTCACCTGAA	GCTAGCCACT	GGCCCCCTGG	GGAGACACTG
	*** ** *	* *****	* *****	***** **	***** *****
MouseScxPromDistal	AGGCAACGGA	CAAGGTCGCT	GTGGCCCCCTG	TAGACCTGCA	GGAGAAGAAG
HumanScxPromDistal	AGGCACCGGA	CAAGGTCACT	GTGGCCCCCTG	TAGACCTGAG	GAGGCGGCGG
RatScxPromDistal	AGGCAACGGA	CAAGGTCGCT	GTGGCCCCCTG	TAGACCTACA	GAAGAGGAAG
	***** *****	***** ** *	***** *****	***** *	* * * *

MouseScxPromDistal	-AGTGAGAAG	ACAGACACAC	AGACACCGGA	GGGCGTTTGC	--A--CCTCT
HumanScxPromDistal	CAGTGAGGAG	TCAGAC----	-----TGGGA	GGGTGCAACC	CCAGCCCCCT
RatScxPromDistal	-AATGAGAAG	TCAGACACAC	AGACGCCTGA	GGGCATTTCG	CCA--CCTCT
	* * * * *	*****	**	***	* * * * *
MouseScxPromDistal	GACTCCACAC	TTACGAGGGC	CTGGCACACA	GGGAAAGGCT	GAAGGTCTCT
HumanScxPromDistal	AGTCTC-CAC	TTACCAGGGC	CTGGCACGTG	GGGAAGGGCT	GCAGGTCCCT
RatScxPromDistal	AATCCACAC	TTACAAGGGC	CTGGCACACA	GGGAAAGGCT	GAAGGTCTCT
	* * *	*****	*****	*****	* * * * *
MouseScxPromDistal	TGGCCGAGGG	AGTTTGGGGA	TGAGGTCTTC	GGGGTCCACA	TTACCTTGGG
HumanScxPromDistal	CGGCCGAGGC	AGCTTGGGGA	TGAGGTCTTC	AGGGTCTACA	TTACCTTGGG
RatScxPromDistal	TGGCCGAGGG	AGCTTGGGGA	TGAGGTCTTC	GGGATCCACA	TTACCTTGGG
	*****	**	*****	**	*****
MouseScxPromDistal	--AAAAGAGC	AC----TGAG	CATCACCTTC	-ACACTACAC	TCCCCAGGA
HumanScxPromDistal	GCAGGAGAGC	GCCAGGTCAG	CCTTGCCCCC	TGTGCCA---	CCCCCTGG-
RatScxPromDistal	GCAAAAGAGC	AT----TTAG	CATTACCCTC	-ATCCTA--A	TTTCCCAGGG
	* * * * *	* * *	* * * * *	* *	*** **
MouseScxPromDistal	CCTGTGCGGG	CTGTCTCAGC	TACCCTCATC	TTGCGTTGCC	GTGGGCACAG
HumanScxPromDistal	--TGTGC---	CACCCCCACA	CACCCTCATC	TTGCGCTGCC	GTGGGCACAG
RatScxPromDistal	CCTCTGGGGG	CTGCTTCACA	TACCCTCATC	TTGCGCTGCC	GTGGGCACAG
	* * *	* * *	*****	*****	*****
MouseScxPromDistal	ATACAAATCG	AGGCAGCGCT	CGAAACGCTC	CTGGATGAAG	CGGCTGTAAG
HumanScxPromDistal	GTACAGGTCA	AGGCAGCGCT	CGAAGCGTTC	CTGGATGAAG	CGTCCGTAGG
RatScxPromDistal	ATACAAATCA	AGACAGCGTT	CAAACGCTC	CTGGATGAAG	CGGCCATAAG
	**** *	**	* * * * *	*****	** * * *
MouseScxPromDistal	CAGGCACTGT	CCTCAAGCTG	GGGAATTTCT	GTGGCAAGAA	GTTAAGCTTC
HumanScxPromDistal	CAGGCACGGC	CCGCAGGCTC	GGGAACCTGC	GTGGCAAAAA	GCTCAGCTTC
RatScxPromDistal	CAGGCACTGT	CCTCAGGCTG	GGGAACCTTC	GTGGTAAGAA	GTTAAGCTTC
	***** *	** * * *	*****	**** * * *	* * * * *
MouseScxPromDistal	CTCTCCACAG	GCTCCTGCTG	CATCCACGCC	GAGCGCTGGG	GAGAC-AGGA
HumanScxPromDistal	CTCTCGCCTG	GCTCCTGCTG	TTCCCACGCC	AAGCGCTGTG	GAGACCAAGA
RatScxPromDistal	CTCTCAATGG	GCTCCTGCTG	CATCCATGCC	AAGCGCTGGG	GACAC-AGGA
	*****	* * * * * *	*** **	***** *	** * * *
MouseScxPromDistal	GGCAGG--TG	AGAAGGG--A	TCGGGAAACA	TGGC--CGGG	GGCCACACCC
HumanScxPromDistal	CACAGGGGTG	GGGAGGGTCA	CAGGGAAGCA	TGGGGACAGG	GTCC-CGGCC
RatScxPromDistal	GATTAG--TG	AGATGGG--A	TCAGGAGACA	TGGC--TGGG	GGCCACTCCC
	* * *	* * * *	*** **	***	* * * * *
MouseScxPromDistal	ACCTCAGCGC	AGGCCACCT	CCTCCTCAGT	GGGCAGGTAC	TCGGGAGGTG
HumanScxPromDistal	CCCAGGATGC	AGGCCACCT	CCTCCTCGCT	GAGCAGGTAT	TCAGGGGGTG
RatScxPromDistal	ACCTCAGTGC	AGGCCACCT	CCTCCTCAGT	GGGCAGGTAC	TCGGGAGGTG
	**	**	*****	***** *	** * * *
MouseScxPromDistal	GGTTGTAAGA	CTCGGCATGG	CCAGGCAAGG	CCAGCTTGGG	GGCAGGCACA
HumanScxPromDistal	GGTTGTACGA	CTCGGCGTGG	CCTGGCAGGG	CCAGCTTGGG	AGCAGGTACG
RatScxPromDistal	GGTTGTAAGA	CTCCGCATGG	CCGGGCAGGG	CCAGCTTGGG	TGCAGGCACG
	*****	** * * *	** * * *	*****	*****
MouseScxPromDistal	TGCATCTTGT	GGCGTCCCAA	CACAGCATTC	GGATCCTCCT	GGGCCCACAG
HumanScxPromDistal	TGCATCTTGT	GGCGCCCGAG	CACGGCGTTG	GGGTCTCCT	GGGCCCACAG
RatScxPromDistal	TGCATCTTGT	GGCGCCCAA	CACAGCATTT	GGATCCTCCT	GGGCCCACAG
	*****	*****	*** **	**	*****
MouseScxPromDistal	GTCATAGAAG	CTAGGGGTGG	GGTCGTGGGG	CCGTCGAGGC	TTGATCCACC
HumanScxPromDistal	GTCATAGAAG	CTGGGGGTGG	GGTCTCGGGG	CCGGCGAGGC	TTGATCCAGC
RatScxPromDistal	GTCATAGAAG	CTAGGAGTGG	AGTCGGGGGG	CCGTCGAGGC	TTGATCCAAC
	*****	** * * *	*** **	*****	* * * * *
MouseScxPromDistal	CCATCTTGAT	GGCATGTACC	ATTCGAGACA	CCTGCACACA	AAGGTAAGGT
HumanScxPromDistal	CCATCTTGAT	GGCGTGCACC	ATGCAGAGAG	CCTGCATAGA	CAGCCGG-GT
RatScxPromDistal	CCATCTTGAT	GGCATGCACC	AATCGAGACA	CCTGCACACA	GAGCTAACGT
	*****	*** * *	* * * * *	***** *	** **

