Antifibrotic properties of c-Ski and its regulation of cardiac myofibroblast phenotype and contractility

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Cunnington RH, Wang B, Ghavami S, Bathe KL, Rattan SG, Dixon IM. Antifibrotic properties of c-Ski and its regulation of cardiac myofibroblast phenotype and contractility. Am J Physiol Cell Physiol 300: C176–C186, 2011. First published October 13, 2010; doi:10.1152/ajpcell.00050.2010.—Cardiac myofibroblasts are key players in chronic remodeling of the cardiac extracelluar matrix, which is mediated in part by elevated transforming growth factor-β1 (TGF-β1). The c-Ski proto-oncoprotein has been shown to modify TGF-β1 post-receptor signaling through receptor-activated Smads (R-Smads); however, little is known about how c-Ski regulates fibroblast phenotype and function. We sought to elucidate the function of c-Ski in primary cardiac myofibroblasts using a c-Ski overexpression system. Cardiac myofibroblasts expressed three forms of c-Ski with the predominant band at 105 kDa, and adenoviral c-Ski treatment resulted in overexpression of 95-kDa c-Ski in cellular nuclei. Exogenous c-Ski led to significant inhibition of type I collagen secretion and myofibroblast contractility using two-dimensional semi-floating gel contraction assay in both basal and with TGF-β1 (10 ng/ml for 24 h) stimulation. Overexpressed c-Ski did not inhibit nuclear translocation of phosphorylated R-Smad2, despite their binding, as demonstrated by immunoprecipitation. Acute treatment of primary myofibroblasts with TGF-β1 in vitro revealed a marked nuclear shuttling of c-Ski at 24 and 48 h following stimulation. Remarkably, overexpression of c-Ski led to a stepwise reduction of the myofibroblast marker α-smooth muscle actin with increasing multiplicity of infection, and these results indicate that 95-kDa c-Ski overexpression may effect a loss of the myofibroblastic phenotype. Furthermore, adenovirus (Ad) for hemagglutinin-tagged c-Ski infection led to a reduction in the number of myofibroblasts versus Ad-LacZ-infected and uninfected controls, due to induction of apoptosis. Finally, we observed a significant increase in 105-kDa c-Ski in the cytosolic fraction of cells of the infarct scar and adjacent remnant myocardium vs. noninfarcted controls.

Cardiac fibroblasts are the main regulators of matrix homeostasis in the heart. Fibroblasts are phenotypically diverse and exhibit significant differences among organs and tissues (4). For example, expression of collagens type I and V is robust in fetal skin fibroblasts but weak in fetal lung fibroblasts (4), and desmin is a specific marker for skin fibroblasts compared with cardiac fibroblasts and myofibroblasts (25). Fibroblast diversity occurs among atria and ventricles, because fibroblasts from these tissues respond differentially to pathological stimuli (3).

The biology of cardiac fibroblasts and myofibroblasts is incompletely studied in health and disease, respectively. In diseased hearts, fibroblasts may undergo phenotypic conversion to myofibroblasts (15), which are hypersecretory (6), express α-smooth muscle actin (α-SMA) (8), and ED-A fibronectin markers (43). Transforming growth factor-β1 (TGF-β1) induces the myofibroblastic phenotype (9, 40), and elevated levels of this cytokine are associated with matrix remodeling (22, 39). Receptor-activated Smads (R-Smads) are the canonical post-receptor signaling proteins for TGF-β in heart, and they are implicated in profibrotic events in heart disease (2, 12). TGF-β1 binds to transforming growth factor-β receptor type II and ultimately causes phosphorylation of R-Smads, which hetero-oligomerize with co-mediator Smad4 and translocate into the nucleus, where they may effect gene expression (37, 54). R-Smads are overexpressed in fibrosed hearts and are implicated in regulation of cardiac fibroblast function (12, 22). Endogenous inhibitors of this pathway exist in inhibitory Smad7 (I-Smad7) and c-Ski. I-Smad7 functions via negative feedback through recruitment of Smad-ubiquitin regulatory factor 2 (Smurf2), targeting the type I receptor for degradation (26) thereby affecting downstream signal transduction and gene targets of TGF-β1. Expression of I-Smad7 is reduced following myocardial infarction (MI) thereby releasing negative feedback in wound healing (49); however, c-Ski has not been examined in this context.

c-Ski is a proto-oncoprotein that is highly conserved in vertebrate evolution (38) and expressed in a variety of tissues including skeletal muscle and heart (5, 30). c-Ski knockout mice exhibit defects in the cranial neural tube, reduction in skeletal muscle mass, and undergo perinatal lethality (1). While in the nucleus, c-Ski may exert transcriptional regulatory functions (7) and be involved in mitosis (31). Several hypotheses exist to explain c-Ski-mediated inhibition of the TGF-β/Smad signal. Suzuki et al. (46) showed that c-Ski functions at the transcriptional level as a repressor by binding of activated Smad complexes on DNA serving as a “disrupting bridge.” In this hypothesis, activated Smad complexes move into the nucleus following TGF-β ligand binding; however,
c-Ski binding of the Smad complexes on DNA within the nucleus prevents normal transcription of TGF-β target genes (46). Others have proposed that c-Ski prevents phosphorylation of cytosolic R-Smads, preventing activation of Smad complexes in cell lines (41). However, neither the effect(s) nor mechanism(s) of c-Ski actions have been studied in cardiac myofibroblasts. Because these cells play a critical role in expansion of cardiac matrix remodeling in a variety of heart failure etiologies, we examined the effects of c-Ski on myofibroblast collagen type I synthesis/secretion and contractility. Because TGF-β1 is a known stimulus for phenocversion of fibroblasts to myofibroblasts, we also examined the effect of c-Ski overexpression on myofibroblastic phenotype to test whether regression of the latter phenotype ensues.

