The role of Ski protein in the modulation of cardiac myofibroblast phenotype: MMP expression and function

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Abstract

Cardiac fibrosis results from excessive formation of the extracellular matrix by activated cardiac myofibroblasts. Ski, an endogenous repressor of the profibrotic factor transforming growth factor-β1, has been shown to attenuate the myofibroblast phenotype. We demonstrate that Ski regulates rat cardiac myofibroblast's capacity for ECM remodeling, further solidifying its putative role as an endogenous anti-fibrotic TGF-β1 repressor. We show that Ski overexpression alters matrix metalloproteinase-2 and 9 expression and activity via immunoblotting and zymography. We also observe an attenuation of paxillin, a focal adhesion associated protein, and FAK (Tyr 397) expression by immunoblotting. Additionally, myofibroblast motility is reduced by Ski overexpression via transwell migration and scratch assay. We suggest that Ski may exert multiple effects on adverse ECM remodeling by altering the expression and function of the ECM proteases. The effects of Ski on cell motility also represent a putative mechanism for modulation of myofibroblast function in progression of cardiac fibrosis.

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List of Abbreviations

αSMA	Alpha-smooth muscle actin
Ad	Adenovirus
AngII	Angiotensin II
Arp2/3	Actin related protein 2/3
BSA	Bovine serum albumin
CDK1-cyclin B	Cyclin Dependent Kinase 1
Cdc2-cyclin B	Cell division control protein kinase 2
Cell-ECM	Cell- Extracellular matrix
C-Terminal	Carboxyl-terminal
DAB	3, 3'-diaminobenzidine
DCM	Dilated cardiomyopathy
DMEM-F12	Dulbecco's Modified Eagle Medium-Nutrient Mixture-F12
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
ED-A Fn	Extracellular domain-A splice variant of fibronectin
Eef-2	Eukaryotic elongation factor-2
EGTA	Ethylene glycol tetraacetic acid
EMT	Epithelial to mesenchymal transition
FAs	Focal adhesions
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
GPCR	G-protein-coupled receptors
GTPase	Guanosine triphosphatase
HEK	Human Embryonic Kidney
HF	Heart Failure
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IL	Interleukin
Ins 2	Insulin 2
I-Smad	Inhibitory Smad
JAK	Janus-associated kinase
LAP	Latency-associated peptide
LD	leucine-aspartate repeat
LLC	Large latent complex
LTBP	Latent TGF-β-binding protein
LV	Left ventricle
LVH	Left ventricular hypertrophy
MAPK	Mitogen-activated protein kinase
MEKK1	Mitogen-activate protein kinase kinase kinase-1
MI	Myocardial infarction
MMPs	Matrix metalloproteinases
MMP-1	Matrix metalloproteinase-1
MMP-2	Matrix metalloproteinase-2

MMP-9	Matrix metalloproteinase-9
MMP-13	Matrix metalloproteinase-13
MMP-14	Matrix metalloproteinase-14
MT-MMP	Membrane Type MMP
MT1-MMP	Membrane Type 1- MMP
MOI	Multiplicity of infection
N-terminal	Amine terminal
P0/P1	Passage 0/ Passage 1
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline-Tween 20
PDGF	Platelet-derived growth factor
pFAK	Phosphorylated focal adhesion kinase
PI3K	Phosphatidylinositol 3- kinase
PI3K-AKT	Phosphatidylinositol 3- kinase and Protein Kinase B
PIP5KIγ	Type Iγ phosphatidylinositol-4-phosphate 5-kinase
PtdIns(4,5)P2	Phosphatidylinositol 4,5-bisphosphate
PVDF	Polyvinylidene fluoride
PYK2	Proline-rich tyrosine kinase-2
PYK2 (Y402)	PYK2 phorphorylated at tyrosine 402
RIPA	Radioimmunoprecipitation assay
co-Smad	Common mediator Smad
R-Smad	Receptor Smad
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFKs	Src-family kinases
SMEM	Serum-free MEM (Minimum Essential Media)
SMemb	Embryonic non-muscle myosin heavy chain
SnoN	Ski-related novel protein-N
TAB	TAK-1-binding protein
TAK-1	TGF-β-activated kinase-1
TGF-β	Transforming growth factor-β
TIMPs	Tissue inhibitor of matrix metalloproteinases
TIMP-1	Tissue inhibitor of matrix metalloproteinase-1
TIMP-2	Tissue inhibitor of matrix metalloproteinase-2
TIMP-3	Tissue inhibitor of matrix metalloproteinase-3
TIMP-4	Tissue inhibitor of matrix metalloproteinase-4
TSP1	Thrombospondin-1
TβRI/II	TGF-β receptor-I/-II
WT	wild-type
Y397	Tyrosine 397
Y402	Tyrosine 402

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Introduction

Cardiac fibrosis is an consequence of a variety of cardiac pathologies [1] such as myocardial infarction (MI), hypertension and diabetic cardiomyopathy. Subsequent to myocardial injury the heart undergoes extensive remodeling leading the accumulation of fibrous tissue in both the infarcted and non-infarcted myocardium [2]. In cardiac fibrosis, excess production of the extracellular matrix (ECM) components increases passive myocardial stiffness, which may impair diastolic and/or systolic function. In addition to increased stiffness, accumulated fibrotic tissue also distorts myocardial structure, both of which in combination can lead to the development of arrhythmias, ventricular dysfunction and eventually heart failure (HF). Generally, HF is evidenced by dysfunction in either systolic ejection, diastolic filling, or a combination of both. Proportional to ventricular dysfunction, progressive clinical signs and symptoms develop, including dyspnea, edema, and fatigue. Heart failure is a leading cause of morbidity and mortality all around the world, afflicting millions of patients annually, and is associated with an extremely high economic burden [3]. Currently used conservative treatments only improve the quality of life in patients with heart failure, without targeting the underlying causes.

In spite of extensive research focused on underlying mechanisms of cardiac fibrosis, no specific preventive therapy for heart failure in the context of remodeling matrix has been discovered. The fundamental perception of cardiac fibrosis is based on molecular mechanisms of adverse ECM remodeling wherein fibroblasts play numerous functions. Understanding of the signaling pathways regarding cardiac fibroblast function including ECM degradation and production, cell proliferation, migration, infiltration into the infarcted area, and phenotype conversion represent critical points of investigation for achieving novel and effective anti-

fibrotic treatments. Recent literature points to burgeoning results to support the relevance of matrix remodeling as a primary cause of heart failure of different etiologies. Our work presented here will indicate a possible role by which Ski modifies ECM remodeling through alteration of the myofibroblast phenotype and hence may modulate the progression of cardiac fibrosis.

1. LITERATURE REVIEW

1.1. Fibroblasts and the myocardium

Cardiomyocytes comprise approximately 75% of the normal tissue volume but only 30-40% of cell number of the heart [4]. The majority of the non-myocyte cell population in the ventricular myocardium consists of fibroblasts, which are located throughout the cardiac interstitium and are the most numerous cell type in the heart [5]. Thus, each cardiomyocyte is in a close contact with fibroblasts [6]. Endothelial cells, vascular smooth muscle cells and neuronal cells account for the smaller populations [7]. Though previously thought to provide only structural support, more evidence has shown that fibroblasts play multiple important roles in the myocardium, mainly through maintaining cardiac ECM homeostasis.

1.2. Fibroblasts and the extracellular matrix

The cardiac ECM provides an organized scaffold which maintains cardiomyocyte adhesion, association, coordinated function, and stable boundaries for force transmission between cardiomyocytes [8]. The highly organized structure of the ECM is essential for heart and vascular tissue functional and structural integrity [5, 9]. Cardiac fibroblasts contribute to a variety of cardiac features including structural, biochemical and electromechanical functions. Cardiac fibroblasts are the fundamental source of the cardiac ECM and are responsible for synthesis of basement membrane type IV collagen, structural or fibrous proteins including fibrillar collagens (type I and type III), elastin, laminin, and fibronectin [9, 10]. Therefore, cardiac fibroblasts play an important role in providing a spatial coordination of the ECM. In addition to structural proteins, the ECM consists of nonstructural proteins including glycoproteins, proteoglycans, and glycosaminoglycans [11]. Aside from the production of the

ECM proteins, fibroblasts are responsible for synthesis and secretion of numerous known cytokines, peptides, and enzymes. The signaling pathways in which these molecules are involved influence cell fate including motility, proliferation, and apoptosis [12-16, 17, 18, 19]. The cardiac ECM is a dynamic network which undergoes constant turnover as the heart is subjected to continuous stress. Normal cardiac development and optimized function is based on a balance between synthesis and degradation of the ECM that maintains intact myocardial structure [12, 20, 21]. The intact structure and homeostasis of the cardiac ECM is preserved by the precise function of a group of proteolytic enzymes called matrix metalloproteinases (MMPs) and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) [19 , 22].

1.2.1. Matrix Metalloproteinases (MMPs)

Degradation of a variety of the ECM proteins is mediated by a family of MMPs [23, 24], named as such since their ability to degrade ECM is dependent on metal ions including Zn and Co [25, 26]. Beside this well-described activity, MMPs participate in the regulation of cell behaviour through the cleavage of non-ECM proteins and cell surface molecules [27, 28]. MMPs are categorized as collagenases, gelatinases, stromelysins, matrilysins as well as the membrane-bound types [29]. The gelatinases (MMP-2 and MMP-9) are the most abundant MMPs in cardiac tissue and are critical components of the ECM remodeling response [30], although a number of other MMP types are also expressed in the myocardium, including the collagenases (MMP-1 and MMP-13), stromelysin (MMP-3), and membrane type 1 (MT1)-MMP, MMP-14 [31]. Along with fibroblasts, cardiomyocytes and smooth muscle cells also express MMPs [31]. MMPs are involved in inflammatory cell migration which leads to tissue inflammation [32] while they can even modify the biological activity of their regulator chemokines [33]. MMPs are regulated at the transcriptional level by a variety of cytokines, hormones, growth factors and receptor-

mediated interactions between cells and matrix [34]. Secretion of MMPs is regulated by external signals as well as by cellular differentiation [35], and their effectivity undergoes changes by release of growth factors and cytokines from their cleaved substrate [36].

1.2.1.1. General Structure of MMPs

Belonging to a family of 28 structurally relevant enzymes [37], MMPs generally contain the following domains: a signal peptide to direct their secretion, an amino-terminal pro-peptide for maintaining their latency which contains a conserved catalytic Zn^{2+} binding site, a carboxy-terminal hemopexin domain which determines the substrate specificity and interacts with endogenous inhibitors, and also a linker peptide of varying length (hinge region) [38, 39]. Additionally, the two gelatinases (MMP-2 and MMP-9) possess three fibronectin type II modules that facilitate a dense collagen binding domain [40]. The MT-MMPs possess an additional basic residue between the pro-peptide and the catalytic domain. MMPs are produced in the form of pro-MMPs or zymogens [41-43]. Specific MMPs are stored in intracellular granules as pro-enzymes and external signals stimulate their exocytosis and release. In general, latent MMPs are activated intracellularly by pro-protein convertases like furin in their secretory trail, or extracellularly by other proteases. Moreover, activation of MMPs can take place at the cell surface e.g. activation of pro-MMP-2 *via* formation of trimolecular complex of MMP-14–TIMP-2–pro-MMP-2. Ultimately, active MMPs are endogenously inhibited by tissue inhibitors of metalloproteinases (TIMPs) in the extracellular milieu [44].

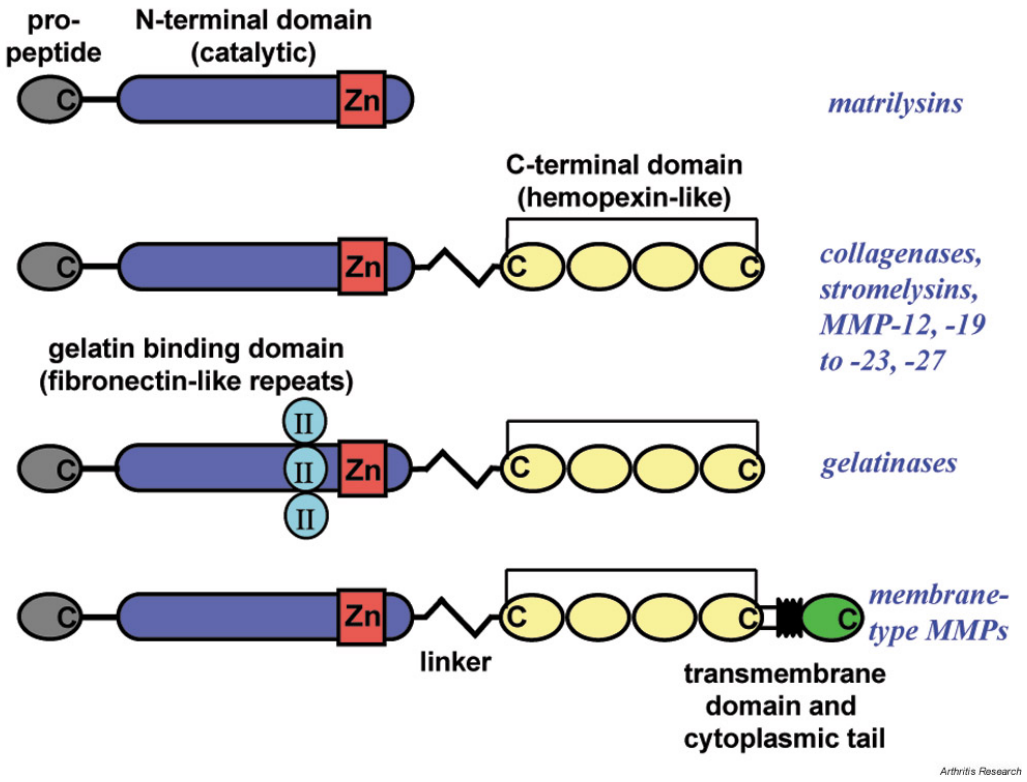


Figure 1. Model of basic domain structures of the MMPs. Here is a cartoon representation of the difference structural classes of MMPs; they all contain a propeptide that preserves enzyme latency (grey), a catalytic domain with an active site (Blue), and a catalytic zinc site (Red). In the case of the Gelatinases, they contain an additional insert of 3 fibronectin type II repeats within the catalytic domain. With the exception of the matrilysins the 3 other structural classes contain a central linker domain that links the N-terminus to the C-terminal domain. The C-Terminal domain has homology with the serum protein hemopexin (Yellow). The Membrane-type MMPs contain a C-terminal transmembrane domain (Black) connected to the intracellular cytoplasmic tail (green). Reproduced with permission from "Murphy G, *et al*. Matrix metalloproteinases in arthritic disease. Arthritis Res. 2002; 4 Suppl 3:S39

1.2.1.2. MMPs in Cardiovascular Physiology

In addition to maintaining myocardial homeostasis through continuous ECM turnover, MMPs regulate cardiovascular function as well as embryogenesis and angiogenesis. During growth and development, physiological vascular matrix remodeling and angiogenesis is facilitated by vascular smooth muscle cells and endothelial cells. Endothelial cells also regulate wound healing and granulation tissue formation [45]. There is evidence of expression of MMP-1, -2, -3 and -9 by these cells during these processes [46, 47]. In the adult heart, MMPs facilitate the turnover of the ECM proteins through degradation, and are inhibited by TIMPs. Gelatinases (MMP-2 and-9) are the core regulators of the ECM homeostasis in the heart, due to their specific affinity for denatured fibrillar collagen. They also possess an ability to degrade elastin and proteoglycans [48]. MMP-2 is detected in normal murine LV extracts and is expressed by cardiomyocytes, endothelial cells, vascular smooth muscle cells, fibroblasts and myofibroblasts [49].

1.2.1.3. MMPs in Cardiovascular Pathology

Following myocardial injury, such as an MI, the activity of MMPs increases during the initial inflammatory phase, which degrades the cardiac matrix, allowing for infiltration of inflammatory cells to clear dead cardiomyocytes [50]. Inflammatory response mediators such as Interleukin-1 (IL-1) and IL-6 augment inflammatory cell influx, as well as fibroblast proliferation and migration to the injured area, followed by conversion of fibroblasts to the myofibroblast phenotype. Moreover, the ECM components are essentially involved in inflammatory pathways [50]. In turn, inflammatory cells and converted myofibroblasts induce MMP activity resulting in further proteolysis of the ECM components [51]. Interestingly, MMP-

2 also cleaves non-ECM protein such as cell surface receptors and intracellular proteins, therefore it is involved in cardiac and vascular dysfunction *via* other mechanisms [52]. Altered MMP activity can trigger pro-fibrotic events, resulting in cardiac fibrosis and ultimately heart failure. During post-MI remodeling, relatively high levels of MMP-1, MMP-2, MMP-3 and MMP-9 expression and activity exist in human, rat and porcine hearts [53, 54]. In murine post-MI hearts, there is a transient rise in collagenase activity compared to sham controls. Collagenase activity increases 4.5-fold 2 days post-MI, peaking at day 7 with a 6.5-fold increase in activity and subsequently decreasing. This transient increase in collagenase activity was also associated with increased gelatinase (MMP-2 and MMP-9) activity [55]. Studies in transgenic reporter mice have also demonstrated increased MMP-2 and MMP-9 promoter activation in the border zone of infarcted hearts. Moreover, attenuation in left ventricular remodeling has been observed by using MMP-2 and MMP-9 knock out transgenic mice [56]. In a diabetic heterozygous insulin 2 ($Ins2^{-/+}$), MMP-9-null ($MMP\text{-}9^{-/+}$) mouse model, reduced collagen accumulation was observed post-MI, as well as improved survival of exogenous stem cell transplants and their differentiation into cardiomyocytes [57]. MMP-2-deficient mice displayed noticeably decreased expression of elastin and collagen I in response to Angiotensin II treatment, indicating that MMPs, in particular gelatinases, may play an important role in regulating remodeling, as well as facilitating the ECM degradation [58]. In fact, this view has now begun to become generally accepted in this field of study. The pro-fibrotic effects of elevated MMP levels post-MI may also be due to the ability of MMPs to cleave and thereby activate pro-fibrotic factors and cytokines such as transforming growth factor-β (TGF-β). Cell surface localization of MMP-9 by CD44 leads to activation of TGF-β through the proteolytic degradation of a latency-associated peptide (LAP) to which inactive TGF-β is bound in the ECM [59]. There is also evidence that TGF-β1 induces MMP

activity in cardiac fibroblasts, which in turn increases fibroblast migration. This likely occurs through amplification of pro-protein convertases like furin, which is required for MT1-MMP and MMP-2 activation in cardiac fibroblasts. Thus, MMPs also play a significant role in fibroblast and myofibroblast migration [60].

1.2.2. Tissue Inhibitor of Metalloproteinases (TIMPs)

Tissue inhibitors of Metalloproteinases (TIMPs) have been identified as the most potent physiological inhibitors of MMPs in the tissue compartment. To date, four TIMPs have been described TIMP-1-4, and they have a molecular weight of 21-30 kDa [22, 61].

1.2.2.1. The molecular structure of TIMPs

TIMPs contain 12 conserved cysteine residues responsible for establishing six disulfide bonds that fold the protein into two domains. Each domain consists of three loops, with six total per TIMP protein. Loops 1-3 are contained within the N-terminal region (also the MMP inhibitor domain) and the other three (loops 4-6) are found within the C-terminal domain [62]. TIMPs are produced in either O-linked or N-linked glycosylated forms [10, 43]. While TIMP glycosylation changes their molecular weight, this process does not affect their MMPs inhibitory properties [63, 64], and in fact it has been shown that O-linked glycosylation regulates autolysis of membrane type-1 matrix metalloproteinase (MT1-MMP) [65]. The exception is TIMP-4, the largest identified human TIMP, which is only produced in a non-glycosylated form [66]. Glycosylation also varies between TIMPs; TIMP-1 has two N-linked glycosylation sites required for proper folding and stabilization, which are not present in TIMP-2 [64]. TIMP-3, an ECM-bound TIMP, is also N-glycosylated, though glycosylation has no effect on its binding to ECM or with its MMP-inhibitors function [43, 63]. Glycosylation of TIMP-4 takes place through an O-

linked site, which alters its conformation and turnover rate but does not affect MMP inhibition [10, 67].

1.2.2.2. TIMPs in Cardiovascular Physiology

TIMP-2, TIMP-3 and TIMP-4 are expressed in heart in the normal physiological state. Although TIMP-1 expression is low in the normal heart, its expression level increases in a pathological state [19, 68-70]. Cardiac-enriched TIMP-4 plays an important role in the ECM homeostasis through inhibition of MMP-9 activity [71]. TIMP-4 has been identified as a widely expressed TIMP in the cardiovascular system, and has been shown to regulate activity of both MT1-MMP and MMP-2 [72, 73]. In addition to MMP inhibition, TIMPs also regulate other cell activities such as growth, proliferation [74] and apoptosis [75, 76]. TIMP-1 and TIMP-2 promote cell proliferation [77]. In some cell types, TIMP-1 possesses anti-apoptotic activities [78, 79]. TIMP-2 can interact with MT1-MMP, leading to activation of pro-MMP-2 [80-82]. Conversely, TIMP-4 induces apoptosis in fibroblasts [83]. TIMP-3 can either promote or inhibit apoptosis according to the cell type as it has represented variable activities in different experimental models [34, 84].