MouseScxPromDistal	CAGGT--GGC	AGCGTGGAGA	CCTTA--GAG	TGTGCAGGAG	GAG-----GA
HumanScxPromDistal	CAGGACGGGC	AGTGCGGGGC	GGTCA-----	-GCCCAGGCC	AAGCCCCAGG
RatScxPromDistal	CAGGC--GGT	AGCATGGAAA	CCTTACAAAG	TGTACTAGAG	AAG-----GA
	****	**	**	**	*
MouseScxPromDistal	TACCAGGGTT	CCCACCTTCT	CCTTTTCAAC	TAGGGATGGG	ATGAAACTAC
HumanScxPromDistal	GGCTGTGTGC	CCCACCTTCT	CCTTCTCCAC	CAGGGAGGGG	ATGAAGCTGC
RatScxPromDistal	TGATGGGGCT	CCCACCTTCT	CCTTCTCAAC	TAGGGACGGG	ATGAAACTAC
	*	*****	****	**	*
MouseScxPromDistal	GCTTGTTCAGC	TGGGCGGTTG			
HumanScxPromDistal	GCTTGTTCGGC	CGG-----			
RatScxPromDistal	GTTTGTTCAGC	TGG-----			
	*	*****	**		

Figure A-2. Clustal W of the Mouse, Human, and Rat Scleraxis Distal Promoter. Alignment of the mouse, human, and rat Scleraxis proximal promoter regions was conducted using Clustal W software from Expasy. The star (*) beneath the alignment marks bp that are conserved between both species. Pink highlighted sequences indicated the locations of potential SBE.