MATERIALS AND METHODS

Cell isolation/culture. Cardiac fibroblasts were isolated as previously described (21). Briefly, hearts from adult male Sprague-Dawley rats (150–200 g) were subjected to Langendorff perfusion with DMEM-F-12 (GIBCO) followed by serum-free MEM (SMEM; GIBCO). Perfused hearts were digested with 0.1% wt/vol collagenase type 2 (Worthington) in SMEM for 20 min. Hearts were minced in dilute collagenase solution (0.05% wt/vol collagenase type 2 in SMEM) for a further 15 min before addition of growth media—DMEM-F-12 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin (GIBCO-BRL), 100 μg/ml streptomycin (GIBCO BRL), and 1 μM ascorbic acid (Sigma-Aldrich). Upon settling of large tissue pieces to the bottom of a 50-ml tube, supernatant was centrifuged at 2,000 rpm for 5 min. Cell pellets were resuspended in growth media and plated on 75-cm² culture flasks. Cells were allowed to grow for 3–4 days before passaging into first passage (P1) for experiments. Media were changed the following day, and cells were allowed to grow for 3–4 days before passaging into first passage (P1) for experiments. P1 myofibroblasts were infected with adenovirus at the time of plating. P0 fibroblasts were infected with retrovirus 24–48 h following isolation and then passaged as normal for experiments.

Experimental rat model of myocardial infarction. Experimental protocols for animal studies were approved by the Animal Care Committee of the University of Manitoba, Canada, following guidelines established by the Canadian Institutes of Health Research and the Canadian Council of Animal Care (2001). MI model was produced in male Sprague-Dawley rats (150–175 g) by surgical occlusion of the left coronary artery, as described previously (11). The mortality of the animals operated on in this fashion was ~30% within 48 h. Experimental animals were killed after 48 h, 2 wk, or 4 wk, and cardiac tissue was isolated from two left ventricular (LV) regions: remnant/noninfarcted MI. Tissues from these regions and from sham-operated rats were used for Western blot analysis to quantify c-Ski protein expression.

Retro- and adenovirus plasmid construction. Plasmids containing recombinant DNA for human c-Ski was obtained from Dr. Shunsuke Ishii. Human c-Ski was tagged at the 5’-end (NH₂ terminus) with a hemagglutinin (HA) tag (YPYDVPDYA) by insertion into pcDNA3.1N-HA (obtained from Dr. Michael Czubry), and then adenovirus for HA-tagged c-Ski (Ad-HA-Ski) was constructed using the pAdenoX adenovirus construction kit (Clontech) following the kit instructions. Retroviral HA-tagged c-Ski was constructed by removal of the gene from the pcDNA3.1N-HA-Ski plasmid and insertion into the pMXie-enhanced green fluorescent protein (eGFP) retroviral backbone (obtained from Dr. Jeffrey Wigle). This plasmid contains the eGFP reporter gene that is not linked to the gene of interest, pMXie or pMXie-HA-Ski was transfected into Ecotropic Phoenix packaging cell line using CalPhos Mammalian Transfection kit (Clontech). Control retrovirus (Rv-pMXie) or HA-Ski retrovirus (Rv-HA-Ski) was concentrated 100× by centrifugation and titered using NIH 3T3 cells (American Type Culture Collection). The titer was measured using flow cytometry for determination of the percentage of eGFP-expressing cells.

Reagents/antibodies. c-Ski antibody (Upstate/Millipore), HA antibody (Rockland Immunochemicals), phospho-Smad2 Ser450/467 antibody (Upstate/Millipore), α-SMA (Sigma), non-muscle myosin heavy chain b (SMemb) (Abcam), eukaryotic elongation factor 2 (eEF2) (Cell Signaling), β-tubulin (Abcam), procollagen type I antibody (sp1LD) (Hybridoma Bank), antibodies for lamin A and heat shock cognate 70 (Hsc70) (Abcam), TGF-β1, cytokine (Cell Signaling), collagen gel solution (Stem Cell Technologies/Advance Biomatrix Purcol), and procollagen type I amino terminal peptide enzyme immunoassay (PINP EIA) from Immuno-Diagnostic Systems.

Protein isolation. Following treatment of cells in 100-mm² dishes, cells were washed twice with PBS. RIPA lysis buffer containing protease inhibitor cocktail (Sigma) and phosphatase inhibitors (10 mmol/l NaF, 1 mmol/l sodium orthovanadate, and 20 mmol/l β-glycorophosphate) was used to lyse cells, and, following mechanical scraping, cell lysates were incubated on ice for 1.5 h. Lysates were then sonicated for 10 s three times and then centrifuged at 14,000 rpm at 4°C for 15 min. Supernatants were transferred to new tubes, and protein assay was performed using the bicinchoninic acid method (44).