1.2.2.3. TIMPs in Cardiovascular Pathology

An imbalance of the MMP/TIMP ratio has been associated with structural and functional changes in hypertensive heart disease. Increase in TIMPs and decrease in MMPs lead to decrease in collagen degradation and increase in collagen accumulation which are associated with left Left ventricular hypertrophy (LVH) and diastolic dysfunction. [85, 86]. In patients with dilated cardiomyopathy (DCM), cardiac levels of TIMP-1, TIMP-3, and TIMP-4 protein were reduced, although TIMP-2 levels were not altered [87]. Furthermore, TIMPs are implicated in

angiogenesis [22, 88, 89] as well as the development of cardiac fibrosis [90]. Therefore, TIMPs have been considered as potential anti-fibrotic targets in different animal models [91, 92]. In mice post-MI, increased plasma levels of TIMP-1 and MMP-9 are directly correlated with adverse LV remodeling and dysfunction [93]. In TIMP-1-null mice, MI induces increased LV dilation and end-diastolic volume compared to wild-type (WT) mice, indicating a role for TIMP-1 in regulating the ECM remodeling in the post-MI ventricle [94]. Another study showed that TIMP-2-null mice display non-uniform ECM remodeling, degradation of cell membrane integrins, and impaired ECM-cardiomyocyte adhesion, which may be some of the underlying mechanisms responsible for LV dilatation and dysfunction in this animal model [95]. In TIMP-3-mull mice, Angiotensin II-induced hypertension is suppressed, suggesting TIMP-3 regulates arterial ECM responses to Angiotensin II, as well as inducing vascular remodeling [96]. TIMP-4 appears to be especially important in the post-MI response. In a mature Yorkshire pig animal model of MI, TIMP-4 levels are initially reduced within the infarct and border zone of the heart [97], and subsequent TIMP-4 induction reduces levels of MMP-2 and MMP-9, as well as upregulating TGF-β1 receptor levels, increasing fibroblast proliferation, and augmenting the ECM synthesis [98]. In turn, deletion of TIMP-4 has been demonstrated to exacerbate post-MI remodeling, resulting in rapid LV rupture in the absence of LV dilation or dysfunction [71]. The importance of TIMP-4 during the post-MI remodeling phase indicates significant importance of this TIMP in the timely inhibition of MMPs. In post-MI patients, plasma TIMP-4 levels were reduced even months after infarct [99], further emphasizing the involvement of TIMPs in cardiovascular pathology. *In vitro*, increased expression of TIMP-1-4 *via* use of recombinant TIMPs have been found to enhance expression of the myofibroblast marker α-smooth muscle actin (α-SMA) in cardiac fibroblasts. Additionally, TIMP-1 and TIMP-3 were found to increase

phosphorylation of Smad3 (a member of the canonical TGF-β signaling pathway in fibroblasts), as well as phosphorylation of the TGF-β1 receptor [100].

1.3. Fibroblast Adhesion and Motility

Communication between cardiac fibroblasts and the ECM *via* cell-matrix adhesions are critical to the regulation of cell morphology, growth, differentiation and migration. Weak cellular-ECM junctions are composed of actin cytoskeleton that interacts with matrix fibronectin filaments at discrete sites (adhesions) on the fibroblast cell surface [101, 102]. These fibrillar adhesions typically are extended structures originating in the cardiac fibroblast interior which are composed of numerous adhesion and scaffolding proteins, including tensin and integrin α5β1. Another type of fibroblast-matrix adhesion, which is slightly increased in its strength and size compared to fibrillar adhesions, is called the focal contact. Focal contacts are typically composed of α-actinin, vinculin, talin, integrin, and tyrosine-phosphorylated proteins. These components link the microfilament bundles to the plasma membrane [103, 104]. These structures are located in the cell periphery and interact with the ECM *via* integrins, primarily the α5β1 and αvβ3 isotypes [105] representing plasma membrane receptors for matrix. Focal contacts are typically small (< 1μm) and transitory adhesions found beneath the marginal lamellipodium of migrating cells [106]. With conversion to the myofibroblast phenotype, focal contacts mature into stronger and larger focal adhesions. These multi-molecular complexes are located where integrins attach to the adjacent extracellular matrix [107].

Although cell adhesions are important in maintaining cell-ECM contact, they are also critical in the process of cell motility, a dynamic process of adhesion formation and dissolution [108]. Cell motility is important in cell fate, development, ECM remodeling, cancer cell metastasis, immune responses, and wound healing. A number of overlapping steps are involved

in cell motility: protrusion of the cell leading edge, ECM attachment, cell cytoskeleton contraction, and detachment of the cell at the rear (trailing) edge [109, 110]. Protrusion of the leading edge involves the expansion of lamellopodia, which are protrusions of congregated actin filaments [111]. This process is performed *via* polymerization of actin filaments adjacent to the leading edge site [111-113]. Contraction involves the condensation of actin filaments within the cell, and retraction of the trailing edge is performed through a process of actin degradation called retrograde actin flow. Therefore, cell migration is determined by the interplay of actin cytoskeleton force generated in the cell leading edge to incorporate with rear end dissociation from the ECM *via* integrin-based focal adhesion interactions [106]. During both adhesion formation and motility, a variety of cytoskeletal proteins (vinculin, talin, alpha actinin, paxillin) as well as numerous cytoplasmic protein tyrosine kinases, e.g. members of the Src family and focal adhesion kinase (FAK) become associated into complexes which are connected to the ECM primarily through integrins [114-116]. Moreover, biochemical signals are transduced towards the cell through tyrosine 397 autophosphorylation of FAK [117]. Dynamic focal contacts exist closely to the leading edge of protrusions in a motile cell [118] and commonly include paxillin, talin, and the phosphorylated FAK (Tyr 397), which are involved in downstream signaling [106, 119, 120] . Mechanotransduction, the process of conversion of mechanical force to biochemical signaling pathways, induces activation of MAPK signaling through recruitment of integrins [121], independently of FAK. Therefore, existence of variable signaling mechanisms through recruitment of integrins can be concluded [122].

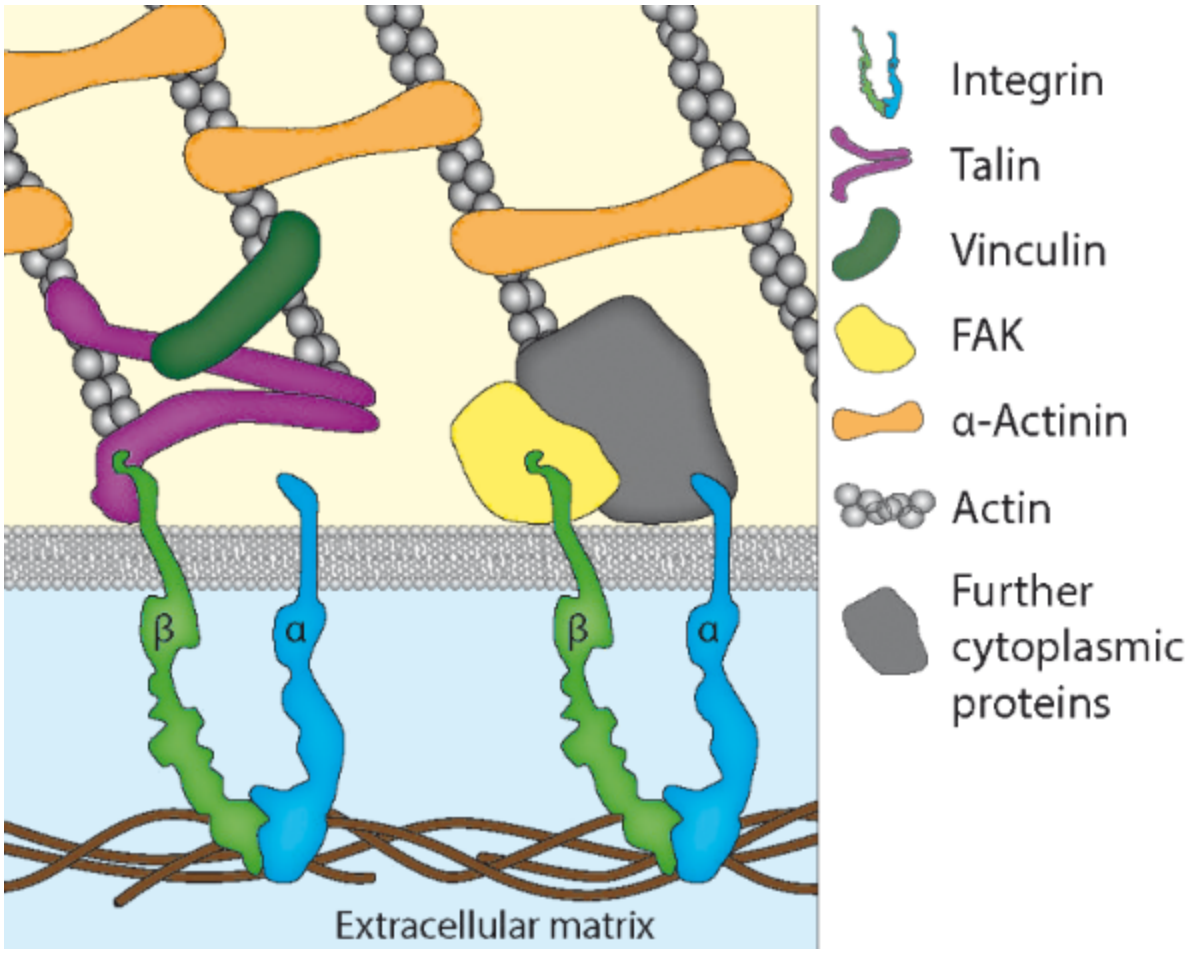


Figure 2. Model of cell adhesion to the ECM. Integrin molecules link the ECM to the actin cytoskeleton. ECM molecules attach to the heads of the integrin molecules as their ligands. The intracellular domain of integrin attaches to the proteins like talin and FAK. FAK develop a linkage to the actin accompanied by additional cytoplasmic proteins. These intracellular anchor proteins, including vinculin and α-actinin, facilitate and reinforce the actin–integrin linkage. Reproduced with permission from "Brüggemann D, *et al*. Model systems for studying cell adhesion and biomimetic actin networks. Beilstein J Nanotechnol. 2014 Aug 1;5:1193-202

1.3.1. Integrins

Integrins are a family of heterodimeric receptors that span the plasma membrane and communicate with the ECM constituents on the cell exterior to mediate cell adhesion, as well as other intracellular signaling pathways [123, 124]. Integrins are composed of an α and a β chain, both of which may be present in various isoforms. Numerous dimerization patterns of different α and β chains induce 24 diverse α/β heterodimers due to existence of 8 potential α chains and 18 β chains [125]. On the cell interior, integrins interact with actin filament bundles to invoke the formation of focal complexes and adhesions during cell attachment and motility [114]. Cardiac fibroblasts express an inventory of α subunits except α6 and α7 but αv and the collagen-exclusive α2 subunit are only expressed by the cardiac fibroblasts [126]. β1 integrins are correlated to the hypertrophic response of cultured ventricular myocytes and knockout mice lacking β1 integrin developed postnatal cardiac fibrosis. This is suggestive of a β1 integrin crucial role in the myocardium and its association with cardiac fibrosis and cardiomyopathies [127]. The most important and common integrin expressed in fibroblast adhesions is integrin α5β1 which is also the main fibronectin receptor [128, 129].

1.3.2. Focal Adhesion (FAs)

Focal adhesions (FAs) are mature cell-matrix assemblies which are localized mainly at the cell margin [128]. The intracellualr domain of FA ensures interactions with the ends of actin stress fibers which provide such stable plaque for the cell body to attach to the surrounding milieu in culture [130-132]. Furthermore, during the homeostasis state, the creation and destruction of adhesion sites are required to be synchronized [133]. Like focal contacts, FAs are composed of linker proteins such as talin and paxillin, as well as regulatory proteins including FAK and Src, where cytokines and growth factor-dependent signaling transduction occur.

Integrins co-localize with FAK at focal adhesions; this process is a requirement for activation of FAK signaling which depends on cell adhesion [123]. Although FAs maintain stronger connections to the ECM, and are critical in contraction, they are also found in migrating cells, and thus play a number of important roles in cardiac fibroblast biology.

1.3.2.1. paxillin

Paxillin is a 559 amino acid (68-70 kDa) focal adhesion associated adaptor protein activated by tyrosine phosphorylation, and is the main scaffolding protein of FAs [134, 135]. Paxillin acts as a docking protein and serves to recruit signaling complexes to focal adhesions, as needed [136]. The N-terminal end of paxillin contains a highly conserved leucine-rich sequence of repeats, termed the LD motif [137]. These motifs contain tyrosine and serine phosphorylation sites for the non-receptor kinases such as FAK and PYK2 and the other structural proteins such as vinculin [138-140]. The C-terminal end of paxillin proteins is constituted of four double zinc finger LIM domains, which are involved in paxillin targeting to FAs [138, 141, 142].

1.3.2.2. vinculin

Vinculin is a 117 kDa protein present in both integrin-based cell-ECM adhesions, as well as cadherin-based cell-cell junctions [115]. Vinculin is a critical regulator of cell adhesion and migration, and its recruitment is often used as a marker of maturation of focal contacts into FAs [119, 143]. Vinculin acts as an essential mediator of cytoskeletal actin polymerization, an important process in both contraction and lamellopodia formation [109]. Vinculin is positioned between integrins and polymerized, filamentous actin (F-actin). Vinculin binds both F-actin and talin, providing structural support to FAs during force transmission [144-146]. During FA maturation, vinculin forms a complex with FAK, inducing phosphorylation of paxillin *via*

myosin II activity, further promoting recruitment of vinculin to FAs to strengthen the cell cytoskeleton linkage to the ECM [147]. Vinculin also interacts with paxillin, Arp2/3, and vasodilator-stimulated phosphoprotein during lamellopodia formation [109, 115].

1.3.3. Focal adhesion Kinase (FAK)

Focal adhesion kinase (FAK) is a 125-kDa cytoplasmic tyrosine kinase which is associated with cytoskeleton alteration and turnover of cell adhesions during migration. It is an essential signaling protein in both focal complexes and focal adhesions. Tyrosine phosphorylation of FAK is an early, transient event during the creation of focal contacts [148]. It mainly is associated with the signaling pathways originated from integrins [149, 150]. However, association of FAK with integrins is indirect, occurring through linker proteins such as paxillin and talin [149]. The ECM interaction with integrin leads to FAK recruitment to the focal adhesion complex and its subsequent activation via autophosphorylation at tyrosine 397. FAK activation through integrin ligation can explain involvement of FAK in the β1 integrin/PI3K/Akt viability pathway mechanisms [149, 151]. Furthermore, FAK transmits signaling induced by growth factors and cytokines such as TGF-β and platelet derived growth factor (PDGF) in fibroblasts [150, 152]. FAK is also involved in Rho-family GTPases regulation [150, 153]. Moreover, FAK plays a role in signaling pathways by G-protein-coupled receptors e.g. angiotensin II as well as receptor tyrosine kinases, e.g. the platelet-derived growth factor (PDGF) receptor [154]. Additionally, FAK acts as a proximal sensor of conducting mechanotransduction towards the downstream molecules in fibroblasts; as previously described, phosphorylation of FAK at Tyr397 is a necessity for the assembly of actin stress fibers. This mechanism involves the interaction of PIP5KIγ with FAK, generation of PtdIns(4)P2, and subsequent activation of gelsolin at FAs [155]. In addition to its crucial role in the development and turnover of FAs, FAK has also been

shown to be associated with the processes of cell proliferation and apoptosis [117, 149, 154]. Moreover, phosphorylation of FAK (Tyr397) has a key role in regulation of MMP-2 induced collagen I synthesis in rat cardiac fibroblasts [117, 156].

1.3.3.1. PYK2

Proline-rich tyrosine kinase-2 (PYK2) is a cytoplasmic tyrosine kinase with high sequence similarity and function as FAK [157, 158]. For example, PYK2, like FAK, is associated with cell proliferation and migration [159, 160]. PYK2 shares a partially similar ~ 45% amino acid sequence and with FAK including FERM, kinase, proline-rich and FAT domains [149, 150]. It also possesses similar phosphorylation sites as FAK does [149]. PYK2 participates in linking integrins with G-protein coupled receptor signaling and the actin cytoskeleton [161]. Moreover, it has been shown that PYK2 is the main mediator of extracellular stimulus-induced activation of the MAPK and Janus-associated kinase (JAK) pathways [158, 162]. However, their divergent functions in cells can be considered as a result of variable bindings of proteins to the FERM domain of FAK and to the FAT domain of PYK2 [149]. There is evidence of increase PYK2 expression as a compensatory response in fibroblast and endothelial cells from FAK knockout mouse embryos [163, 164]. Unlike FAK, the main PYK2 autophosphorylation site is tyrosine 402, between the FERM and catalytic domains [157]. Tyrosine 402 phosphorylation of PYK2 provides a binding site for Src family kinase (SFK), that subsequently phosphorylates other tyrosine residues within Pyk2 (Tyr-579, 580, and 881), further promoting PYK2 activation [149]. Nonetheless, PYK2, like FAK, is activated by several extracellular stimuli such as cell attachment to the ECM and integrin activation, providing redundancy for processes in which FAK plays a crucial role [161].

1.4. Myofibroblasts and Cardiac Pathology

The majority of cardiac pathologies, such as MI, hypertension, diabetes, and congenital cardiomyopathies are associated with adverse ECM remodeling, which eventually evolves into cardiac fibrosis [165-167]. The clinical manifestations that contribute to changes in LV function and subsequent progression to HF are comprised of changes in the structure and constitution of the LV myocardium, its constituent cells, and the associated vasculature. During cardiac remodeling, dead cardiomyocytes are replaced by fibrotic tissue produced by myofibroblasts. In the healthy heart, fibroblasts residing in the cardiac interstitium preserve matrix homeostasis through a constant, slow turnover of the majority of the ECM proteins (mainly fibrillar collagens type I and III). However, in response to cardiac injury and corresponding disruption of the ECM, ordinarily quiescent fibroblasts are exposed to mechanical stress and TGF-β stimulation [168, 169]. Mechanical stress induces conversion of fibroblasts to myofibroblasts, a cell type that is marked by increased α-SMA and selective activation of MAPKs [170].

Myofibroblasts are a highly contractile, hypersynthetic and hypersecretory cell phenotype, critical to the proper formation of an infarct scar. There is evidence that as the injury stabilizes and a permanent scar forms, some of the myofibroblasts undergo senescence [171] and/or apoptosis [172, 173]. However, persistence of myofibroblasts in the myocardium for months or years after wound healing is the basis of cardiac fibrosis [174]. Myofibroblasts may originate from a variety of sources, including resident fibroblasts, bone marrow-derived cells, circulating fibrocytes [175], peripheral vascular pericytes [176], and fibroblasts produced through differentiation of epithelial cells through a process called epithelial-mesenchymal transition (EMT) [177-179]. Alternatively, some studies have suggested that the majority of myofibroblasts are derived from endothelial cells, induced through TGF-β1-mediated

endothelial-mesenchymal transition [180]. The myofibroblastic phenotype is characterized by increased expression of α-SMA and collagen [181, 182], the extra domain A (ED-A) splice variant of cellular fibronectin [183], and non-muscle myosin heavy chain B (SMemb) [16]. Myofibroblasts are also characterized by increased contractile force, which is necessary to mediate wound contraction post-injury [184]. Thus, migration of fibroblasts to the site of injury and their phenoconversion to the myofibroblast impairs the balance of the ECM turn over. In the acute phase of injury, e.g. early after MI, fibroblasts and early myofibroblasts secrete pro-inflammatory cytokines and chemokines which induce the ECM degradation *via* increased MMP expression and activity [185]. Subsequently, in the granulation and scar maturation phases, the disruption of the ECM structure is induced by excessive production of the ECM proteins (mainly collagens type I and III), adverse deposition of other ECM proteins, as well as unnecessary contraction of the extracellular matrix structure [186]. Moreover, the proportion of MMPs to their endogenous inhibitors, TIMPs production and activity become imbalanced, further promoting adverse ECM remodeling [12, 49].

1.5. Cardiac Fibrosis

Fibrosis of the heart, as well as other tissues such as lung, liver, and skin, is the outcome of excessive and disorganized deposition and contraction of the ECM [186]. Enhanced fibrillar collagen synthesis coupled with reduced degradation and turnover results in excess collagen accumulation [187], which results in disruption of normal tissue architecture and homeostasis that contributes to organ dysfunction [14]. The ECM remodeling may initially be regarded as an adaptive response to increased wall stress and neurohormonal activation. A critical therapeutic consideration is the degree to which cardiac fibrosis is reversible and if myofibroblasts can revert to a quiescent state or dedifferentiate back to their original cell type. Myofibroblasts and the pro-

fibrotic cytokine TGF-β that they produce and activate play primary roles in a wide range of pathological conditions associated with fibrosis [188]. Recent studies in humans and experimental models have shown increased myocardial TGF-β expression during cardiac hypertrophy and fibrosis [15, 189]. In addition, TGF-β exerts potent matrix-preserving actions by suppressing the activity of matrix metalloproteinases (MMP) and by inducing synthesis of protease inhibitors, such as TIMPs and Plasminogen activator inhibitor-1 [190].

