Mechanisms of Ski-induced apoptosis in cardiac fibroblasts and myofibroblasts

by

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Abstract

One of the hallmarks of chronic cardiac disease is the excessive formation of fibrous extracellular matrix. This inappropriate remodeling is mediated in large part by cardiac fibroblasts and phenoconverted myofibroblasts. The production of matrix proteins by these cells is triggered by a variety of stimuli, both chemical and mechanical. The protooncoprotein Ski has previously been described as possessing anti-fibrotic properties within the myocardium, in addition to triggering apoptosis when overexpressed. We initially hypothesized that overexpression of Ski in myofibroblasts would induce an apoptotic response, which would either be supported, or hindered by autophagic flux. In the current study, we found that overexpression of Ski results in a set of distinct morphological and biochemical changes within primary cardiac myofibroblasts that is indicative of apoptosis. Its upregulation is associated with the expression of pro-apoptotic factors such as Bax and Bak, as well as caspase-9 and -7. In all, our results indicate that Ski triggers a pro-death mechanism in primary rat cardiac myofibroblasts that is mediated through the intrinsic apoptotic pathway. The survival of these cells appears to be prolonged by a pro-survival autophagic response as apoptosis is hastened when autophagy is inhibited. The observed cell death response is likely working in parallel with the previously observed anti-fibrotic properties of Ski within this cell type. As myofibroblast cells are the engines of matrix expansion in heart failure, we suggest that using Ski or a projected Ski-mimetic to induce graded apoptosis in myofibroblasts within the failing heart may be a novel therapeutic mechanism of controlling cardiac fibrosis. Future studies will need to examine the potential effects of

Ski overexpression on other cell types in the heart, as apoptosis of cardiomyocytes, for example, would be detrimental to an already weakened post-MI heart.

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List of Abbreviations

AIF – Apoptosis Inducing Factor

Apaf-1 – Apoptotic protease activating factor 1

ATG – Autophagy Related Gene

Bak – Bcl-2 homologous antagonist killer

Bax – Bcl-2-associated X protein

Bcl-2 – B-cell lymphoma-2

Bcl-xL – B-cell lymphoma-extra large

BMP – Bone morphogenetic protein

CDK – Cyclin-dependent kinase

cDNA – Complementary deoxyribonucleic acid

CNS – Central nervous system

DNA – Deoxyribonucleic acid

dsDNA – Double stranded deoxyribonucleic acid

ECM – Extracellular matrix

EDA-fibronectin – Extra domain-A fibronectin

EMT – Epithelial-to-mesenchymal transition

FACS – Fluorescent automated cell sorting

FasL – Fas ligand

FasR – Fas receptor

HDAC1 – Histone deacetylase 1

I-Smad7 – Inhibitory-Smad7

MEF – Mouse embryonic fibroblasts

MI – Myocardial Infarction

NADPH – Nicotinamide adenine dinucleotide phosphate

NOXA – NADPH oxidase activator

Mad – Mothers against decapentaplegic

MPTP – Mitochondrial permeability transition pore

mRNA – Messenger RNA

N-CoR – Nuclear receptor co-repressor

PCD – Programmed cell death

PNS – Peripheral nervous systen

PUMA – p53 upregulated modulator of apoptosis

RAS – Rat sarcoma

RIP – Receptor-interacting protein kinase

RNA – Ribonucleic acid

ROS – Reactive oxygen species

Smac – Second mitochondrial-derived activator of caspases

SnoN – Ski-related novel protein

TGF-β1 – Transforming growth factor- β1

TNF-α – Tumor necrosis factor-alpha

TNFR1 – Tumor necrosis factor receptor-1

TUNEL – Terminal deoxynucleotidyl transferase dUTP nick end labeling

VSMCs – Vascular smooth muscle cells

I. Introduction/Statement of the Problem

The two leading causes of death in Canada are heart disease and stroke[1]. Cardiovascular disease (encompassing both heart disease and stroke) in Canada claims the life of one person approximately every 7 minutes, accounting for almost 70 000 deaths every year, or nearly 30% of the total mortality rate[1]. The burden is felt not only at a societal level, but also a financial one, costing Canadian society almost $21 billion each year in physician compensation, health care wages, and decreased economic productivity[2]. This problem is neither exclusively a Canadian one, nor a North American one. In 2004, the WHO calculated that over 17 million deaths each year could be attributed to cardiovascular disease, the majority occurring in low and middle income countries[3].

Cardiovascular disease is a general term and accounts for a variety of heart and vascular related pathologies including, cardiomyopathy, hypertension, ischemic heart disease and myocardial infarction (MI), among many more. These pathologies are accompanied by a process (often reparative and reactive in acute wound healing) known as fibrosis, which is characterized by the excess synthesis and deposition of fibrillar extracellular matrix (ECM) proteins such as collagen and fibronectin. The cells responsible for this repair response are fibroblasts and their phenoconverted counterparts, myofibroblasts.

This thesis summarizes the results of our basic science studies to elucidate the role of the evolutionarily conserved protein, Ski in initiating programmed cell death (PCD) within cardiac myofibroblasts. Using an *in vitro* overexpression model, we have examined the ability of Ski to trigger apoptotic and autophagic responses in rat cardiac

myofibroblasts. Humans have failed to evolve a specific ability to attenuate post-MI repair during the normal lifespan, so there is little cellular dropout or loss of myofibroblasts after would healing in post-MI hearts. As a result, myofibroblasts may be present for up to decades after MI, and thus there is a continual synthesis and deposition of ECM proteins by these cells. This thesis specifically addresses initiation of PCD mechanisms within these cells, as it represents a novel way to ameliorate the natural fibrotic response that occurs in the injured heart, opening the door to the potential for new therapeutic strategies to combat this global health burden.

II. Literature Review

1. Fibrosis in Cardiac Disease

Fibrosis can be characterized by the maladaptive accumulation of extracellular proteins (eg. collagens) within the interstitium. Fibrosis is a common secondary consequence of diagnosed cardiac related diseases including, but not limited to, diabetes[4], hypertension[5], and myocardial infarctions[6]. More recently, studies have suggested that fibrosis may not just be a consequence of other present pathologies but may have potential to represent a primary pathological disorder, which is potentially regulated through microRNAs[7,8]. Regardless of the etiology, there are several characteristics that all fibrotic disorders share. Collagen turnover in the heart is regulated by cardiac fibroblasts and when there is dysfunction in that process, there arises structural and functional consequences for the heart and the organism. Fibrosis has been shown to disrupt excitation-contraction coupling in the heart thereby impairing systolic and diastolic function[9]. Fibrosis has also been implicated as being pro-arrhythmogenic, via a reduction in the velocity of electrical conduction and generation of re-entrant arrhythmias[10].

2. Post-MI Wound Remodeling

Proper functioning of the heart requires perfusion of the myocardium by the coronary arteries. When blood flow to the tissue is impeded by the progression of atherosclerosis or a thromboembolism, the heart becomes starved of oxygen and nutrients. If blockage of flow is prolonged, massive myocardial cell death occurs as a myocardial infarction (MI) is sustained. To prevent the dilation and rupture of the

chamber walls associated with the insult, the heart must repair itself in a process called wound remodeling. The repair process can generally be divided into 4 different stages: hemostasis, an inflammatory response, proliferation, and then the remodeling process/scar formation[11]. During the initial stages of an infarct, it has long been believed that loss of cardiomyocytes occurred via necrosis but evidence has demonstrated the presence of apoptosis in the borderzone of the infarct, a region between the infarcted area and the remaining healthy myocardium[12]. Following the initial ischemic insult, there is a secondary necrotic response as cells are unable to phagocytose the large amount of apoptotic cardiomyocytes. The release of intracellular contents triggers an acute inflammatory response, signaling the onset of the second phase of wound healing[13]. Cells involved in the inflammatory response (eg. macrophages) migrate to the infarcted area and release cytokines and growth factors to attract cells (eg. fibroblasts and myofibroblast precursors), which will repopulate the infarcted area[13]. To facilitate the migration of these cells to the infarcted area, the expression and activity of matrix metalloproteinase enzymes are increased - these enzymes degrade the extracellular matrix[14]. The third stage of wound healing is characterized by the increased presence of myofibroblasts, the secretion of matrix proteins such as collagens (type I and III), and the subsequent cross-linking of collagen fibrils[13]. During the final remodeling stage - scar formation, there is a decrease in the number of cells within the scar due to apoptosis[15], although a population of myofibroblasts remains in the scar for decades after the healing process has been completed[16].

3. The Role & Origins of Cardiac Fibroblasts and Myofibroblasts

Cardiac fibroblasts are quiescent, spindle-shaped cells that are derived from the mesenchyme[17]. This cell type represents up to two-thirds of the total number of cells in a healthy heart[18]. Fibroblasts have been shown to function in a variety of capacities, including mechanotransduction, chemical signaling, and in the electrophysiology of the heart[19]. Fibroblasts form a highly organized network within the heart, associated intimately with the surrounding extracellular matrix; an association that allows for the rapid transmission of mechanical signals from the surrounding matrix. Traditionally, the myocytes of the heart have been thought to be the only electrically active cells within the heart; the remaining non-myocyte population being believed to be electrophysiologically 'inert'. This viewpoint has proven to be inaccurate, as cardiomyocyte-fibroblast cell coupling has now been extensively studied[20]. While cardiac fibroblasts themselves are thought to play only a minor role in the electrophysiology of the heart, myofibroblasts have potential to induce arrhythmias; either by modification of cardiomyocyte potential, or by delaying the propagation of electrical signals in an injured heart[21].

In response to stress, injury, or stimulation, these quiescent fibroblasts undergo a phenoconversion into myofibroblasts, a larger and more hypersynthetic phenotype. Potent pro-remodeling cytokines that induce this conversion include angiotensin II[22], endothelin-1[23], and TGF- β_1[24]. During this conversion, myofibroblasts begin to adopt a smooth muscle cell-like phenotype and express markers such as alpha-smooth muscle actin and vimentin[25]. Myofibroblasts are absent in the healthy heart, with the exception of valve leaflets where a constant remodeling response is required to maintain their integrity and functionality[26]. These myofibroblasts are less motile than their quiescent

counterparts and are capable of generating the tensile strength required to heal an infarct[25]. Studies have shown that the myofibroblast population peaks at 1-2 weeks following myocardial injury, with the population decreasing at 3 weeks[27]. This hypothesis has been debated, as other studies have shown differing expression patterns[28].

For many years, the accepted source of myofibroblasts in the injured region of the heart was the resident fibroblasts in the surrounding myocardium. Since then, studies have shown that migration of adjacent fibroblasts make up only a fraction of the myofibroblasts present. The heterogeneous origins of the myofibroblast population that is present in the infarcted myocardium has been partially elucidated. Cell linage studies have found that up to 50% of myofibroblasts may come from fibrocytes, a bone marrow derived circulating leukocyte[29,30]. Myofibroblasts have also been shown to arise out of endothelial cells in a process known as epithelial-to-mesenchymal transition (EMT). In studies using pressure overload models in mice, it was found that resident endothelial cells underwent EndoMT and contributed to cardiac fibrosis in a process that was induced by TGF- β_1[31]. Other sources of myofibroblasts in the heart include pericytes, smooth muscle cells, and epicardial derived cells[32].

Fibroblasts and myofibroblasts represent a diverse group of cells, playing a heterogeneous role depending on where they are found within the body[33]. In most tissues, when the remodeling process has been completed, myofibroblasts undergo apoptosis and are removed them from the tissue, preventing the development of fibrosis[15]. Although a similar event has been seen to occur in cardiac tissue, activated myofibroblasts have been shown to persist for decades after the initial insult to the heart, leading to the development of cardiac fibrosis and later, heart failure[16]. More recent work

has looked at modulation of myofibroblast phenotype through manipulation of the TGF-β signaling pathway. It was shown that Ski overexpression is associated with overt reversion of myofibroblasts back to a quiescent fibroblast phenotype, and that this is associated with decreased contractility and collagen production[34].

4. The Extracellular Matrix

The extracellular matrix (ECM) is an acellular scaffold that is a component of, and surrounds all organs and tissues. ECM is primarily made up of proteins and proteoglycans which together, function to transmit biological and mechanical signals to the cell[35]. The hallmark of cardiac fibrosis, independent of the underlying cause is the accumulation of fibrillar collagens and other matrix proteins, primarily collagen I and collagen III[36]. Several studies have shown that collagen I is subject to a more dramatic upregulation as compared to collagen III while other studies have shown that may not always be the case, suggesting a context dependent response depending on the etiology of the disease and other surrounding factors[37, 38]. Once synthesized and secreted by activated myofibroblasts, collagens are assembled into fibrils and then begin crosslinking with each other, a process that has been linked to the development of diastolic dysfunction[39]. Other non-fibrillar collagens have also been linked to the progression of fibrosis. In an infarct model of cardiac fibrosis, collagen VI is capable of stimulating conversion of fibroblasts to myofibroblasts although its role in other fibrosis models remains to be seen[40].

Other matrix components that are known to mediate various aspects of fibrosis are fibrin and fibronectin, which are thought to form a matrix that allows for fibroblast

proliferation and migration[41]. In addition to these functions, the fibronectin splice variant extra domain-A (EDA) has been linked to TGF-β mediated phenoconversion and acquisition of a myofibroblastic phenotype[42]. In addition to alpha smooth muscle actin expression, EDA-fibronectin is consistently up-regulated in models of fibrosis and is commonly used as a marker for the myofibroblasts.

5. Ski

5.1. Discovery and Characterization

Ski was initially identified as an oncogene via the isolation of Sloan-Kettering retroviruses from chicken embryos that had been infected with an avian leukosis virus. Further analysis showed that the Ski protein was conserved not only in avian evolution, but in mammalian evolution as well [43]. The use of multiple chicken cDNA libraries resulted in the identification of 3 distinct sequences of the chicken Ski gene, which are the result of alternative mRNA splicing [44]. All three variants, which are differentially phosphorylated, are primarily localized to the nucleus, with evidence of differential sub-nuclear localization between the variants [45]. The genomic locus that houses the c-Ski gene is no shorter than 65 kb within the chicken genome, and it has been determined that c-Ski cDNA is encoded by at least eight exons [46]. Also discovered was the related oncogene product v-Ski. It encodes a shorter nuclear protein, 428 amino acids opposed to 750, and is a weaker transforming/differentiating agent [47].

Ski is capable of dimerization, forming both homo/heterodimers and homo/heterotrimers with the related protein SnoN [48]. Using deletion analysis, it was shown that the region crucial for the dimerization process lies in the C-terminal portion of

the protein, which consists of a series of tandem repeats and a leucine zipper motif. This C-terminal region in c-Ski is absent from the v-Ski protein and may explain the enhanced transcriptional activity of c-Ski [47, 49]. *In vitro,* preferential Ski-Sno heterodimerization occurs, resulting in binding to the GTCTAGAC element and a greater transcriptional activity than other homodimer combinations (Ski-Ski or Sno-Sno) or the constituent monomers alone[50].

5.2. Transcriptional Activity

Ski was first shown to have the ability to bind to dsDNA in a Molt4 cancer cell line. Using deletion mutants, two regions of Ski were identified as being necessary components in DNA binding: 1) the N-proximal domain, rich in cysteine and histidine residues, and 2) a C-terminal region rich in basic amino acids. It was determined that a complex containing Ski and another protein factor was responsible for DNA binding[51]. During skeletal muscle hypertrophy caused by Ski overexpression[52], it was found that increased Ski levels are capable of stimulating regulatory elements for the muscle genes megakaryoblastic leukemia (MLK) 1/3 and muscle creatine kinase (MCK), a result that was seen both *in vitro* and *in vivo*[53]. It has also been shown that Ski is capable of binding to nuclear factor I (NFI) proteins and activating the transcriptional activity of NFI reporters[54].

In addition to its ability to trigger transcriptional activation, Ski is also capable of repressing gene expression[55]. It was shown that Ski binds to the sequence GTCTAGAC not by itself, but with the help of protein components (including N-CoR and Mad[56]), acting as a transcriptional co-repressor[55]. Other studies have revealed that Ski interacts

with many other co-repressors including Sin3A, SMRT, and HDAC 1[57]. Subsequent studies have shown Ski's ability to interact with Rb[56], MeCP2[58], PML[59], Gli-3[60], HIPK2[61] and perhaps most extensively studied, Smad proteins[62,63,64,65].