Western blot analysis. SDS-PAGE of 20–40 μg of protein was performed on 6–8% gels using SeeBlue Plus2 Pre-Prepared Stained (Bio-Rad) as a molecular weight marker. Proteins were transferred onto 0.45-μM polyvinylidene difluoride membrane and blocked in Tris-buffered saline with 0.2% Tween 20 (TBS-T) containing 10% skim milk for 1.5 h at room temperature with constant shaking. Primary antibodies were diluted in TBS-T with 3% skim milk as follows: anti-Ski (1:1,000) (when using Ski antibody, PBS containing 0.05% Tween 20 was used to prepare skim milk, wash, and dilute antibodies in place of 0.2% TBS-T), β-tubulin (1:5,000), α-SMA (1:5,000), eEF2 (1:3,000), P-Smad2 (1:1,000), lamin A (1:500), and Hsc70 (1:1,000). Membranes were incubated for 1.5 h at room temperature or overnight at 4°C with shaking. Secondary antibodies horseradish peroxidase (HRP)-labeled anti-rabbit and HRP-labeled anti-mouse were diluted 1:10,000 with TBS-T with 3% skim milk and incubated for 1.5 h at room temperature with shaking. Protein bands on Western blots were visualized using ECL Plus (Amersham, Arlington Heights, IL) according to the manufacturer’s instructions and were developed on X-ray film. Equal protein loading was confirmed using β-tubulin or Coomassie blue staining.

Nuclear/cytoplasmic fractionation. P1 cardiac myofibroblasts were plated on 100-mm dishes and allowed to adhere and grow overnight until ~75% confluence was achieved. Fractionation was then carried out using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) according to the manufacturer’s instructions.

Collagen secretion. P1 cardiac myofibroblasts were seeded on 100-mm² cell culture dishes and either left uninfected or infected with LacZ adenovirus (Ad-LacZ) [100 multiplicity of infection (MOI)] or Ad-HA-Ski (100 MOI) adenoviruses and allowed to grow overnight to ~75% confluence. Cells were then serum starved for 24 h by washing twice with PBS and adding starvation media (serum-free DMEM-F-12 with 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 μM ascorbic acid). Next, cells were either treated with TGF-β1 (10 ng/ml) for 24 h or left untreated. Once the treatment period was complete, media were removed to a 15-ml tube and cells from that plate were scraped and added to the tube with the media. Next, tubes were subjected to five rapid freeze thaws in liquid nitrogen/37°C water bath. Five microliters of this media/cell solution were used for the PINP EIA following kit instructions.
Immunofluorescence. P1 cardiac myofibroblasts were seeded onto coverslips in six-well cell culture dishes and either left uninfected or infected with Ad-LacZ (100 MOI) or Ad-HA-Ski (100 MOI) adenoviruses and allowed to grow overnight to ~75% confluency. Cells were then serum starved by washing twice with PBS, adding starvation media, and incubating for 24 h. Treatments were added directly to each well. After duration of treatment was complete, cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. Cells were then washed three times with PBS and permeabilized with 0.1% Triton X-100 followed by three additional washes. Slides were blocked with 10% bovine serum albumin in PBS for 30 min, washed, and incubated overnight in primary antibody diluted in 1% BSA in PBS. Again, cells were washed three times with PBS and incubated with Alexa Fluor-conjugated secondary antibody for 1.5 h followed by another wash cycle. Coverslips were dried, using a vacuum, and mounted on slides using Slowfade gold with DAPI mounting media and then sealed with nail polish. Immunofluorescence of Rv-HA-Ski-infected cells was conducted following the same procedure except for the time of infection (Rv-HA-Ski or Rv-pMrxie was applied ~24 h following isolation for a duration of 24 h. Cells were then allowed to grow to confluency and then passaged for experiments.

Immunoprecipitation. P1 cardiac myofibroblasts were plated and infected with Ad-HA-c-Ski (100 MOI) or Ad-LacZ control virus. Following 24 h of incubation, cells were washed twice with PBS and then lysed with 500 µl of mild RIPA lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, pH 8) containing protease inhibitor cocktail (Sigma) and phosphatase inhibitors (10 mM NaF, 1 mM sodium orthovanadate, and 20 mM β-glycerophosphate). Cell lysates were used for immunoprecipitation using Dynabeads Protein G (Invitrogen) following the manufacturer’s protocol using gentle elution to remove protein complexes bound to beads.

Collagen gel contraction. Collagen gels were made by combining 7 ml of cold collagen solution (Stem Cell Technologies) with 2 ml 5× concentrated DMEM-F-12 and adjusting the pH to ~7.4. Total volume was brought to 10 ml with sterile double-distilled water. Six hundred microliters of the mixture were added to each well of a 24-well dish and allowed to solidify a minimum of 3 h to overnight at 37°C in a 5% CO2 incubator. P1 cardiac myofibroblasts were seeded onto solidified collagen gels at 1×10^5 cells per well and either left uninfected or infected with Ad-LacZ (100 MOI) or Ad-HA-Ski (100 MOI) at the time of plating. Cells were allowed to grow and adhere for ~24 h and then were serum starved for 24 h. Before addition of TGF-β1 (10 ng/ml) to the appropriate wells, gels were released from the walls of the dish using a scalpel blade or circular cutting tool. Once gels were released around the edges, cytokine was added and pictures were taken (time 0). Pictures were then taken 24 h following the initial treatment and analyzed using IDL Measure gel software to determine gel surface area at each time point.