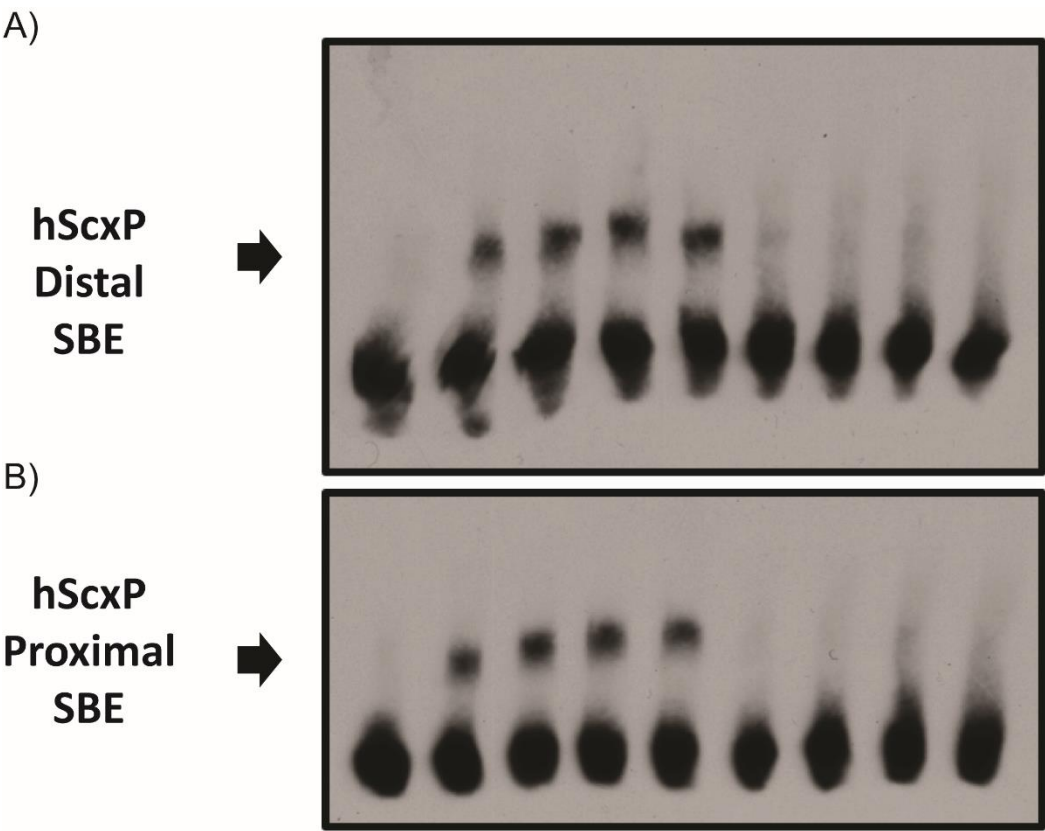
Appendix B

Fractionation of Cos7 Cells



Figure B-1. Fractionation of Cos7 Cells. Cos7 cells were fractionated and assayed for purity of the fractionation process. Nuclear extracts were enriched with nuclear proteins including Smad, Ski, and lamin A/C proteins. Purity of the nuclear fraction was determined by the expression of GAPDH within the nuclear fraction. Western blot shows little nuclear staining of the cytoplasmic protein GAPDH, indicating that our fractionation process was successful. WCL – Whole Cell Lysate.

Electrophoretic Mobility Gel Shift Assay of the Distal and Proximal SBE of the Human Scleraxis Promoter in Cos7 Cells.



Input		+	+	+	+	+	+	+	+
Anti-Smad 2			+						
Anti-Smad 3				+					
Normal Rabbit IgG					+				
100x Cold						+			
500x Cold							+		
100x Mutant Cold								+	
500x Mutant Cold									+
Probe	+	+	+	+	+	+	+	+	+

Figure B-2. Electrophoretic Mobility Gel Shift Assay of the Distal and Proximal SBE of the Human Scleraxis Promoter in Cos7 Cells. Similar to that seen in figure 20 of this thesis, nuclear lysate isolated from Cos7 cells were incubated with biotinylated probes encompassing the 2 putative hScxP SBE. As was with nuclear lysate from primary cardiac myofibroblasts, Cos7 nuclear lysate was incubated overnight with antibodies against Smad2, Smad3, or rabbit IgG control. Incubation the biotinylated probe demonstrated a clear interaction with both the **A)** distal, and **B)** proximal SBEs found within the hScxP. Similar to nuclear protein lysate from P1 myofibroblasts, co-incubation of Cos7 nuclear lysate with Smad antibodies was unable to show any R-Smads binding to either probe probes. Furthermore, mutated, unlabelled probes were still capable of outcompeting WT biotinylated probes. Figure is a representative of 3 individual experiments. Arrow indicates DNA:protein bound complexes.