1.5.1. Transforming growth factor β (TGF-β)

Although the source of myofibroblasts in cardiac fibrosis remains a moot point, it has been established that their activation is heavily influenced by TGF-β signaling [191]. In addition to its role in conversion of fibroblasts to myofibroblasts, TGF-β is also a potent mediator of various functions in many cell types, including differentiation, proliferation, and the ECM synthesis (most notably collagen and fibronectin) [192-194]. In addition to stimulating the ECM production, TGF-β also contributes to fibrosis through suppression of proteases that degrade the ECM components [195]. TGF-β is a highly ubiquitous protein produced primarily by myofibroblasts, but also cardiomyocytes, and is a major stimulator of fibrogenesis. Activation of TGF-β through its release from the LAP is highly dependent upon the cellular environment, and is particulary sensitive to myofibroblast-generated contractile forces and increased tissue stiffness [196]. Fibroblasts and myofibroblasts secrete TGF-β1 as part of a large latent complex (LLC) that consists of TGF-β1, LAP, and a latent TGF-β-binding protein-1 (LTBP-1) [197]. This latent complex provides a reservoir of TGF-β1 that can be quickly and easily activated in response to injury or stress, such as MI.

1.5.1.1. The Canonical SMAD-dependent TGF-β Signaling Pathway

TGF-β1 signaling is initiated through ligand binding to a pair of Serine/Threonine kinase heterodimeric TGF-β1 receptors, TβR-I and TβR-II. Binding of TGF-β1 induces TβR-II autophosphorylation, resulting in the recruitment and dimerization with TβR-I, which in turn activates receptor-mediated Smads (R-Smads) [198]. Within this complex, TβR-I phosphorylates one or more R-Smads (e.g. Smad2 and Smad3) at SSXS motifs in their carboxy-terminal tail [199]. Following phosphorylation and activation, these R-Smads then associate with the co-mediator Smad4 to form a complex that translocates to the cell nucleus and directly activates transcription of pro-fibrotic genes such as collagens and α-SMA [199, 200]. This canonical signaling pathway underlies nearly all functions of TGF-β [201].

1.5.1.2. Non-Canonical SMAD-independent TGF-β Signaling Pathway

Though the canonical pathway mediates most of the pro-fibrotic functions of TGF-β signaling, non-canonical pathways may also be involved in cell- or process-specific functions. Non-canonical signaling is also initiated through TβR-II, primarily through recruitment of TGF-β-activated kinase-1 (TAK-1) and TAK-1-binding protein (TAB). These proteins activate various MAPK signaling pathways including FAK-dependent JNK signaling [202] and p38 kinase signaling [203, 204]. Moreover, in response to non-canonical TGF-β signaling, ED-A fibronectin production increases resulting in formation of fibronectin fibrils that associate with α-SMA-positive stress fibers *via* FAs, increasing FA size, and inducing neoexpression of tensin and FAK as well as recruitment of vinculin and paxillin [205]. In a FAK-dependent manner, TAK1/mitogen-activate protein kinase kinase kinase-1 (MEKK-1)/JNK signaling induces myofibroblast differentiation [206], contributing to adverse ECM remodeling [207].

1.5.1.3. TGF-β activation

In addition to mechanical stress, proteases such as plasmin, MMP-2, and MMP-9 liberate and activate TGF-β from the LAP, altering the state of the ECM from that of degradation to one of synthesis [208]. Another matricellular protein, thrombospondin-1 (TSP-1), has been shown to activate TGF-β by binding to the LLC and inducing a conformational change in LAP that prevents its inhibition of TGF-β activity [209]. Additionally, reactive oxygen species generated in response to myocardial injury produce a slightly acidic environment that can also contribute to TGF-β activation. Moreover, integrin activation mediates various interactions that are capable of activating TGF-β, though their specific role likely depends on the cell type and pathological context [210].

1.5.1.4. TGF-β inhibition

Pro-fibrotic TGF-β1 signaling is ultimately a balancing act of stimulatory and inhibitory stimuli. The inhibitory Smad7 of the canonical TGF-β signaling pathway provides a negative feedback mechanism that reduces the effects of TGF-β on the activation of fibrosis-related genes by targeting TβR-I for degradation *via* recruitment of the Smad ubiquitination protein Smurf2 [211-213]. Regulation and inhibition of TGF-β1 signaling is also achieved through the action of anti-fibrotic transcription factors such as SnoN and Ski, discussed in the next sections [214, 215]. As a result of its pleiotropic effects leading to fibrogenesis, several drug companies have synthesized TGF-β inhibitors as novel treatments for cardiac fibrosis. The methods that have been used include anti-ligand antisense oligonucleotides, RNA ligand traps and antibodies targeting ligand or receptor [216-218]. For example, SB-431542 is a small molecule inhibitor that has been identified as an inhibitor of the TGF-β1 receptor in cancer *in vitro* studies [219,

220]; however, no clinical trials have been testing the direct effects of TGF-β inhibition in cardiac fibrosis.

1.6. Ski protein, structure and function

Ski is a highly conserved proto-oncoprotein and versatile regulator of transcription expressed in various tissues such as skeletal and cardiac muscle [221, 222], which is implicated in wound healing as well as the development of cancer [222-225]. Studies in transgenic animal models have shown that Ski upregulation causes skeletal muscle hypertrophy which is accompanied by decreased contractile force [226, 227]. In rat models of skeletal muscle injury, Ski mRNA levels are increased [228]. Human Ski is a 728 amino acid protein, containing a variety of structural domains. In the N-terminal region, Ski possesses a proline-rich region from amino acids 61-89, a cysteine/histidine-rich region, helix-loop-helix motifs, a leucine zipper-like domain, and a region consisting of a sequence of basic amino acids. Ski has been demonstrated to be phosphorylated by the cyclin-dependent protein kinase cdK1-cyclin B also know as cell division control protein kinase 2 (Cdc2-cyclin B) [229], likely regulating its function and/or location within the cell [214, 230]. Ski is also involved in regulation of canonical TGF-β signaling pathway, acting as a co-repressor that can prevent R-Smad phosphorylation in various cell types [231-233]. Ski can also bind activated R-Smad/co-Smad hetertrimer complexes (i.e. Smad2/3/4), preventing their translocation to the nucleus [234]. At the transcriptional level, Ski represses binding of activated Smad complexes to target DNA sequences, subsequent to Smad translocation to the nucleus [235]. Mutant Ski proteins that lack a fully functional nuclear translocation signal are still capable of inhibiting TGF-β signaling, indicating the ability of Ski to regulate pro-fibrotic signals beyond the transcriptional repression [214, 236]. This inhibitory

function has been demonstrated in skeletal myocytes, tumor cells, and fibroblasts, and may also regulate proliferation of vascular smooth muscle cells (VSMCs) following arterial injury.

1.6.1. Ski in the Heart

Ski expression has been detected in both neonatal and adult rat myocardium, as well as isolated cardiac cells, though levels are higher in neonates [222]. Different isoforms, produced through post-translational modifications, have been observed in isolated cardiac fibroblasts and human ventricular biopsies, including 95-, 105-, and 115-kDa variants [215, 237]. Such post-translational modifications may include phosphorylation and ubiquitination [215, 229]. In isolated cardiac myofibroblasts, expression of the 95-kDa Ski variant is reduced, indicating possible degradation. Although levels of the 105-kDa variant of Ski increase in post-MI rat infarcts (from 48 hours to 4 weeks) compared to viable regions and sham hearts, the effect of Ski in the cytosol remains unknown *in vivo*.

1.6.2. Ski and Myofibroblasts

Inhibitors of canonical TGF-β signaling, such as Smad7, have been shown to have anti-fibrotic effects *in vitro*, reducing the ECM synthesis and preventing conversion of fibroblasts to myofibroblasts [212]. Ski overexpression in primary cardiac myofibroblasts induces functional and phenotypic changes such as decreased synthesis and secretion of fibrillar type I collagen, decreased contractility, and reduced levels of myofibroblast markers such as α-SMA[215]. Moreover, myofibroblast apoptosis is increased with increasing doses of Ski, indicating the potential for this transcriptional regulator as a novel anti-fibrotic target. More specifically, overexpression of the 95-kDa form of Ski, which localizes to the nucleus, inhibits R-Smad2 in cardiac myofibroblasts, thereby inhibiting phenotypic conversion; collagen secretion and

contractility were reduced following Ski overexpression in these cells [215]. Thus, negative regulation of the myofibroblast phenotype by Ski may be an efficient and specific mechanism to inhibit or reverse the myofibroblast phenotype and resulting the ECM remodeling, cardiac fibrosis and heart failure. Understanding the effect of Ski on cellular functions, such as fibroblast MMP activation and migration, which are critical processes in wound healing post-MI, is a critical first step in determining the effectiveness of Ski as a therapeutic tool and its temporal utility.

2. RATIONALE, HYPOTHESIS AND OBJECTIVES

Proliferation and migration of fibroblasts *in vivo*, although necessary for proper cardiac wound healing, is the first step in a cascade of events leading to adverse ECM remodeling, which eventually progresses to cardiac fibrosis and subsequent heart failure. This fibrotic tissue remodeling process is initially triggered by MMP activation during the acute (inflammatory) phase of wound healing. Although activated MMPs are necessary for normal cardiac function as they degrade, remove and turnover matrix proteins that have reached their biological limit of usefulness cardiac matrix [50, 238], they are also the initial trigger in adverse ECM remodeling and fibrotic events in the heart [239]. Recently, MMPs and TIMPs have been identified as being potential therapeutic targets in cardiac fibrosis[240], and are known regulators of fibroblast migration, a critical process in post-injury remodeling in the heart[60]. More specifically, MMP-2 and -9 have been suggested to play a critical role in adverse remodeling, in part due to their ability to activate matrix-bound TGF-β1 directly by cleaving the latency-associated peptide normally bound to this cytokine in the myocardial interstitium [59, 241, 242].

At the cellular level, TGF-β induces the myofibroblastic phenotype associated with matrix remodeling and deposition of fibrotic tissue [156]. R-Smads are the canonical post-receptor signaling proteins for TGF-β1 in the heart (among other tissues), and are known to be strongly associated with the pro-fibrotic processes in heart disease [50, 238]. R-Smads are up-regulated in fibrosed hearts and are implicated in the regulation of various cardiac fibroblast functions [238, 243]. Endogenous inhibitors of the TGF-β pathway include inhibitory Smad7 (I-Smad7), which inhibits R-Smad activation, as well as the transcription factor Ski [215, 243]. Although the mechanisms involved in Ski inhibition of TGF-β/Smad signaling are not fully understood, its over-expression inhibits the myofibroblast phenotype through reduction in key myofibroblast

markers such as α-smooth muscle actin (α-SMA) and Extra domain-A fibronectin (ED-A FN) [215]. Thus, the strategic use of a TGF-β repressor such as Ski represents a putative novel means to manipulate the cardiac matrix in the disease setting for the direct treatment of cardiac fibrosis, *via* regulation of pro-fibrotic actions in myofibroblasts.

Based on these observations, in this project we investigate the effect of Ski expression on various pro-fibrotic functions, including ECM protein expression and cell migration, of cardiac myofibroblasts, specifically expression and activity of MMPs and their inhibitors, TIMPs.

2.1. General Hypothesis

We tested the hypothesis that in primary rat cardiac myofibroblasts the endogenous TGF-β inhibitor Ski modulates MMP activation, represses TIMP expression, reduces myofibroblast migration, and inhibits the expression and recruitment of key components to focal adhesion complexes in the cardiac myofibroblast. To examine these hypotheses, we outline 5 objectives.

2.1.1. Objective 1.

To investigate the effect of Ski on MMPs:

In vitro analysis of ectopic Ski overexpression on MMP-1, MMP-2, and MMP-9 protein expression in P1 cultured primary adult rat cardiac myofibroblasts using immunoblotting. Functionally the levels of MMP-2 and MMP-9 activity in cardiac myofibroblasts overexpressing Ski will be determined using gelatin zymography.

2.1.2. Objective 2.

To investigate the effect of Ski on TIMPs:

In vitro analysis of the effects of exogenous Ski overexpression on TIMP-3 and TIMP-4 protein expression in cardiac myofibroblasts using immunoblotting.

2.1.3. Objective 3.

To investigate the effect of Ski on cell motility:

In vitro assessment of exogenous Ski overexpression in cardiac myofibroblasts motility using transwell migration and scratch assay.

2.1.4. Objective 4.

To investigate the effect of Ski on focal adhesion associated proteins:

In vitro analysis of focal adhesion associated proteins including paxillin, and vinculin in cardiac myofibroblasts subjected to exogenous Ski overexpression using immunoblotting.

2.1.5. Objective 5.

To investigate the Effect of Ski on focal adhesion kinase:

In vitro analysis of focal adhesion kinase (FAK) family members including phosphorylated FAK [FAK (Tyr 397)] and phosphorylated PYK2 [PYK2 (Y402)] in cardiac myofibroblasts subjected to exogenous Ski overexpression using immunoblotting.

3. METHODS AND MATERIALS

3.1. Cell isolation/Culture

All animal studies protocols were accepted by the Animal Care Committee of the University of Manitoba, in accordance with the Canadian Institute of Health Research and the Canadian Council on Animal Care guidelines. According to our previously-established methods [215, 244, 245], cardiac fibroblasts were isolated from adult male Sprague–Dawley rats (150-200 g). Following heart excision, the removed heart was subjected to retrograde Langendorff perfusion with DMEM-F12 (Gibco, Carlsbad, CA) continued with serum-free MEM (SMEM; Gibco, Carlsbad, CA). Perfused hearts underwent digestion with 0.1% w/v collagenase type 2 (298 U/mg; Worthington)/SMEM followed by an additional digestion by using dilute collagenase solution (0.05% collagenase type II (298 U/mg)/SMEM). Digesting was terminated by adding [DMEM-F12 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin (Gibco, Carlsbad, CA), 100 mg/ml streptomycin (Gibco, Carlsbad, CA) and 1 mM ascorbic acid (Sigma, St Louis, MO)]. Afterwards, the entire suspension was settled to a 50 mL tube fitted with a Fisher brand sterile cell strainer (Fisher Scientific, Rockford, IL). The filtered solution was then centrifuged at 2000 rpm for 7 minutes. The pellet was resuspended in growth media prior to plating in 75 cm^2 culture flasks and kept for 2–3 hours in a 5% CO_2 incubator at 37°C to allow cell anchorage. Consequently, cells were washed twice with phosphate-buffered saline (PBS) to remove non-adherent cells (ie. myocytes). Adherent cells (fibroblasts) were incubated in fresh growth medium containing 10% FBS for approximately 24 hours before growth medium was changed. Cells were left to grow for 3-4 days until they reached the appropriate confluency prior to passaging. First passage (P1) myofibroblasts were utilized to make a suspension containing different cell numbers to perform transwell migration assays and scratch assays, or were plated at

10% confluency in either 100 mm^2 dishes or 6-well plates for use in Western blots, Zymography and Immunofluorescence assays.

3.2. Gene Overexpression

Cells were plated at 10% confluency and were left to grow to a confluency of 70%, at which point they were infected with either an HA-tagged Ski-overexpression adenovirus vector (Ad-HA-Ski) or Lac-Z adenovirus (Ad-Lac Z) and were serum-starved for 48 hours. A variety of multiplicities of infection (MOI) were implemented for each virus: 30, 50, 100 and 150. Non-infected cells were utilized as negative controls. In some experiments, cells were stimulated with TGF-β1 (10 ng/plate) at 24 hours post-infection to promote fibroblast-to-myofibroblast phenoconversion.

3.3. Adenoviral Constructs

HA-Ski adenoviral vector generation has been previously described [215]. The human Ski recombinant DNA inserted in plasmids was obtained from Dr. Shunsuke Ishii (Riken Tsukuba Institute, Ibaraki, Japan). Human Ski was tagged at the N-terminus by a hemagglutinin (HA) tag (YPYDVPDYA) by inserting into a pcDNA3.1N-HA vector (obtained from Dr. Michael Czubryt, University of Manitoba). Consequently, adenovirus for HA-tagged Ski (Ad-HA-Ski) was created by using the Adeno-X™ Expression Kit (Clontech, Mountainview, CA).

3.4. Adenovirus Titration

Adenoviral overexpression vectors were titred using the Adeno-X^{TM} Rapid Titre Kit, following the manufacturer's protocol. In short, HEK 293 ($5x10^5$ cells/ml) were plated in each well of a 12-plate in standard growth medium (10% FBS-DMEM). A set of 10- fold serial dilutions of the

viral samples, either Ad-Lac Z or Ad-HA-Ski, from 10^{-2} to 10^{-6}, were prepared in PBS or media as diluent. Each well was infected with 100 µl of viral dilution, and the cells were incubated at 37°C, in 5% CO_2 for 48 hours. Thereafter, the media was aspirated and cells were fixed with 1 mL 100% ice-cold methanol per well. The plate was incubated at -20°C for 10 minutes. The methanol was then removed, and the wells were gently rinsed with 1 mL PBS+1% bovine serum albumin (BSA). Subsequently, 0.5 mL of mouse anti-Hexon antibody (1:1000, in PBS+ 1% BSA) was added to each well, and incubated for an hour at 37°C. The anti-hexon antibody was removed, and each well was washed 3 times with 1 mL PBS+1% BSA. The secondary, HRP-conjugated rat anti-mouse antibody was then added to each well (0.5 mL; 1:500, in PBS + 1% BSA), and the plate was incubated for another hour at 37°C. DAB (3, 3'-diaminobenzidine) working solution was then prepared by diluting the 10x DAB substrate 1:10 with 1X stable peroxidase buffer. After removing the secondary antibody, each well was again washed 3 times with 1 mL PBS+1% BSA. 500 µl of DAB working solution was then added to each well, and allowed to incubate for 10 minutes at room temperature. Prior to visualizing the cells under a light microscope, the DAB solution was aspirated and 1 mL of PBS was placed in each well. To calculate the titre, a minimum of 3 fields of brown/black-positive cells were counted by using the 20x objective of microscope. The mean number of positive cells in each well obtained, and the infectious units/mL (virus titre) for each well was calculated according to the manual formula.

3.5. Protein Isolation

Forty-eight hours following transduction, conditioned medium was collected into 50 mL Centrifugal Filter Units (Millipore, Billerica, MA) and centrifuged at 4000 g for 20 minutes at 4°C. Thereafter, cells were washed with PBS. Consequently, RIPA lysis buffer (120 µl of lysis buffer per 100 mm plate) including protease inhibitor cocktail (4 µM leupeptin, 1 µM pepstatin

A, and 0.3 μM aprotinin; Sigma) and phosphatase inhibitors (10 mM NaF, 1 mM Na_3Vo_4 , and 1 mM EGTA) was utilized in order to lyse cells. Cell lysates were collected into 1.5 mL microcentrifuge tubes by mechanical scraping with a rubber cell scraper and were incubated on ice for an hour. Concentrated media from the upper side of the filter tubes was collected into 1.5 mL microcentrifuge tubes; protease inhibitor cocktail was added 1:100 (1 μL/100 μL), then stored at -20°C. After an hour incubation on ice, cell lysates were either stored at -20°C or sonicated for 3 x 10 seconds and then centrifuged at 14,000 rpm at 4°C for 15 minutes. The supernatants were carefully collected and transferred into new, autoclaved 1.5 mL microcentrifuge tubes. The samples were either stored at -20°C, or immediately analyzed for protein content.

3.6. Protein assay

All samples including concentrated media (secreted protein) and cell lysates proteins were put on ice to thaw. A standard curve was generated by loading 10 μL of various concentrations of bovine serum albumin (BSA) standard (Thermo scientific, Waltham, MA) in RIPA buffer (0, 0.2, 0.4, 0.8, 1.0, 1.5 and 2.0 μg/μL) into a 96-well plate, in triplicate. Subsequently, 1:10 dilutions of all samples in RIPA buffer were prepared and 10 μL of each diluted sample was loaded into the plate, in triplicate. A solution of 50:1 Bicinchoninic acid to Copper (II) sulfate was made and 200 μL was loaded into each well. The plate was then incubated for 30 minutes at 37°C. Thereafter, samples were analyzed in triplicates and the absorbance was recorded using an iMark Microplate Absorbance Reader (Bio-rad Laboratories, Hercules, CA) at a wavelength of 560 nm. Total protein content of each sample was calculated relative to the BSA standard curve [246].

3.7. Western Blot Analysis of Target Proteins

According to the concentration of proteins determined by protein assay, the loaded volume of each sample was calculated to contain 10-20 µg of protein each. Using RIPA buffer to equalize all the sample volumes, Laemmli buffer (125 mM Tris-HCl (pH6.8), 5 % glycerol, 2.5 %SDS, 5 % 2- mercaptoethanol, and 0.125 % bromophenol blue) was added by a ratio of 1:4. Thereafter, samples were boiled for 5 minutes and were allowed to return to room temperature. Proteins were separated using SDS-PAGE, with 10-20 µg of protein loaded per lane. Spectra Multicolor Broad Range Protein Ladder and Spectra High Range Protein Ladder (Thermo Fisher Scientific, Rockford, IL) standards were also used for identification of protein size. Proteins were separated electrophoretically on 8-12 % SDS polyacrylamide gels using 1X Running Buffer (Tris: 3.0 g, Glycine: 14.4 g, SDS: 1.0 g) at 120V for approximately 1.5 hours. Separated proteins were then transferred onto 0.45 µm polyvinylidene difluoride (PVDF) membrane using 20% 1X Transfer Buffer (Tris: 3.0 g, Glycine: 14.4 g, Methanol: 200 mL) at 100V for 110 minutes. Membranes were blocked in PBS containing 10% skim milk for 1.5 hours on an orbital shaker at room temperature. Subsequently, membranes were probed with primary antibodies diluted according to manufacturer recommendations in PBS with 3% skim milk for either 1.5 hours at room temperature or overnight at 4^{o} C on the shaker. Following 3 10-minute washes with PBS 0.2% Tween 20 (PBS-T), membranes were incubated with secondary antibody (horseradish peroxidase (HRP)-anti-mouse IgG or HRP-anti-rabbit IgG) diluted in PBS with 3 % skim milk for 1 hour at room temperature on the shaker. Membranes were then washed three times with PBS-T for ten minutes. Either Pierce ECL Western Blotting Substrate or SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL)

was used to image protein bands, and blots were developedon CL-XPosure ™ Film (Thermo Fisher Scientific, Rockford, IL). Protein loading was normalized against β-tubulin or eEF-2.