The Smad family of proteins is well known as mediators of the TGF-β signaling pathway[66]. Ski has been identified as an inhibitor of TGF-β signaling. Initial reports suggested that Ski is recruited to the Smad/DNA binding complex, and inhibits Smad3 from interacting with co-activators (p300), in turn recruiting HDAC and repressing transcription of TGF-β target genes[62]. Subsequent studies have shown that a complex exists that consists of Smad3, Smad4 and Ski, with this complex binding to the Smad binding element (SBE) GTCTAGAC[63]. Trapping these inactive Smad complexes on DNA to prevent TGF-β activation has been deemed the 'disrupting bridge' hypothesis[63]. Smad proteins are also known to mediate the BMP signaling pathways. Evidence regarding the role of Ski in the repression of this pathway has been mixed. Early studies showed no association between Ski and the BMP-associated Smad1 & Smad5 proteins[62]. More recent studies have shown that there is a weak interaction between Ski and these Smads, albeit one that is capable of suppressing BMP signaling[67,68,61].

5.3. Cell Fate & Proliferation

Evidence for the effect that Ski has on apoptotic pathways has been seen in multiple models[69,34]. An elevation of Ski levels has been seen in terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive (apoptotic) granulosa cells undergoing follicular atresia[69], and primary cardiac myofibroblasts overexpressing Ski *in vitro* have been shown to have an increased level of apoptosis, as

determined by fluorescnence-activated cell sorting (FACS). Conversely, Ski is capable of reducing Smad3-induced apoptosis in a model of radiation injury in dermal fibroblasts[70]. Given the differing results, it seems highly likely that the apoptotic effect of Ski may be concentration and cell-type specific. With respect to the latter, it is pointed out that fibroblasts from different organs do not share the same morphology or gene expression complement, and that the term "fibroblast" as applied to all related cells may be somewhat naïve[33].

The effect of Ski on proliferation has been mixed as well, with several studies showing it has a positive impact on proliferation and while more recent work shows it inhibits the process. In both skeletal and cardiac muscle cells and tissue, Ski is expressed at a high level in both proliferating, and differentiating cells, suggesting an involvement in these processes[71]. Using an antisense Ski RNA in L6 skeletal muscle cells has shown that you can reduce the positive effect Ski has on proliferation within these cells, highlighting its importance[72]. This protein has even been seen to play a role in the proliferation and differentiation of a number of cells within the central nervous system (CNS) and peripheral nervous system (PNS)[73], as well as to increase the proliferation of dermal fibroblasts[74]. The most recent study to address the issue used a balloon injury model in rats to examine the effect of Ski on proliferation in aortic VSMCs. In a time-dependent manner, overexpression of Ski was able to prevent the TGF-β1 induced proliferative response. Knockdown of Ski enhanced the proliferative effect of TGF-β1. The p38 pathway and its associated CDK inhibitors, p21 and p27, which are unregulated in response to increased Ski expression mediate this change[75]. Due to the complex

effects that Ski seems to have on cell viability and proliferation, further work is required to fully elucidate the full effect that this protein has on a variety of different cell types.

5.4. Other Biological Functions

Ski is known to have important functions during the development of several tissues. In *Xenopus*, it has been shown that Ski is widely present in adult tissues and is maternally regulated during early developmental stages[76]. Overexpression of Ski in *Xenopus* embryos leads to the induction of neural axis formation, as well as expression of neural specific genes and the formation of a neural tube-like structure in ectodermal cells[77]. Further evidence of this protein's importance in neuronal development comes from experiments involving Zebrafish in which the overexpression of two Ski paralogues (skiA and skiB) resulted in a dorsalized phenotype and a disruption of gastrulation[78]. In mice models, a high level of Ski gene expression is detectable throughout the entire neural tube during development and is detected in the cerebellum and hippocampus in postnatal mice[73]. *In vivo,* overexpression of Ski in mice causes hypertrophy of skeletal muscle fibers, specifically type II fast fibers[52,79]. Conversely, $Ski^{-/-}$ mice suffer from exencephaly, leading to perinatal lethality. In addition to several other neurological defects, these mice also have a reduced skeletal muscle mass[80].

Originally identified as a proto-oncogene, the subsequent research into the effect Ski has on cancer biology has been inconclusive, with many studies stating that it promotes oncogenic transformation and growth[82,83,84], while others dispute that claim[85]. Once again, the effects of Ski that are seen may be relevant only in a context-dependent (cell type and concentration) manner.

In the context of the heart, very little is known about Ski. Early studies have shown that it is present within proliferating and differentiating cardiac muscle cells[71]. More recently studies have shown that expression of Ski in cardiac myofibroblasts is capable of controlling phenoconversion, type I collagen secretion, and contractility[34]. Clinical studies have suggested that deletion of Ski, in the form of 1p36 deletion syndrome is associated with the occurrence of congenital heart disease[86].

5.5. Regulation

Due to the effect that irregular expression (overexpression & knockdown/out) has on cellular phenotype, fate, and function, the level of intra- and sub-cellular Ski has to be closely monitored and regulated in order to maintain proper physiological function. Arkadia is an E3 ubiquitin ligase, which functions to enhance TGF-β signaling, doing so by targeting TGF-β inhibitors (c-Ski, I-Smad7, SnoN) [87,88]. It has been shown that Arkadia interacts with Ski in both its free and Smad-bound forms, thus removing inactive R-Smad-Ski complexes from the nucleus, allowing transcriptionally active R-Smads to bind to DNA[89]. Ubiquitination of Ski has been reported, with degradation of the protein being suppressed in the presence of MG132, a proteasome inhibitor, suggesting that Ski is removed by an ubiquitin-mediated proteasomal mechanism[88]. Studies examining the cell cycle of epithelial cells have shown that the enzyme Cdc34 (an ubiquitin-conjugating enzyme) mediates proteolysis of Ski, both *in vitro* and *in vivo*[90]. Although Arkadia mediated proteolysis has not been linked to Cdc34, it may function through a similar enzyme and further studies are required.

Due to the effect on transcriptional activity, the primary research focus of Ski has been on its nuclear localization and function. C184M is a novel cytoplasmic protein with Ski-binding ability[91]. When Ski and C184M are co-overexpressed in CV-1 monkey kidney cells, both proteins are shown to localize within the cytoplasm and normal Smad2/3 translocation to the nucleus is inhibited[91]. Although few studies have examined the role of C184M, it is possible that the sequestration of Ski to the cytoplasm may have a de-repressive effect on TGF- β signaling. It is known that sub-cellular localization (nuclear vs. cytoplasmic) of Ski differentially modulates cellular activity[92]. Using mutations in the nuclear localization sequence (NLS) of Ski studies have shown that when sequestered within the cytoplasm, Ski interacts with R-Smads and Co-Smad to inhibit TGF-β -Smad signaling[92]. Cytoplasmic Ski fails to suppress basal expression of inhibitory-Smad7 (I-Smad7)[92], which is involved in a negative feedback loop to regulate TGF-β -Smad signaling[93].

6. Programmed Cell Death

6.1. Apoptosis

Although scientist Carl Vogt first described the principle of programmed cell death in the mid 1800s, it was not until 1972 that Kerr *et al* first described the process of apoptosis[94]. Subsequent studies by Horvitz, Shulston, and Brenner on the organism *C. elegans* identified critical genes involved in the regulation and control of apoptosis, earning them the Nobel Prize in medicine in 2002.

Apoptosis plays a critical role in normal physiological function and development as well as in disease states. In normal physiological systems, apoptosis balances mitosis

to maintain organ size and function. While the latter is responsible for cell proliferation and growth, apoptosis clears unwanted or damaged cells from the body. It has been estimated that in order to balance out the normal growth of cells in the body, approximately 10 billion cells must undergo apoptosis per day to maintain body homeostasis[95]. In normal physiological situations, apoptosis plays important roles in processes such as digit formation in the fetus[96], sloughing of the inner lining of the uterus during the menstrual cycle[97], neural development[98], immune system maturation[99], and morphogenesis[100], [101]. Dysregulation of apoptosis can lead to diseases such as cancer, AIDS, neurodegenerative diseases (ALS, Parkinson's, Alzheimer's), autoimmune disorders, and many more; some a result of insufficient apoptosis, others the result of excessive cell death[102].

Apoptosis can be identified and characterized by a variety of morphological and biochemical changes that occur. Some of the major morphological changes that distinguish it from other forms of cell death include cell shrinkage, chromosomal condensation, nuclear fragmentation, and the formation of apoptotic bodies through a process called "budding" which minimizes the inflammatory response by sequestering the intracellular contents of dead cells within the apoptotic bodies[103]. The biochemical events that occur during apoptosis are largely mediated by a group of cysteine-aspartic proteases known as caspases. These proteolytic enzymes are widely found through cells existing in an inactive form, called pro-caspases. Once an apoptotic stimulus has been applied to the cells, the enzymes become activated and are able to initiate their respective proteolytic cascade. Although as many as 16 different caspase enzymes have been identified, 10 of these enzymes play major roles and have been broadly categorized into

three distinct groups: executioner caspases (caspase-3, -6 and -7), initiator caspases (caspase-2, -8, -9, and -10), and inflammatory caspases (caspase-1, -4, and -5)[104]. Once caspases are activated, the commitment to apoptosis and cell death is thought to be final and irreversible, although some evidence has suggested that upon removal of the apoptotic stimulus, cells undergoing early p53-induced apoptosis can be rescued from cell death. This rescue appears to be caused by the activation of early DNA repair mechanisms[105].

Apoptosis is a very complex process that can be activated in a variety of ways and triggered by several factors external to the cell. The two main apoptotic pathways that have been described in the literature are the intrinsic, or mitochondrial mediated pathway, and the extrinsic pathway. Another pathway includes the perforin/granzyme pathway. Although these pathways are described separately, there exists a great deal of crosstalk and interaction between them[106].

6.1.1. Intrinsic Apoptotic Pathway

The intrinsic pathway, involving the mitochondria, is a non-receptor mediated apoptotic pathway. Stimuli that trigger this pathway initiate a series of intracellular signals that results in changes in mitochondrial permeability resulting in the release of pro-apoptotic factors, loss of mitochondrial membrane potential, and opening of the mitochondrial permeability transition pore (MPTP). The loss of mitochondrial membrane potential leads to the opening of the MPTP, which allows small molecules and proteins to leave the mitochondria and enter the cytosol of the cell[107]. In addition to the release of small molecules, opening of the MPTP may allow water into the mitochondria,

causing it to swell and rupture the membrane of the organelle, subsequently releasing proteins[108]. The most prevalent protein factors that are released from the mitochondria during apoptosis are cytochrome c[109], Smac/DIABLO[110], and Htr2A/Omi[111]. These proteins function in various manners, promoting apoptosis by either actively stimulating the process or removing inhibitors of apoptosis. Cytochrome c is released from the mitochondria in a manner that is independent of membrane depolarization[109] and moves into the cytosol where it interacts with Apaf-1 and procaspase-9, forming a large quaternary protein structure known as the apoptosome[112]. The apoptosome is the location where procaspase-9 is activated, yielding active caspase-9, which is capable of triggering downstream apoptotic effects such as the activation of caspase-3[113]. Smac/DIABLO and Htr2A/Omi promote apoptosis by interacting with, and inhibiting a group of proteins known as inhibitor a apoptosis proteins (IAP)[107]. PUMA and NOXA are two other important pro-apoptotic proteins that are members of the Bcl-2 family. These proteins are involved in p53-mediated apoptosis and are thought to act through BAX translocation[114,115].

The events that occur at the mitochondrial level are evidently very important to the induction of apoptosis and are therefore highly regulated by in the body. The family of proteins responsible for this regulation is known as the Bcl-2 superfamily, which is composed of over 25 identified genes that are either pro-apoptotic or anti-apoptotic[116]. Even proteins within the same group function in different ways. Some of the most well studied pro-apoptotic proteins Bax and Bak are thought to promote apoptosis by inserting into the mitochondrial membrane and forming oligomers. This process is thought to contribute to mitochondrial permeabilization[117]. Other pro-apoptotic proteins such as

Bad, function by dimerizing with anti-apoptotic proteins (eg. Bcl-2 and Bcl-XL), repressing their protective effect[118]. In the absence of repression by Bad, these two proteins inhibit the release of cytochrome c from the mitochondria[119]. Levels of these apoptotic proteins are critical in regulating apoptosis and evidence exists that members of the same group are reciprocally regulated[120].

6.1.2. Extrinsic Apoptotic Pathway

One of the features distinguishing the extrinsic pathway from the intrinsic one is the fact that the external apoptotic pathway is mediated by ligand-receptor interaction and binding[102]. Although many receptor-ligand combinations have been reported, two of the most comprehensively studied are FasL/FasR, and TNF-α/TNFR1. Receptors are clustered on the surface of the cell and bind their respective ligand, which triggers recruitment of intracellular adaptor proteins, which have corresponding "death domains". Upon binding of FasL to FasR, the adaptor protein Fas-associated death domain (FADD) is recruited to the receptor[121]. A similar event occurs in TNF mediated apoptosis[122]. The death domain of these adaptor proteins can then interact with procaspase-8, resulting in the formation of a death-inducing signaling complex yielding activation of caspase-8 which that then go on to trigger activation of caspase-3[123].

6.1.3. Granzyme B/Perforin Pathway

The granzyme/perforin pathway plays a major role in immunity, as it is the main way in which cytotoxic T lymphocytes (CTLs) clear infected and transformed cells from within the body via apoptosis[124]. CTLs are capable of triggering apoptosis by activating

the extrinsic pathway via interaction with FasL/FasR but they are also capable of inducing cell death in a novel pathway, which involves secretion of a membrane spanning protein known as perforin[124]. Early studies believed that perforin contained lytic properties, inserting itself into a target cell membrane, allowing for the diffusion of granzymes into the cell, activating caspase enzymes and thus triggering apoptosis[125]. More recent studies have hypothesized that the mannose-6-phosphate receptor acts as a cell surface receptor that recognizes a macromolecular complex containing perforin, granzyme B and other molecules and takes these complexes up in a manner that does not rely on significant pore formation by perforin[126].

Once granzyme B is in the target cell, it can induce cell death in a variety of ways. Studies have shown granzyme B is capable of activating pro-caspase 10 by cleaving it at one of its aspartic residues[127]. Other studies have shown that granzyme is capable of utilizing the intrinsic mitochondrial pathway to amplify its signal, inducing release of cytochrome c from the mitochondria[128]. It has also been shown that granzyme B can directly active caspase-3, bypassing the upstream apoptotic signaling[129].

Granzyme A is also capable of inducing apoptosis via CTLs, although this signal is independent of caspase activation. Upon entry into the cell, granzyme A triggers the DNAse enzyme NM23-H1, causing DNA nicks to occur[130]. To prevent a DNA repair mechanism from being triggered, granzyme A also cleaves the protein SET, which normally inhibits the NM23-H1 gene and functions in DNA repair[130].

6.1.4. Caspase-Independent Cell Death

Although traditional apoptosis is considered by many to be mediated through caspase activation, many studies in the past decade have characterized a form of apoptotic cell death that is independent of caspase involvement[131, 132, 133]. In addition to organelles that play a large part in classical apoptosis (ie. the mitochondria), other organelles such as lysosomes, the endoplasmic reticulum (ER), and the plasma membrane may all play a role in PCD independent of caspases. One mitochondrial protein that may play a role in this process is the protease endonuclease G, which is able to cause DNA fragmentation in nuclei independent of caspase activation[134]. Another mitochondrial protein that may be involved is apoptosis inducing factor (AIF), which can trigger cell death. When it is released from the mitochondria into the cytosol, AIF translocates to the nucleus, possibly in conjunction with endonuclease G, and triggers DNA fragmentation and chromatin condensation[135,136].

The endoplasmic reticulum is another important organelle involved in cell death. It monitors cellular stress and if need be, inhibit protein synthesis which initiates a programmed cell death response. This response is mediated by one of two cellular events: the unfolded protein response, or influx of calcium into the cytoplasm[137]. The increase in intracellular calcium triggers a family of proteases called calpains, which are known to be involved in crosstalk with apoptosis pathways through Bax and likely other factors and signals[138].