Clustal W of the Mouse, Human, and Rat Ski Proximal Promoter

225

MouseSkiProm	ACT-----GCCGAGAGGGGCCGAGCTCCGGTACGGGGGAGCCGAGCG---GAGTC-GAGC
HumanSkiProm	ATG-----GCCGAGCGTAGCCGAGTCCCGG-----CCGAGCG---GAGCC-GAGT
RatSkiProm	ACTTCTTCACTTAAACTGTCTGGCTCTGGTT-----TTGAATACCTGTGTCAAGGC
	* * * * *
MouseSkiProm	TGTGGGCGGACAAAGCCGAGCTTGGCCGAGTCCGGTCCGGGCGTAGCCGAGCGCCACTGA
HumanSkiProm	CATGGCAGGGCGGGTCCGAGCGTGGCCGAGTCCCGGGCGAGCGGAGCCGAGCGCGGCCGA
RatSkiProm	CTCTGTTAGCGTTGCCTGCTTTAGTTATCTTTC-TGCAAGACTGTCC---TGCC-TTGA
	* * * * *
MouseSkiProm	GCCCG--GGAGGGGGAGCGCGCTTCTTGAAGAGCCCGCCCG--AGCTGGTCACATTC
HumanSkiProm	GTCCG--GGCGGGG-AGCGCGCTTCTTGAAGAGCCCGCTGGGGAGCGGGTCACATTC
RatSkiProm	TCCCATGTATAGTCAATATA-----TGGAGGGCCATA CATATGCAGATG G---ATAC
	* * * * *
MouseSkiProm	CTGGCCCGCACTCC---GGCTG--AGGCGGACGCACGGCAACAAACAACG--CGGG--A
HumanSkiProm	TTGGCCAGCGCTAG---GGCTG--AGGCGGACGCACGGCAACAAACAGCG--CGGG--G
RatSkiProm	TTGGTTCAGTCTTGGAGGCTGCCAGCCTGAATCACAGAGAATCACAGAGATCGGTCCA
	* * * * *
MouseSkiProm	GGC---GG-----GGCTGGCGCGGG-CGGGGCGG-GGC-----GGTACGG-----
HumanSkiProm	GGC---GG-----GGCCGGCGCGGGGCGGAGCGG-G-C-----GGCGCGG-----
RatSkiProm	AGCTATTGGTTAATGGCTGGCTCAAGAAGGGTCAATAGCTACTAGGTACAGCTTCTTCAA
	* * * * *
MouseSkiProm	GGCGGGGCGGCGCG-CGGCGGCGTGAGCTCAGTCCCGGCGCTCAGT---CCGA---GC
HumanSkiProm	GGCGGGGCGGCGCGGCGGCGGCGTGAGCTCAGCGCCGGGCGCTCAGT---CCGA---GC
RatSkiProm	GGCAGGATTCCGTTCCAAAGTTCCACCTCACTCAACAGGGTCCAGTAGA CAGATG TTGC
	* * * * *
MouseSkiProm	CGGAGC--GAGCCGCCGGAAGATG-TGCGCC-CAGTGCGCAGAGC--CCCGCGCGCCCG
HumanSkiProm	CGGAGC--GAGCCGCCGCGGAGGATG-TGCGCC-GAGCGCCCCGAGC--CCCGCGCGCCCG
RatSkiProm	TGGTACCTATGTCACTGGTGAGAGACTGGATCACAGTTCATGGGGTAGTCCATGTAGC--
	* * * * *
MouseSkiProm	CGGCGCTCTGAGG-CCGCGGGCCAGAGGCGCTCCGCCGTCCCG-----G
HumanSkiProm	CCGCGCTCTGAGGGCCGCGGGCGAGAGGCGCTCCGCCGCCCG-----G
RatSkiProm	CTGCTTTCCACAG-CTGTTTTTCTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTG
	* * * * *
MouseSkiProm	GAGCCGCGCGCGGGGCCAGCGGGCGCCGGGAGCCGGCAGCGCGCGCGGGCGGGCA
HumanSkiProm	AAGCCGGGCGGGCGGGCC-----GCG-----GAGCCGGC-----GGGCGGGCG
RatSkiProm	GAGCTGGGGAC-CGAACCC---AGGGCCTTG CACCTG C-----TAGGCAAGT-
	* * * * *
MouseSkiProm	GGCCGCCAGGGGCCGCGGGCGGCGGCGACGCGGCCGGTCAGCACCGCCGGGACGAGCCG-
HumanSkiProm	GGCCG--GGGGCCGGCGGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG
RatSkiProm	-GCCA----- CAGCTG TATTTCTTATGCATC---TCAGCATCCCCATGTCATGCTGT
	* * * * *
MouseSkiProm	-AGC---GGGCGCCGCG---CGGTGGGAGGAGCGGG-GCAGCGG-CGGCGCGCCGCGC
HumanSkiProm	-GGC---GGGCGCCTCG---CGGTGGGAGGAGCGGG-GCGGCGG-CGGCGCGCCGCGC
RatSkiProm	GAGCTCCTAGGTTCTGCAATCCTGTGTCTTCTTGGGTGCAGAATTCTGTATATCAAGT
	* * * * *
MouseSkiProm	G-----