MTT assay. The effect of Ad-LacZ and Ad-HA-Ski infection on rat ventricular fibroblast proliferation and viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (16, 17, 19). Briefly, the infected cells were seeded in 96-well plates at either 1,000 or 5,000 cells per well in the presence of 10% FBS. After 24 h, cells were serum starved in FBS-free media for a further 24 h before treatment with TGF-β1 (10 ng/ml) in either serum-free media or media supplemented with 1% FBS for 48 h. The cell viability was calculated using mean optical density (OD) (570 nm) of cells.

Measurement of apoptosis by flow cytometry. Apoptosis was measured using the Nicoletti method (18, 23). Briefly, infected cells grown in 12-well plates (prestarved in FBS-free medium for 24 h) were treated with TGF-β1 (10 ng/ml) for 48 h. After being scraped, the cells were harvested by centrifugation at 1,500 g for 5 min, washed once with PBS, and then resuspended in a hypotonic propidium iodide lysis buffer (1% sodium citrate, 0.1% Triton X-100, 0.5 mg/ml RNase A, 40 µg/ml propidium iodide). Cell nuclei were then stained with eEF2 antibody and DAPI nuclear stain. Images are representative of 3 different experiments.

Fig. 1. Characterization of adenovirus for hemagglutinin (HA)-tagged c-Ski (Ad-HA-Ski)-infected first passage (P1) myofibroblasts. A: cardiac myofibroblasts express 115-, 105-, and 95-kDa forms of c-Ski. Expression of endogenous c-Ski was performed using Western blot of normal P1 cardiac myofibroblasts. Ad-HA-Ski overexpression lane was done with multiplicity of infection (MOI) of 100 virus particles per cell. TGF-β1, transforming growth factor-β1, B: Ad-HA-Ski overexpresses 95-kDa c-Ski. P1 cardiac myofibroblasts were left uninfected or infected with Ad-LacZ (100 MOI) or Ad-HA-Ski for 24 h. Total c-Ski expression was examined using Western blot using eukaryotic elongation factor 2 (eEF2) as a loading control. C: exogenously expressed c-Ski is nuclear. P1 cardiac myofibroblasts were uninfected or infected with Ad-LacZ (100 MOI) or Ad-HA-Ski (MOI 100) for 24 h. Cells were fixed and immunostained using HA antibody and DAPI nuclear stain. Images are representative of 3 different experiments.
incubated for 30 min at 30°C and subsequently analyzed by flow cytometry. Nuclei to the left of the G1 peak containing hypodiploid DNA were considered to be apoptotic.

**Statistical analysis.** Data are expressed as means ± SE. Data were entered and processed using the SigmaStat statistics program. Groups were compared using one-way ANOVA with Student-Newman-Keuls post hoc test for multiple group comparisons. Data for MTT and FACS experiments are expressed as means ± SD. These groups were compared using one-way ANOVA with Bonferroni post hoc test for multiple group comparisons. A value of \( P < 0.05 \) was considered to be significant for all experiments.

**RESULTS**

Adenoviral c-Ski overexpression increases 95-kDa c-Ski and is localized to the nucleus. Previous studies of c-Ski have been conducted mainly in sundry cell lines, with very little work in primary cells and in heart. Myofibroblasts are key players in cardiac fibrosis, and their function is tied to Smad signaling (22, 49). Here we characterized the expression of this protein in P1 cardiac myofibroblasts. Using Hela nuclear extract as a standard for c-Ski expression, we observed a strong c-Ski banding at 105 kDa irrespective of treatment (serum-starved cells, 10% FBS treated or 10 ng/ml treated TGF-\( \beta_1 \)) (Fig. 1A). Longer exposure times revealed 115-kDa and 95-kDa bands in addition to the 105-kDa band (data not shown). To examine the effects of c-Ski overexpression, we infected P1 cardiac myofibroblasts with an HA-tagged human c-Ski adenovirus (Ad-HA-Ski). Using various MOIs, we observed a significant increase in the level of the 95-kDa form of the c-Ski in comparison to control uninfected cells and adenoviral LacZ (Ad-LacZ)-infected cells (Fig. 1, A and B). Furthermore, immunofluorescent analysis of our c-Ski-overexpressing cells (HA antibody) revealed strong nuclear localization of HA-Ski compared with uninfected and Ad-LacZ-infected controls. To delineate the entire cell outline, phalloidin was used to stain the actin cytoskeleton (Fig. 1C).