Another organelle that appears to play a key role in apoptosis is the lysosome. Although the relative contribution of lysosomal mediated apoptosis versus caspase

mediated apoptosis remains unclear, it is evident that lysosomal permeabilization leads to apoptotic activation[139].

6.2. Autophagy

The other major form of programmed cell death, often referred to as programmed cell death type II, is autophagy. The term autophagy refers to degradation of cytoplasmic contents within lysosomes, although it is quite distinct from the endocytosis-mediated degradation of extracellular components, which also occurs within the lysosome[140]. In fact, autophagy is largely mediated by a structure known as the autophagosome, as well as by a family of genes known as autophagy-related genes (ATG). Autophagy is a general term, often used to describe the most studied form of autophagy, macroautophagy, although other forms do exist, including: microautophagy, mitophagy, chaperone-mediated autophagy, macropexophagy, micropexophagy, as well as piecemeal microautophagy of the nucleus[141]. The role that autophagy plays within the body is very complex, and appears to be involved in many physiological and pathophysiological situations, active at both a basal rate, and capable of being induced[142].

Once autophagy is induced, the first step in the sequestration of organelles is the formation of a membranous structure known as the phagophore. Elongation of this structure and complete sequestration of the components to be degraded yields what is known as the autophagosome[143]. Of the 31 ATG genes that have been identified, 18 of them are known to be involved in autophagosomal formation[144]. Autophagosomes next fuse with lysosomes forming an autophagolysosome[140], exposing their contents to lysosomal hydrolases and other catabolic enzymes. This fusion results in the breakdown

of the organelles and macromolecules that were initially isolated and sequestered by the phagophore/autophagosome. Autophagy functions to recycle cellular contents so once the organelles are degraded within the autophagolysosome, the monomeric remains are released into the cytosol where they can be utilized again by the cell for its needs during a specific response[145].

Autophagy has been described as both a pro-survival process (for its role in nutrient starvation) and a pro-death process as it has been shown to mediate apoptosis in certain circumstances[146]. This apparent duality has made autophagy the focus of many studies, with more research being needed to fully elucidate its role in normal cell physiology as well as pathophysiology.

6.3. Other Programmed Cell Death Mechanisms

Although PCD research has primarily focused on the role of apoptosis and autophagy in health and disease, recent discoveries have revealed the existence and importance of alternative pathways in how cellular environment and other stressors can determine cell fate. Among these novel pathways are entosis, ferroptosis, and necroptosis.

Detachment of a cell from its surrounding ECM, a process called anoikis, was first seen to trigger an apoptotic response in both epithelial[147], and endothelial cells[148]. A recent study designed to track mammary epithelial cell fate was able to show that following detachment from the ECM, a significant portion of cells were internalized by neighboring epithelial cells, a process independent of apoptosis – which they named entosis[149]. Although apoptotic cells that expose phosphatidylserine on the external face of the plasma membrane may also be internalized by macrophages[150], one of the primary

distinctions between the two processes lies in the cellular fate after internalization. Cells undergoing entosis have been shown to be fully functional, capable of participating in cellular division during engulfment, and are even able to be released from the cell in which they are engulfed[149]. The mechanism and cytological features that occur during entosis have been used to explain the 'cannablistic' behavior of cancer cell lines that has been previously reported[151], [152], [153]. The potential for anchorage-independent cell growth and survival has made entosis a research focus in the field of cancer biology [154], [155], [156], [157].

Reactive oxygen species are capable of inducing cell death pathways, including apoptosis[158], necrosis[159] and autophagy[160]. A recent study has shed light on another form of non-apoptotic cell death that relies on ROS produced by NADPH oxidases to induce cell death[161]. This novel pathway, ferroptosis, is triggered by the RAS-selective lethal agent erastin, which induces an intracellular-iron dependent programmed cell death pathway that is distinct from all other known forms of cell death[161]. Subsequent studies have shown that ferroptosis may be the primary pathway that certain cancer drugs use to target cancerous cells[162].

Perhaps the most well-known and well-characterized cell death mechanism is a non-programmed pathway called necrosis, which results from irreversible trauma to the cell caused by external factors. This process results in the spilling of the cell's intracellular contents into the extracellular environment, eliciting an inflammatory immune response[163]. Recent evidence has revealed an additional, programmed form of necrosis, called necroptosis, which is regulated in a RIP1-/RIP3-dependent manner[163].

The involvement and importance of this novel process has already been identified in a plethora of organs and tissues, as well as diseases, including cardiac I/R injury[164].

III. Hypothesis and Rationale

Myofibroblasts appear in infarct scar soon after the infarct occurs and this is followed by rapid wound healing, which is complete within weeks of the initial injury. Myofibroblasts do not disappear after completion of this initial stage of wound healing but rather persist in the infarct scar. These cells contribute to progressive hypertrophic scar remodeling at the site of infarction and then subsequently to overt expansion of the interstitium (cardiac fibrosis) of the remnant left ventricle and the right ventricle, and these events are associated with cardiac decompensation and frank heart failure. Recently published data from our lab has identified a correlation between Ski protein overexpression in primary P1 rat cardiac myofibroblasts and their subsequent death. The purpose of this research is to further characterize this phenomenon and to elucidate the mechanism that is responsible for this process. If Ski modulates myofibroblast cell death, then this mode of action may represent a novel means of therapy in controlling matrix remodeling in the post–MI heart.

Research Question: What is the link between cell fate and Ski protein expression in primary cardiac myofibroblasts?

Hypothesis: Overexpression of Ski protein in primary P1 rat cardiac myofibroblasts will activate programmed cell death mechanisms resulting in cell death.

Objectives:

1) Investigate the role Ski protein plays in regulating the cell viability of primary P1 cardiac myofibroblasts.
2) If cell viability is affected, determine the specific apoptotic pathway that mediates the effects that are seen.
3) Determine whether multiple programmed cell death pathways (e.g. apoptosis and autophagy) act synergistically or antagonistically to mediate the cell death effect seen in primary cardiac myofibroblasts overexpressing Ski.

IV. Materials and Methods

1. Isolation of primary fibroblasts/myofibroblasts

Injections of a Ketamine/Xylazine cocktail were administered to adult Sprague Dawley rats (150-200g) via an intra-peritoneal injection into the right iliac region of the abdomen to anesthetize them. Hind limb withdrawal reflex was used to test the effectiveness of the anesthesia. Absence of this reflex was a sufficient result to conclude adequate anesthesia. Heparin was then injected into the right femoral vein. If venous access was unattainable, the heparin was injected directly into the ventricles of the heart. A thoracotomy was then performed and the heart resected from the animal. Once resected, the heart was placed into a 100 mm dish containing 20 mL DMEM-F12. The heart was then hung via the aorta on a cannula attached to a Langendorff retrograde perfusion apparatus. Non-absorbable 3-0 braided silk sutures were used to secure the heart in place on the cannula and to prevent detachment during perfusion. DMEM-F12 media was perfused through the coronary arteries for 5 minutes in an effort to rid the heart of any remaining blood. Following DMEM-F12 perfusion, SMEM was perfused through the heart for 6 minutes in order to stop the heart from beating. Following SMEM perfusion, type II collagenase (0.1%) in SMEM was run through the coronary vasculature for 20 minutes to degrade the collagen within the cardiac tissue. Once sufficient tissue degradation had occurred, the heart was removed from the Langendorff apparatus and placed into a 100mm culture dish containing a type II collagenase (0.05%)/SMEM solution. The partially digested heart tissue was then mechanically broken down using tweezers, liberating as many fibroblasts as possible and maximizing the digestion process. These dishes were then incubated at 37°C for 15 minutes. Digestion was halted

by adding 10 mL DMEM-F12 containing FBS (10%). To remove undigested portions of the tissue, all of the contents of the 100mm culture dishes were passed through a 70-micron strainer and into a 50 mL tube. This tube was centrifuged at 2000 rpm for 7 minutes to separate the cells (pellet) from the supernatant. The supernatant was then carefully suctioned off in an effort to not disturb the remaining pellet. This pellet was then re-suspended in 40 mL 10% DMEM-F12 with FBS growth media. Media was supplemented with antibiotics (100 U/mL penicillin & 100 μg/mL streptomycin) as well as sodium bicarbonate (1.2 g/L) and ascorbic acid (1 μM). Each re-suspensded pellet was plated in T-75 culture flasks (10 mL/flask) and placed in the incubator at 37°C for 2 hours. Following this, the cells were washed 2x with 1X PBS and fresh culture media was added. These freshly plated cells were considered to be 'P0' cardiac fibroblasts. The day after digestion and plating, the cells were once again washed 2x with 1X PBS and new growth media (10% DMEM-F12 with FBS) was added and left to grow for 72 hours or once 80-90% confluency was reached before being passaged into 'P1' myofibroblasts.

2. Adenoviral Amplification & Titration

Amplification

HEK293 cells were plated in T175 flasks and allowed to grow until a confluency of 70-80% was reached. The growth media (DMEM + 10% FBS) was then replaced with 10 ml of fresh media containing adenovirus (5-10 MOI). After 5 hours of incubation at 37°C, an additional 5 ml of growth media was added to the flask. These flasks were incubated at 3-4 days at 37°C or until approximately 80% of the cells had detached from the flask. When 80% of the cells had detached, cells were collected from the T175 flask and

transferred to a 50 ml tube. The suspension was centrifuged at 1000 rpm for room temperature for 5 minutes. The supernatant was collected a stored at -20°C. The resultant pellet was resuspended in 10 ml of media. This cell-containing solution is lysed by 3 consecutive freeze-thaw cycles. Freezing was accomplished via liquid nitrogen and the cells were thawed using a water bath. After the final freeze-thaw cycle, the tube was once again centrifuged at 1000 rpm for 5 minutes. The supernatant was collected and stored at -80°C while the pellet was discarded.

Titration

1 ml (5×10^5 cells) of healthy HEK 293 cells in log phase of growth was seeded into a 12-well plate with growth medium (DMEM + 10% FBS). Using PBS, 10-fold serial dilutions of the viral sample was prepared. 100 µl of viral dilution was added to each well. The cells were incubated for 48 hours at 37°C in 5% CO_2. After incubation, media in the wells was aspirated and the cells were left in the culture hood to air dry. Cells were fixed by gently adding 1 ml cold 100% methanol to each well. Plates were then incubated for 10 minutes at -20°C. The methanol was then aspirated and wells were rinsed 3x with 1 ml PBS + 1% BSA. After aspirating the PBS, 0.5 ml of Rat Anti-Hexon dilution (1:1000 in PBS + 1% BSA) was added to each well and incubated for 1 hour at 37°C. This dilution was then aspirated and the wells were washed 3 times as described previously with PBS + 1% BSA. After removal of the final wash, 0.5 ml Rat Anti-Mouse antibody (HRP conjugate) dilution (1:500 in PBS + 1% BSA) was added to the wells. The plate was incubated for another hour at 37°C. The dilution was aspirated and the wells were once again washed 3 times. After completion of the wash cycle, 500 µl DAB working solution was added to each well and incubated a room temperature for 10

minutes. Dab was the aspirated and 1 ml of PBS was added to each well. A minimum of three fields of brown/black cells was counted using a microscope (20x magnification), and mean number of positive cells was calculated for each well. The infectious units for each well was calculated as follows:

$$\frac{\text{(Infected cells/field) x (Fields/ well)}}{\text{Volume virus (ml) x (Dilution factor)}}$$

3. Protein Isolation

Primary cardiac fibroblasts were plated as P1 cells on 100 mm plastic culture dishes and left to grow in 10% DMEM-F12 with FBS until a confluency of 70-80% was reached. Overexpression was achieved by infecting the cells at the time of plating with adenoviruses (Ad-LacZ or Ad-HA-Ski). Once the desired confluency was reached, cells were washed twice with 1X PBS and starved with serum-free DMEM-F12 for up to 96 hours. Once experimental time points had elapsed, the plates were removed from the incubator, the media was aspirated, the plates were washed once with 1X PBS and then left sitting in 1X PBS on ice. If sufficient cell death had occurred and there were a significant number of floating (dead) cells in the media, the media was removed and put into a 15 mL tube for later preparations. The remaining 1X PBS was washed from the plates on ice and 1 mL of cold 1X PBS was added to each plate for scraping. Cells were collected and put into a 1.5 mL Eppendorf tube. Another 0.5 mL cold 1X PBS was added to the plate for further scraping, and added to the same tube. Cells were spun down at 1500xg (~5500 rpm) for 5-6 minutes at 4°C in the cold room (both cells from the plates and from the media in the 15 mL tubes) . The resulting supernatant was removed and the pellet was resuspended in RIPA lysis buffer pH = 7.6 containing 150 mM NaCl, 1.0%

nonidet P-40 (NP-40), 0.5% deoxcholate, 0.1% sodium dodecyl sufate (SDS), 50 mM Tris, phosphatase inhibitors (10 mM NaF, 1 mM Na_3VO_4, and 1 mM EGTA), and protease inhibitors (4 μM leupeptin, 1 μM pepstatin A, and 0.3 μM aprotinin). Cells were then left on ice for up to 1 hour. Next, cells were sonicated 3X for 10 seconds/time, and then spun down at 14000 rpm for 15 mins at 4°C in a microcentrifuge. The resulting protein-containing supernatant was collected and transferred to a new Eppendorf tube. The pellet was discarded. Samples were stored at -20°C until quantification of protein content could be determined.

3. Protein Assay

Protein samples were removed from the -20°C freezer and put on ice to thaw. Samples were vortexed and then diluted using RIPA buffer (1:3), allowing for 10 μL diluted sample per well in a 96-well plate. Protein samples were analyzed in a triplicate manner. Protein standards were prepared by diluting bovine serum albumin protein (Pierce) in RIPA buffer to yield standard concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1.0 & 2.0 μg/μL. Protein standards were also loaded in triplicate fashion on the 96-well plate. 200 μL of a solution of bicinchoninic acid and copper (II) sulfate (50:1 dilution) was added to each well containing sample or standard. The 96-well plate was wrapped in parafilm and placed into the 37°C incubator for 30 minutes. Once the incubation was complete, the absorbance of the plate was detected at 550 nm on a microplate reader. A standard deviation of less than 0.100 was considered desirable for each protein sample and therefore outliers may have been removed from the final calculation of protein concentration. A linear correlation coefficient of ≥0.995 for the protein standards was also desirable.

4. SDS-PAGE

After the protein concentration was determined, the protein lysates were subjected to SDS-polyacrylamide gel electrophoresis. Protein samples were prepared by combining equal concentrations of protein (10-20μg) with Laemmli buffer (125 mM Tris-HCl (pH6.8), 5 % glycerol, 2.5 %SDS, 5% 2-mercaptoethanol, and 0.125 % bromophenol blue) and boiling for 5 minutes. These samples were cooled and then loaded onto SDS-polyacrylamide gels (6-15% polyacrylamide). Pre-stained protein ladders (Bio-Rad) were added to the gel in order to easily identify target proteins by size. Proteins were separated by running the gel at 180V until the desired separation had been achieved. The gel was then removed from the running tank and proteins were transferred onto a 0.45 μm polyvinylidene difluoride (PVDF) membrane at 150V for 90 minutes at 4°C.

5. Western Blotting

PVDF membranes were blocked either for 90 mins at room temperature or overnight at 4°C in a 10% skim milk powder solution made in phosphate-buffered saline (PBS). Membranes were washed with 3x for 10 min with PBS containing 0.2% Tween-20 (PBS-T). Primary antibodies were diluted in a 3% skim milk solution in PBS and incubated for 90 mins at room temperature or overnight at 4°C. Primary was then removed and the membrane was washed 3x for 10 min with PBS-T. Secondary antibodies (horseradish peroxidase [HRP]-labeled anti-mouse IgG or HRP-labeled anti-rabbit IgG) were diluted in PBS with 3% skim milk and incubated at room temperature

for 1 hour. Secondary was removed and the membrane was washed 3x for 10 min with PBS-T. Enhanced chemiluminescent substrate was added to the membrane for 5 minutes to assist in protein visualization. The membranes were then developed on blue X-ray film. Equal protein loading was confirmed by using β-tubulin as a loading control.