3.8. Gelatin Zymography

Sample volumes used for zymography were calculated to contain 10-15 µg of protein each. Using of RIPA buffer to equalize all the sample volumes, 4X zymography sample buffer(250 mM Tris-HCl (pH 6.8, 40% v/v glycerol, 8% w/v SDS; and 0.01% w/v bromophenol blue) was added by a ratio of 1:4. Thereafter, samples were vortexed and loaded on either Novex® 10% Zymogram (Life technologies, Grand Island, NY) or gelatin substrate acrylamide gels manually made in lab. The Spectra Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific, Rockford, IL) was used for identification of protein size along with using 3-5 µL of native human MMP-2 protein as a positive control. Zymography gels were run electrophoretically at a constant voltage (125 V, approx. starting current: 30–40 mA/gel) for approximately 3 hours using running buffer (25 mM Tris base, 192m M glycine (pH 8.3) and 0.1% w/v SDS). The gel was carefully removed from the cassette and placed in a plastic tray containing 50 mL of renaturing solution (25% v/v Triton X-100 in ddH2O). The gel was incubated for 30 minutes at room temperature with gentle agitation. The renaturing solution was discarded and the gel was rinsed with 100 mL of ddH_2O. The gel was incubated for an additional 30 minutes in a plastic tray containing 50 mL of developing buffer (500 mM Tris-HCl (pH 7.8), 2 M NaCl, 50 mM CaCl2, and 0.2% v/v Brij 35) at room temperature with gentle agitation. The developing buffer was replaced with 50 mL of fresh developing buffer and gel was incubated at 37°C for approximately 16 hours in a closed tray. Thereafter, the developing buffer was discarded and the gel was rinsed with ddH_2O. Gels were stained for at least 1 hour or until the gels were evenly dark blue, using a gel staining solution (0.5% w/v Coomassie blue R-250, 5% v/v methanol, and

10% v/v acetic acid in ddH_2O). Subsequently, the gel was destained using a destaining solution (10% v/v methanol and 5% v/v acetic acid in ddH2O) until the areas of gelatinolytic activity were observable as clear bands over the blue background. Thus, gels were carefully wrapped in gel wrappers, avoiding air bubble formation. Finally, zymography gels were framed and allowed to fully dry prior to imaging.

3.9. Transwell® Migration Assay

Upon passaging P0 cells, a suspension of 6 $X10^5$ P1 myofibroblasts in ~ 9 mL of 10% DMEM-F12 was prepared. 1.5 mL/well of this suspension (1X 10^5 cell/well) was placed in the upper chambers of a 6-well – Costar® Transwell® plate (Corning Inc, Corning, NY), a transmembrane, and two-chamber system which separates the cells from the lower chamber by an 8 µm porous polycarbonate membrane. Additionally, 2.5 mL/well of serum-free DMEM/F12 was added to each lower chamber. The cells were left for 2-3 hours to fully cover the transmembrane upper surface. Thereafter, cells were subjected to either Ad-HA-Ski or Ad-LacZ transduction with a MOI of 50. Some cells were left untreated as control. Twenty four hours after transduction, the lower chamber media was changed to 10% DMEM-F12 containing 20 ng/ml PDGF-BB (Platelet-Derived Growth Factor-BB; R&D Systems, Minneapolis, MN). The procedure was performed as previously described, with alterations[247]. Following additional 24-hour incubation, cells migrated through the membrane pores due to the chemoattractant gradient and became adherent to the lower surface of the membranes and at the bottom of the lower chambers. Thus, upper chamber media was carefully aspirated and washed once with PBS to remove all the floating cells. Using a cotton swab, remaining cells attached to the upper surface of each chamber were removed. All the inserts were placed into another 6-well plate containing 2 mL/well of 4% paraformaldehyde in PBS and were incubated at room temperature

for 5 minutes. Thereafter, the inserts were rinsed with PBS and soaked in 0.5 % Crystal violet solution for 3 minutes. The inserts were then rinsed with ddH_2O for 3 times or until the water no longer runs dark. The inserts were left to dry and their lower surfaces were imaged by a light microscope at the objectives of 4X and 10X. Along with imaging of the cells attached to the lower surface of the membrane, the floating cells in the media of each lower chamber were counted using a Moxi™ Z Mini Automated Cell Counter (Orflo Technologies, Hailey, ID) with a medium size cassette. Following aspiration of media from the chambers and subsequent washing with PBS, chambers surfaces were treated with 1.5 mL/well tripLE™ 1X (Life technologies, Grand Island, NY) for 10-15 minutes at 37°C and then neutralized with equal volumes of DMEM/F12. The cell suspensions were counted once again using the Moxi™ Z Mini Automated Cell Counter (Orflo Technologies) to identify the number of cells that migrated and adhered to the bottom surface of lower chambers.

3.10. Scratch Assay

Upon passaging P0 cells, a suspension of $3x10^5$ P1 myofibroblasts in 1 mL of DMEM-F12 supplemented with 10% FBS was prepared. Following settling, silicone culture inserts (Ibidi, Martinsried, Germany) were placed into each well of a 6-well plate; 70 µL of cell suspension was transferred to each side of the silicone divider. The area surrounding each culture insert was carefully covered with 1.8 mL of 10% DMEM-F12. Cells were allowed to form a confluent monolayer on each side of silicone dividers for 24 hours. Thereafter, all culture media was changed to 2% DMEM-F12, at which point cells were either infected with Ad-HA Ski or Ad-LacZ, or kept untreated as a control group. Forty-eight hours after, all media was aspirated and cells were washed once with PBS to remove all floating cells. Inserts were carefully removed using forceps and 2 mL of fresh 2% DMEM-F12 was added to each well. Cells were imaged

using a light microscope using a 10X objective for the time t = 0. Photographs were taken every 3 hours for a period of 24 hours or until the gaps were fully covered with migrated cells. Scratch area percentage was obtained by Wimasis image analysis program (Wimasis Image analysis, Munich, Germany).

3.11. Live/Dead assay

Upon passaging P0 rat cardiac fibroblasts, cells were plated in a 6-well plate, containing coverslips, at 10% confluency in 10% DMEM-F12. The cells were then left to reach a confluency of 60-70%. Subsequently, the cells were either infected with Ad-LacZ or Ad-Ski, or were left uninfected as a negative control. Following 48 hours of incubation, a dead control group was obtained by incubating on well of cells plated in 100% methanol for 20 minutes. A live/dead assay was performed by using the LIVE/DEAD® Viability/ Cytotoxicity Kit for mammalian cells (Invitrogen, Waltham, MA). In accordance with the manufacturer's recommendations, 20 µL of 2 mM EthD-1 stock solution (Component B) was added to 10 mL of sterile PBS, and mixed thoroughly. Thereafter, 5 µL of 4 mM calcein AM stock solution (Component A) was transferred to the 10 mL EthD-1 solution and was fully mixed. Subsequently, 100–150 µL of the combined LIVE/DEAD® assay reagents was added to the surface of a 22 mm square coverslip, covering all cells with the solution. The treated coverslips were then incubated for 30–45 minutes at room temperature. Following incubation, 10 µL of the fresh LIVE/ DEAD® reagent solution was added to clean microscope slides and wet coverslips were inverted and mounted on the slide by using forceps. The coverslip was then sealed to the glass slide by using nail polish. The cells were viewed by fluorescence microscopy, acquiring

images using fluorescein and rhodamine optical filters. Cells were counted as "dead" if they demonstrated intense fluorescence at a wavelength of 600 nm and little fluorescence at 530 nm.

3.12. Antibodies

Anti-Ski (Upstate/Millipore), anti-HA epitope tag (Rockland Immunochemicals), anti-β-tubulin, anti-MMP-1(ab806), anti-MMP-2 C-terminal (ab79781), anti-MMP-9 [EP1254], anti-Integrin-β1 [EPR16895], anti-p-PYK2 [Tyr 402], anti-PYK2 [EP206Y] (Abcam), anti-paxillin [5H11], anti-vinculin [V284] (Millipore), anti-TIMP-3 (Chemicon), anti-TIMP-4 (Chemicon), anti-FAK (Biosource), anti-p-FAK [Tyr 397] (Biosource).

Biological Reagents Native human MMP-2 Protein (Abcam); TGF-β1 cytokine (R & D System)

3.13. Statistical Analysis

The reported values for all data correspond to means ± standard error of means. All the experiments were repeated with a minimum of 3 replicates (n=3) with actual cells taken and cultured separately from three different rats for building the statistical cohort (otherwise mentioned in the figure legends). For the all experimental data with multiple samples, the statistical significance of differences between means was concluded by repeated measures one-way analysis of variance (ANOVA) with a Brown-Forsythe test for equal variance and Tukey post-hoc analysis. Differences were considered to be statistically significant when $p \leq 0.05$.

4. RESULTS

4.1. Adenoviral transduction and P1 rat cardiac myofibroblasts viability

Considering the possible effect of adenoviral transduction to reduce cell viability, a Live-Dead assay was performed to assess the effect of Ski overexpression through adenoviral transduction on rat cardiac myofibroblasts viability. We observed a trend towards a slight reduction in cell viability in cells subjected to adenoviral transduction over a 48 hours duration in camparison with non-infected control cells. However, this effect, as per preliminary visual examination, appears to be similar between the Ad-HA-Ski and Ad-LacZ infected goups (Figure 3).

Figure. 3

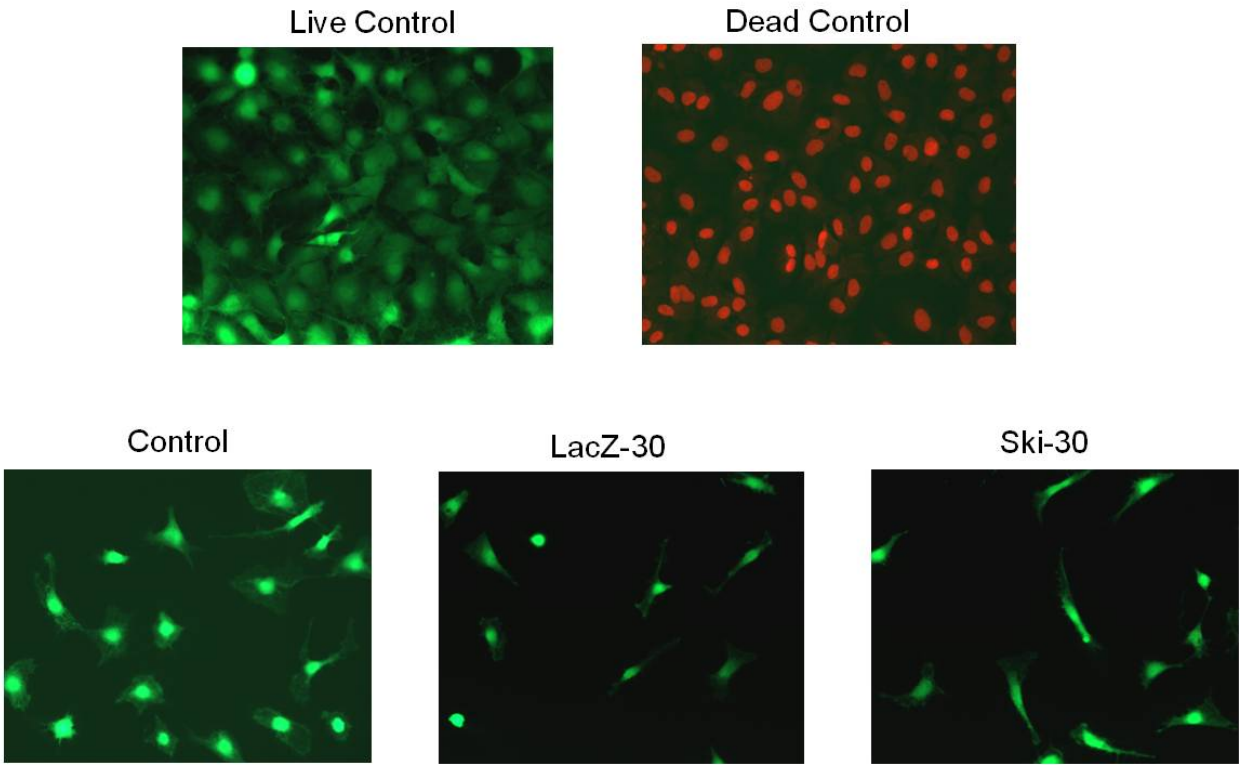


Figure 3. Adenoviral transduction affects cardiac myofibroblast viability. First passage (P1) rat cardiac myofibroblasts were grown to 60-70% confluency, starved, and infected with either Ad-HA-Ski or Ad-LacZ (MOI of 30) for 48 hours. Control cells were starved but not infected. Subsequently, a live/dead assay was performed by using LIVE/DEAD® Viability/ Cytotoxicity Kit and the cells were viewed by fluorescence microscopy. Preliminary results indicate a possible trend towards decreased viability in adenovirus-infected cells compared to uninfected controls; however the effect may potentially be equal between adenoviral treatment groups(n=1).

4.2. Ski overexpression in P1 rat cardiac myofibroblasts

To investigate the effect of Ski on a variety of target proteins, we overexpressed Ski by infecting P1 cardiac myofibroblasts with an HA-tagged human Ski adenovirus (Ad-HA-Ski) at 100 MOI (Ski-100). The 95-kDa form of Ski protein, normalized to internal control β-tubulin, was markedly increased in comparison to uninfected and Lac-Z expression adenovirus (Ad-LacZ) controls (Figure 4).

Figure. 4-A

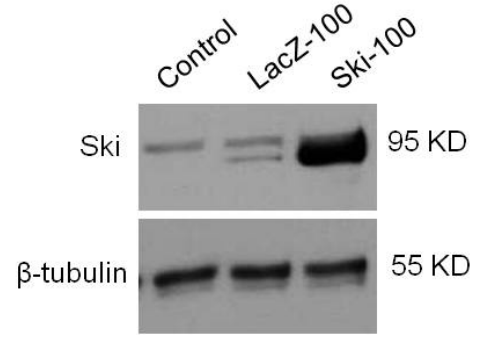


Figure. 4-B

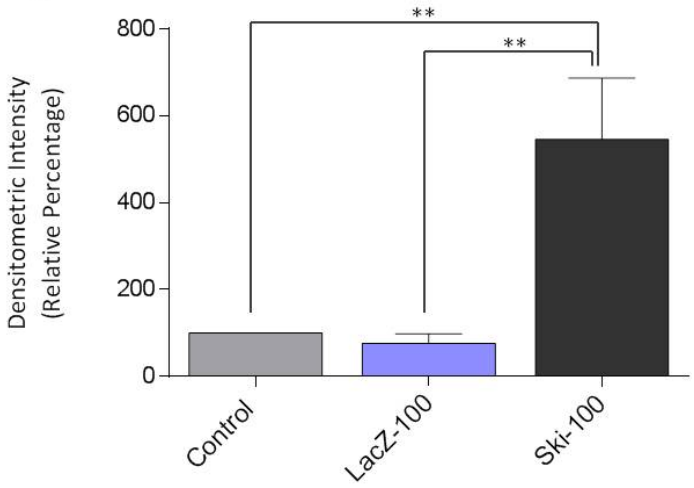


Figure 4. Overexpression of Ski in primary rat cardiac myofibroblasts. First passage (P1) cardiac myofibroblasts were grown to 70% confluency, and infected with either Ad-LacZ (LacZ-100) or Ad-HA-Ski (Ski-100) at MOI of 100 for 48 hours. Untreated control lysates were starved but not infected with either virus. **(A)** Representative immunoblots of total cell lysates probed for Ski, and β-tubulin (control). **(B)** Histogram showing data obtained by demsitometry of blots as in A. The level of Ski was significantly increased in response to Ski overexpression, compared to compared to Ad-LacZ-infected and untreated controls. (n=4, repeated measures one-way ANOVA, Tukey post-hoc analysis; $p \leq 0.01$ (**), compared to Ad-LacZ-infected and uninfected controls, mean ± SEM)

4.3. Ski overexpression and intracellular MMP-2 expression in P1 rat cardiac myofibroblasts

Ski overexpression induced a significant increase in intracellular MMP-2 protein abundance in comparison to uninfected control (Figure 5).

Figure. 5-A

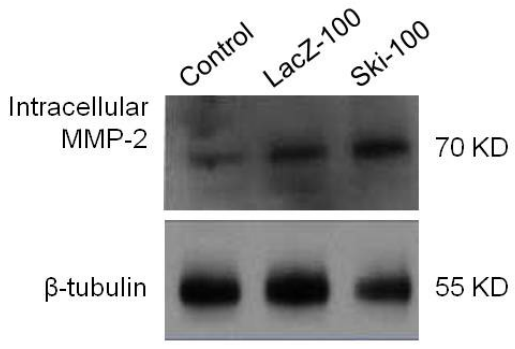


Figure. 5-B

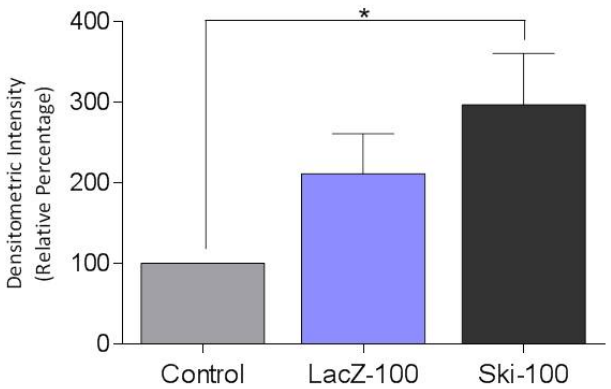


Figure 5. Overexpression of Ski increases intracellular MMP-2 expression in primary rat cardiac myofibroblasts. First passage (P1) cardiac myofibroblasts were grown to 70% confluency, and infected with either Ad-LacZ (LacZ-100) or Ad-HA-Ski (Ski-100) at MOI of 100 for 48 hours. Untreated control lysates were starved but not infected with either virus. **(A)** Representative immunoblots of total cell lysates probed for intracellular MMP-2, Ski, and β-tubulin (control). **(B)** Histogram showing data obtained by demsitometry of blots as in A. The level of intracellular MMP-2 was significantly increased in response to Ski overexpression, compared to untreated controls. (n=4, repeated measures one-way ANOVA, Tukey post-hoc analysis; $p \leq 0.05$ (*), compared to uninfected control, mean ± SEM)

4.4. Ski overexpression and intracellular MMP-2 activity in P1 rat cardiac myofibroblasts

To examine the effects of Ski overexpression on intracellular MMP-2 activity, primary rat cardiac myofibroblasts were infected with Ad-Ski at either 50 or 100 MOI for 48 hours. Activity of intracellular MMP-2 was assessed using gelatin zymography. MMP-2 activity was significantly increased by Ski overexpression compared to uninfected and Ad-LacZ-infeced controls (Figure 6).

Figure. 6-A

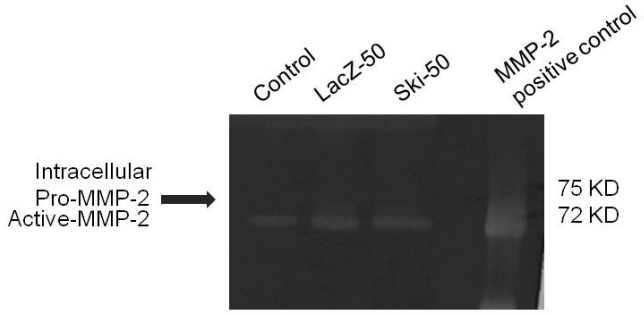


Figure. 6-B

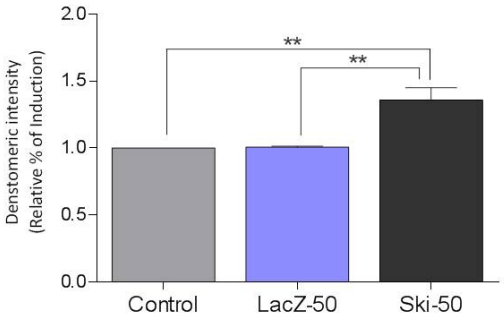


Figure. 6-C

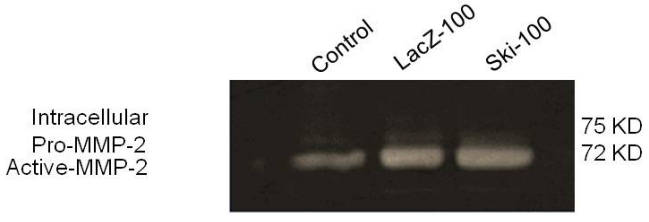


Figure. 6-D

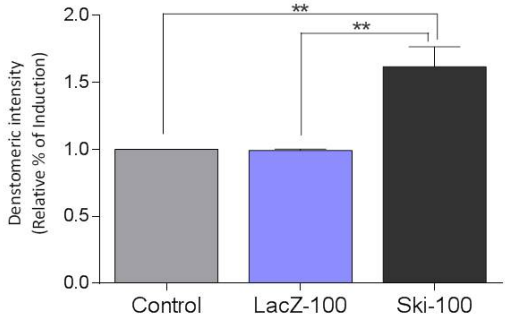


Figure 6. Overexpression of Ski increases intracellular MMP-2 activity in rat cardiac myofibroblasts. First passage (P1) primary cardiac myofibroblasts were grown to 70% confluency, and infected with either Ad-LacZ or Ad-Ski at 50 and 100 MOI for 48 hours. Untreated control lysates were starved but not infected with either virus. **(A and C)** Representative gelatin zymographs of total cell lysates for intracellular MMP-2. Expression of exogenous HA-tagged Ski was characterized using anti-Ski antibody by Western blot (data not shown). Native human MMP-2 protein (5 ug) was used as a positive control. **(B and D)** Histogram showing data obtained in A and C, respectively. The level of intracellular MMP-2 activity was significantly increased in response to Ski overexpression, compared to Ad-LacZ and untreated controls. (n=3, repeated measures one-way ANOVA, Tukey post-hoc analysis; $p \leq 0.01$(**), compared to uninfected control and Ad-LacZ, mean ± SEM).