c-Ski overexpression reduces myofibroblast collagen synthesis/secretion and contractility. c-Ski has been shown to be a TGF-\( \beta \) inhibitor (46). TGF-\( \beta_1 \) itself is a potent inducer of collagen type I secretion (13) and myofibroblast contraction of collagen gel matrices (33). We immunostained using procollagen type I antibody (sp1.D8) to assess monomeric procollagen expression within cells and collagen secretion using PINP EIA to measure the globular NH2-terminal heads cleaved in the secretion of trimeric mature type I collagen. We observed significant inhibition of procollagen type I expression in Rv-HA-Ski-infected cells compared with uninfected and Rv-pMxie-infected cells (Fig. 2A). Furthermore, collagen type I secretion of Ad-HA-Ski-infected cells was reduced in both the basal state and in TGF-\( \beta_1 \)-stimulated (10 ng/ml, 24 h) cells compared with Ad-LacZ and uninfected controls (Fig. 2B). To assess myofibroblast contractility, we used semifloating two-dimensional gel contraction assays and observed similar marked inhibition of basal and TGF-\( \beta_1 \)-induced contractility in c-Ski-overexpressing cells compared with uninfected and Ad-LacZ-infected cells (Fig. 3, A and B). These data, taken with Ad-HA-Ski characterization data (Fig. 1), suggest that 95-kDa c-Ski of nuclear origin plays a significant role in modulation of cardiac myofibroblast function, i.e., physical remodeling of extracellular matrix.

![Fig. 2. Overexpression of c-Ski inhibits collagen type I synthesis (A)/secretion (B). A: retrovirus (Rv)-infected cells and uninfected (mock) control cells were stimulated with TGF-\( \beta_1 \) (10 ng/ml, 30 min), fixed, and immunostained with procollagen type I antibody (sp1.D8) with enhanced green fluorescent protein (eGFP) as a reporter for infection. Arrows indicate infected cells. B: uninfected (control) or Ad-LacZ/Ad-HA-Ski-infected cells were serum starved for 24 h and then treated with TGF-\( \beta_1 \) (10 ng/ml) for 24 h or left unstimulated. Cleared amino globular heads of procollagen type I molecules were assayed using procollagen type I amino terminal peptide enzyme immunoassay (PINP EIA) on the media/cell solution. Data were normalized to control within each group. Data are means ± SE; \( n = 6 \); \( \ast P < 0.001 \) vs. control; †\( P < 0.01 \) vs. control + TGF-\( \beta_1 \).](http://ajpcell.physiology.org/ by 10.220.33.4 on October 20, 2017)
c-Ski does not impair nuclear translocation of P-Smad2 on TGF-β1 stimulation. Multiple possible mechanisms for c-Ski-mediated inhibition of TGF-β1 have been described using immortalized cell lines (41, 46). To identify the likely mode of c-Ski action in primary cardiac myofibroblasts, we examined the effect of c-Ski overexpression on P-Smad2 nuclear shuttling following TGF-β1 stimulation. Using P1 primary cells, we examined the effect of c-Ski overexpression on nuclear translocation of P-Smad2 using immunofluorescence and nuclear/cytoplasmic fractionation. Ad-HA-Ski infection did not prevent P-Smad2 from accessing the nucleus as evidenced through strong nuclear staining of P-Smad2 (Fig. 4A) following TGF-β1 stimulation (10 ng/ml, 30 min), indicating that 95-kDa c-Ski does not prevent phosphorylation and translocation of P-Smad2. These data were confirmed with Western blot analysis wherein we found that nuclear/cytoplasmic fractions (Fig. 4, C and D) exhibited robust induction of P-Smad2 in the nuclei of all cells, including control, Ad-LacZ-infected, and Ad-HA-Ski-infected cells. We observed a trend toward reduced P-Smad2 in the nuclei of c-Ski-overexpressing cells compared with uninfected and Ad-LacZ-infected controls (not significant), and there was no observable increase in P-Smad2 levels in the cytoplasm of Ad-HA-Ski-infected cells (Fig. 4, C and D).

c-Ski and P-Smad2 are binding partners. To identify the mode of action of c-Ski in cardiac myofibroblasts, we investigated whether c-Ski bound P-Smad2 in primary myofibroblasts. Using immunoprecipitation, we assessed the binding ability of c-Ski and P-Smad2 in P1 rat cardiac myofibroblasts (Fig. 4B). On immunoprecipitation with c-Ski antibody we observed the marked presence of the P-Smad2 band on Western blots of lanes of uninfected cell extracts as well as a relatively intense band for Ad-HA-Ski-infected cells. Thus the mechanism of 95-kDa c-Ski/P-Smad2 binding, taken with the observations above, favors the “disrupting bridge” hypothesis of c-Ski action, wherein binding of phosphorylated R-Smads occurs in cardiac myofibroblasts.

c-Ski expression pattern in cardiac fibroblast to myofibroblast differentiation. Cardiac fibroblasts phenoconvert to myofibroblasts with TGF-β1 stimulation (9, 40), and plating at low density (32, 51, 52) on a rigid substrate exerts the same effects (51). Here we confirm in vitro phenoconversion of cardiac fibroblasts (P0) to myofibroblasts (P1 and P2) under similar conditions. In addition to elevated α-SMA in the latter, these cells also express SMemb in healing infarcted cardiac tissue (14). Western analysis to characterize fibroblast phenoconversion in in vitro primary cell cultures was performed and demonstrated low levels of α-SMA and SMemb expression in P0 cells while being significantly upregulated in P1 and P2 cells (Fig. 5). Relative levels of c-Ski revealed marked increase in expression of 105-kDa c-Ski in P1 and P2 myofibroblasts vs. P0 fibroblasts (Fig. 5). Equal loading was confirmed with Coomassie blue staining.