6. MTT Assay

First passage (P1) rat cardiac myofibroblasts were infected with 30 MOI Ad-LacZ or Ad-HA-Ski and then plated at 7500 cells/well in 96-well plates. Each treatment was performed multiple times. Cells were allowed to grow/adhere for 24 hours in the presence of 10% FBS, allowing them to grow up to approximately 70% confluency. Upon reaching 70% confluency, the cells were washed twice with 200 µL 1X PBS and the media was changed to 200 µl serum free DMEM-F12. After 72 and 96 hours of starvation, 20 µl MTT solution (5 mg/ml MTT in PBS, sterile filtered with 0.45 µm filter) was added to each well and incubated at 37°C for a further 3 hours. The media/MTT mixture was then removed by pipetting and replaced with 200 µl dimethyl sulfoxide (DMSO) and then the plates were allowed to sit for 5 min. Each well was then mixed by pipetting and absorbance was measured via microplate reader at 570 nm to calculate cell viability.

7. Caspase-GLO® Assay

First passage (P1) rat cardiac myofibroblasts were infected with 30 MOI Ad-LacZ or Ad-HA-Ski and then plated at 7500 cells/well in 96-well plates. Cells were allowed to grow for 24 hours in the presence of 10% FBS, allowing them to reach a confluency of approximately 70%. They were then starved for 96 hours in serum free DMEM-F12.

Caspase-GLO substrate and buffer was equilibrated to room temperature and the lyophilized substrate was re-suspended in the buffer. Approximately 100 μL starvation media was removed from the experimental wells and 100 μL Caspase-GLO solution was added to these wells. The plate was gently mixed on a plate shaker and incubated at room temperature for 30 minutes. A luminometer was then used to measure the luminescence of the experimental wells.

8. LIVE/DEAD® Cytotoxicity Assay

P1 rat cardiac myofibroblasts were plated at a confluency of 30% in a 6-well culture plate containing sterile glass coverslips and infected with either 30 MOI Ad-LacZ or 30 MOI Ad-HA-Ski, or left untreated. Cells were grown to 70% confluency in the presence of DMEM-F12 media with 10% FBS. Cells were then washed 2X with 0.02% PBS-T to remove growth media and were starved in serum free DMEM-F12 media for 96 hours. At the desired time-point, each well was washed 2X with 0.02% PBS-T. PBS-T was removed and 100-150 μL combined LIVE/DEAD® assay reagent, using optimized concentrations, was added to the surface of the coverslip. The cells were then incubated at room temperature for 30-45 minutes. Cells were then gently washed 2X with PBS and the wet coverslips were then inverted onto a clean microscope slide and imaged using a Nikon TE 3000 microscope. Images thought to be representative of the entire sample were taken. As per the untreated control and negative control, live cells stained green while dead cells stained red in color.

9. Immunofluorescence

P1 adult cardiac myofibroblasts were plated in 6 well dishes onto glass coverslips. Cells were seeded into the wells at 20% and cultured in DMEM-F12 with 10% FBS at 37°C until a confluency of 60% was reached. At the time of plating, cells were either left untreated, infected with 30 MOI LacZ virus or 30 MOI Ski virus. Once target confluency was reached, cells were washed with 1X PBS and serum-free DMEM-F12 was added to the wells, starving the cells for up to 96 hours. After starvation, media was aspirated and cells were washed 3x with cold 1X PBS. Cells were fixed by adding 4% paraformaldehyde to the wells and incubating them for 10 minutes at room temperature. Cells were once again washed 3x with cold 1X PBS. 0.1% Triton-X100 was added to the wells for 15 minutes at room temperature in order to permeabilize the cells. 1X PBS was used to wash the cells as previously described. 10% bovine serum albumin in PBS was added to the wells for 30 minutes at 37°C to block non-specific binding sites. After blocking, cells were once again washed 3x with 1X PBS. Primary antibody diluted in 1% BSA was then gently spread over the coverslips and incubated at 4°C overnight in darkness. Cells were then washed 3x with 1X PBS. Secondary antibody was then added and left on the cells for 90 minutes. One final wash was applied and coverslips were removed from the well and gently aspirated. A drop of Slowfade Gold with DAPI was applied to the coverslips and they were placed on glass microscope slides and mounted cells down. Nail polish was used to secure the coverslips to the slides and slides were then stored in an opaque box at 4°C until ready to image. Pictures that were considered to be representative of the entire sample were taken using a Nikon D90 camera on a Nikon E600 microscope.

10. Reagents & Antibodies

DMEM-F12 (Hyclone), SMEM (Gibco), Collagenase (Worthington Biochemical Corp.), Caspase-7 antibody (Cell Signaling), Caspase-9 antibody (Cell Signaling), Bax antibody (Cell Signaling), Cytochrome C antibody (Cell Signaling), β-tubulin antibody (Abcam), Caspase-GLO® (Promega), MTT, PVDF (Millipore), Secondary antibodies, ECL, Albumin Protein standard, Pencillin & Streptomycin (Gibco), Gentamycin (Gibco), Antibiotic-Antimycotic (Gibco), Fetal Bovine Serum (Wisent), Ski antibody (Upstate/Millipore), Western blot ladders (BIO-RAD)

11. Statistical Analysis

All data is expressed as mean ± standard error. Data comparisons were conducted using a two-sided paired t-test or a one-way analysis of variance (ANOVA) with a Student-Newman-Keuls *post hoc* test. A p-value less than 0.05 ($p<0.05$) was considered to be statistically significant unless otherwise indicated in the figure legend.

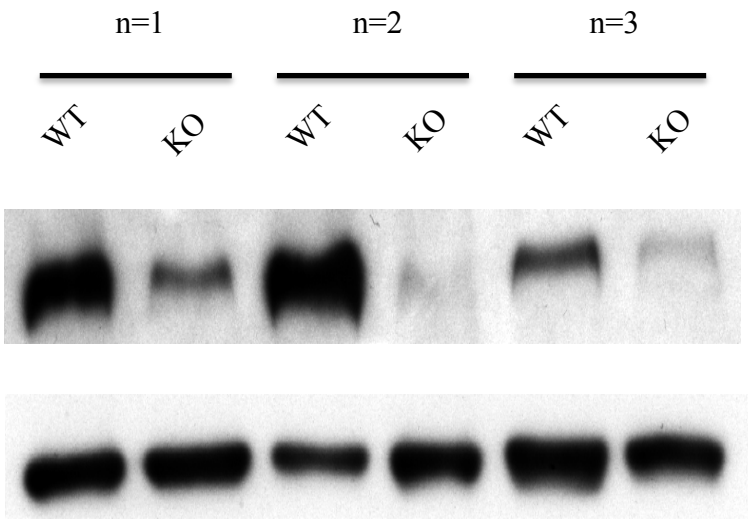
V. Results

The link between Ski and cell death – reciprocal regulation?

Recently published data from our lab has identified a correlation between Ski protein overexpression in primary rat cardiac myofibroblasts and cell death. The purpose of this research is to identify if this correlation is physiologically relevant and to elucidate the mechanism that is responsible for this process.

While this research primarily utilizes rat primary cardiac myofibroblasts derived from rat heart, a small, initial experiment was performed using embryonic mouse cells. Preliminary studies utilizing a Bax/Bak knockout (KO) model in mouse embryonic fibroblast (MEF) cells showed that the absence of the pro-apoptotic proteins Bax/Bak from these particular cells correlated with a decreased Ski expression (Figure 1), roughly a 60% decrease in protein expression compared to wild type (WT) MEF cells. This data, along with the previously reported Ski-overexpression data from our laboratory suggests a possible correlation between Ski and programmed cell death, primarily apoptosis. Although there are many benefits to using MEF cells, the nature of them being derived from embryonic tissues can cause problems when you are trying to examine the physiology and pathophysiology of adult tissues. First hand experience in our laboratory has shown that results seen in embryonic cells can differ immensely from the results seen in primary adult cells lines thus limiting the potential and the value of using MEF cells for further experiments.

A.



B.

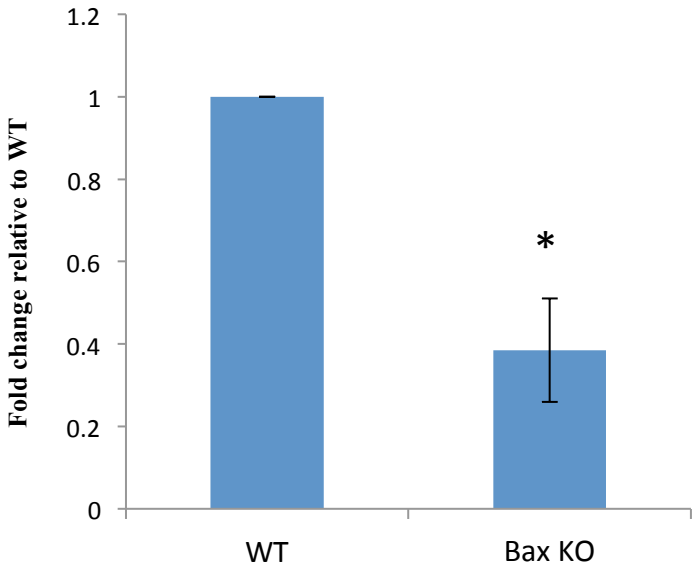


Figure 1. **Ski expression is decreased in Bax/Bak MEF KO cells.** Wild type and Bax/Bak knockout mouse embryonic fibroblasts were cultured and grown to 80% confluency before protein was isolated for Western blot analysis. β-tubulin is used as a loading control. Fold change is relative to each individual control. Panel B: Histogram representation of data from images in A. Image is representative of n=3. *$p<0.05$ vs wild-type controls.

In order to test the potency of our adenovirus, Ski was overexpressed using an adenoviral vector in passage 1 (P1) cardiac myofibroblasts isolated within our laboratory. Overexpression with 30 viral particles/cell, or 30 multiplicity of infection (MOI) yielded an overall increase in Ski protein expression that was approximately 2-3 fold higher than uninfected cells, and the viral control, LacZ infected cells (Figure 2).

Cell viability is a time dependent process

Induction of cell death in primary P1 cardiac myofibroblasts appears to be a time dependent process. We have routinely seen that Ski overexpression for 24 - 48 hours is not associated with significant cell death or any phenotypic changes suggestive of an injured or dying cell. Cells during these time points have a normal fibroblastic morphology: spindle-shaped branching cells with regular, oval nuclei. Upon reaching a confluency of ~70-80%, all treatment groups (control, LacZ, Ski) of P1 cardiac myofibroblasts were starved in serum free DMEM-F12 for up to 96 hours. Morphological changes that suggest apoptotic activation are present at 96 hours of starvation (Figure 3). Viewed under 4x magnification, control and LacZ-infected cells exhibit normal spindle-shaped myofibroblast morphology, indicative of healthy cells. Ski-infected cells are seen to be losing their cytoplasmic extensions and appear to be blebbing – traits indicative of a cell undergoing programmed cell death.

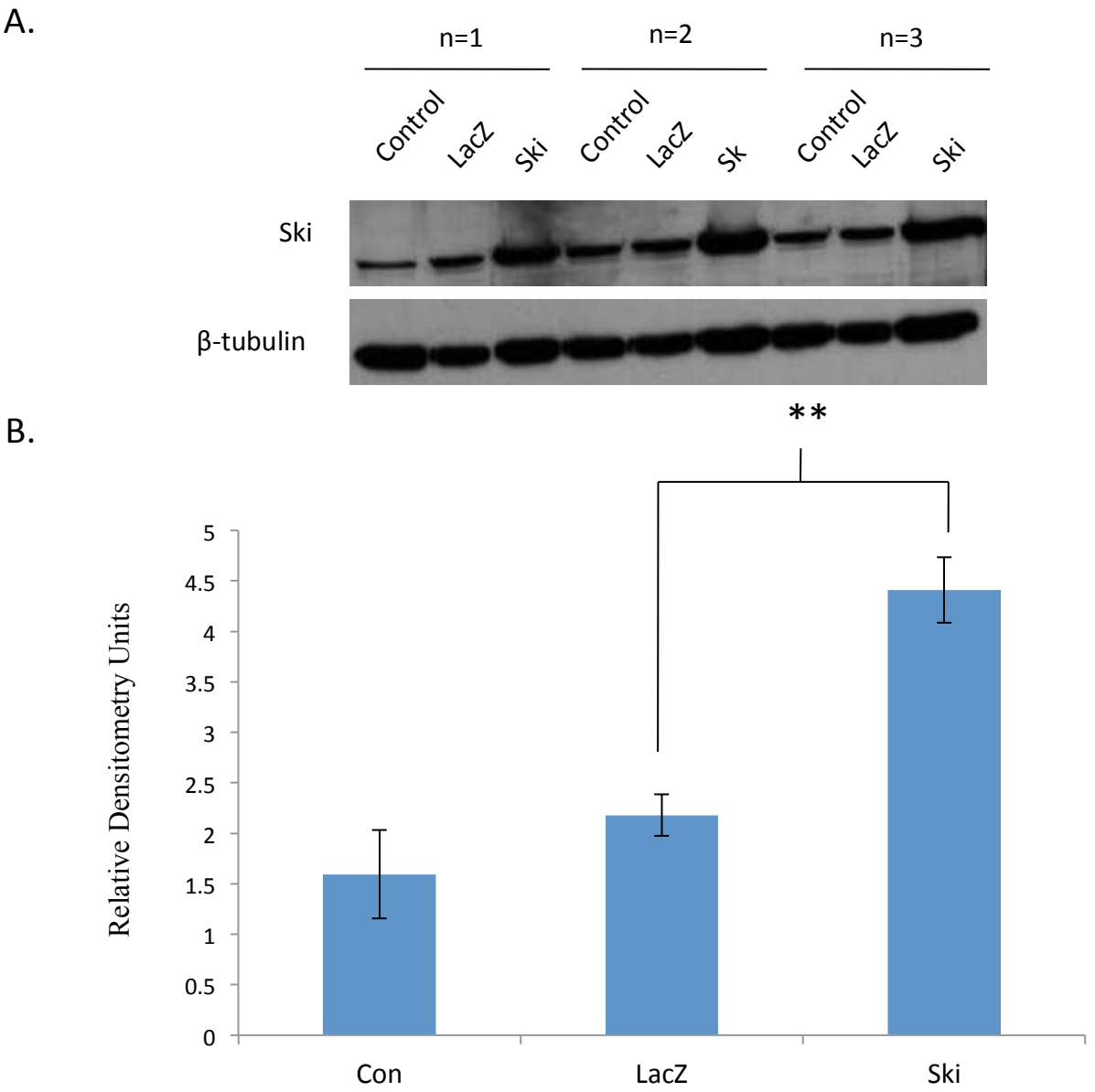


Figure 2. Ski overexpression in P1 cardiac myofibroblasts. P1 primary cardiac myofibroblasts were infected with either LacZ (30 MOI) or Ski (30 MOI) at the time of plating. Uninfected cells were used as a control. Cells were harvested at 80% confluency for protein isolation. Expression of Ski was examined by Western blot. β-tubulin is used as a loading control. Image is representative of n=3. Panel B: Histogram representation of data from images in A. **$p<0.005$

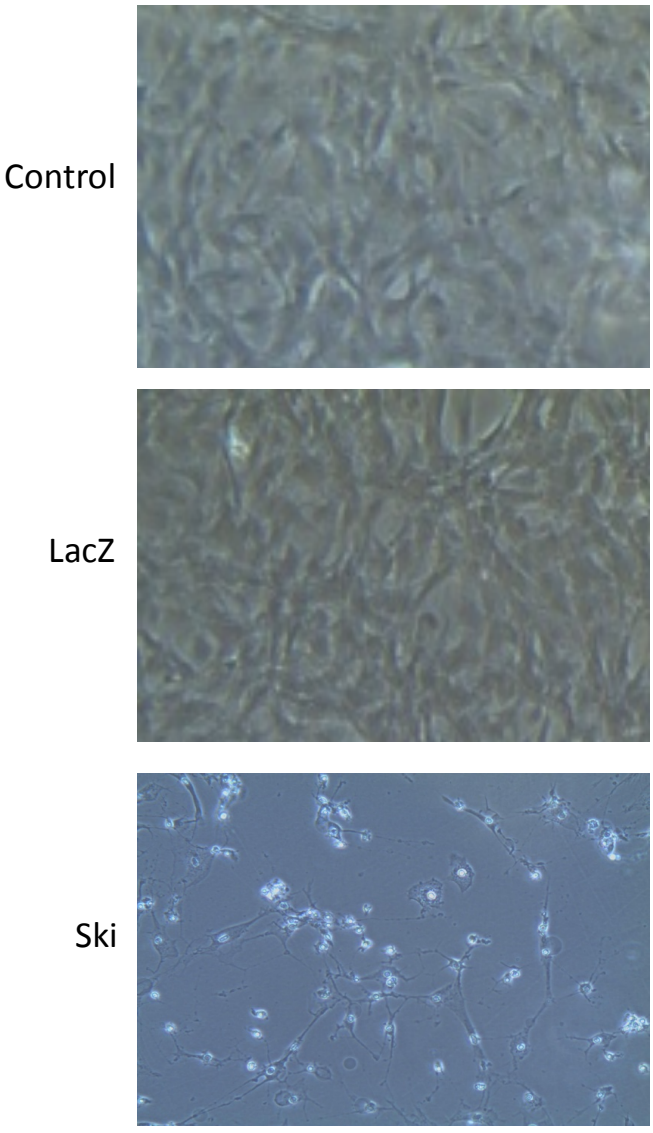


Figure 3. **Morphological changes in cardiac myofibroblasts overexpressing Ski**. Primary cardiac fibroblasts (P1) were cultured and infected with Ad-Ski (30 MOI) for up to 96 hrs. Control and LacZ infected cells exhibit typical spindle shaped morphology while cells overexpressing Ski are morphologically similar to cells undergoing apoptosis and exhibit features such as blebbing, granularity, loss of cytoplasmic extensions, and condensation.