4.5. Ski overexpression and MMP-2 secretion by P1 rat cardiac myofibroblasts

In order to investigate the effects of Ski expression on MMP-2 secretion, we assayed P1 rat cardiac myofibroblast media for MMP-2 abundance using immunoblotting. We observed a significant reduction in the level of secreted MMP-2 in Ad-Ski-infected cells, compared to Ad-LacZ-infected and uninfected controls (Figure 7).

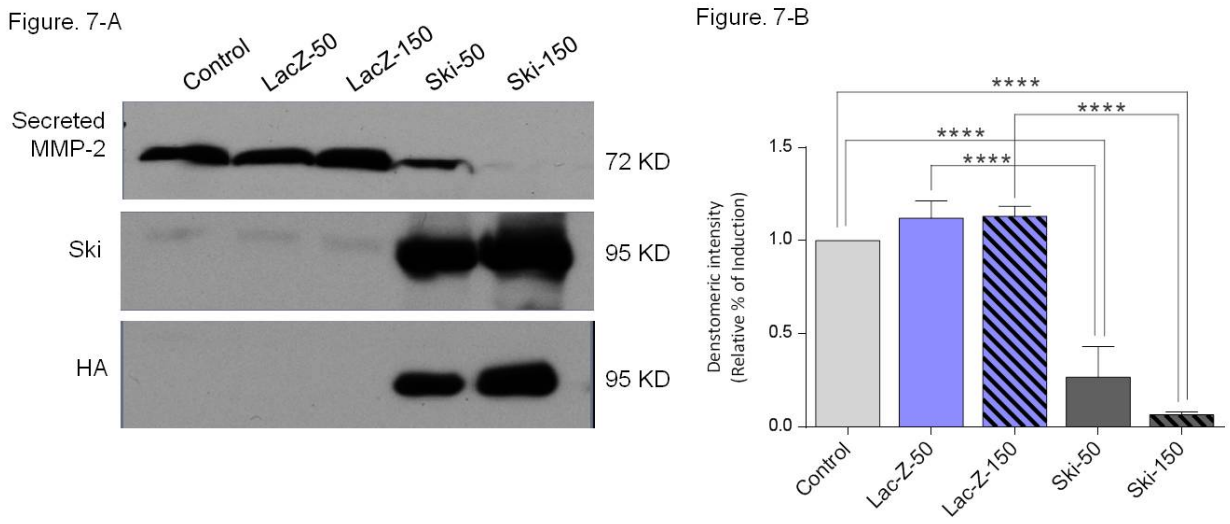


Figure 7. Overexpression of Ski decreases MMP-2 secretion in rat cardiac myofibroblasts. First passage (P1) primary rat cardiac myofibroblasts were grown to 70% confluency, and infected with either Ad-LacZ or Ad-Ski at 50 and 150 MOI for 48 hours. Untreated control lysates were starved but not infected with either virus. Total cell lysates were assayed for Ski and HA flag to confirm successful overexpression. Conditioned media was assayed for MMP-2 protein via immunoblotting. **(A)** Representative immunoblots of concentrated conditioned media (20µg/lane) for secreted MMP-2, as well as cell lysates for HA-tagged Ski. **(B)** Histogram showing data obtained in A. The level of secreted MMP-2 was significantly attenuated in response to Ad-Ski infection at 50 and 150 MOI. (n=3, repeated measures one-way ANOVA, Tukey post-hoc analysis; $p \leq 0.0001$(****), compared to control, LacZ-50 and 150, mean ± SEM)

4.6. Ski overexpression and secreted MMP-2 activity in P1 rat cardiac myofibroblasts

Ski-overexpressing cells exhibited significantly reduction in MMP-2 activity at a MOI of 150 in comparison with Ad-LacZ-infected control cells by gelatin zymography (Figure 8).

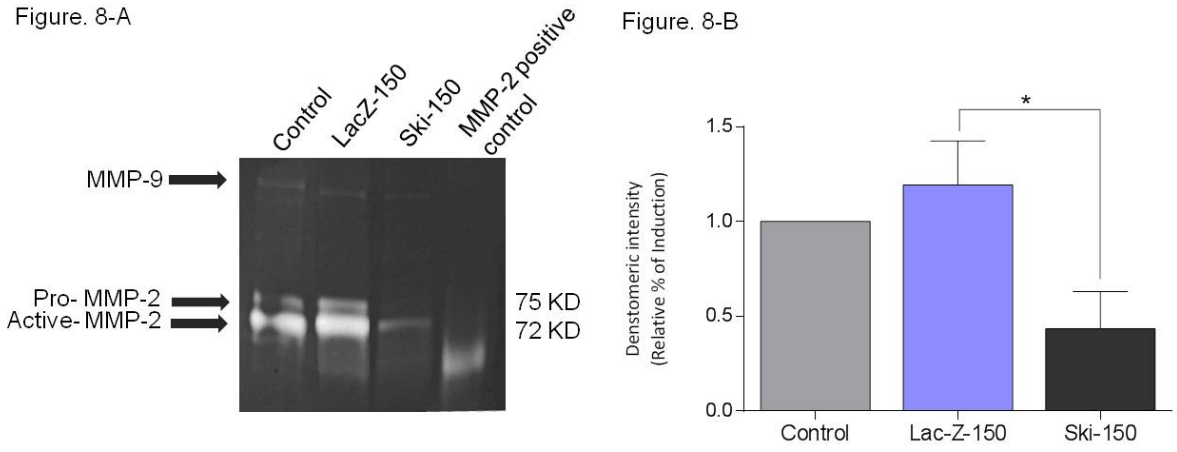


Figure 8. Overexpression of Ski downregulates secreted MMP-2 activity by rat cardiac myofibroblasts. First passage (P1) primary cardiac myofibroblasts were grown to 70% confluency, starved, and infected with either Ad-LacZ or Ad-Ski at 150 MOI for 48 hours. Untreated controls were starved but not infected with either virus. Concentrated conditioned media (10-15ug/lane) from all groups were examined for MMP-2 activity by gelatin zymography using native human MMP-2 protein (5 ug) as a positive control. **(A)** Representative gelatin zymography of concentrated conditioned media for secreted MMP-2. Expression of exogenous HA-tagged Ski was confirmed using anti-Ski antibody by Western blot (data not shown). **(B)** Histogram showing data obtained in A. The level of secreted MMP-2 activity was significantly attenuated in response to Ski overexpression at MOI of 150, relative to LacZ-infected controls

(n=3, repeated measures one-way ANOVA, Tukey post-hoc analysis; $p \leq 0.05$(*), compared to LacZ-150, mean ± SEM).

4.7. Ski overexpression and intracellular MMP-9 expression in P1 rat cardiac myofibroblasts

To assess the effect of Ski on intracellular MMP-9 abundance, levels of this protein were examined using immunoblotting subsequent to Ad-Ski infection (MOI of 100, 48 hours) in P1 cardiac myofibroblasts. We observed no significant difference in intracellular MMP-9 protein levels between Ad-Ski-infected lysates and those of Ad-LacZ-infected and uninfected controls (Figure 9).

Figure. 9-A

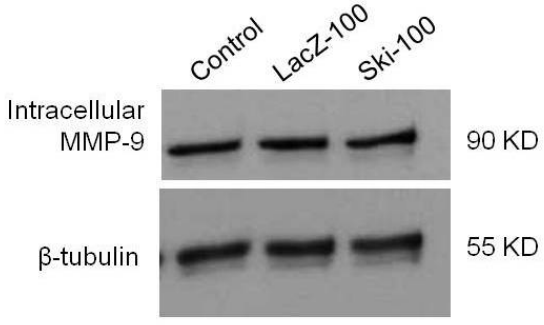


Figure. 9-B

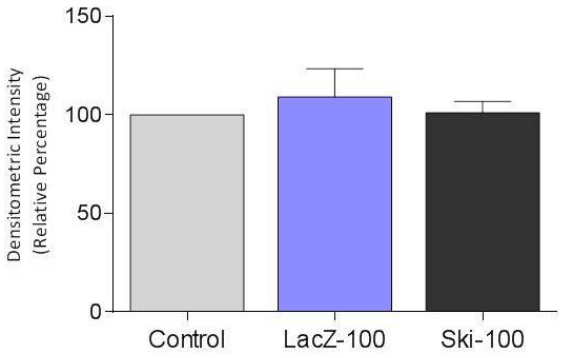


Figure 9. Overexpression of Ski does not alter intracellular MMP-9 expression in rat cardiac myofibroblasts. First passage (P1) cardiac myofibroblasts were grown to reach 70% confluency and infected with either Ad-LacZ or Ad-HA-Ski (MOI of 100) for 48 hours.

Untreated control lysates were starved but not infected with either virus. **(A)** Representative immunoblots of total cell lysates probed for intracellular MMP-9. Expression of exogenous HA-tagged Ski was characterized using anti-Ski antibody (Fig. 4) and β-tubulin was used as a loading control. **(B)** Histogram showing data obtained in **A**. The level of intracellular MMP-9 was not altered in response to Ski overexpression (Ski-100). (n=8, repeated measures one-way ANOVA, Tukey post-hoc analysis; p= 0.7394, mean ± SEM)

4.8. Ski overexpression and MMP-9 secretion by P1 rat cardiac myofibroblasts

In addition to MMP-9 expression within cells, we also assessed MMP-9 secretion in response to overexpression of Ski in P1 cardiac myofibroblasts. As demonstrated by immunoblotting of conditioned cell media after 48 hours of infection with Ad-Ski or Ad-LacZ (MOI of 50 or 150), MMP-9 secretion is significantly increased in response to Ski overexpression (Figure 10).

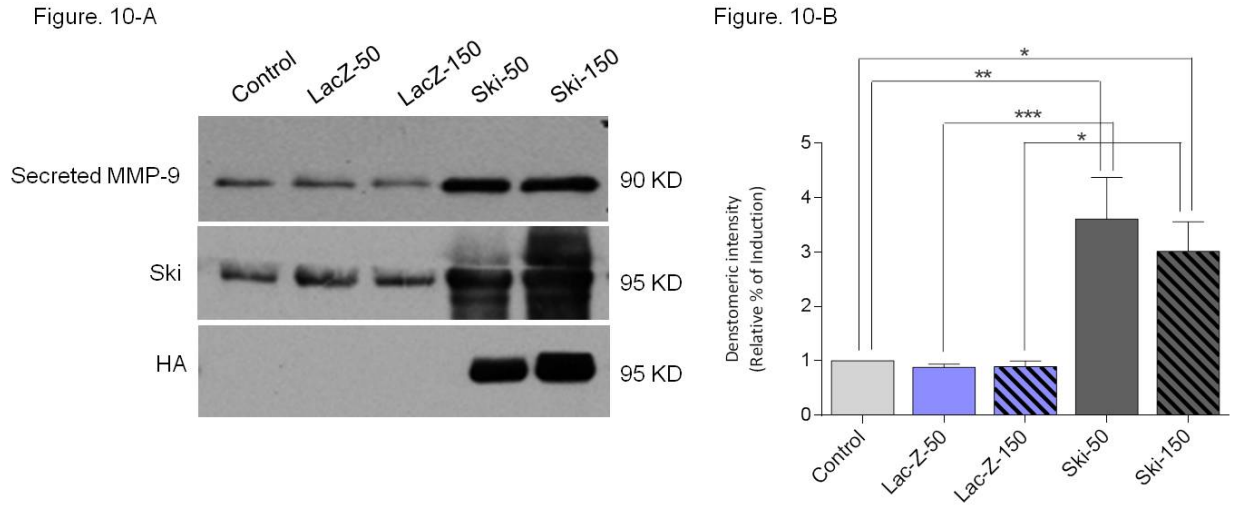


Figure 10. Overexpression of Ski increases MMP-9 secretion by rat cardiac myofibroblasts. First passage (P1) cardiac myofibroblasts were grown to reach 70% confluency and infected with either Ad-LacZ or Ad-HA-Ski (MOI of 50 or 150) for 48 hours. Untreated control cells were starved but not infected with either virus. **(A)** Representative immunoblots of conditioned media probed for MMP-9. Concentrated conditioned media (20ug/lane) of all groups were assessed for MMP-9 secretion using anti-MMP-9 antibody by Western blot. Expression of exogenous HA-tagged Ski in total cell lysates was characterized using anti-Ski and anti-HA antibodies. (B) Histogram showing data obtained in A. The level of secreted MMP-9 was significantly increased in Ad-Ski-infected cells compared to Ad-LacZ-infected controls. (n=7, repeated measures one-way ANOVA, Tukey post-hoc analysis; At MOI of 50, $p \leq 0.01$ (**) compared to control and $p \leq 0.001$ (***) compared to LacZ-50 and at MOI of 150, $p \leq 0.05$ (*) compared to control and LacZ-150, mean ± SEM)

4.9. MMP-9 secretion over time in response to Ski overexpression in P1 rat cardiac myofibroblasts

We assayed MMP-9 secretion at various timepoints following Ad-Ski infection (MOI of 50). We observed significant increases in MMP-9 secretion at all durations (24, 48, 72, and 96 hours) of Ad-Ski infection, as compared to Ad-LacZ-infected and untreated controls. MMP-9 secretion induced by Ad-Ski infection did not increase significantly between 24 and 48 hours of infection. Cells infected with Ad-Ski for 72 hours showed significantly greater levels of MMP-9 secretion compared to that of 48 and 24 hours. However, increased duration (96 hours) of Ad-Ski infection did not further augment MMP-9 secretion (Figure 11).

Figure. 11-A

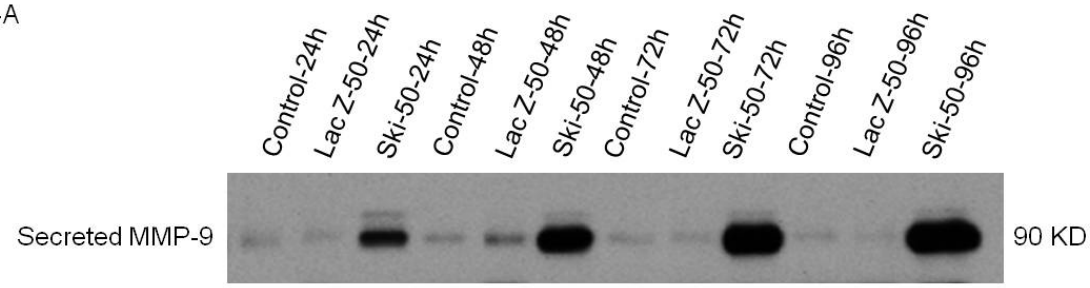


Figure. 11-B

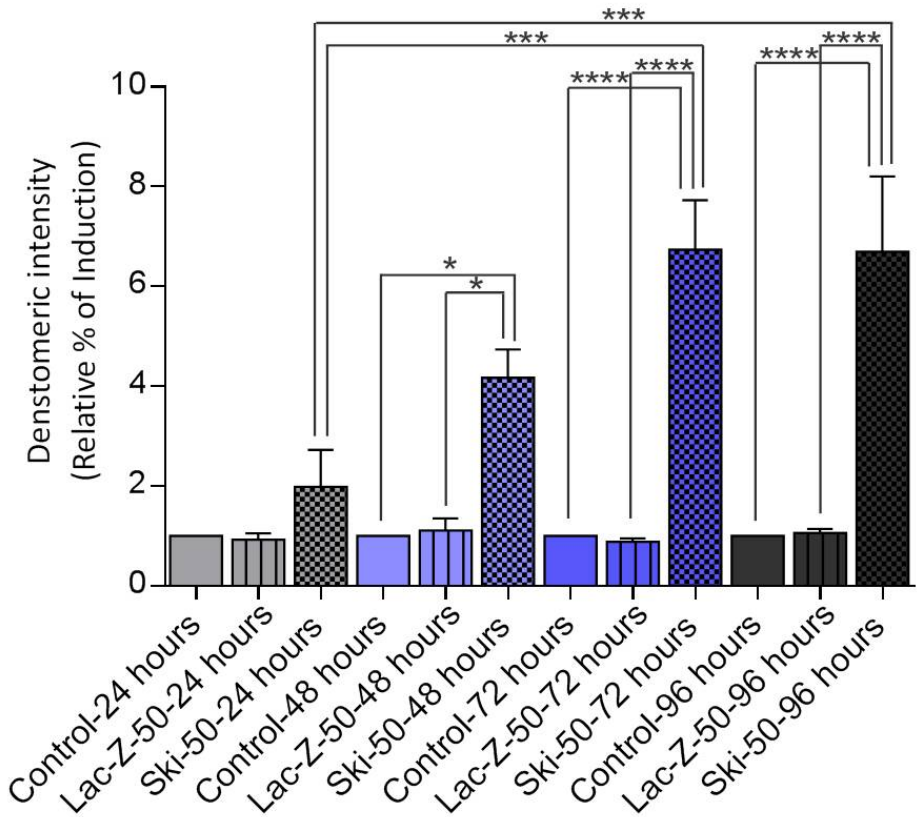


Figure 11. MMP-9 secretion is gradually augmented over time in Ski overexpressing rat cardiac myofibroblasts. First passage (P1) cardiac myofibroblasts were grown to 70% confluency and infected with either Ad-LacZ or Ad-HA-Ski (MOI of 50) for 24, 48, 72, or 96 hours. Untreated control cells were starved but not infected with either virus. **(A)** Representative immunoblot of concentrated conditioned media probed for MMP-9. Expression of exogenous HA-tagged Ski was characterized using anti-Ski and anti-HA antibody in total cell lysates (data

not shown). **(B)** Histogram showing data obtained in A. The level of secreted MMP-9 was significantly increased in response to Ski overexpression at 48, 72, 96 hours-post infection. (n=3, repeated measures one-way ANOVA, Tukey post-hoc analysis; $p \leq 0.05$ (*) for 24 hours and $p \leq 0.0001$ (****) for 72 and 96 hours compared to control and LacZ-50, and $p \leq 0.001$ (***) for Ski-24 hours compared to Ski-72 and 96 hours, mean ± SEM)

4.10. Ski overexpression and secreted MMP-9 activity in P1 rat cardiac myofibroblasts

In consideration of our finding that Ski-overexpressing cardiac myofibroblasts exhibited significant increases in MMP-9 secretion, the activity of secreted MMP-9 was assessed using gelatin zymography. Ad-Ski infection (50 MOI, 48 hours) induced an increase in MMP-9 activity, as compared to Ad-LacZ-infected and uninfected controls (Figure 12).

Figure. 12-A

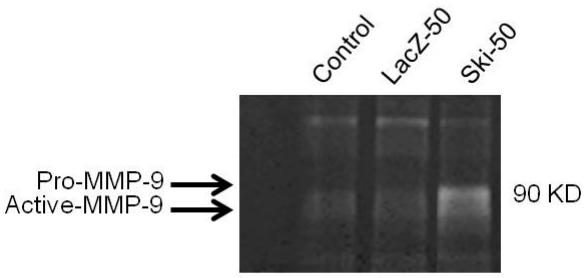


Figure. 12-B

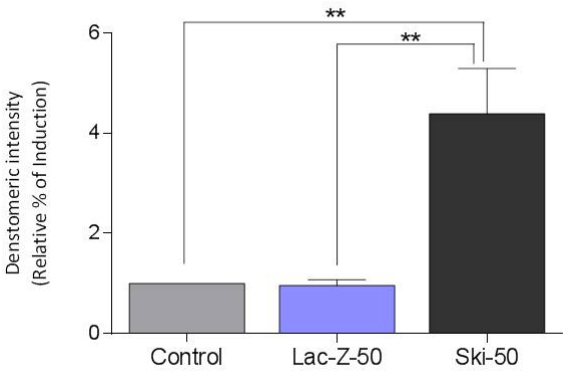


Figure 12. Overexpression of Ski increases MMP-9 activity in rat cardiac myofibroblasts. First passage (P1) cardiac myofibroblasts were grown to 70% confluency and infected with either Ad-LacZ or Ad-HA-Ski (MOI of 50) for 48 hours. Untreated control cells were starved but not infected with either virus. Expression of exogenous HA-tagged Ski was characterized using

anti-Ski antibody in total cell lysates (data not shown). (A) Representative gelatin zymography of concentrated conditioned media (25ug/lane) from all groups for MMP-9 activity. (B) Histogram showing data obtained in A. MMP-9 activity is significantly increased in response to Ad-Ski infection, compared to Ad-LacZ-infected and uninfected controls (n=3, repeated measures one-way ANOVA, Tukey post-hoc analysis; $p \leq 0.01$(**) compared to control and LacZ-50, mean ± SEM)

4.11. Ski overexpression and intracellular MMP-1 expression in P1 rat cardiac myofibroblasts

Since MMP-1 (collagenase) is also expressed in myocardium and involved in degradation of ECM components, we assayed the effect of Ski over-expression on levels of MMP-1 in rat cardiac myofibroblasts. Intracellular MMP-1 expression was not significantly altered by Ski overexpression, compared to Ad-LacZ-infected and untreated controls (Figure 13).