Overexpression of c-Ski results in diminution of myofibroblastic phenotype in transfected cells. We have demonstrated above that 95-kDa c-Ski is associated with impaired P-Smad2 function (but not nuclear localization). Because R-Smads are implicated in the transduction of TGF-β signal resulting in the phenoconversion of fibroblasts into myofibroblasts (9, 12, 13), we examined the effects of elevated...
95-kDa c-Ski on myofibroblastic phenotype by examining α-SMA expression. Ad-HA-Ski at various MOIs was associated with significant reduction of α-SMA expression in P1 myofibroblasts (Fig. 6, A and B) compared with uninfected and Ad-LacZ-infected controls. Because we also observed increased expression of 105-kDa c-Ski with passage, these data support the hypothesis that 115-kDa, 105-kDa, and 95-kDa forms of c-Ski exert differential effects in myofibroblasts.

Overexpression of c-Ski reduces cardiac myofibroblast viability through induction of apoptosis. To examine the effect of c-Ski on cellular viability, we performed MTT assays of P1 cells with or without TGF-β1 in the presence of serum-free media for 48 h. Under these conditions, we show that Ad-HA-Ski infection is associated with a significant dropout of cell numbers versus uninfected and Ad-LacZ-infected controls. Because we also observed increased expression of 105-kDa c-Ski with passage, these data support the hypothesis that 115-kDa, 105-kDa, and 95-kDa forms of c-Ski exert differential effects in myofibroblasts.

Nuclear localization of endogenous c-Ski protein following 48 h TGF-β1 treatment in vitro. We employed immunocytochemistry to determine the effects of acute TGF-β1 treatment on c-Ski localization in cultured cardiac myofibroblasts in vitro. Stained myofibroblasts revealed that c-Ski exhibited strong cytoplasmic to nuclear shuttling following TGF-β1 stimulation (10 ng/ml) beginning 24 h poststimulation with marked nuclear translocation at 48 h (Fig. 8). Earlier time points were associated with diffuse endogenous c-Ski staining throughout the nucleus and cytoplasm with slightly more intense staining in the cytoplasm.

c-Ski accumulates in the cytoplasm following myocardial infarction. Because TGF-β1 is elevated following MI and is important in the phenocconversion of cardiac fibroblasts into myofibroblasts during cardiac wound healing (22, 39), we examined c-Ski expression in an in vivo coronary ligation model of MI. We observed a significant increase in cytosolic 105-kDa c-Ski at 48 h post-MI through to 4 wk post-MI (Fig. 9) in the scar portion of the heart compared with sham heart and viable tissue. We also noted that at 48 h post-MI, cytosolic 105-kDa c-Ski in viable tissue was not significantly different from sham hearts, yet at 2 and 4 wk, cytosolic 105-kDa c-Ski...
was notably increased relative to sham hearts (although not as high as in infarct scar) (Fig. 9).

DISCUSSION

Myofibroblast transdifferentiation is now recognized as a hallmark event in the pathogenesis of cardiac fibrosis from a variety of cardiac diseases (15). While it is well recognized that these cells are hypersecretory for a number of key matrix proteins and that TGF-β/R-Smads are key mediators of cardiac fibrosis in heart failure etiologies (12, 22, 42), the study of endogenous Smad-associated proteins that may inhibit these profibrotic signals is in its infancy. We have previously described the antifibrotic actions of Smad7 in plated primary cardiac myofibroblasts, and its diminished expression in healing myocardial infarcts (49, 50), but the function(s) of other members of this unique group of proteins, e.g., c-Ski and Sno-N, remains unclear in this context. Furthermore, an understanding of the effects of c-Ski isoforms in cardiac fibroblasts may assist in determining the physiological role of this protein in healthy and diseased heart. Herein we have characterized the functional and phenotypic changes in primary cardiac myofibroblasts induced by c-Ski overexpression and suggest a mechanism for the mode of action of this protein. We found that c-Ski overexpression is correlated to 1) a significant decrease in type I collagen synthesis and secretion; 2) decreased myofibroblast contractility; 3) suppression of the myofibroblast phenotype, as reflected by reduced α-SMA expression; and 4) significant loss of myofibroblast cell viability due to apoptosis at higher cell seeding densities. Furthermore, we have begun to characterize the in vivo localization of c-Ski following myocardial infarction and observed a dramatic cytosolic accumulation of c-Ski in infarct scar portion of the post-MI heart.

There are few reports of multiple forms of c-Ski in primary cells, e.g., 95-, 105-, and 115-kDa forms that we observed in primary cardiac cells, but it is likely that posttranslational modifications give rise to this diversity (36). While multiple phosphorylation states of this protein are known (31, 45), there are other possibilities, e.g., ubiquitination, for changes in apparent protein molecular weight.