To examine if loss of cell viability was occurring in these primary P1 cardiac myofibroblasts, a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed by plating control and LacZ and Ski infected P1 cells in a 96 well dish and then serum starving them for four time points: 24 hours, 48 hours, 72 hours and 96 hours. Cells were then analyzed using a microplate reader. Ski-infected myofibroblasts showed a significant decrease in viability at 72 hours, and a reduction in viability of approximately 50%, compared to LacZ control, after 96 hours of starvation (Figure 4). At 24 and 48 hours, statistically insignificant decreases in viability were observed (data not shown)

The MTT assay measures the NAD(P)H-dependent oxidoreductase activity within the cell and is therefore an indicator of overall cell viability. This assay does not differentiate how cell viability is affected either: either increased cell death or a lack of proliferation. To distinguish between these two factors affecting cell viability, a mammalian LIVE/DEAD® cytotoxicity kit was used to detect cell death. Following 96 hours starvation, Ski infected cells showed an increased proportion of dead cells (red), relative to viable cells (green), compared to LacZ infected and control samples (Figure 5).

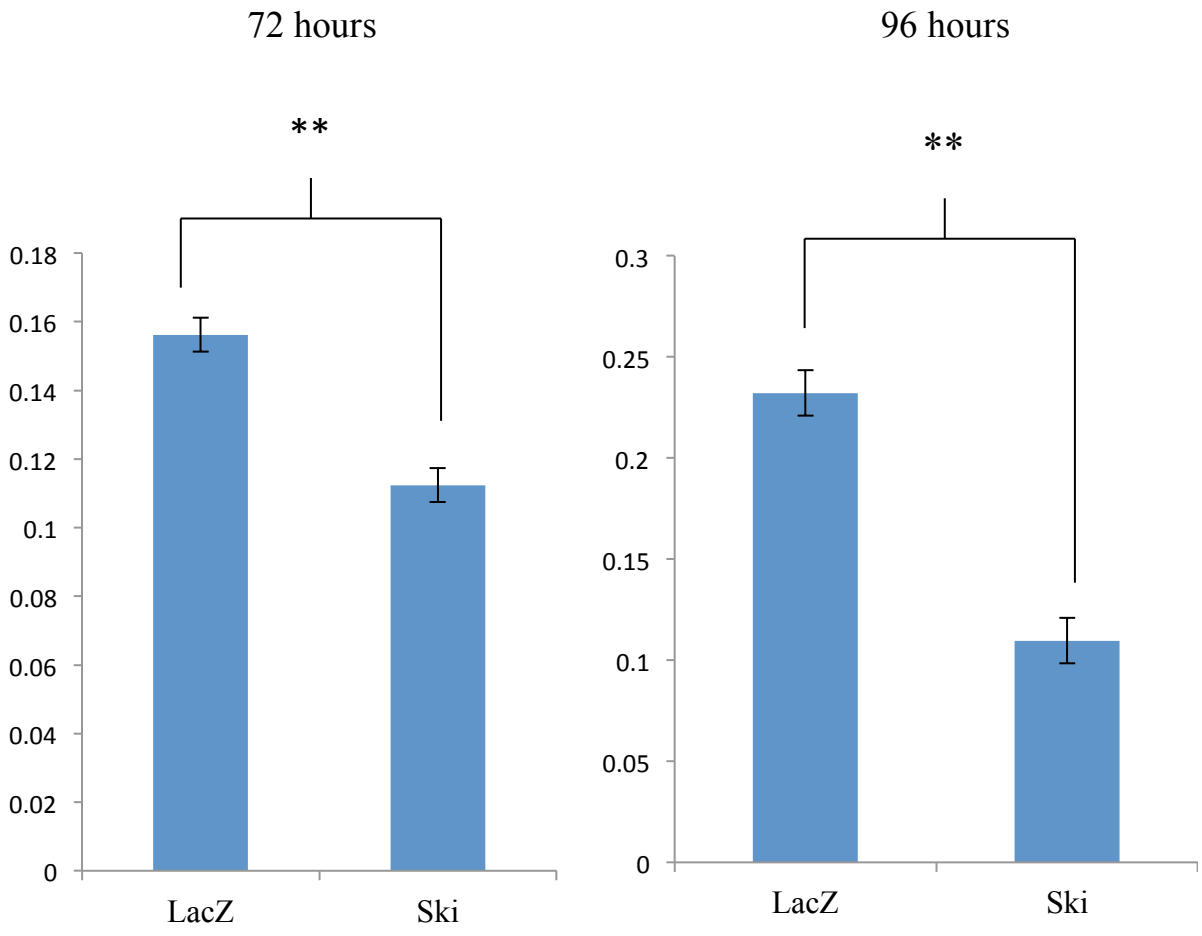


Figure 4. **Cell viability is decreased with Ski overexpression in P1 cardiac myofibroblasts**. Primary P1 cardiac myofibroblasts cultured in 96-well plates were grown to 70% confluency and starved for 72 & 96 hours. Cells overexpressing Ski showed a decrease in overall cell viability compared to their LacZ control counterparts at both time points. Histogram is representative of n=3 (**$p<0.001$)

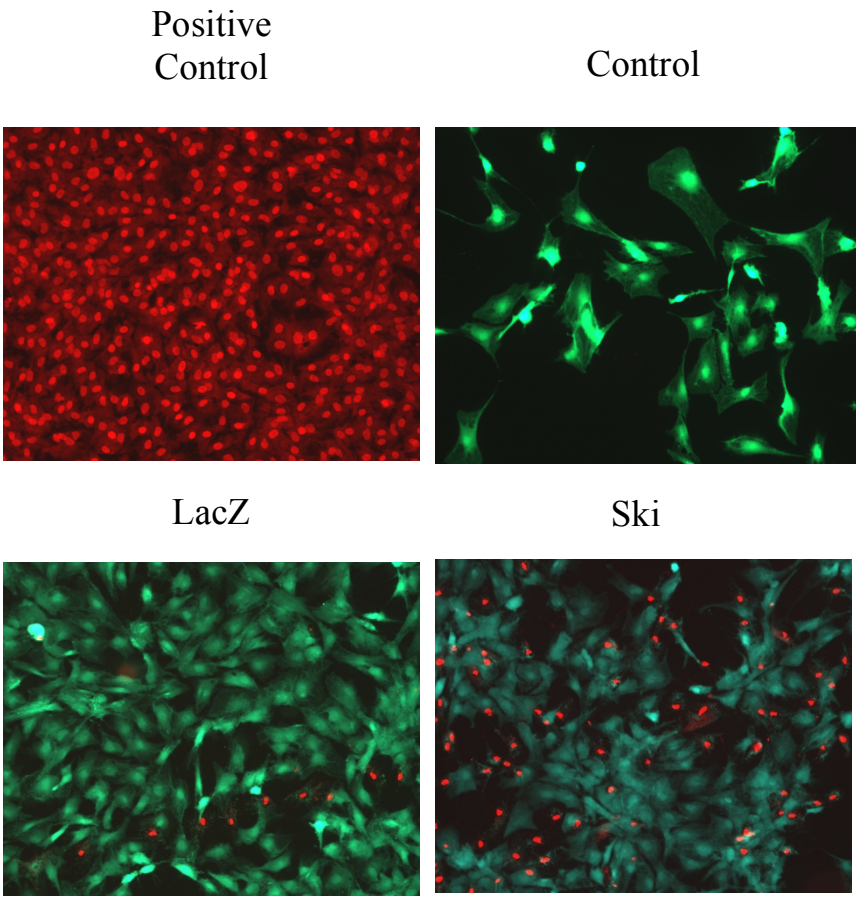


Figure 5. LIVE/DEAD assay of primary cardiac myofibroblasts infected with Ski adenovirus. Cells were infected at the time of plating with either Ad-LacZ (30 MOI) or Ad-HA-Ski (30 MOI). Cells were allowed to grow until 70-80% confluency. Test cells were then starved for 96h in serum free DMEM-F12. Following starvation cells underwent a Live/Dead assay using the Live/DEAD Viability/Cytoxicity Kit for mammalian cells and imaged on a Nikon TE 3000. The increase in red staining in the Ski infected cells is indicative of an increase in the amount of dead cells. Live cells = green, dead cells = red. 75% ethanol was used to induce cell death in the positive control sample.

Ski overexpression triggers the intrinsic apoptotic pathway

Programmed cell death can occur by a variety of mechanisms that can be classified by the distinct morphological and biochemical changes that occur in the dying cells. These processes are unique to the type of cell death, and can be examined and measured by looking at specific mediators of the process. The three most common mechanisms of cell death, and certainly the best studied to date are necrosis, apoptosis and autophagy. Each of these processes is mediated by distinct cellular changes and the activation of unique proteins and enzymes. To determine what kind of cell death was occurring in our samples, the critical mediators of apoptosis - caspases, were examined. Cleavage of procaspase-7 is a hallmark of apoptosis as it is one of the 'executioner' caspases, responsible for causing the biochemical effects that lead to cell death. Cleavage of procaspase-7 to its active form, caspase-7, is evident after 96 hours of starvation in P1 cardiac myofibroblasts overexpressing Ski. Cleavage of this protein is absent from uninfected control and viral (LacZ-infected) control groups examined at the same 96-hour time point (Figure 6). The process of apoptosis itself can be further subdivided into the extrinsic and the intrinsic pathways. Although the end result of both pathways is the same (activation of executioner caspases), they are triggered and mediated by different processes. The extrinsic pathway is largely mediated through the activation of caspase-8 while the intrinsic pathway is largely mediated through the activation of caspase-9 and formation of the apoptosome. In order to elucidate which pathway is implicated in triggering apoptosis via Ski overexpression, cleavage of other caspase enzymes was examined. Cleavage of caspase-9 was detected in cells overexpressing Ski, indicative of mediation through the intrinsic apoptotic pathway (Figure 7). Cleaved caspase-8 protein was absent in our samples (data not shown).

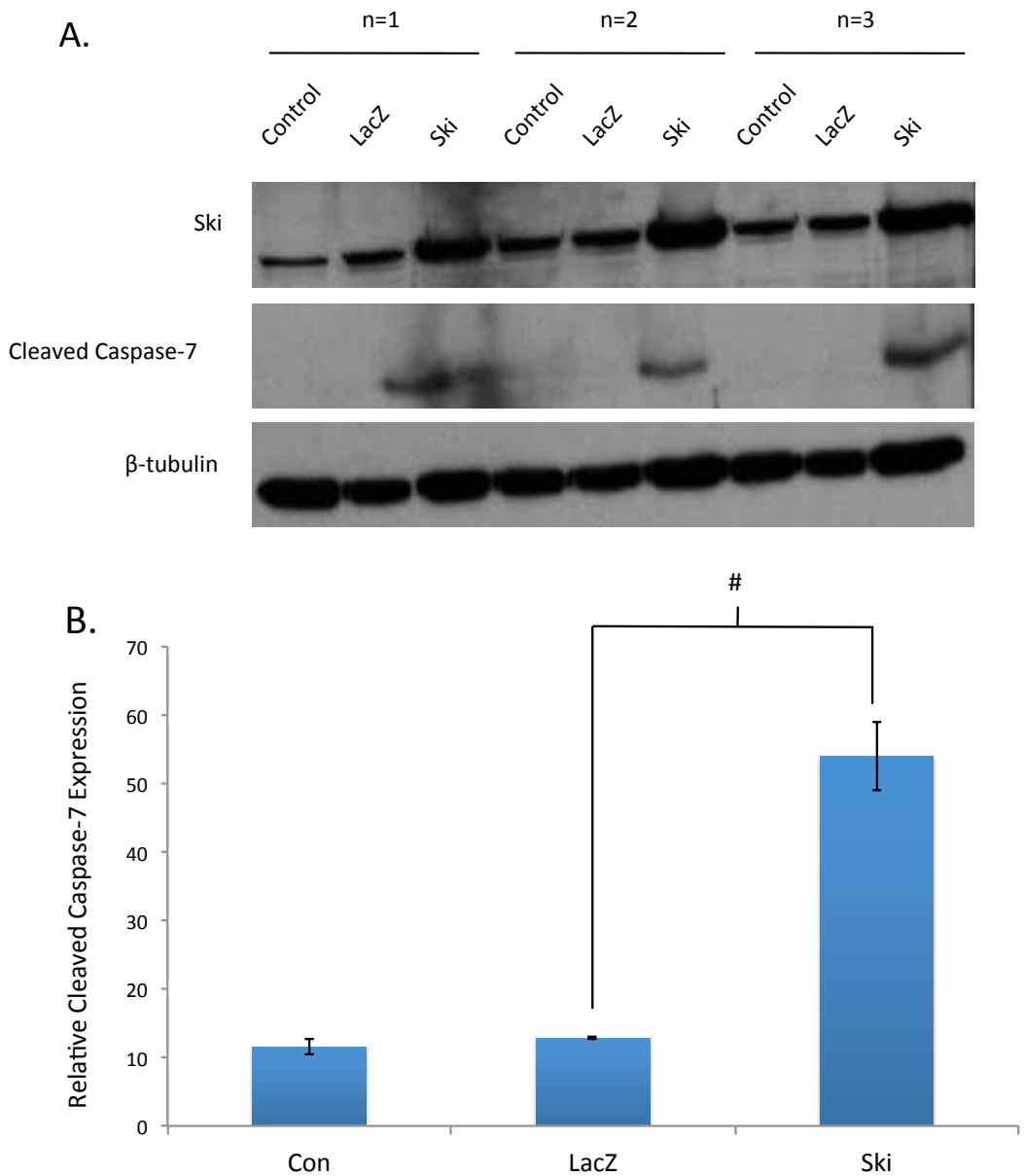


Figure 6. Caspase-7 is activated in P1 primary myofibroblasts overexpressing Ski. P1 cardiac myofibroblasts were infected with either Ski or LacZ (30 MOI), or left uninfected for 24 hours and then serum starved for an additional 96 hours before proteins were isolated. Cleaved caspase-7 was probed, using β-tubulin as a loading control (A). Panel B: Histological representation of A. (#$p<0.001$)