HumanSkiProm	GAGGACGGCCCGGGGCCCGCGCCCGCGCCCGCTCCTCCGGGCCCTCGGCCTCG
RatSkiProm	GGTATCTCTC-----
	*
MouseSkiProm	-----
HumanSkiProm	GCCGCGCGCGGATTGCGGCTCGCGGCGCCGG CACCTG CCCGCGCGCCCCCGCGAGCC
RatSkiProm	-----
MouseSkiProm	-----
HumanSkiProm	CCGGGCCCGGAGCGTTGGCGTTGGCGTTGGCGGCGCGCGCCGGGGCATGCCCCGCGCT
RatSkiProm	-----

```

MouseSkiProm      -----CGCGGGGGGCCCGGGCGGCGGGCGCGGCGC-
HumanSkiProm      AGAGCCCGGGGGCGCGCGGGGACGCGCGGGGGGCCCGGGCGGCGGGCGCGGCGC-
RatSkiProm        -----TACACATGGAACCCAGAAGTACTTTGTGCAGTCCT
                      * * * * *

MouseSkiProm      -----GGGGCGCGTGGATGTGGCGCCG--GGCCCGGGCGGCGGGGCTCCAGCGG
HumanSkiProm      -----GGGGCGCGTGGATGTGGCGCCG--GGCCCGGGCGGCGGGGCTCCAGCGG
RatSkiProm        TCTGCAATGGAGACCTGGGTGGTCTTTGTGAGGTCTGGTTGTG-TAGGCAT-----A
                      * * * * *

MouseSkiProm      CGGGACCCCTTCGCCCCGCCCGCTTCGCCTTCGCGCCCCGGGCGAGGCCGAGGCCGGG
HumanSkiProm      CGGGACCCCTTCGCCCCGCCCGCTTCCTTCGCGCCCCGGGCGAGGCCGCGG-----
RatSkiProm        TGGGGT---TTTGTGCCATCTTC--TCATCATAGAGTAACCTGGCGTGAGTGTT-----
                      ***      * * * * *

MouseSkiProm      GCCGTGATCGGCCGTGAACCGGCGACGGGGGGCG-GTCGTGGCCCGGTCGCGGGCGGCG
HumanSkiProm      -CCGTGATCGGCCGTGAGCCGGCGGGGGGGCG-GCCGGGGTCGGGGCCGCGGGCGCCG
RatSkiProm        -CTGTGGT-GTCCTTTATGTAACC-TGGGATGCGCGTTGTGTTCCCTTATCATATGGAG
                      * * * * *

MouseSkiProm      GCGGGGTGCGCGGGGCGGCGGGGAGGCCGTGGGGGCCCGGGCGCACGGGAGCGGGAGCG
HumanSkiProm      CCGGGGCGCGCGGGGCGGCGGGGCGGGGGGCCCGGGCGCGCGGGAGCGGGAGCG
RatSkiProm        ACAGGG-GTGTGTGGAGACCGTAGGGTGCTCGAGACGTTCTTGATCAGAACCCGAATG
                      * * * * *

MouseSkiProm      GCGGGGGAGCCCGATCGCACCATGGAAGCTGCAGCGGCGCGGGCGGCTTCCAGCC
HumanSkiProm      GCGGGGGAGCCCGAGCGCACCATGAGGCGGCGGCGGGCGGCTGTTTCCAGCC
RatSkiProm        TTTTGTGGCATC---CTTGCTATGAGGGAGGGAAC-----CTGGGTTTGTGTTCTA-TG
                      * * * * *

MouseSkiProm      GCACCCGGGGCTGCAGAAGACGC-TGGAGCAGTTCCACCTGAGCTCCATGAGCTCGCTGG
HumanSkiProm      GCACCCGGGGCTGCAGAAGACGC-TGGAGCAGTTCCACCTGAGCTCCATGAGCTCGCTGG
RatSkiProm        GTGTCCTTG-TTACGAATGTCTCGTGAGCA-----TGACTTCCAGCAG-----TGG
                      * * * * *

MouseSkiProm      GCGGCCCGGCGCTTTCTCGGCGCG-CTGGGCGCAG-GAGGCCTACAAGAAGGAGAGCGC
HumanSkiProm      GCGGCCCGGCGCTTTCTCGGCGCG-CTGGGCGCAG-GAGGCCTACAAGAAGGAGAGCGC
RatSkiProm        GGGGAGTGAAGGTTACTTCGGAGTGTCTCCGTAGAATGGGGACCCCGGATGAAGGCGCG
                      * * * * *

MouseSkiProm      CAA-GGAGG-CGGGTGCGG---CCACAGTGCCGCGCCGGTGCCCACTGCCGCGGAGCC
HumanSkiProm      CAA-G-----
RatSkiProm        CAGCGGAGATCTGCTGCGGACCGCCACTCAGCTGGC--CTGTG---GTTGTGGCGGGGG-
                      ** *