Figure. 13-A

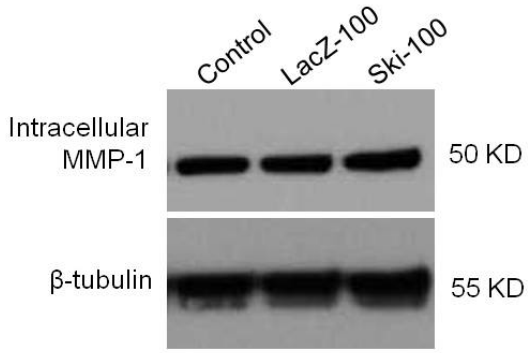


Figure. 13-B

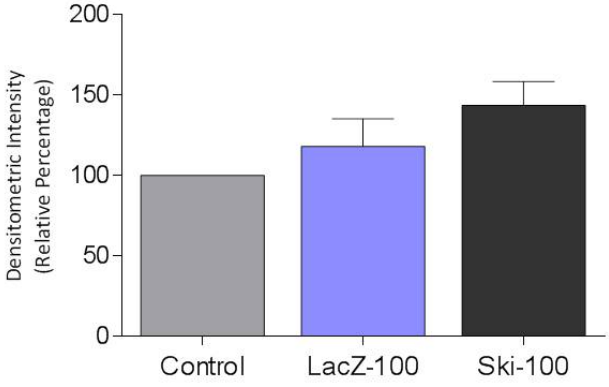


Figure 13. Overexpression of Ski does not alter intracellular MMP-1 expression in rat cardiac myofibroblasts. First passage (P1) cardiac myofibroblasts were grown to reach 70% confluency and infected with either Ad-LacZ or Ad-HA-Ski (MOI of 100) for 48hours. Untreated control lysates were starved but not infected with either virus. **(A)** Representative immunoblots of total cell lysates probed for intracellular MMP-1 48 hours post-infection. Expression of exogenous Ski was characterized using anti-Ski antibody (Fig. 4); β-tubulin was used as a loading control. **(B)** Histogram showing data obtained in A. The level of intracellular

MMP-1 did not show significant changes in response to Ski overexpression after 48 hours. (n=8, repeated measures one-way ANOVA, Tukey post-hoc analysis; p=0.0880, mean ± SEM)

4.12. Ski overexpression and TIMP-3 secretion by P1 rat cardiac myofibroblasts

The activity of MMPs is not the sole determinant of cardiac myofibroblast-mediated remodeling. The levels of MMP inhibitors (TIMPs) will affect how MMP expression, secretion, and activity will affect overall myofibroblast function. As TIMP-3 and TIMP-4 are mainly involved in cardiac pathologies and are responsible for inhibiting MMP-2 and MMP-9, we assayed the secretion of these TIMPs in response to Ad-Ski infection (at MOI of 50 and 150 for 48 hours) in primary cardiac myofibroblasts. TIMP-3 secretion, determined by probing of the conditioned cell media, was significantly decreased in response to Ad-Ski infection, as compared to Ad-LacZ-infected and untreated controls (Figure 14).

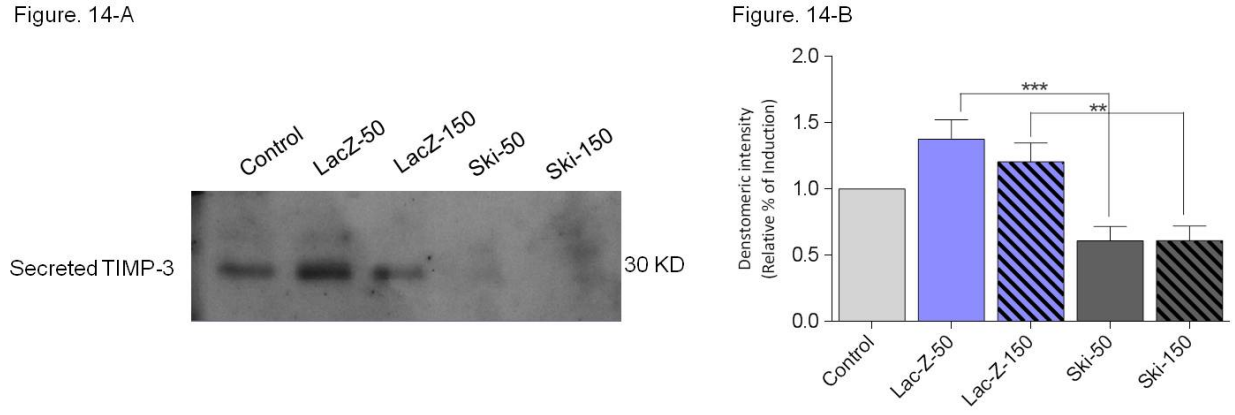


Figure 14. TIMP-3 secretion is attenuated by Ski overexpression. First passage (P1) cardiac myofibroblasts were grown to 70% confluency and infected with either Ad-LacZ or Ad-HA-Ski (MOI of 50 or 150) for 48 hours. Untreated control cells were starved but not infected with either virus. Expression of exogenous HA-tagged Ski was confirmed using anti-Ski and anti-HA

antibody in total cell lysates (data not shown). **(A)** Representative immunoblot of conditioned media for TIMP-3. Concentrated conditioned media (20ug/lane) of all groups were assessed for TIMP-3 secretion using TIMP-3 antibody. **(B)** Histogram showing data obtained in A. Secreted TIMP-3 levels are significantly decreased in response to Ad-Ski infection, compared to Ad-LacZ and uninfected controls. (n=6, repeated measures one-way ANOVA, Tukey post-hoc analysis; $p \leq 0.001$ (***) compared to LacZ-50 and $p \leq 0.01$ (**) compared to LacZ-150, mean ± SEM)

4.13. Ski overexpression and TIMP-4 secretion in P1 rat cardiac myofibroblasts

As for TIMP-3, we assayed for TIMP-4 secretion levels in P1 cardiac myofibroblasts infected with either Ad-Ski or Ad-LacZ at MOI of 50 or 150 for 48 hours. In agreement with previous reports, TIMP-4 was observed in 2 molecular weight forms, 23 and 28 kDa, which are most likely representative of the non-glycosylated and glycosylated forms of TIMP-4, respectively [67]. Interestingly, glycosylated TIMP-4 secretion was significantly reduced in Ski-overexpressing cells at a MOI of 50 and 150, compared to uninfected and Ad-LacZ-infected controls; however, secretion of non-glycosylated TIMP-4 was not altered by Ski overexpression (Figure 15).

Figure. 15-A

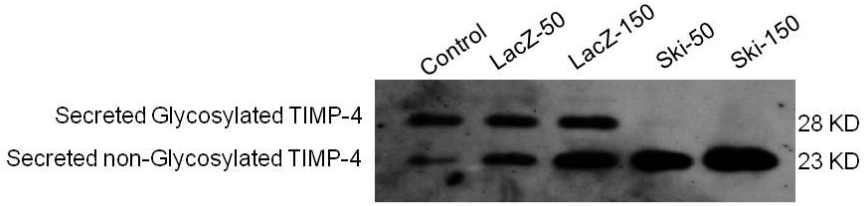


Figure. 15-B

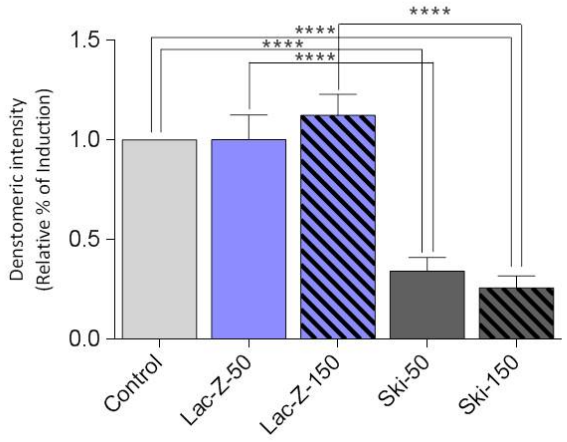


Figure. 15-C

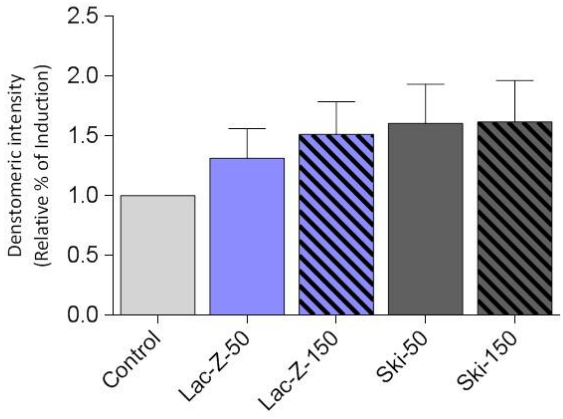


Figure 15. Glycosylated TIMP-4 secretion is attenuated by Ski overexpressing cells. First passage (P1) cardiac myofibroblasts were grown to 70% confluency and infected with either Ad-LacZ or Ad-HA-Ski (MOI of 50 or 150) for 48 hours. Untreated controls were starved but not infected with either virus. Expression of exogenous HA-tagged Ski was confirmed using anti-Ski and anti-HA antibody in total cell lysates (data not shown). **(A)** Representative immunoblot of conditioned media probed for TIMP-4. Concentrated conditioned media (20ug/lane) of all groups were assessed for both glycosylated and non-glycosylated TIMP-4 secretion using anti-TIMP-4 antibody. **(B)** Histogram showing data obtained in A for glycosylated TIMP-4. Protein levels of secreted glycosylated TIMP-4 were significantly reduced with Ad-Ski infection, compared to Ad-LacZ-infected and uninfected controls. (n=7, repeated measures one-way ANOVA, Tukey post-hoc analysis; $p \leq 0.0001$ (****) compared to control, LacZ-50 and 150). **(C)** Histogram showing data obtained in A for non-glycosylated TIMP-4. No changes in protein levels of non-

glycosylated TIMP-4 were observed between treatment groups. (n=7, repeated measures one-way ANOVA, Tukey post-hoc analysis; p= 0.4653, mean ± SEM)

4.14. Ski overexpression and P1 rat cardiac myofibroblasts motility

Considering the identified role for Ski as a modulator of fibroblast phenotype, we sought to examine the possibility of Ski-induced modulation of myofibroblast motility by wound healing (scratch) assay. After achieving a confluent layer of cells seeded in each side of culture inserts in a 6-well plate, P1 myofibroblasts were starved and then subjected to either Ad-Ski or Ad-LacZ (50 MOI) infection, or left untreated for 48 hours. Subsequently, the inserts were removed and cell imaging was conducted over a period of 24 hours. It was revealed that Ski-overexpressing cells had both slower motility and gap-closing rate, versus uninfected and Ad-LacZ-infected controls (Figure 16).

Figure. 16-A

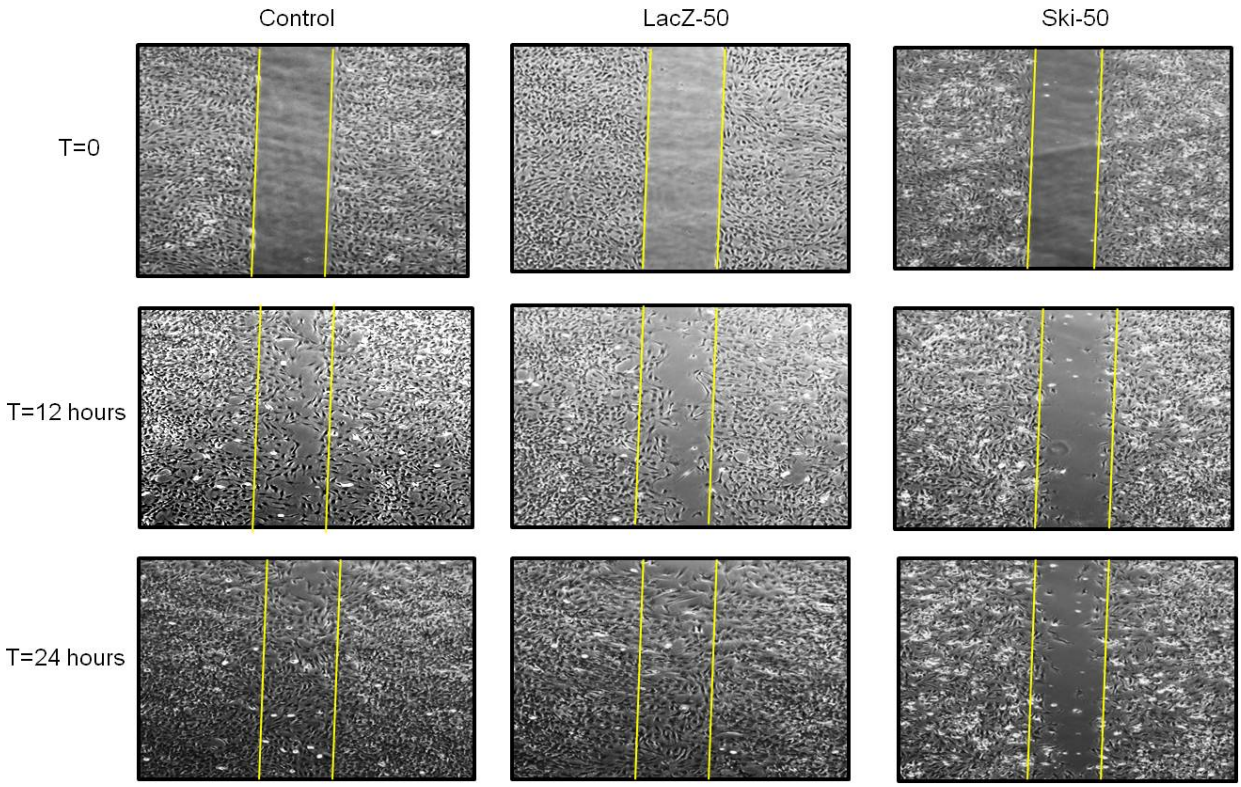


Figure. 16-B

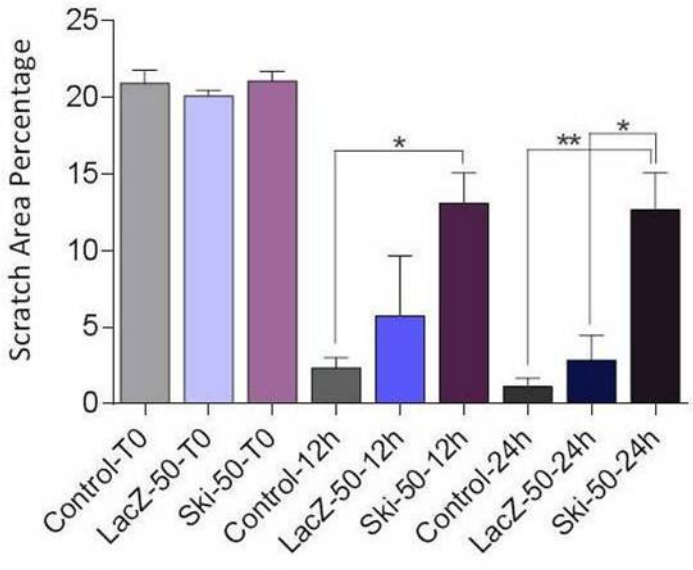


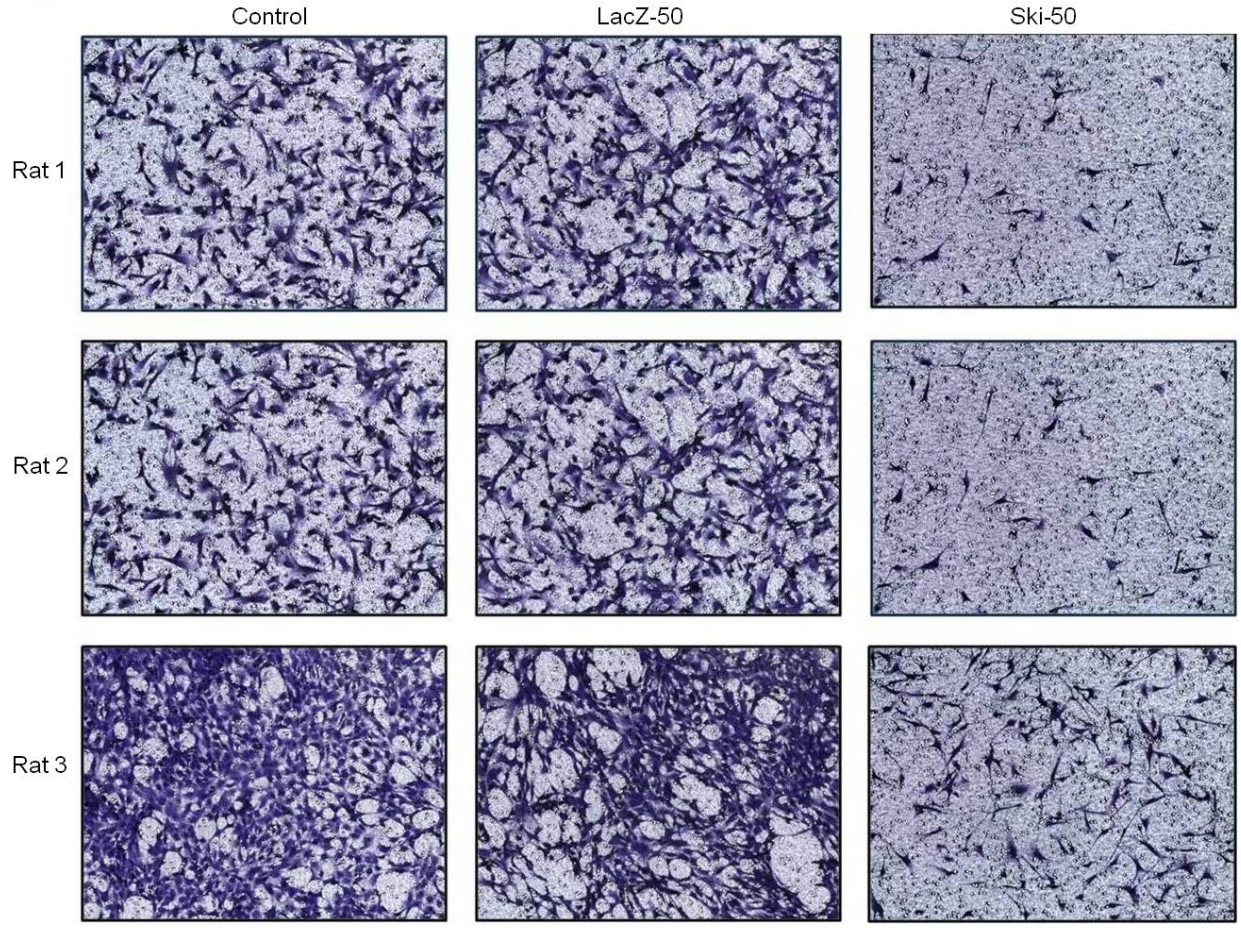
Figure 16. Overexpression of Ski affects rat cardiac myofibroblast motility. Following achievement of a confluent layer of P1 cardiac myofibroblasts on each side of silicone cell culture inserts, cells were infected with either Ad-LacZ or Ad-HA-Ski at 50 MOI for 48 hours. Untreated controls were starved but not infected with either virus. Expression of exogenous HA-tagged Ski was characterized using anti-HA antibody by Western blot (data not shown). **(A)** Cells were imaged upon removing the inserts (time t=0) and at 3-hour intervals for 24 hours using a Nikon light microscope at 10X magnification. Compared to Ad-LacZ-infected and uninfected controls, gap closure induced by Ad-Ski infection was visibly reduced. **(B)** Histogram showing data obtained in A. Gap closure was significantly reduced by Ski over-expression at 12 hours (compared to untreated, but not Ad-LacZ-infected controls) and at 24 hours (compared to both control groups). (n=3, repeated measures one-way ANOVA, Tukey post-hoc analysis; $p \leq 0.05$ (*) compared to control at 12 hours and $p \leq 0.01$ (**) compared to control, and $p \leq 0.05$ (*) compared to LacZ-50 at 24 hours, mean ± SEM)

4.15. Ski overexpression and P1 rat cardiac myofibroblast migration towards PDGF

In addition to the wound healing assay, we used Transwell migration assays to examine whether Ski affects myofibroblast migratory characteristics. In the Transwell assay, an equal number of P1 cardiac myofibroblasts were plated in each upper chamber of the porous (8 μm) well insert. Subsequent to infection with Ad-HA-Ski or Ad-LacZ (50 MOI, 48 hours), along with the addition of 20 ng/mL PDGF-BB as a chemoattractant to the lower chambers, fewer cells were observed on the bottom side of the membrane in the Ski-overexpressing group compared to the other groups (Figure 17-A). Cell counts of attached and suspended cells in the lower chamber containing PDGF also indicated a significant decrease in the number of migrated cells in

response to Ad-Ski infection, compared to Ad-LacZ-infected and uninfected controls (Figure 17-B)

Figure. 17-A



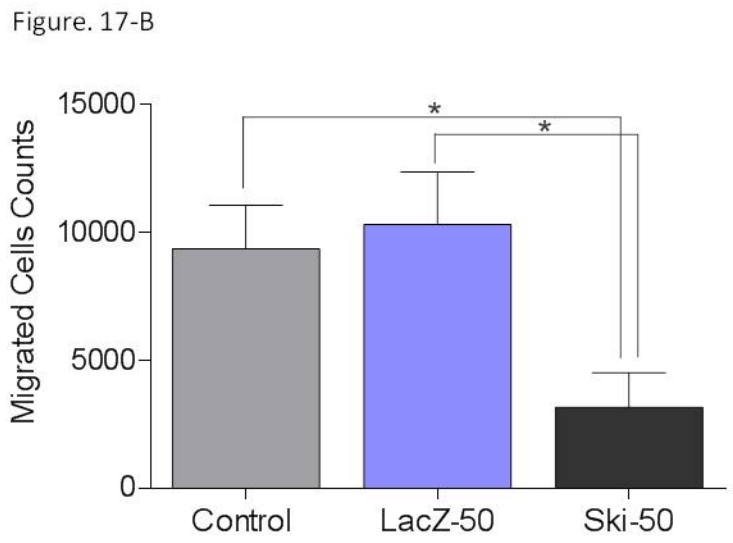


Figure 17. Overexpression of Ski inhibits cardiac myofibroblast migration in Transwell plates. First passage (P1) cardiac myofibroblasts were plated at a density of 1.0 x 10^5 cells/insert in the upper chamber of Transwell® inserts, starved, and infected with either Ad-LacZ or Ad-HA-Ski (MOI of 50) for 48 hours. Uninfected controls were starved but not infected with either virus. **(A)** Representative image of crystal violet-stained myofibroblasts (visualized on the underside of the porous membrane of the insert) for the different treatment groups. The background tan-colored region is the membrane upon which the violet-stained cells appear. Pores in the membrane appear as light blue circles. Visual examination reveals an apparent decrease in the number of migrated cells in response to Ad-Ski infection (n=3). **(B)** Histogram showing data obtained in A. Cell counts of attached and floating cells in the bottom chamber of the Transwell® apparatus reveal a significant decrease in the number of cells migrated in response to PDGF chemoattractant following infection with Ad-Ski, as compared to Ad-LacZ-infected and untreated groups (n=3, repeated measures one-way ANOVA, Tukey post-hoc analysis; $p \leq 0.05$ (*) compared to control and LacZ-50, mean ± SEM).