In primary cardiac myofibroblasts, we have selectively overexpressed the 95-kDa form and noted its marked nuclear localization in vitro. c-Ski overexpression led to reduced contractility and collagen secretion when compared with uninfected controls, and these findings indicate a homeostatic role for this protein. Notably, we have also observed 95-, 105-, and 115-kDa forms of c-Ski in human ventricular cardiac myofibroblasts (data not shown), and thus the relevance of these findings to human fibroblast function is supported.

While we have begun to address the effects of 95-kDa c-Ski, the functions and physiologic relevance of other c-Ski forms,
e.g., 105- and 115-kDa forms of c-Ski, are unclear. Nonetheless, the correlation of increased 105-kDa c-Ski expression with progressive myofibroblast passage may point to a significant role for this form. We suggest 105- and 115-kDa c-Ski bands represent a latent cellular pool of c-Ski that may be activated through dephosphorylation, or other modifications. While it has been reported that c-Ski is targeted for removal by Arkadia protein (34), the relevance of the different forms of c-Ski to its removal is unclear. In contrast, 95-kDa c-Ski is minimally expressed in cardiac myofibroblasts, indicating either a rapid synthesis and modification cycle or a swift degradation. Further investigation is required to elucidate the importance of each form of c-Ski.

c-Ski overexpression does not inhibit nuclear translocation of P-Smad2 despite their direct interaction and subsequent R-Smad “trapping” by c-Ski on DNA in nonfunctional transcription complexes, preventing activation of target genes (46). Furthermore, we found nuclear c-Ski translocation following

Fig. 7. c-Ski overexpression reduces rat ventricular myofibroblast viability through induction of apoptosis. A and B: cells were seeded at 1,000 cells/well (A) or 5,000 cells/well (B) in 96-well plates, were left uninfected or infected with Ad-LacZ (100 MOI) or Ad-HA-Ski (100 MOI), and were allowed to grow and adhere overnight. Cells were then serum starved for 24 h before treatment with TGF-β1 (10 ng/ml) for a further 48 h. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are expressed as percentage of corresponding control and represent the means ± SD of 9 independent experiments in 3 different sets of rat ventricular cells. (A, *P < 0.01 vs. control; #P < 0.05 vs. control + TGF-β1; B, *P < 0.01 vs. control; #P < 0.001 vs. control + TGF-β1). WT, wild type. C: DNA histogram of rat ventricular cells (Nicoletti method) from FACS analysis of uninfected, Ad-LacZ-infected (100 MOI), and Ad-HA-Ski-infected (100 MOI) P1 cardiac myofibroblasts. M2 (statistical marker) has been placed to mark subdiploid DNA that is low on control histograms. The diploid (G1) and tetraploid (G2) DNA is visible in a form of two peaks in the far right of the histograms. G1 and G2 peaks are still preserved, and subdiploid peak corresponding to apoptotic cells is also clearly visible to the left from both peaks that represent normal cells. D: histographical representation of FACS data in C. *P < 0.01 vs. control; #P < 0.01 vs. control + TGF-β1; n = 3.

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acute stimulation with TGF-β1, suggesting a negative autoregulation similar to I-Smad7 in healthy cells. We suggest that inhibition of R-Smads by c-Ski is a major avenue of regulation of fibroblast function. Frangogiannis and coworkers recently identified the critical nature of R-Smad3 in regulation of fibroblast phenotype, increased migration, and extracellular matrix protein expression (12). While the current study addresses c-Ski association with R-Smad2, our data indicate that it is very likely that both R-Smads contribute to regulation of cardiac fibroblast phenotype and function. Thus their inhibition may be a critical point in controlling functionality of these cells.

Fibroblast phenoconversion is a feature of cardiac fibrosis whereby induction of myofibroblast phenotype is characterized by increased α-SMA expression and type I collagen synthesis (6, 8). While some evidence supports the suggestion that this conversion may be reversible in vitro (20, 51), whether it occurs in vivo is at present an understudied topic. Liu et al. (29) found that overexpression of c-Ski in rat dermal fibroblasts of passage 3–8 leads to a reduction of type I collagen synthesis yet had no effect on α-SMA expression. Because fibroblasts are a diverse group of cells with topographic differentiation from organ to organ including heart (3, 4), the effect(s) of c-Ski may be quite different in fibroblasts from skin vs. heart. Further dermal fibroblasts, like smooth muscle, express desmin and thus are fundamentally different from cardiac fibroblasts (25).

For the first time, we report a significant reduction in type I collagen synthesis/secretion in cardiac myofibroblasts overexpressing c-Ski coupled with a marked reduction in contractility and diminution in α-SMA expression in these cells. The lack of response of α-SMA expression in previous work may be ascribed to specific cell type (desmin-positive dermal myofibroblasts vs. desmin-negative cardiac myofibroblasts), passage number (P3–P8 vs. P1 cells), and the relative level of c-Ski overexpression. Furthermore, because myofibroblasts reside permanently in the infarct scar in damaged heart, in contrast to dermal wound healing, the latter characterized by rapid disappearance of these cells following completion of wound healing (10, 53), we suggest that negative regulation of the myofibroblast phenotype by c-Ski is a primary mechanism to suppress inappropriate matrix remodeling and collagen deposition.