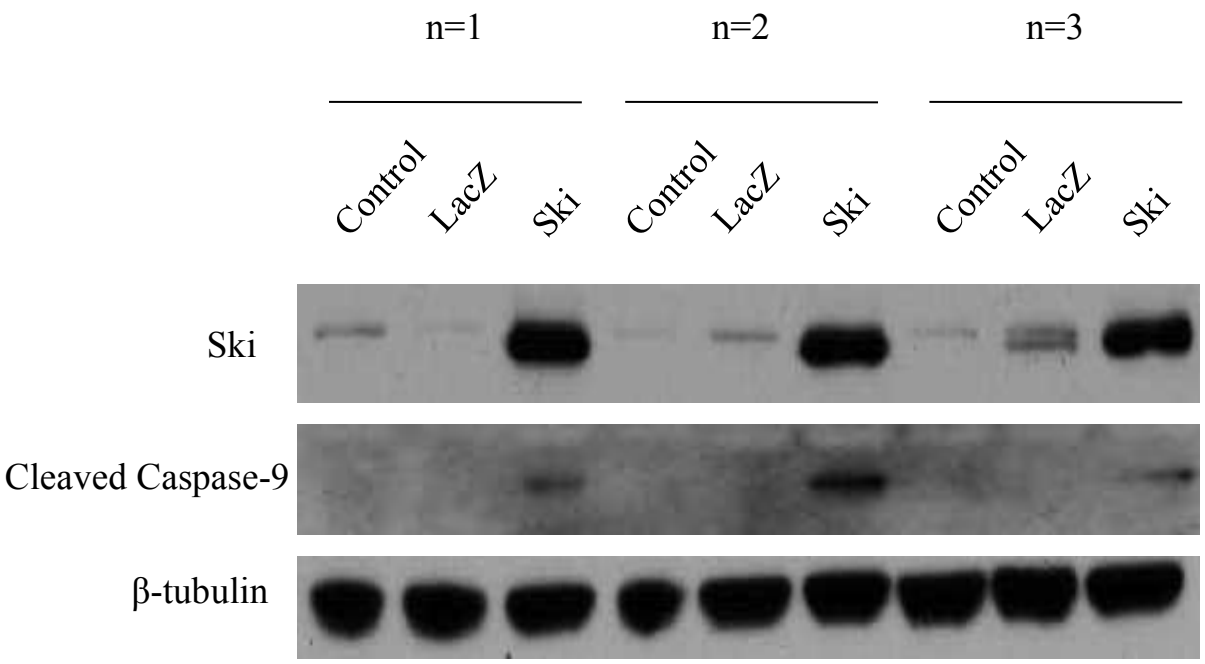


Figure 7. **Exogenous Ski expression triggers the cleavage of caspase-9.** P1 cardiac myofibroblasts were infected with either Ski or LacZ (30 MOI), or left uninfected for 24 hours and then serum starved for an additional 96 hours before proteins were isolated. Cleaved caspase-9 was probed, using β-tubulin as a loading control (n=3)

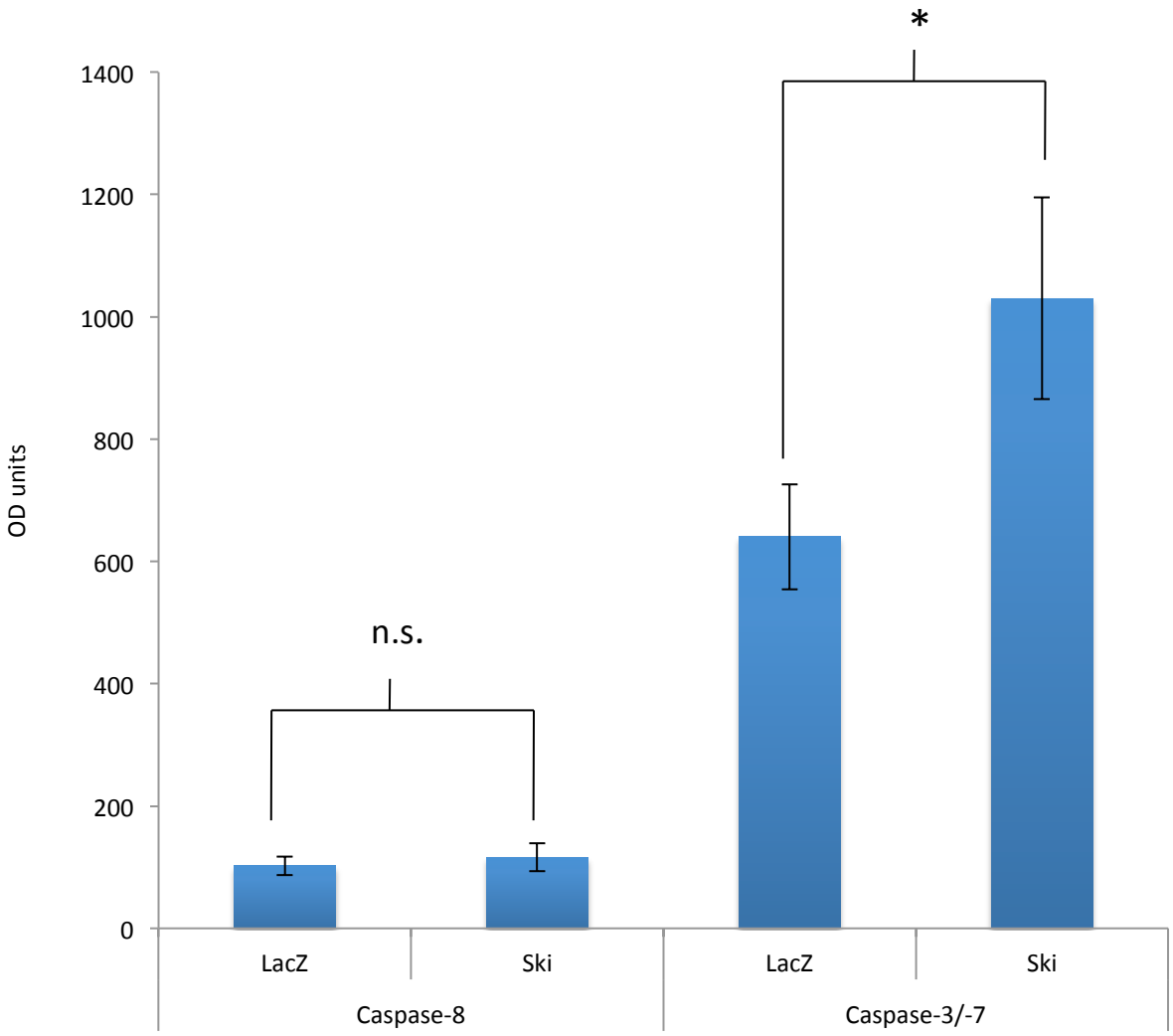


Figure 8. Caspase -3/-7 activity increases with overexpression of Ski. P1 cardiac myofibroblasts, cultured in a 96-well plate, were starved for 96 hours when a confluency of 70-80% was reached. Caspase activity was measured by adding enzyme substrate to the wells and measuring the luminescence produced by substrate cleavage. Histogram representative of n=4. *$p<0.05$ (n.s.= not significant)

Measuring protein level assesses the overall expression of a certain protein but it does not measure the biological function and activity of that protein. In order to confirm that the caspase enzymes were actually exerting their biological function, we measured their activity level. Using a commercial Caspase-Glo® kit, the activity of caspase-8, and caspases-3 and -7 were measured using this luminometric technique. P1 cardiac myofibroblasts were cultured in a 96 well plate and infected with LacZ or Ski. At 96 hours starvation, caspase enzyme substrate was added to the wells and then incubated as per the protocol. Using a microplate reader, the luminescence was measured. The activity of caspase 8 was insignificantly increased compared to the LacZ control. When cells overexpressed Ski protein, their caspase-3/-7 levels were significantly increased compared to the LacZ infected control cells (Figure 8).

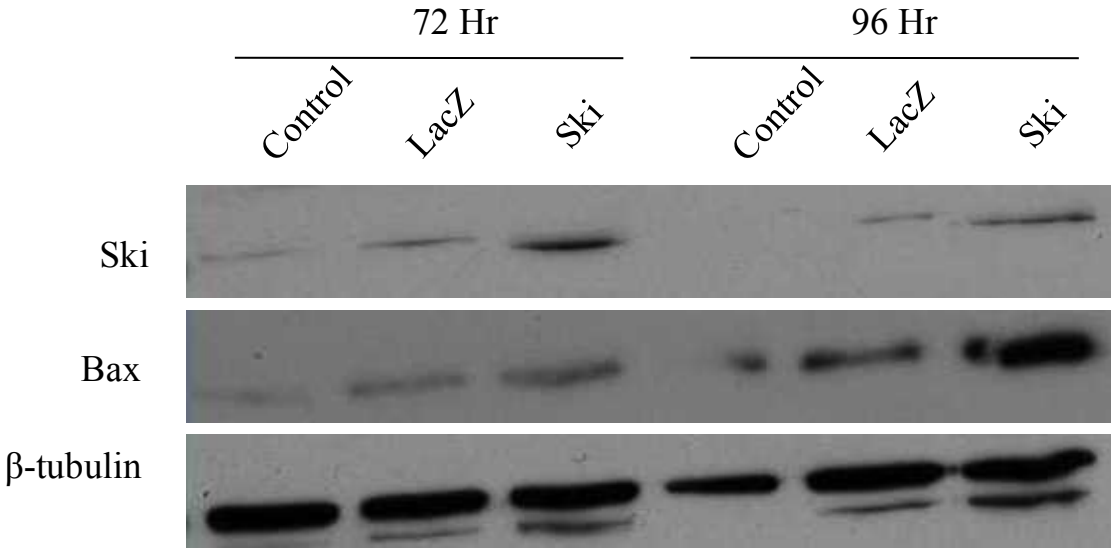


Figure 9. The pro-apoptotic protein Bax is upregulated in P1 myofibroblasts overexpressing Ski. Cells were starved for 72 & 96 hours in serum-free media and expression of Bax at both 72 & 96 hours appeared to increase compared to control, and LacZ infected cells. β-tubulin was used as a loading control. (n=1)

The canonical method of activating procaspase-9 is accomplished via the release of cytochrome C from the mitochondria. Cytochrome C is located within the intermembrane space of the mitochondria and is loosely associated with the inner membrane. When cytochrome C is liberated, it will interact with the procaspase-9 zymogen and APAF-1, forming a complex known as the apoptosome, which results in cleavage of procaspase-9 into active caspase-9 enzyme. The release of cytochrome C is a critical step in this intrinsic pathway and is mediated by the translocation of pro-apoptotic, pore-forming members of the Bcl-2 superfamily. One of the most potent pro-apoptotic members of this family is Bax. Figure 9 shows an increase in Bax expression in cells that overexpress Ski at 72 hours and 96 hours. This increased expression, apparently increased at 96 hours, may provide a mechanism by which cleavage of caspase-9 can be accomplished.

Although an increase in Bax expression is an important hint as to the mechanism of apoptosis, localization of these pro-apoptotic proteins are arguably more important in determining their role. Immunofluorescence imaging in primary P1 cardiac myofibroblasts overexpressing Ski shows a release of cytochrome C from the mitochondria and a co-localization with Bax protein, suggestive of Bax forming a pore in the outer mitochondrial membrane allowing release of cytochrome C (Figure 10). This further adds to the evidence of Ski triggering an intrinsic apoptotic pathway that leads to cell death in these cells.

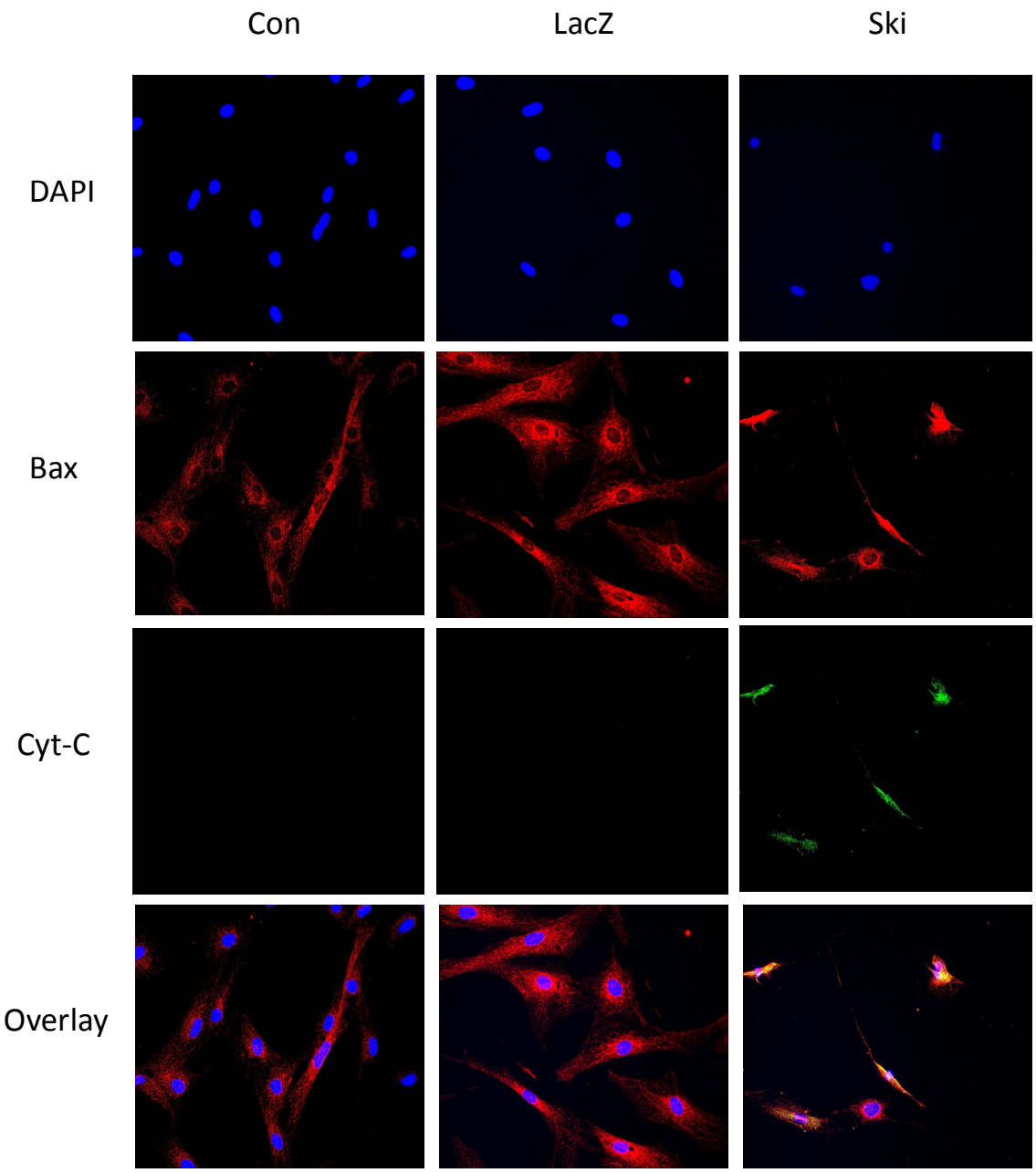


Figure 10. Co-localization of Bax and cytochrome C in Ski overexpressing myofibroblasts. P1 cardiac myofibroblasts overexpressing Ski were stained for immunofluorescence using anti-Bax (red), anti-Cytochrome C (green) and DAPI (blue) stains. Overlay of images shows that when these cells are infected with Ski there is a release of cytochrome C from the mitochondria that is not present in control and Lac-Z infected cells. There is a distinctive co-localization of these proteins (composite image of Ski infected cells) that supports the hypothesis of an intrinsic apoptotic pathway that is dependent on the release of cytochrome C from the mitochondria.