4.16. Ski overexpression and paxillin in P1 rat cardiac myofibrobalsts

To examine changes in expression of focal adhesion-related proteins, a key aspect of the myofibroblast phenotype, we assayed cardiac myofibroblasts for the expression of paxillin, a critical focal adhesion protein that provides a scaffold for recruitment of other focal adhesion proteins, in response to infection with either Ad-HA-Ski or Ad-LacZ (MOI of 50 or 150 for 48 hours). Immunoblot analysis of total cell lysates from these cells revealed a significant reduction in the paxillin protein level following Ski overexpression, in comparison with uninfected and Ad-LacZ-infected controls (Figure 18).

Figure. 18-A

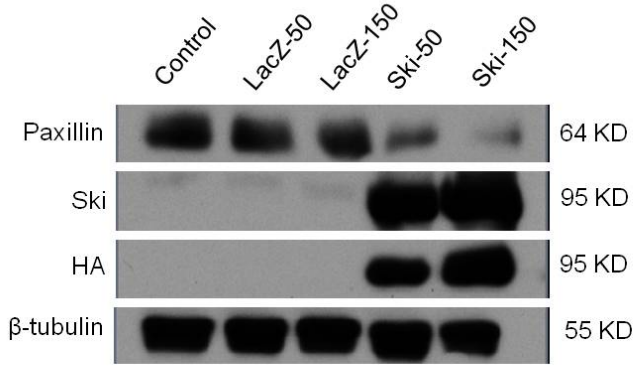


Figure. 18-B

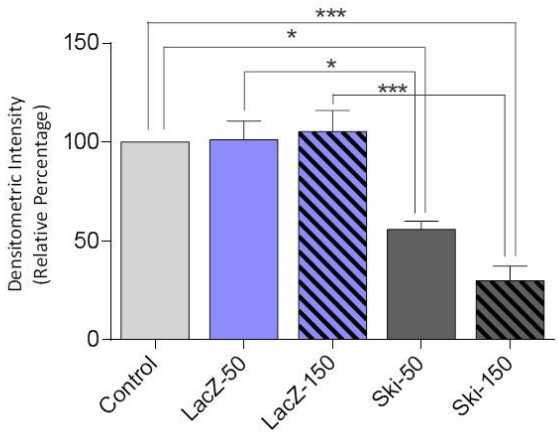


Figure 18. Overexpression of Ski downregulates paxillin expression in rat cardiac myofibroblasts. First passage (P1) cardiac myofibroblasts were grown to reach 70% confluency and infected with either Ad-LacZ or Ad-HA-Ski (MOI of 50 or 150) for 48 hours. Untreated controls were starved but not infected with either virus. **(A)** Representative immunoblots of total cell lysates probed for paxillin. Expression of exogenous Ski was characterized using anti-Ski and anti-HA antibodies; β-tubulin was used as a loading control. **(B)** Histogram showing data obtained in A. paxillin protein levels are significantly decreased in response to Ad-Ski infection, as compared to Ad-LacZ-infected and uninfected controls (n=3, repeated measures one-way ANOVA, Tukey post-hoc analysis; $p \leq 0.05$ (*) for Ski-50 compared to control and LacZ-50, $p \leq 0.001$ (***) for Ski-150 compared to control and LacZ-150, mean ± SEM).

4.17. Ski overexpression and phosphorylated FAK (Tyr 397) expression in P1 rat cardiac myofibroblasts

Phosphorylation of FAK at tyrosine 397 (Tyr 397) is indicative of increased FAK activity. Similar to results obtained when probing for focal adhesion component, paxillin, Tyr 397 phosphorylation of FAK was significantly decreased in response to overexpression of Ski (Ad-HA-Ski, 150 MOI for 48 hours), as compared to uninfected controls (Figure 19).

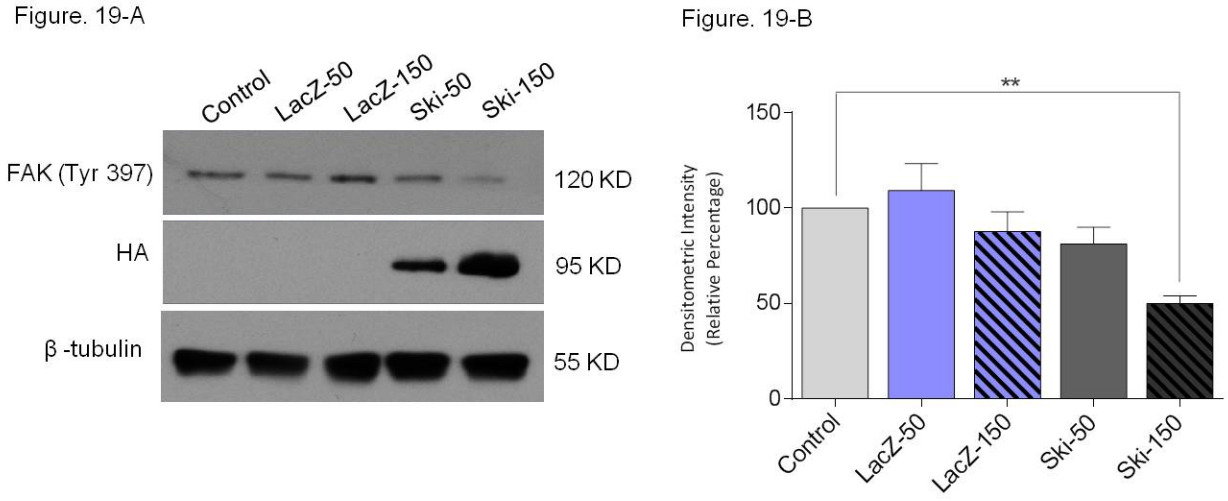


Figure 19. Overexpression of Ski downregulates FAK (Tyr 397) expression in rat cardiac myofibroblasts. First passage (P1) cardiac myofibroblasts were grown to 70% confluency and infected with either Ad-LacZ or Ad-HA-Ski (MOI of 50 or 150) for 48 hours. Untreated controls were starved but not infected with either virus. **(A)** Representative immunoblots of total cell lysates probed for FAK (Tyr 397) antibody. Expression of exogenous HA-tagged Ski was confirmed using anti-HA antibodies; β-tubulin was used as a loading control. **(B)** Histogram showing data obtained in A. FAK phosphorylation (Tyr 397) is significantly decreased with Ad-Ski infection at 150 MOI, compared to uninfected controls (n=4, repeated measures one-way ANOVA, Tukey post-hoc analysis; $p \leq 0.01$ (**) for Ski-150 compared to control, mean ± SEM).

4.18. Ski overexpression and PYK2 (Y402) expression in P1 rat cardiac myofibroblasts

PYK2 is another tyrosine kinase of the focal adhesion kinase family. Phosphorylation of PYK2 is associated with its activation and focal adhesion formation. We assayed phosphorylation of PYK2 at Tyrosine 402 (Y402) and observed no significant differences between Ad-HA-Ski, Ad-LacZ, or untreated control groups (Figure 20).

Figure. 20-A

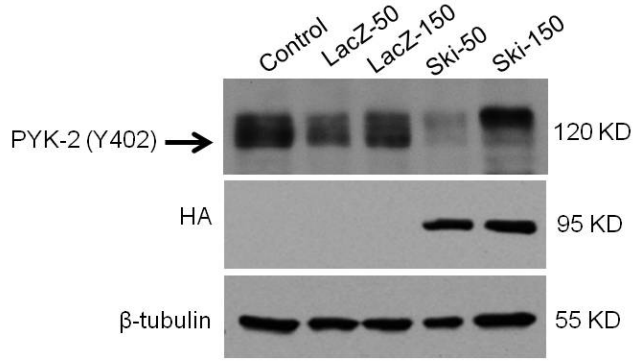


Figure. 20-B

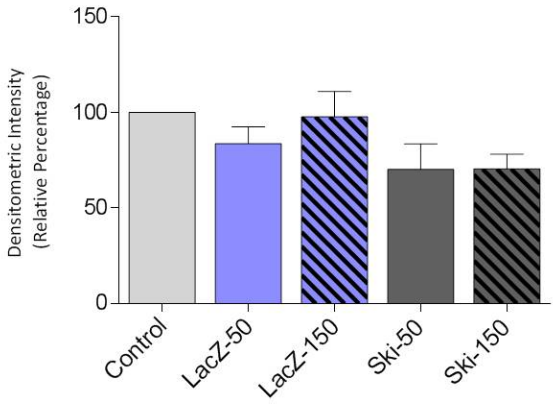


Figure 20. Overexpression of Ski does not alter the expression of PYK2 (Y402) in rat cardiac myofibroblasts. First passage (P1) cardiac myofibroblasts were grown to 70% confluency and infected with either Ad-LacZ or Ad-HA-Ski (MOI of 50 or 150) for 48 hours. Untreated controls were starved but not infected with either virus. **(A)** Representative immunoblots of total cell lysates probed for PYK2 (Y402) antibody. Expression of exogenous HA-tagged Ski was confirmed using anti-HA antibodies; β-tubulin was probed as a loading control. **(B)** Histogram showing data obtained in A. No significant differences are induced by any of the treatment groups (n=4, repeated measures one-way ANOVA, Tukey post-hoc analysis; p=0.124, mean ± SEM).

4.19. Ski overexpression and vinculin expression in P1 rat cardiac myofibroblasts

Vinculin is a focal adhesion protein that links these complexes to the actin cytoskeleton of myofibroblasts. To determine the effect of Ski overexpression on vinculin expression, we assayed vinculin protein levels in Ad-HA-Ski and Ad-LacZ-infected cardiac myofibroblasts (100 MOI, 48 hours). Vinculin levels were not significantly changed in response to any treatment group (Figure 21).

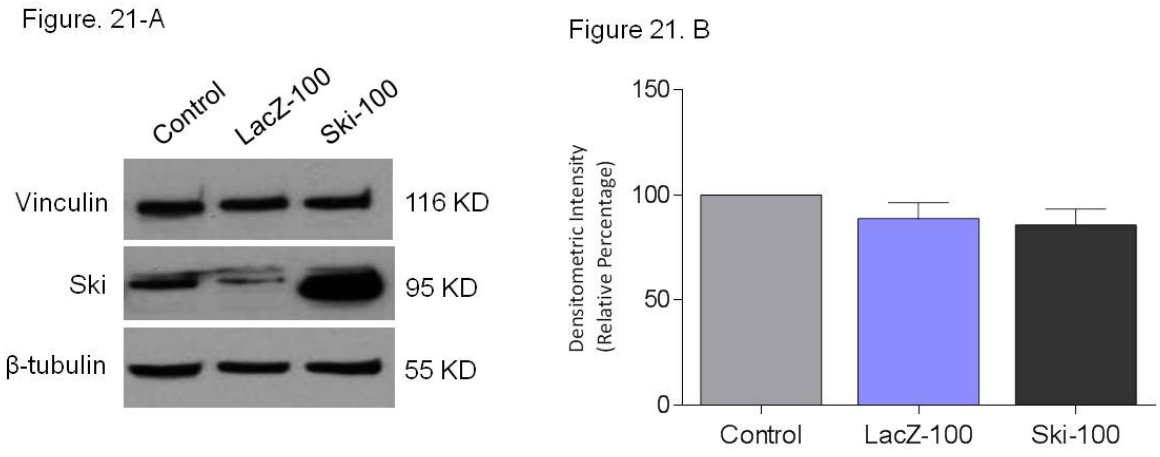


Figure 21. Overexpression of Ski does not modify vinculin expression in rat cardiac myofibroblasts. First passage (P1) cardiac myofibroblasts were grown to 70% confluency and infected with either Ad-LacZ or Ad-HA-Ski (MOI of 100) for 48 hours. Untreated controls were starved but not infected with either virus. **(A)** Representative immunoblots of total cell lysates probed for vinculin antibody. Expression of exogenous HA-tagged Ski was characterized using anti-Ski antibody; β-tubulin was probed as a loading control. **(B)** Histogram showing data obtained in A. No significant differences were observed among treatment groups (n=8, repeated

measures one-way ANOVA, Tukey post-hoc analysis; though the difference was not significant among the groups, p=0.2619, mean ± SEM).

4.20. Ski overexpression and integrin-β1 expression in P1 rat cardiac myofibroblasts

Intracellular focal adhesions are linked to extracellular matrix components primarily through integrins. The primary β subunit of integrins that link focal adhesions to extracellular fibronectin is the β1 subunit [248]. Thus, we probed lysates of cardiac myofibroblasts infected with either Ad-HA-Ski or Ad-LacZ (50 or 150 MOI, 48 hours) for integrin-β1 protein. However, immunoblot analysis did not reveal any alteration in the expression of integrin-β1 by Ski overexpression compared to uninfected and Ad-LacZ-infected controls (Figure 22).

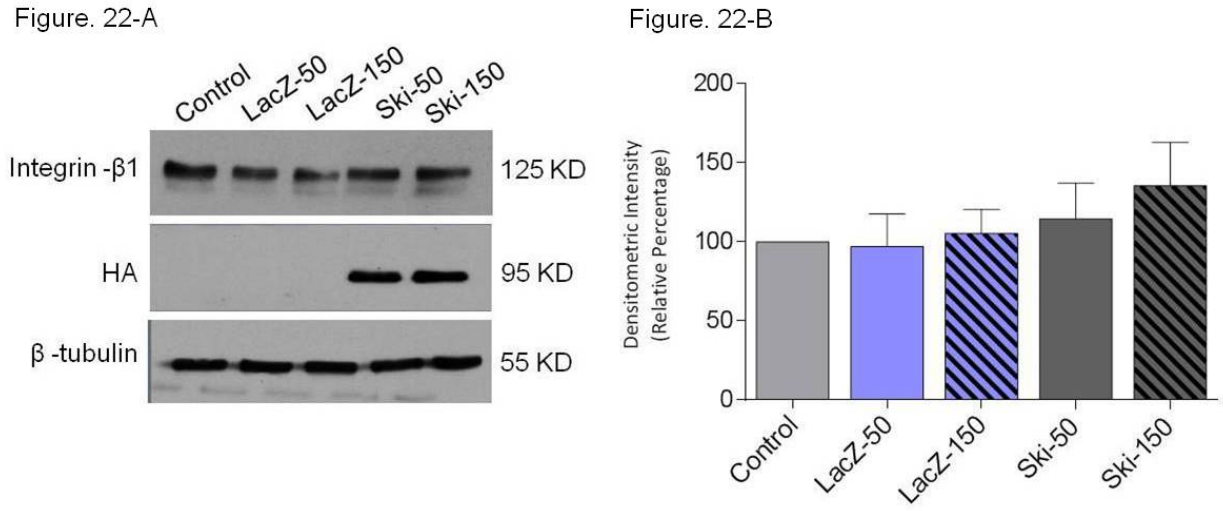


Figure 22. Overexpression of Ski does not affect integrin-β1 expression in rat cardiac myofibroblasts. P1 cardiac myofibroblasts were grown to reach 70% confluency and were infected with either Ad-LacZ (MOI of 50 and 150) or Ad-HA-Ski (MOI of 50 and 150) for 48

hours. Untreated controls were starved but not infected with either virus. **(A)** Representative immunoblots of total cell lysates for integrin-β1 antibody. Expression of exogenous Ski was characterized using anti-HA antibody followed by anti-integrin-β1 antibody; β-tubulin was probed as a loading control. **(B)** Histogram showing data obtained in A (n=4, repeated measures one-way ANOVA, Tukey post-hoc analysis; though the difference was not significant among the groups, p=0.6506, mean ± SEM).

5. DISCUSSION

Adverse cardiac remodeling, an imbalance of the ECM synthesis and turnover, eventually leads to fibrosis [249]. The fibrosing myocardium is subject to impingement of growth factors including TGF-β1 which facilitate the synthesis and deposition of matrix protein production in the expansion of the cardiac interstitium, as well as the expression of MMPs, which are responsible for tissue breakdown [249, 250]. *In vitro*, TGF-β1 stimulation of the cell is associated with activation of the myofibroblast phenotype eg, phenoconversion of cardiac fibroblasts and concomitantly with increased collagen synthesis [169]. Blockade of TGF-β1 signaling can prevent cardiac fibrosis in the pressure-overloaded rats [15]. Ski has been shown to operate as an endogenous inhibitor of TGF-β signaling through R-Smad and Co-Smad signaling. The expression of the 95 kDa form of c-Ski protein is associated with impaired p-Smad2 function, as previously characterized by our lab [215]. Furthermore the effects of Ski overexpression on the myofibroblastic phenotype were previously assessed by us, and Ski overexpression is attended by the decreased prevalence of the activated myofibroblast phenotype along with a decrease in collagen synthesis and secretion by these cells, thereby affecting pathologic ECM remodelling [215]. This work notwithstanding, the effects of Ski on the ECM turnover *via* MMPs have not been investigated previously and the effect of Ski on these aspects of myofibroblast function is unknown.

MMP-2 is a ubiquitous metalloproteinase enzyme that is relatively highly expressed *versus* other MMPs, and is constitutively secreted by cardiac fibroblasts [8]. In addition, MMP-2 secretion is enhanced by pro-fibrotic factors such as TGF-β1, thus this enzyme is thought to play a significant role in wound healing and heart failure in the heart, especially in relation to ischemia/reperfusion injury [11, 251-253]. Although MMP-2 is localized near the exterior of

cardiomyocyte cell membranes, Z-lines, and myofilaments [254], its existence and co-localization with fibroblast proteins and those of other cardiac cells remains unknown. A number of *in vivo* studies have shown that cardiac fibrosis associated with diabetic cardiomyopathy which is in turn, linked to decreased MMP-2 expression and activity in the myocardium [255-257]. In the current study we show that Ski overexpression is associated with increases in both intracellular MMP-2 expression and activity, as assessed *via* zymography (Figures 5 and 6). In contrast, secreted levels and activity of secreted MMP-2 was significantly decreased with Ski overexpression *versus* the LacZ control group (Figures 7 and 8). The complex effects of Ski on intracellular and secreted MMP-2 levels and activity indicates a possible unknown mechanism whereby Ski inhibits the secretion of MMP-2 in primary rat cardiac myofibroblasts. However, this effect appears to be temporally restricted, as secreted MMP-2 levels were only reduced after 48 hours of Ski overexpression, and remained unchanged at other time points assayed (24, 72, and 96 hours) (Data are not shown here). This difference in timing of the effects of Ski expression on MMP-2 secretion suggest that there may be a transient regulatory role for Ski in the ECM remodeling process mediated by MMP-2 following cardiac injury which may become less pronounced over the duration of wound healing and scar formation in the heart. MMPs are potential therapeutic targets in the treatment of cardiac fibrosis and subsequent heart failure [258] and are differentially affected by TGF-β. It has previously been observed that TGF-β induces MMP-2 activity in oral gingival fibroblasts [259]; thus the reduction in MMP-2 secretion in Ski overexpressing cardiac myofibroblasts is in agreement with the anti-fibrotic role for Ski as a repressor of TGF-β signalling in the myocardium. Therefore, Ski may function as a multifunctional anti-fibroproliferative agent in the heart, and possibly other tissues.