![Fig. 8](image8.png)

**Fig. 8.** TGF-β1 stimulates nuclear translocation of c-Ski in P1 cardiac myofibroblasts. P1 cardiac myofibroblasts at ~75% confluency were serum starved for 24 h before treatment with TGF-β1 (10 ng/ml) for various times (t). Immunofluorescent staining of c-Ski in serum-starved cells (DAPI nuclear stain) at selected time points under oil immersion (magnification, ×400). Pictures are representative of n = 4.

![Fig. 9](image9.png)

**Fig. 9.** Expression of c-Ski in post-myocardial infarction (MI) cardiac tissues. Representative Western blots show cytosolic and total 105-kDa c-Ski protein obtained from sham-operated control hearts and remnant tissue as well as tissue from the infarct zone in 48 h (A), 2 wk (B), and 4 wk (C) post-MI left ventricular samples. In A–C, top representative Western blot shows cytosolic-105 kDa c-Ski expression, middle images are Coomassie blue stained to verify lane loading, and bottom Western blot shows total 105-kDa c-Ski expression in samples. Histogramical representations of cytosolic 105-kDa c-Ski data from samples normalized to load control are shown below the blots. A, *P ≤ 0.01 vs. sham; C and D, *P ≤ 0.001 vs. sham, #P ≤ 0.001 vs. viable tissue; E, *P ≤ 0.001 vs. sham. Data are means ± SE of 4–6 experiments.
Myofibroblasts express increased α-SMA (28), and upregulation of this protein is associated with increased contractility (24). Because TGF-β1-mediated fibroblast phenconversion appears to be regulated in large part by R-Smad2 (13), we suggest that increased levels of 95-kDa c-Ski are associated with diminution of myofibroblast phenotype and inhibition of R-Smad2 signal. Furthermore, reduced α-SMA expression in c-Ski-overexpressing cardiac myofibroblasts may contribute to reduced contractility of infected cells. Myofibroblast contractility is characterized by tonic contraction in contrast to phasic contraction by cardiac myocytes (48). Myofibroblasts manipulate and remodel the existing matrix, and this depends on connection of matrix to the cell using numerous fibronectin that consist of focal adhesion-associated proteins not highly expressed in fibroblasts (42, 48). We suggest that c-Ski-mediated diminution of myofibroblast contractility may decrease the ability of these cells to physically remodel the extracellular matrix.

While increased expression of 95-kDa c-Ski attended the conversion fibroblasts to hypersecretory myofibroblasts in culture, we speculate that this reflects elevated autoregulation of a potential “brake” for R-Smad signaling. The current data highlighting nuclear localization, and showing Smad2 and c-Ski interaction in cardiac cells, support the suggestion that c-Ski provides a molecular brake to disrupt R-Smad2 function. Because c-Ski was seen to induce apoptosis of myofibroblasts in serum-free conditions at higher seeding densities, this effect could be argued to influence net collagen secretion and contractility, but not on phenotypic reversion of these cells, because equal amounts of protein were used to test phenotypic markers. Interestingly, when fewer cells were seeded in 96-well culture plates, the apoptotic effect of Ad-HA-Ski infection was negligible, suggesting that c-Ski-induced effect may be dependent in part on the environment of the cell.

It has been previously reported that increased apoptosis in cranial neuroepithelium is associated with c-Ski-deficient embryonic mice (1). However, we observe an induction of apoptosis with overexpression of c-Ski (Fig. 7, C and D) in adult myofibroblasts. These conflicting results may highlight the changing role of c-Ski through development and with respect to cell type because c-Ski deficiency was associated with apoptosis in embryonic mice (1), whereas our observations were made with adult cells. The mechanisms of c-Ski-mediated apoptosis in cardiac myofibroblasts warrants further study.

In the context of chronic post-MI wound healing, the role of c-Ski becomes complex. An apparent cytosolic accumulation of 105-kDa c-Ski occurs in all post-MI experimental groups in vivo. While our in vitro data describe clear antifibrotic and anticontractile effects of 95-kDa c-Ski (which may be a function of diminished myofibroblastic phenotype), the integrated effect of cytosolic c-Ski function in vivo is uncertain. Nonetheless, reduced nuclear localization of c-Ski may facilitate inappropriate wound healing through relatively unimpeded TGF-β signaling. Although negative regulation of TGF-β functions by cytosolic c-Ski has been described (41), similar functions in the post-MI heart appear to be limited. One possibility for the translocation of c-Ski into the cytoplasm following MI may be due to proteosomal degradation of c-Ski (35). Further research is required to elucidate the physiological role of c-Ski in the infarcted heart.

In summary, the 95-kDa form of c-Ski diminishes cardiac myofibroblast phenotype in association with inhibition of R-Smad2, and its overexpression leads to significantly reduced contractility and collagen secretion of cardiac myofibroblasts. Immunoprecipitation of c-Ski and phosphorylated Smad2 in these primary cells supports the disrupting bridge model of c-Ski-mediated inhibition through P-Smad2 trapping in cellular nuclei. The complex nature of c-Ski function in cardiac myofibroblasts is revealed with in vitro studies, and the data indicate that abnormal functioning of this protein in cardiac myofibroblasts in fibrosed hearts may contribute to adverse matrix remodeling and expansion in heart failure.

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No conflicts of interest, financial or otherwise, are declared by the author(s).

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