```

Figure C-1. Clustal W of the Mouse, Human, and Rat Ski Proximal Promoter. Alignment of the mouse, human, and rat Ski proximal promoter regions was conducted using Clustal W software from ExPasy. The star (*) beneath the alignment marks bp that are conserved between both species. Underlined sequence marks the Ski ORF. Green highlighted text marks the beginning of the ORF for each species. Yellow, teal, and grey highlighted sequences indicated the locations of potential NF-κB, E-Box, and CAAT binding sites, respectively.

Clustal W of the Mouse and Human Ski Proximal Promoter

```

MouseSkiProm      -----TCTGTATATCA--CTACACATGG-----AAACC-----CAGAAGT
HumanSkiProm      CGCCGGCCCGAACGCCGGGCCCGGGGCCGAAGTCCTGATAAGCCACCTGGCAGGAGT
                  * * * * * * * * * * * * * * * * * * * * * * * * * *
MouseSkiProm      ACTTTGGGCAGTCCTGCTCTG---AAGACCTTGAGTGGTTCTTTGTGCAGGCACATGGGT
HumanSkiProm      GGCT--GGCAGACCGGCTGCGTCTGGGGCACC GGCGCCGCCCTCCAGGCCCG--AAG
                  * * * * * * * * * * * * * * * * * * * * * * * * * *
MouseSkiProm      TTTTGAGCTATCTTCTCAACATAAGAGTAACCTGGCATGAGTGTTCT--TGTGGTGTCTT
HumanSkiProm      CCCC GG---CCCGCGCAGC--AGGGACACTCCGCCAGGGGCCGGCGGGCGGGCGGGG
                  * * * * * * * * * * * * * * * * * * * * * * * * * *
MouseSkiProm      TTATGTACCTGGAAT-GCG--TGTTTGTTGCC-----CCTATCATATGGGGGTAGG
HumanSkiProm      CGGCGCGGCCGGGAGCCGCGGACACTCG-GCCACCGAGCCACGGCCACGGCGCAGCGGG
                  * * * * * * * * * * * * * * * * * * * * * * * * * *
MouseSkiProm      AACTTCGGTGTGTGGCGACCCTAGGGTGCTTTGAGACGTT-CTGTATCAGAACCCGAA
HumanSkiProm      GGCC--GCCGGGCTGCGCGCCAGGGTCACGCGACGCGGTGCCCGTCTCGGCGC--AG
                  * * * * * * * * * * * * * * * * * * * * * * * * * *
MouseSkiProm      TGTTTTGTGGCATCCTTGCTAT-----GAGGAAGGGAACCTGGGTTTGTTTTGTGGT
HumanSkiProm      GGTCCCTGGGCGTGGCCGCTGTCCCCCTCGTGTGTGGGACCCAGGT--CACCGTGTGGC
                  ** * * * * * * * * * * * * * * * * * * * * * * * * * *
MouseSkiProm      GTCCTTGTTATGAGTGTCTCGTGGAGCACGACGTCCAGCAGTGTGGGAGTGAAGGTTAC
HumanSkiProm      GTCCCGGT---AGTGT---GTGGGAC-----CCCAGAGGCGTCCGGGGCTCC-CGCG
                  **** * * * * * * * * * * * * * * * * * * * * * * * * *
MouseSkiProm      TTCACAGTGTCT-ACCTAGAACGGGAACCCCGGGATGTAGGGCGGCAGCGCGCAGATCT
HumanSkiProm      TGTGTGGTGTCCACCCCTGG--GAGAACCCCGGG-TGCGTGGCG---TGGC-----
                  * * * * * * * * * * * * * * * * * * * * * * * * * *
MouseSkiProm      ACTGCGGACCGCCGCTCAGCTGGCCTGTGTTGTGGCAGGGGCCCTGTGGGTTTTCGGGGG
HumanSkiProm      -CCCCCGGCCGCC-CCAGCTGGTTTGCAGTGTGTGGCGGGGGCCT-GTGGGTTTCTGGGC
                  * * * * * * * * * * * * * * * * * * * * * * * * * *
MouseSkiProm      CCCCAAGACCGTGGTGGCAGCAGGACCGAGTCTTCCAGCGCGCAGGAAGCGGCCTCG
HumanSkiProm      CCC-----GACGCGCGCAGACGGGCGCCTTCCAGCGCGCAGGAAGCGGCCTCG
                  *** * * * * * * * * * * * * * * * * * * * * * * * * *
MouseSkiProm      GCCGGAAGGGGGGCCCCAGGCGCCTCCGCTGTGGCCGCTCCAGTGGCCTCCACAAGGCG
HumanSkiProm      GCCGGAAGGGGGGCCCCGGCGCCACCGC-----CGCCTCC---GCCTCCGC---CC
                  **** * * * * * * * * * * * * * * * * * * * * * * * * *
MouseSkiProm      GGGCCGAGCGAGACCGAGTAGGGCCGAGCAGGGCCGAGCCGTTACCGAGTAGGGCCGAGC