Previous studies have shown that extracellular MMP-2 activation is accompanied by accelerated fibrillar type I collagen synthesis in rat cardiac fibroblasts (likely a heterogenous mix of myofibroblasts, as the experimental conditions were carried out in standard cell culture), and that FAK phosphorylation (FAK -Tyr397) is fundamental in MMP-2-induced fibrillar type I collagen synthesis [117]. Thus decreased secreted MMP-2 activity observed in Ski-overexpressing cells in the current study emphasizes the putative anti-fibroproliferative effects of Ski. Less fibrillar collagen type I synthesis may occur due to decreased secreted MMP-2 activity by these cells (Figure 8). Moreover, phosphorylation of FAK (Tyr 397) is attenuated in Ski-overexpressing cells (Figure 19). The additive nature of these effects could reinforce the validity of the suggestion that Ski may also attenuate the myofibroblast phenotype by reducing its ability to synthesize fibrillar collagen type I, *via* a reduction in MMP-2 activation. Thus, Ski may also act to suppress one or more additional pathways within cardiac fibroblasts and myofibroblasts in addition to suppression of TGF-β/Smad signaling.

Data produced previously in our laboratory underscored the likelihood of increased MMP-9 secretion by cultured adult cardiac myofibroblasts following inhibition of collagen deposition *via* inhibition of prolyl 4-hydroxylase with mimosine [244]. Inhibition of collagen deposition *via* attenuation of hydroxylation of the collagen monomers by decreased activity of prolyl 4-hydroxylase, might show a similar trend in MMP-9 secretion by Ski overexpresion. Previous work from our lab has observed decreased in collagen production in Ski overexpressed cells via the inhibition of TGF-β/Smad signalling pathway [215]. For that reason we sought to assess the expression and activity of intracellular and secreted MMP-9 in primary myofibroblasts that were overexpressing Ski. We found that MMP-9 secretion was significantly augmented in these cells *versus* controls (Figure 10), in agreement with previous findings from our lab.

However, the increase in MMP-9 secretion can also be explained by a decrease in its degradation. MMP-9 secretion was enhanced gradually over time in our Ski overexpressing cells (Figure 11), and we suggest that sustained induction of MMP-9 secretion in response to Ski may reflect a *de facto* increase in MMP-9 half life; however this effect was not observed with MMP-2. This observation, combined with the finding that intracellular MMP-9 was not affected by Ski overexpression (Figure 9), suggests that Ski may specifically regulate the MMP-9 secretory pathway in cardiac myofibroblasts. We also found that the intracellular expression of MMP-1, which targets fibrillar collagens such as those found in the infarct scar, was not altered in response to Ski overexpression (Figure 13), although secreted levels of MMP-1 and its activity were not concurrently examined. It may be that similar to MMP-9, Ski expression affects secretion and extracellular activity levels of MMP-1, as opposed to its prevalence inside the cardiac myofibroblast. It is also possible that there is a compensatory mechanism that exists among MMP-2 and MMP-9 function which is regulated by Ski expression. The putative mechanism notwithstanding, our current results clearly demonstrate that Ski expression plays a role in the modulation of MMP expression, secretion, and activity in cardiac myofibroblasts. In the context of the ECM remodeling, TIMPs must be considered, as they are potent inhibitors of MMPs [22] We examined TIMP-3 and TIMP-4, both of which are known to act on MMP-2 and -9. TIMP-3 is a commonly expressed TIMP found in the ECM of many tissues, and appears to play a critical role in cardiac remodeling in response to injury. There is evidence demonstrating that signaling through TGF-β involves alteration in the ratio of MMPs and TIMPs, with profibrotic effects being due to increased levels of TIMPs, in comparison to MMPs [260]. Our results indicate decreased levels of secreted TIMP-3 in cells subjected to forced Ski

overexpression and therein support the suggestion that this phosphoprotein is an endogenous anti-fibroproliferative agent (Figure 14).

TIMP-4 is involved in the control of the ECM degradation under normal conditions, and plays a crucial role in regulating cardiac cell phenotype [83]. TIMP-4 overexpression results in activation of signaling pathways associated with fibrosis and inflammation [22], though reduced TIMP-4 levels are associated with cellular transformation, potentially leading to adverse ECM remodeling and cardiac hypertrophy [83]. Though overall levels of TIMP-4 were not significantly altered by Ski overexpression in cardiac myofibroblasts (Figure 15), TIMP-4 glycosylation in rat primary myofibroblasts subjected to Ski overexpression was reduced. Conversely, the expression of non-glycosylated TIMP-4 was unchanged among experimental and control myofibroblasts. These results indicate that Ski may not directly regulate TIMP-4 transcription, but may rather be involved in modulating post-translational modification (i.e. glycosylation levels), either in the Golgi apparatus or ER. Together, our results support the suggestion that a novel role exists for Ski in the regulation not only of TGF-β signaling, but perhaps also those involved in modulating post-translational modification, secretion, and activity of these enzymes. Although Ski expression was not associated with increased MMP-2 secretion and extracellular activity, we did observe increased intracellular MMP-2 levels and activity, suggesting that exogenous Ski may interfere with constitutive MMP-2 secretion. Overall, it would appear that the anti-fibrotic effects of Ski extend to the ECM proteases and their inhibitors.

As MMPs are involved in regulating cardiac fibroblast migration [261], and Ski expression alters MMP activity, we sought to determine the effect of Ski overexpression on cardiac myofibroblast migration. TGF-β promotes cell adhesion and migration in many cell

types, including fibroblasts [262]. Our findings linking Ski overexpression to the inhibition of cellular myofibroblast motility, (both in a scratch assays (Figure 16) and the Transwell system for determining migration (Figure 17), agrees with the role of Ski as an endogenous inhibitor of TGF-β-Smad-dependent signalling and associated cell migration. Moreover, it has been shown before that FAK expression is essential for both ERK and p38 activation, which are requirements for TGF-β1-induced α-SMA expression and fibroblast-to-myofibroblasts conversion [263]. Hence we focused to determine the putative mechanism(s) underlying Ski overexpression-induced migratory characteristics. Specifically, we sought to elucidate whether Ski modulates cell movement by altering the expression of focal adhesion associated proteins and their kinases. According to published evidence, the migratory features of myofibroblasts are controlled by focal adhesion-associated proteins [103, 263, 264]. We have previously observed an increase in focal adhesion-associated proteins expression with increase in passage number of myofibroblasts, which is associated with increased expression of myofibroblast markers such as α-SMA and ED-A fibronectin [103]. We observed a downregulation in the focal adhesion protein paxillin (Figure 18), as well as phosphorylation at tyrosine 397, required for activation of FAK, (Figure 19), which was in agreement with our previous findings. Interestingly, this effect does not show the same trend on PYK2 (Y402) by Ski overexpressing cells (Figure 20). Despite the fact that PYK2 posses a comparative structure to FAK, its variable binding sites appear to account for the different response to Ski overexpression. Perhaps Ski binds to FAT domain of PYK2 while it binds to the FERM domain of FAK [149] and it may lead to different outcome observed in our cells. On the other hand, we also found that vinculin did not change in cells subjected to Ski overexpression (Figure 21). However, vinculin is an identified binding partner

for paxillin, Ski did not show a similar trend in affecting vinculin expression in our primary rat cardiac myofibroblasts.

The group of β1 integrins are the main ECM receptors found on fibroblast cell membranes. In addition to arbitrating a variety of cell processes (e.g. cell adhesions and migration) [265, 266], integrin-β1 activates TGF-β signalling [267]. According to previous work, TGF-β expression is upregulated by overexpression of integrin α5β1 [268]. Conversely, fibroblasts stimulated by TGF-β showed an upregulation of integrin α5β1 expression [269]. With these reported observations in consideration, we focused on measuring the expression of integrin-β; however Ski overexpression had no effect on integrin-β1 expression (Figure 22). Thus, we suggest that modulation of myofibroblast motility by Ski overexpression does not appear to be arbitrated through the β1 integrins.

While the use of adenoviruses including Ad-LacZ and Ad-HA-Ski as vehicles to overexpress key genes in our experimental system showed a mild trend to the induction of apoptosis (Figure 3), this effect is similar between these groups and it is unlikely that the effects observed in Ski overexpression (at our MOI and duration) studies are due simply to adenoviral effects. We suggest that changes in protein levels and activity induced by Ski are specific and linked to suppression of TGF-β/Smad signaling.

Our current findings support the suggestion that Ski has multiple cellular functions. The mechanisms by which Ski modulates multiple functions in myofibroblasts are beyond its role as an endogenous inhibitor of TGF-β. It is possible that Ski is a scaffolding protein that may bind to several proteins leading to their activation or deactivation by sequestration, thereby affecting different pathways in myofibroblasts. Previous studies confirmed that Ski binds to a number of transcription factors, altering its function as a co-activaor or co-repressor, depending on its

specific binding partner [270-272]. For example, Ski activates the myogenin promoter independently of regulating endogenous TGF-β signaling which induces myogenic differentiation by inhibiting HDAC activity in the nucleus of myogenic cells [273]. Additionally, it has been shown that Ski stimulates growth *in vivo* by activating the Wnt signaling pathway through interaction with FHL2, thus potentiation of β-catenin function in human melanoma cells [274]. We conclude that Ski's multiple functions present novel mechanisms for the modulation of myofibroblast function and phenotype, and that loss of normal Ski expression, such as is seen in myofibroblasts of the infarct scar of post-MI hearts [215] may underpin the adverse ECM remodeling and facilitate ongoing cardiac fibrosis in the diseased myocardium.

6. CONCLUSIONS

In summary, we have demonstrated these findings:

- Overexpression of Ski increases intracellular MMP-2 expression and activity.
- Overexpression of Ski decreases MMP-2 secretion and activity.
- Overexpression of Ski does not alter intracellular MMP-9 expression.
- Overexpression of Ski increases MMP-9 secretion and activity.
- The effect of Ski on MMP-9 secretion increases over time.
- Overexpression of Ski, does not alter intracellular MMP-1 expression.
- Overexpression of Ski downregulates TIMP-3 secretion.
- Overexpression of Ski downregulates TIMP-4 glycosylation; however the overall level of TIMP-4 is not altered by Ski.

Therefore, we can conclude that the anti-fibrotic effects of Ski extend to the ECM proteases and their inhibitors.

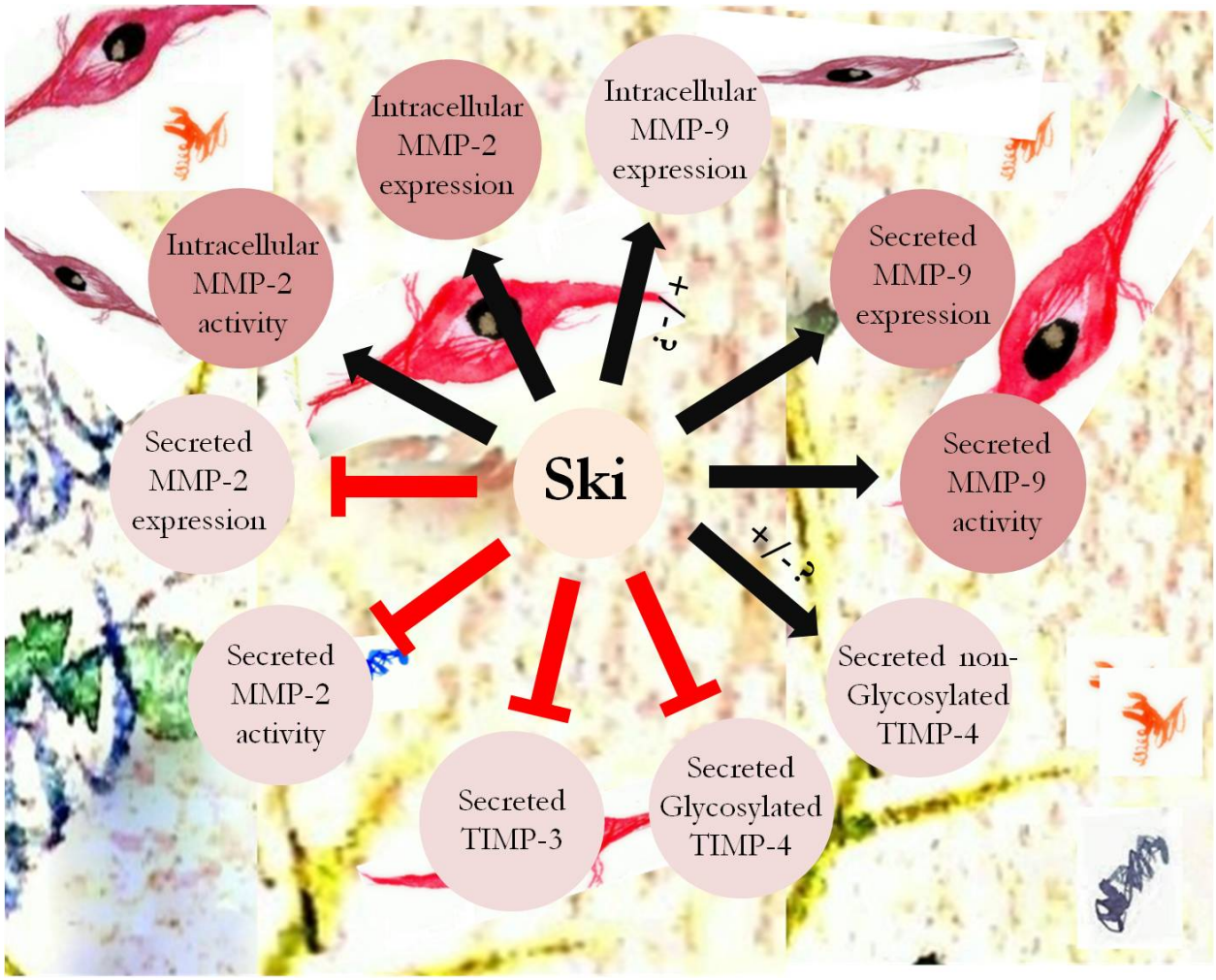


Figure 23. Proposed model of Ski regulation of cardiac ECM via modulation of MMPs and TIMPs. We suggest that Ski overexpression modulates the ECM proteases and their inhibitors. Thus, Ski plays a role in modification of cardiac fibrosis.

Furthermore,

- Overexpression of Ski attenuates cell motility.
- Overexpression of Ski downregulates paxillin and FAK (Tyr 397).
- Overexpression of Ski does not alter vinculin expression.
- Overexpression of Ski does not alter PYK2 (Y402) expression.
- Overexpression of Ski does not modify integrin-β1 expression.

Thus Ski affects the motility characteristics of myofibroblasts through downregulation of paxillin and FAK (Tyr 397), although this effect does not involve PYK2 (Y402), vinculin and integrin-β1.

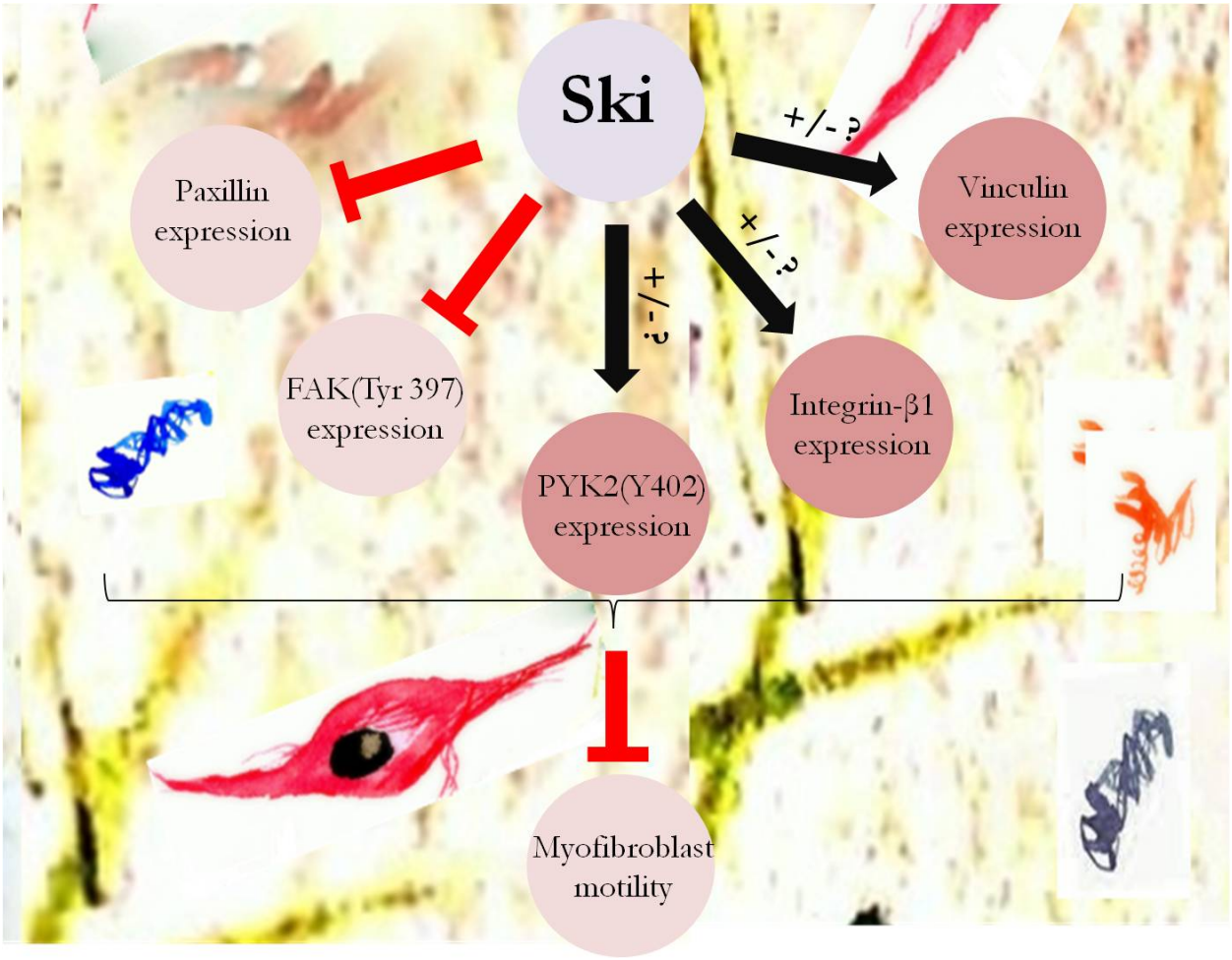


Figure 24. Proposed model of Ski regulation of cardiac myofibroblast motility. We propose that Ski overexpression attenuates myofibroblast motility via modulation of paxillin and FAK (Tyr 397). Thus, Ski plays a role in modification of cardiac fibrosis.

Overall, our findings support the contention that Ski is a multifunctional protein. Ski appear to modify several mechanisms in cardiac myofibroblasts that are involved in the ECM remodeling, although a clear understanding of these mechanisms is the subject of further research.

7. SIGNIFICANCE AND FUTURE DIRECTIONS

Cardiac fibrosis is a consequence of adverse ECM remodeling in response to a broad range of cardiovascular pathologies. Although no effective treatment to abrogate or even to slow this cumulative process have been identified, understanding the underlying mechanisms that modify myofibroblast phenotype and function is a critical step in the future development of targeted therapies to treat cardiac fibrosis. Subsequent to myocardial injury, wound healing of the infarcted zone of myocardium is dependent on fibroblast activation and phenoconversion to myofibroblasts. It is critical to identify unique target to control ongoing ECM remodeling beyond the acute phase of normal wound healing and thereby restore the basal level of fibroblast-mediated turnover of matrix. Ski is a putative novel regulator of myofibroblast phenotype and function and our current results allow us to speculate that Ski-specific regulation of MMPs and TIMPs is a worthy of further investigation. We suggest that studies to extend the current work of Ski's effect on MMP expression and function and especially on the group of so-called gelatinases, would be valuable. Further assessment of TIMP activity in the face of the influence of Ski, including the evaluation TIMP-1and TIMP- 2 expression, will be necessary to understand a more defined mechanism of Ski's effects on these molecules. There also remains uncertainty regarding the mechanisms by which Ski modulates myofibroblastic motility. Although we observed an alteration in paxillin and FAK (Tyr 397), further imaging analysis is required to determine the specific cellular location of the components that are involved in motility in cells subjected to Ski overexpression. The initiation and termination of cell movement recruits a number of other components and further assessment of focal adhesion-associated proteins and FAK in response to Ski is warranted. In addition, the effects of Ski overexpression on actin filaments, microtubules, and cycling lipid vesicles (all of which

participate in the cell's ability to form processes that arbitrate migration) have yet to be assessed in a systematic manner in the face of Ski overexpression (and underexpression). Moreover, the identification of the relevance of MMPs' and TIMPs' involvement with cell motility, and the regulatory effects of Ski should also be investigated. Finally, we have evidence from our recent work that knockdown of Ski may be critical to all of the above cellular parameters, and thus assessment in of loss-of-function in myofibroblasts subjected to Ski knockdown are also warranted. In addition to all these *in vitro* studies, injecting a Ski-overexpressing adenovirus directly to injured and viable myocardial tissue remains to be attempted *in vivo*. In the latter study, the effect of cells affected in proximity to the site of injection could be assessed by laser dissecting microscopic techniques.

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