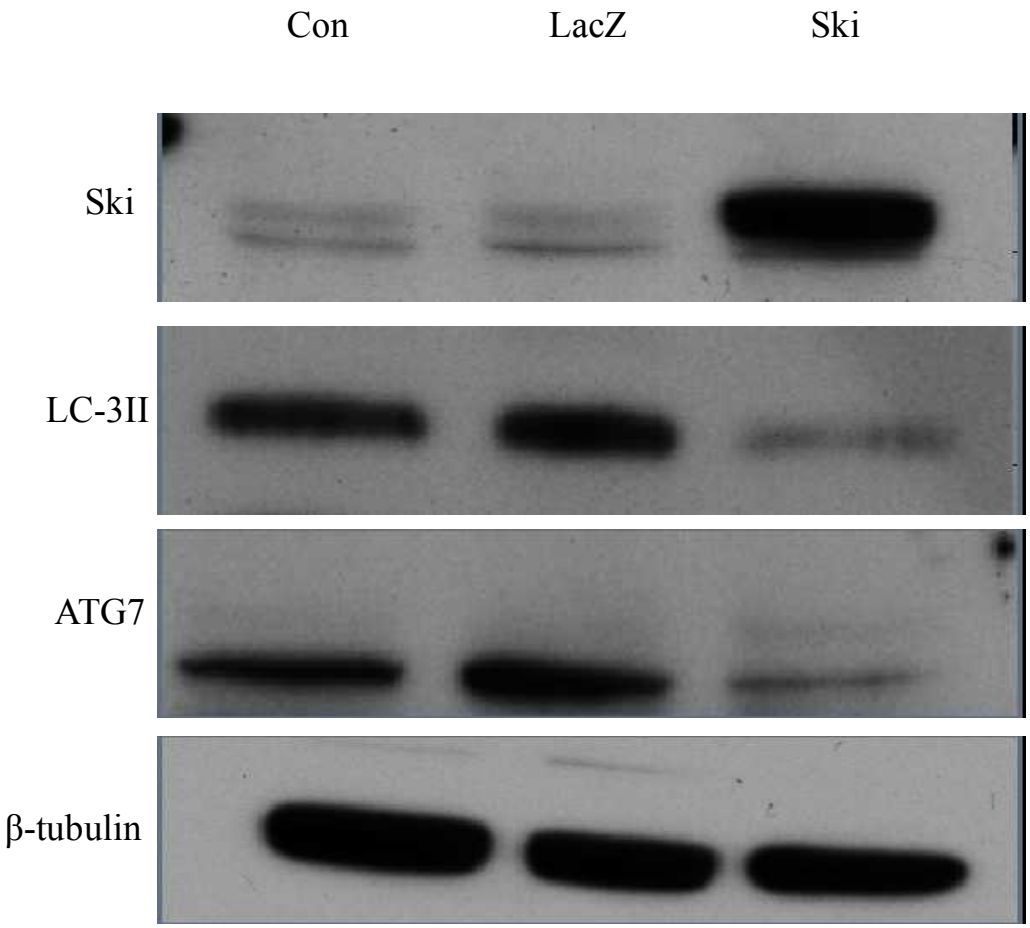


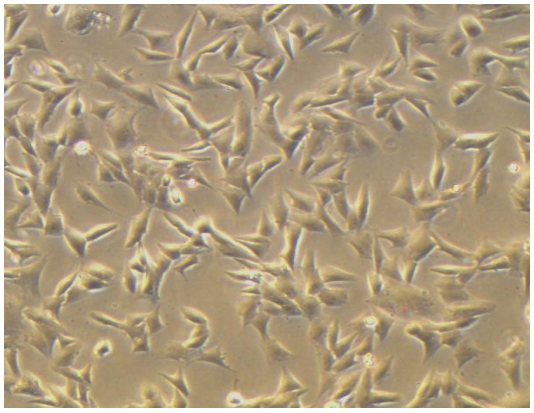
Figure 11. Ski overexpression results in a decreased expression of autophagic markers. When compared to control and Lac-Z infected cells, P1 cardiac myofibroblasts overexpressing c-Ski showed a decreased expression of the autophagy markers LC-3II and ATG7 as seen via immunoblotting. β-tubulin was used as a loading control. (n=1)

The role of autophagy: protective or pathogenic?

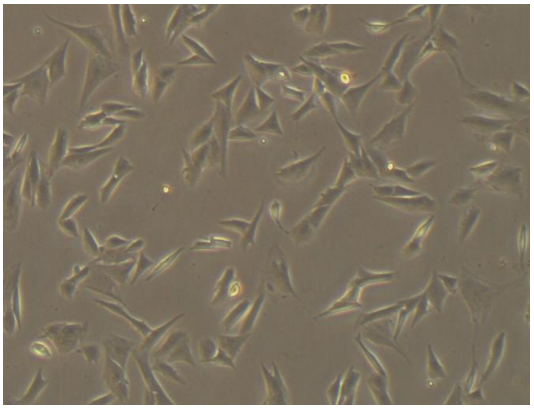
Autophagy has previously been described as a pro-survival signal that can mediate apoptotic cell death[146]. Our next experiments sought to define autophagic activation or deactivation in the presence of increased Ski expression. To achieve this goal we assessed the expression of autophagic markers, LC-3II and ATG7 in P1 primary cardiac myofibroblasts. After 96 hours of serum starvation, control and LacZ-infected cells strongly expressed these markers (expected due to the fact that starvation is one of the primary ways in which autophagy can be triggered) (Figure 11). Conversely, cardiac myofibroblasts overexpressing Ski show a marked decrease in the expression of these two markers, suggesting that autophagy is downregulated (perhaps resulting in increased apoptotic cell death).

In order to measure autophagic flux, an inhibitor of this process must be incorporated into the experimental design. In this series of experiments, bafilomycin-A1 (Baf-A1), a lysosomal proton pump inhibitor, was used to provide further information on the role of autophagy in the observed cell death response when Ski is overexpressed in these cells.

Control



LacZ



Ski

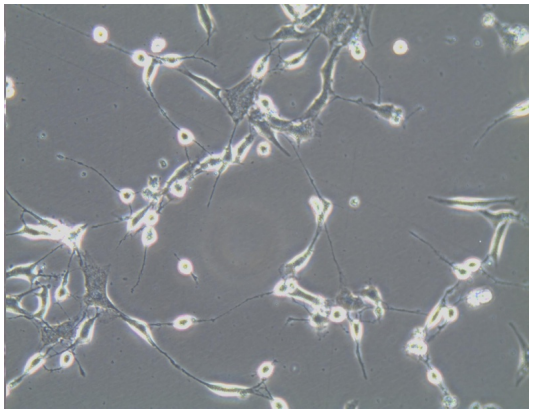


Figure 12. The effect of Ski overexpression and Baf-A1 treatment on primary P1 cardiac myofibroblasts. P1 cells were infected with LacZ or Ski, or left uninfected. They were cultured until 70-80% confluency was achieved before being starved in serum free DMEM-F12 media. Upon starvation, cells were treated with 7.5 nM Baf-A1 to inhibit autophagic flux. Distinct morphological changes such as blebbing, granularity and loss of cytoplasmic extensions are evident 18 hrs into the starvation treatment.

A concentration of 7.5nM Baf-A1 was added to starvation media to inhibit autophagic flux and primary P1 cardiac myofibroblasts were incubated until their cellular morphology began to change as in previous experiments described above. With the addition of Baf-A1 to Ski-overexpressing cells, it took just 18 hours to see significant morphological changes. Control and LacZ-infected cells exhibited normal spindle shaped morphology, consistent with a healthy, viable cell population (Figure 12). P1 cardiac myofibroblasts overexpressing Ski showed a morphology that was similar to the morphology of P1 cells at 96 hours starvation – blebbing, loss of cytoplasmic extensions, and chromatin condensation - all indicative of apoptosis. For consistency, these cells were left in serum starvation conditions for a total of 96 hours. The control and LacZ-infected cells maintained a normal phenotype while the Ski-overexpressing cells were non-viable and were floating in suspension.

Corroborating experiments were performed to examine the protein expression of markers of autophagy, including LC-3II, in the presence of Baf-A1. Although the decrease in LC-3II expression is not as robust compared to samples not treated with Baf-A1, there was found to be a statistically significant decrease in LC-3 lipidation in P1 cells overexpressing Ski (Figure 13).

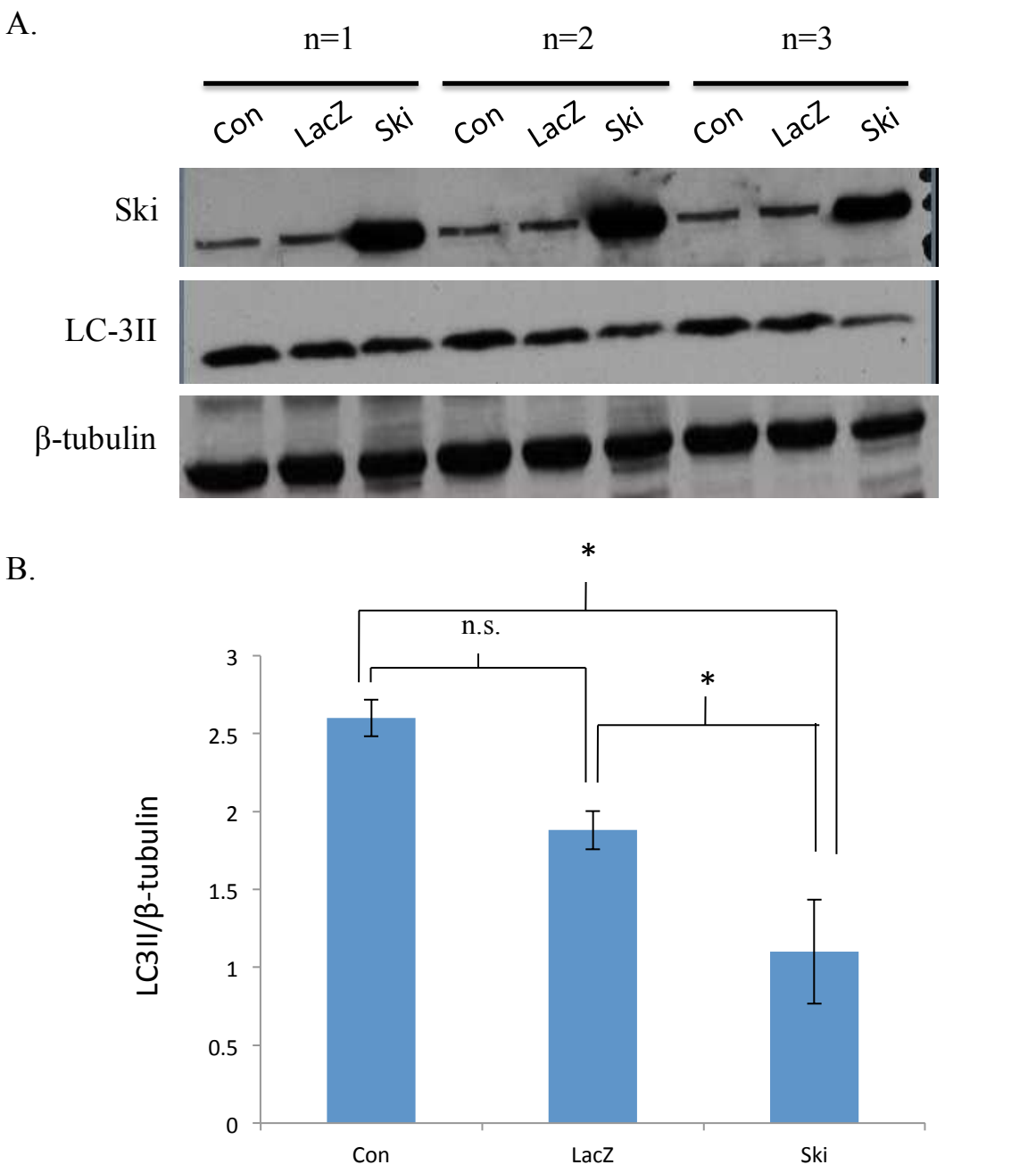


Figure 13. **The effect of Ski expression on LC-3II: repression of autophagy in myofibroblasts.** Overexpression of Ski at 96 hours starvation and treatment with Baf-A1yields a decreased expression of LC-3II, indicating a decreased autophagic response. Ski treatment yielded statistically significant differences compared to control and LacZ control cells (*$p<0.05$) while the difference between control and LacZ was found to be not significant (n.s.). β-tubulin is used as a loading control. Panel B: Histological representation of data presented in A. n=3

Protein expression of ATG7 in Ski overexpressing cells was also decreased but was found to be statistically insignificant compared to control, and LacZ infected cells (not shown). The decrease in the expression of these markers potentially suggests that autophagy provides a pro-survival response to Ski infected cells in the first 96 hours of starvation but "gives up", and downregulates that signal at 96 hours, leading the way to cell death via the intrinsic apoptotic pathway.

VI. Discussion

The mechanisms of myocardial wound remodeling in the heart are not completely understood and have garnered growing attention in the past decade. As this field of research develops, novel theories as to how to ameliorate the maladaptive remodeling process have become clearer. The persistence of myofibroblasts in the remodeling heart results in the continual production of matrix proteins, a process that leads to eventual stiffening of the myocardium, decreased cardiac performance, and ultimately heart failure[15]. While the initial remodeling that these cells mediate is a critical step in the healing process, myofibroblasts can persists in the scar for decades following injury to the myocardium, turning an adaptive response into a maladaptive process[16]. While many therapies and treatments target the production of collagen and other matrix proteins from cardiac myofibroblasts, the selective removal of these cells from the scar represents an approach to fibrosis that warrants further investigation moving forward.

For the first time, this research investigates the role of Ski, a negative regulator of TGF-β signaling, as a potential way to mediate and limit the persistence of activated myofibroblasts within the injured myocardium. Herein, we demonstrate that overexpression of Ski in cardiac myofibroblasts initiates a significant and specific apoptotic response.

The presence of c-Ski within the myocardium has been known for almost 30 years when among other proto-oncogenes, c-Ski was found to be expressed in both proliferating and terminally differentiated cardiac muscle cells[71]. More recently, studies have begun to examine the role of this protein in cell cycle regulation. Ski has been shown to promote dermal fibroblast proliferation[74], while other studies have also shown that Ski inhibits the

proliferation of vascular smooth muscle cells following balloon injury[75]. Interestingly, both studies cite the suppression of Smad3 signaling as crucial to their observed effects. This apparent dichotomy regarding proliferation may be explained by the functional and topographical diversity of the body's fibroblast population[33]. As such, it makes it difficult for researchers to hypothesize as to the effect of Ski on a separate subset of fibroblasts in a different region of the body. Although this study is the first to examine the effects of Ski on programmed cell death in the heart, it is a possible that this event is preceded by a cell cycle arrest mechanism and studies need to be carried out to elucidate if such a mechanism is present in cardiac myofibroblasts.

Both of the viral Ski vectors utilized in this study have proven to overexpress the 95-kDa form of c-Ski, which has previously been shown to function as an anti-fibrotic protein within the heart[34]. The functions of other Ski isoforms within cardiac myofibroblasts, the 105-kDa and 115-kDa, remain unclear but it is possible that these larger isoforms represent a latent pool of Ski protein that has undergone post-translation modifications such as phosphorylation and ubiquitination. Although our model looked exclusively at the 95-kDa isoform, it would be interesting to see if the other isoforms of Ski would produce a similar effect regarding cell fate. The previously reported[34] localization of the 95-kDa isoform to the nucleus may allow a unique privilege of this isoform to trigger cell death through nuclear condensation and fragmentation.

We have shown that Ski triggers apoptosis through the mitochondrial mediated intrinsic apoptotic pathway, through activation of regulators of mitochondrial permeability. Preliminary studies using a Bax/Bak KO MEF cell line showed that when Bax and Bak were decreased in fibroblasts, the expression of Ski was also reduced. This may suggest

that these proteins are either regulated by Ski, or alternatively perhaps regulating Ski expression. Bax and Bak are two pro-apoptotic proteins that belong to the larger Bcl-2 superfamily of proteins, which regulate mitochondrial permeability. These two pro-apoptotic proteins are known to dimerize and insert into the outer mitochondrial membrane allowing for the release of cytochrome c into the cytosol, mediating the formation of the apoptosome and triggering the intrinsic apoptotic pathway.

To investigate the potential link between these proteins and Ski further, primary P1 cardiac fibroblasts were isolated, cultured and infected with Ad-Ski. Initial overexpression of Ski within these cells yielded rapid results. The confluency of Ski treated cells was much lower than control and LacZ-infected cells at the same time-point during incubation. To quantify this discrepancy, MTT assays were utilized to measure the percentage of viable cells that remained after each treatment. Both 72 hours and 96 hours showed a significant decrease in the amount cell viability in the Ski infected cells, a decrease of approximately 50% at 96 hours. As MTT assays only measure the relative amount of viable cells, a decreased absorbance in the assay is caused by either the increased death of cells, or a decreased proliferative capacity of the treated cells. Coupled with a decrease in cell viability was a change in cellular morphology in Ad-Ski infected myofibroblasts.