HumanSkiProm      GCGCCAGCG-GCCC--TCGG--CGGCGGGGCCGAGC-----GGGGCCGAGC
                  * * * * * * * * * * * * * * * * * * * * * * * * * *
MouseSkiProm      GGGGCCGAGCACTGCCAGAGGGGCCGAGCTCCGGTACGGGGAGCCGAGCGGAGTCGAG
HumanSkiProm      GGAGTCGAGCATGGCCGAGCTAGCCGAGTCCCGG-----CCGAGCGGAGCCGAG
                  ** * * * * * * * * * * * * * * * * * * * * * * * * * *
MouseSkiProm      CTGTGGGCGGACAAAGCCGAGCTTGCCGAGTCCGGTCCGGCGTAGCCGAGCGCCACTG
HumanSkiProm      TCATGGCAGGGCGGGTCCGAGCGTGGCCGAGTCCCGGGCGAGCGGAGCCGAGCGCGGCCG
                  ** * * * * * * * * * * * * * * * * * * * * * * * * * *
MouseSkiProm      AGCCCGGGAGGGGGAGCGCGCTTCTTGGAAGAGCCCGCCCG--AGCTGGTCACATTCC
HumanSkiProm      AGTCCGGGCGGGG-AGCGCGCGTTCTTGGAAGAGCCCGGCTGGGGAGCGGGTACATTCT
                  ** * * * * * * * * * * * * * * * * * * * * * * * * * *
MouseSkiProm      TGGCCCGCACTCCGGCTGAGGCGGACGCACGGCAACAACAACGCGGGAGGCGGGGCTGG
HumanSkiProm      TGGCCAGCGCTAGGGCTGAGGCGGACGCACGGCAACAACAACGCGGGGGGCGGGGCCGG
                  **** * * * * * * * * * * * * * * * * * * * * * * * * *
MouseSkiProm      CGCGGG-CGGGGCGGGGCGGTACGGGGCGGGGCGGCGCG-CGGCGGCGTGAGCTCAGCTC
HumanSkiProm      CGCGGGGCGGAGCGGG-CGGCGCGGGGCGGGGCGGCGCGGCGGCGGCGTGAGCTCAGCGC
                  **** * * * * * * * * * * * * * * * * * * * * * * * * *

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MouseSkiProm	CCGGCGCTCAGTCCGAGCCGGAGCGAGCCGCCGAAAGATGTGCGCCAGTGCGCAGAGC
HumanSkiProm	CGGGCGCTCAGTCCGAGCCGGAGCGAGCCGCCGGGAGGATGTGCGCCAGCGCCCCGAGC
	* * * * *
MouseSkiProm	CCCGCGCCGCGCGCGCTCTGAGG-CCGCGGGCCAGAGGCGCTCCGCGTCCCGGGAG
HumanSkiProm	CCCGCGCCGCGCGCGCTCTGAGGGCCGCGGGCAGAGGCGCTCCGCGCCCCGGAAG
	* * * * *
MouseSkiProm	CCGCGCGCGCGGGCCAGCGGGCGGCCGGGAGCCGGCAGGCGGCCGCGGGCGGGCAGGC
HumanSkiProm	CCGGCGGGCGGGCC-----GCG-----GAGCCGGC-----GGGCGGGCGGGC
	* * * * *
MouseSkiProm	CGCCAGGGGCCGGCGGGCGCGACGCGGCCGGTCAGCACCGCCGGGACGAGCCGAGCG
HumanSkiProm	CG---GGGGCCGGCGGGCGCGACGCGGCCGGCCAGCGCCGCGGGACGAGCCGGGCG
	* * * * *
MouseSkiProm	GGCGCCGCGCGGTGGGAGGAGCGGGCAGCGCGCGCGCGCCGCGCG-----
HumanSkiProm	GGCGCCTCGCGGTGGGAGGAGCGGGCGCGCGCGCGCGCGCGAGGACGGCCCCGGCG
	* * * * *
MouseSkiProm	-----
HumanSkiProm	GGCCCCGCGCCCGCGCCCCGCTCCTCCCGGGCCCTCGGCCTCGGCCGCGCGCGCATT
MouseSkiProm	-----
HumanSkiProm	CGCGCCTCGCGGCGCGG CACCTG CCCGCGCGCCCCCGCGAGCCCCGGGCCGCGAGCG
MouseSkiProm	-----
HumanSkiProm	TTGGCGTTGGCGTTGGCGGCGCGCGCGGGGCATGCCCGCGCCTAGAGCCCGGGGGCG
MouseSkiProm	-----CGCGGGGGGCCCGGGCGGGCGGGCGCGGGCGCGGGCGCGTGGATGTG
HumanSkiProm	CGCGGGGACGCGCGGGGGGCCCGGGCGGGCGGGCGCGGGCGCGGGGCGCGTGGATGTG
	* * * * *
MouseSkiProm	GCGCCGGGCCCGGGCGCGCGGGCTCCAGCGCGGGACCCCTTCGCCCCCGCCGCTTC
HumanSkiProm	GCGCCGGGCCCGGGCGCGCGGGCTCCAGCGCGGGACCCCTTCGCCCCCGCCGCTTC
	* * * * *
MouseSkiProm	GCCTTCGCGCCCCCGGGCGAGGCCGAGGCCGGGCGGTGATCGGCCGTGAACGGCGACG
HumanSkiProm	CCCTTCGCGCCCCCGGGCGAGGCCGCGG-----CCGTGATCGGCCGTGAGCCGCGGCG
	* * * * *
MouseSkiProm	GGGGCGGTCTGTGGCGCGGTCTCGGGCGGCGCGGGGTGCGCGGGCGGGCGGGGAGGC
HumanSkiProm	GGGGCGGGCCGGGTCTGGGGCGCGGGCGCGCGGGGCGCGGGGCGCGCGGGGGC
	* * * * *
MouseSkiProm	CGTGGGGGCCCGGGCGCACGGGAGCGGGAGCGGCCGGGGAGCCGATCGAC ATGGAA
HumanSkiProm	CGGGGGGCGCGGGCGCGGGAGCGGGAGCGGCCGGGGAGCCGAGCGCACCATGGAG
	* * * * *
MouseSkiProm	GCTGCAGCGGCCGCGGGCGGGCTTCAGCCGCACCCGGGGCTGCAGAAGACGCTGGAG
HumanSkiProm	GCGCGGGCAGGCGGCGCGCTGTTTCAGCCGCACCCGGGGCTGCAGAAGACGCTGGAG
	* * * * *
MouseSkiProm	CAGTTCCACCTGAGCTCCATGAGCTCGCTGGGCGGCCCGCGCTTCTCGGCGCGCTGG
HumanSkiProm	CAGTTCCACCTGAGCTCCATGAGCTCGCTGGGCGGCCCGCGCTTCTCGGCGCGCTGG
	* * * * *
MouseSkiProm	GCGCAGGAGCCTACAAGAAGGAGAGCGCCAAGGAGGCGGGTGCGGCCACAGTGCCGGCG
HumanSkiProm	GCGCAGGAGCCTACAAGAAGGAGAGCGCCAAG-----

Figure C-2. Clustal W of the Mouse and Human Ski Proximal Promoter. Alignment of the mouse and human Ski proximal promoter regions was conducted using Clustal W software from ExPASy. The star (*) beneath the alignment marks bp

that are conserved between both species. Underlined sequence marks the Ski ORF. Green highlighted text marks the beginning of the ORF. Yellow and teal highlighted sequences indicated the locations of potential NF- κ B and E-Box binding sites, respectively.

Appendix D

Chromatin Immunoprecipitation of the Ski Proximal Promoter

1.0×10^6 Cos7 cells were seeded onto a 100 mm cell culture dish with 10% DMEM-FBS and allowed to adhere and grow for 24 hours. The next day the cells were washed in 1x PBS and 5 mL of Opti-MEM I added to each plate. Cells were then transfected with 5 μ g of either empty vector pCMV4 or p65-pCMV4 using Lipofectamine 3000 as per manufactures protocol. Twenty-four hours later ChIP analysis was performed as outlined on page 77-79. Each pellet was suspended in 50 μ L of nuclease free water (Ambion) and the DNA concentration determined spectrophotometrically. Samples were amplified *via* PCR (Table C-1) using the primers listed in table C-2, separated on a 2.0% agarose gel at 130 volts for 65 minutes, and visualized using a Gel Doc system (BioRad).

<u>Primer</u>	<u>Sequence (5' – 3')</u>
Ski Proximal Promoter κ B Forward	CAGCTGGTTTGCGGTCGTGG
Ski Proximal Promoter κ B Reverse	CCATGCTCGACTCCGCTC

Table D-1. Ski Proximal Promoter ChIP Primers.

<u>Step</u>	<u>Event</u>
1	50°C 5 minutes 1x
2	95°C 5 minutes 1x
3	95°C 15 seconds
4	63.1°C 30 seconds – Plate Read
5	3-4 45x
6	65°C → 100°C – Melt Curve
7	End

Table D-2. Ski Proximal Promoter ChIP PCR Profile.

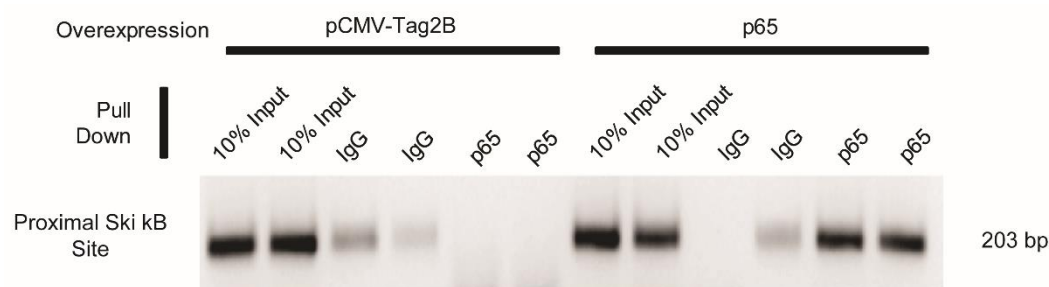


Figure D-1. ChIP Analysis of the Interaction of the NF- κ B p65 Subunit with the Cos7 Ski Proximal Promoter. Preliminary data shows a direct interaction between the Ski proximal promoter and p65. p65 was overexpressed in Cos7 cells for 24 hours. Cells were then fixed in 1.0% formaldehyde for 15 minutes at room temperature to cross-link DNA:protein complexes. Cells were then isolated for ChIP analysis. The Ski proximal promoter was precipitated with p65 in p65 overexpressing cells. This interaction was not present in empty vector control treated cells when precipitated with an antibody against p65. Normal rabbit IgG was used as a control for the protein:antibody precipitation. There was a small amount of background in the IgG controls following PCR, that was taken to indicate non-specific binding to the agarose beads. Better pre-clearing of the cell lysate and shorter PCR cycles may remove this background. 10% of the total cell lysate was removed from the sample prior to the precipitation and was used as a positive control for the region of interest.