Consistent with published reports on apoptotic cellular morphology[103], the Ski-overexpressing cells showed evidence of a decrease in cellular size and blebbing of the membrane, forming smaller, apoptotic bodies. The final confirmation of the induction of apoptosis in the Ski-infected cells was the LIVE/DEAD® assay that showed an increased number of dead myofibroblasts in cells overexpressing Ski, compared to the uninfected, and LacZ-infected control treatments.

The mediators of apoptosis on a macromolecular level are a large family of cysteine-aspartic proteases known as caspases[105]. Although apoptosis can occur via a caspase-independent manner, the traditional apoptotic pathways (intrinsic and extrinsic) all are mediated by these caspase enzymes[102]. The caspase enzymes have a variety of functions within the cell, depending on their localization and the environment they find themselves in. In the cell death process, caspase-3, caspase-6, and caspase-7 are known as effector caspases and mediate the underlying cellular events that precede the morphological changes characteristic of cellular death. The overexpression of c-Ski in P1 cardiac myofibroblasts clearly induced the cleavage of the caspase-7 protein, while cleaved protein product was absent from the other treatment and control groups. This indicates that c-Ski myofibroblasts are the only treatment group undergoing significant amounts of apoptosis at the timepoints examined. Although caspase-7 is not the only effector caspase that is involved in mediated cell death, apoptosis is able to progress with activation of only one of the effector enzymes. Further studies will hopefully be able to elucidate the full apoptotic response that is triggered during c-Ski overexpression in regards to the other effector caspase enzymes (caspase-3 and caspase-6).

Although the end result of apoptotic activation is the same, there are multiple ways in which apoptotic cell death can be mediated. The two most studied pathways include the intrinsic (or mitochondrial mediated) pathway, and the extrinsic (receptor-mediated) pathway[106]. The intrinsic pathway, triggered by factors intrinsic to the cell, is largely mediated by changes in mitochondrial permeability and cleavage of procaspase-9. When factors within the cell cause the cell to die, pro-apoptotic proteins such as Bax and Bak insert into the outer mitochondrial membrane and allow for the release of cytochrome c

from the mitochondria to the cytosol where they interact with other proteins resulting in the cleavage of procaspase-9 and subsequent activation of effector caspases[113]. This differs from the external pathway, which is largely mediated by ligand-receptor interactions (eg. FasL-FasR and TNFα-TNFR). Unlike the intrinsic pathway, which is mediated through involvement of caspase-9, the extrinsic pathway is devoid of caspase-9 activity and relies instead on caspase-8 to trigger downstream effector caspase events[102]. Overexpression of c-Ski in cardiac myofibroblasts was not associated with increased caspase-8 activation as seen by Western blot. Involvement of other caspase enzymes was not investigated and future studies will be required to elucidate the full activation of the caspase family of enzymes.

Levels of Ski protein in the shown Western blots vary between different figures in this thesis, despite a constant multiplicity of infection of 30 viral particles per cell. This can be attributed to the need to switch viruses in the middle of the project. Although it seems that one virus produces much more relative Ski protein compared to the other, the biological effect on the cells appeared to remain constant. The infected myofibroblasts still required a similar time point of 96 hours to show overt signs of apoptotic activity. It is possible that intracellular levels of Ski protein are required to reach a critical threshold before cell death occurs, and the effect beyond that threshold is homogeneous despite the absolute level of protein being present.

Although our current study is focused on the expression of these apoptotic proteins, this may not be an accurate indication of the degree of apoptotic activity that is occurring within our cells. Studies using Western blots allow for indications regarding the relative quantity of a protein or enzyme but are unable to distinguish or measure the absence or

presence of activity that those enzymes may mediate. Enzyme activity can be measured using a variety of different techniques, including fluorometric, chemiluminescent, and spectrophotometric measures. We utilized a commercially available Caspase-GLO® luminescent assay to measure the activity of key apoptotic enzymes including caspase-8, caspase-3, and caspase-7. Enzyme substrate was added to the cultured cells at specific time points consistent with increased apoptotic protein expression. Substrate cleavage products were subsequently measured via luminescence. It was shown that, compared to LacZ-infected control cells, Ski overexpressing P1 cardiac myofibroblasts showed an increase of approximately 60% in caspase-3/-7 activity. Caspase-8 enzyme activity increased approximately 10% compared to LacZ-infected control cells although it is thought that this apparent increase was insignificant and perhaps artificial, as caspase-8 protein was not detected in our Western blot experiments.

Cellular fate (ie. determining whether a cell lives or dies) is a complex process and requires tight regulation at all levels of cellular homeostasis[95]. As described previously, the mitochondria are the primary cellular organelles involved in regulating intrinsic apoptotic cell death. The Bcl-2 superfamily of proteins are key regulators of mitochondrial dysfunction, and an imbalance of these proteins can tilt the fate of an individual cell towards cell death[117]. Members of this superfamily share common structural regions called Bcl-2 homology (BH) domains. A total of four homology domains have been identified: BH1, BH2, BH3, & BH4[102]. While some Bcl-2 family proteins contain all four of these domains, other members have been found to lack one or more domains. The structure of these proteins has been shown to have a strong link to their cellular function and what role they play in mediating programmed cell death. Within the Bcl-2 superfamily, some

members have been identified as being capable of promoting apoptosis, including Bax, Bak, Bad, & Bid, while others have been shown to play anti-apoptotic roles[102]. Relating structure to function, it has been shown that anti-apoptotic proteins in this superfamily must have at least 3 of the BH domains: BH1, BH2, & BH3, while members of the pro-apoptotic side have the minimal requirement of containing the BH3 domain[165]. The evidence of multiple proteins regulating the same biological function emphasizes the importance of this superfamily in regulating survival in times of cellular stress.

Pro-apoptotic proteins, such as Bax and Bak, exert their biological effects through dimerization within the cytosol and subsequent insertion into the mitochondria membrane, allowing the release of cytochrome C. We have shown that consistent with a decrease in cell viability in Ski-infected P1 cardiac myofibroblasts, there is an increase in the expression of the pro-apoptotic protein Bax at both 72 and 96 hours starvation, compared to LacZ-infected and uninfected control cells. This represents the potential mechanism by which apoptosis is triggered within Ski-infected myofibroblasts. The subsequent immunofluorescence performed seems to support the role of these proteins in the initiation of apoptosis. There is a distinct co-localization of Bax and cytochrome c in Ski-infected cells that is not present in untreated Control and LacZ infected cells. Whether this co-localization truly represents the movement of cytochrome c from the mitochondrial intermembrane space to the cytosol is unclear, and a fractionation experiment should be performed to definitively prove this shift. The absence of cytochrome c staining in the Control and LacZ infected samples is of interest as all cells should contain this crucial protein. It is possible that cytochrome c in these cells is adherent to the inner

mitochondrial membrane and as such, much more difficult for immunofluorescent antibodies to bind to, resulting in a decreased signal strength when visualized.

Autophagy is a highly conserved and regulated process that is initiated by the formation of autophagosomes and subsequent sequestration and degradation of damaged or unneeded organelles and macromolecules[166]. The duality of the role of autophagy, as both a pro-survival[167, 168] and pro-death mechanism[169, 170], has been well established in many studies across several disciplines. The apparent contradictory role autophagy is capable of playing lends evidence as to the complexity of its regulation within the cell and it's environment. The role of that autophagy plays within cardiac myofibroblast cell fate is a field that requires further attention and research.

Using the same set of experimental parameters as for the apoptosis experiments, we sought to elucidate the role that autophagy was playing within the cell death process being observed in P1 cardiac myofibroblasts overexpressing Ski. Following the 96 hours starvation period, pro-autophagic markers LC-3II and ATG7 were significantly decreased compared to LacZ-infected and control cells. ATG7, an E1 enzyme, plays an integral role in the activation of both ATG8 (and its mammalian homologue LC3), and ATG12[171] – both of which contribute to the recruitment of LC3-II to the autophagosomal membrane and initiation of autophagy[172]. The decreased expression of these autophagic markers suggests a decreased formation of autophagosomes and therefore a decreased role for autophagy within this process.

This is not the first study to examine the role of c-Ski in regulation of autophagy. Li *et al.* have observed that in rat aortic A10 vascular smooth muscle cells, c-Ski was capable of suppressing platelet-derived growth factor and oxidized low-density lipoprotein-induced

autophagy[180]. With knowledge of the duality surrounding the role of autophagy, it is likely that this process is cell and situation dependent. There is also the possibility that autophagy is capable of playing both a pro- and anti-cell death role in same cell type under evolving conditions. Autophagy may initially play a supportive role in the recycling of intracellular organelles and contents in an effort to try to preserve normal cellular homeostasis, a role that is gives up as cellular damage is beyond repair and it adopts a role mediating programmed cell death.

Given that autophagy is not a static physiological response[172, 173], it is important to accurately measure the autophagic flux that is occurring under our experimental conditions. In order for this to occur, we had to inhibit the flux by treating our cells with an inhibitor of autophagy, bafilomycin-A1 (Baf-A1)[173]. Baf-A1, which is a member of the macrolide family of antibiotics, is a vacuolar proton pump inhibitor - inhibiting H^+-ATPase, which serves to prevent the fusion between lysosomes and autophagosomes[173].

When the c-Ski-infected P1 cardiac myofibroblasts were treated with a low concentration of Baf-A1 at the time of plating (7.5nM), the time for morphological changes to occur was drastically reduced, from 96 hours to 18 hours. Changes that occurred were consistent with changes seen during apoptosis, including blebbing, loss of cytoplasmic extensions, fragmentation, and chromatin condensation. The morphology of the uninfected control, and LacZ-infected cells were similar to the 96-hour starvation time point – healthy and viable with no significant morphological changes. The more rapid appearance of apoptotic features in cells overexpressing Ski when autophagy is inhibited suggests that autophagy may be playing a protective, pro-survival role during this process.

These P1 cells were starved for 96 hours and protein was harvested for the autophagic markers ATG7 and LC-3II. With Baf-A1, LC-3II was significantly decreased, as was the case in c-Ski infected cells cultured without Baf-A1. Changes in ATG7 expression was insignificant throughout the 3 treatment groups, which differed from expression of autophagic markers in cells without Baf-A1 treatment. Although no significant change in ATG7 expression was apparent, LC3 lipidation was significantly decreased which is suggestive of a decreased formation of autophagosomes, and subsequent autophagic activity.

Apoptosis and autophagy are distinct processes but may occur concurrently with each other[175]. Triggered by, and regulated by similar upstream factors, autophagy may play a role in the progression, or resistance to apoptotic cell death[174]. This fact has led to extensive research into the crosstalk between these two processes. Bcl-2 and Beclin-1 family members are thought to serve a critical role in the cross talk between apoptosis and autophagy[175]. Evidence suggests that beclin-1 directly interacts with anti-apoptotic proteins such as Bcl-2 and Bcl-xL, while Bcl-2 plays a role in the inhibition of beclin-1 mediated autophagy[176].

Recent work has also shown another link to exist between these two processes, one that is mediated by caspase-9[177]. Although the primary role of caspase-9 is its function within the intrinsic apoptotic pathways, many caspases have non-apoptotic functions. This recent work shows that caspase-9 in capable of interacting with ATG7, enhancing ATG-mediated formation of LC3-II and facilitating the formation of autophagosomes within the cell. It also proposes that this interaction represses the apoptotic activity of caspase-9[177].

The ability to trigger programmed cell death in primary cardiac myofibroblasts has the potential to represent a novel way to reduce the fibrotic response of the myocardium following injury. The initial response of these cells is critical to maintain function of the weakened heart, but soon switches from an appropriate response to one that is maladaptive and contributes to pathology. This switch represents a potential target for interventions by clinicians and researchers. The targeted overexpression of Ski in myofibroblasts or the use of a small molecule Ski mimetic may epitomize these interventions, although several concerns must be addressed before use in human subjects. As the role of Ski overexpression in other cells within the heart (ie. cardiomyocytes) has not been studied, this treatment has potential to cause further loss of contractile ability and result in subsequent iatrogenic heart failure. Specific delivery methods of such agents must be perfected as to minimize the off-target effects of these therapies not only in the heart, but systemically as well.

This thesis outlines an early mechanism by which Ski overexpression in P1 cardiac myofibroblasts triggers cell death. We demonstrate that autophagy and apoptosis occur in a coordinated fashion to mediate the effects observed. Whether these events play a critical role in post-MI wound remodeling remains unclear and will require further investigation by future studies.

VII. Future Directions

The preliminary mechanism by which c-Ski induces apoptosis and the role that autophagy plays in that process needs to be further elucidated through future studies. Although cleaved caspase-9 is indicative of activation of the intrinsic apoptotic pathways, it is important to elucidate upstream events that trigger caspase cleavage. Changes in mitochondrial permeability can lead to the release of factors that lead to the cleavage of caspase-9, including Smac/Diablo and cytochrome c. Measurement of the changes in transmembrane mitochondrial potential are suggestive of pore formation within the mitochondria, allowing the release of cytochrome c and formation of the apoptosome on which caspase-9 becomes cleaved and activated. Although protein isolation from whole cells will not yield evidence of cytochrome c release, fractionation of cells and subsequent immunoblotting should show evidence of cytochrome c moving from within the mitochondrial fraction to the cytosolic fractions in cells overexpressing Ski. Similarly, movement of Smac/Diablo out of the mitochondria is also indicative of the progression of apoptosis and should be explored to thoroughly elucidate the mechanism by which Ski is capable of triggering apoptosis in cardiac myofibroblasts. In addition to these suggestions, a more thorough analysis of the Bcl2 superfamily of proteins should be examined, as there is complex interplay between many of the members of this family in the regulation of mitochondrial permeability.

Autophagy is a complex process that is under tight regulation within the cell, which has been shown to antagonize, and support apoptotic cell death under certain circumstances. This thesis examined autophagy by measuring autophagic markers LC3-II and ATG7. In addition to these proteins, a plethora of other autophagy proteins should be

examined to fully elucidate its role in the cell fate of cardiac myofibroblasts. Immunofluorescence of these proteins in Ski infected cells should show evidence of autophagy and support our findings. Electron microscopy may also be used to confirm the presence of autophagosomes or other subcellular structures within these cells.

Any disturbance to the normal biological functions of the endoplasmic reticulum will result in the activation of the evolutionarily conserved unfolded protein response. The initial response of this system is to compensate for damage, but if endoplasmic reticulum dysfunction is prolonged, can lead to cell death[178]. Studies have previously shown coordinated regulation of apoptosis, autophagy and endoplasmic reticulum stress within human atrial fibroblasts[179]. An examination of the unfolded protein response and its role in activation of cell death in rat myofibroblasts overexpressing Ski is warranted.

Regulation of cell fate is, as this work shows, dependent on excess levels of Ski protein, but similar experiments requiring the silencing of Ski expression within these cells needs to be performed. Precise control of expression of this protein may suggest a role as a cell cycle regulator within these cells, and cell cycle markers should be examined as well.

Ski has been shown to exist in multiple isoforms within cardiac myofibroblasts[34]. Our study specifically examined the effects of the overexpression of the 95 kDa variant of Ski. For completeness, studies should be included to examine overexpression of other isoforms (105 kDa and 115 kDa) in order to see if the effect on cell viability is similar. These larger isoforms may represent modifications to the protein that may alter its biological function.

Beyond the scope of gene modulation in cell culture, the overexpression and silencing of Ski should ultimately be performed within *in vivo* models in order to

understand the effect on whole organisms. Ski overexpression has effects on skeletal muscle and a variety of other tissue types and these effects need to be fully elucidated if this protein or a Ski mimetic is to be used in clinical practice. Even with targeted delivery of a potential therapy to the myocardium, the possible apoptotic effect on other cells types within the myocardium (ie, cardiomyocytes) may prove to be a fatal blow to the already weakened cardiac muscle of a post-MI patient. Thus, it is imperative that these studies be completed in order to evaluate the potential risks and benefits of using Ski as an agent to reduce cardiac fibrosis and delay/inhibit the progression of heart failure.

VIII. References

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