Regulation of collagen synthesis by inhibitory Smad7 in cardiac myofibroblasts

Baiqiu Wang, Amer Omar, Tatjana Angelovska, Vanja Drobic, Sunil G. Rattan, Stephen C. Jones, and Ian M. C. Dixon

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Wang B, Omar A, Angelovska T, Drobic V, Rattan SG, Jones SC, Dixon IM. Regulation of collagen synthesis by inhibitory Smad7 in cardiac myofibroblasts. Am J Physiol Heart Circ Physiol 293: H1282–H1290, 2007. First published May 18, 2007; doi:10.1152/ajpheart.00910.2006.—Transforming growth factor-β (TGF-β) signal and downstream Smads play an important role in tissue fibrosis and matrix remodeling in various etiologies of heart failure. Inhibitory Smad7 (I-Smad7) is an inducible regulatory Smad protein that antagonizes TGF-β signal mediated via direct abrogation of R-Smad phosphorylation. The effect of ectopic I-Smad7 on net collagen production was investigated using hydroxyproline assay. Adenovirus-mediated I-Smad7 gene (at 100 multiplicity of infection) transfer was associated with significant decrease of collagen synthesis in the presence and absence of TGF-β, in primary rat cardiac myofibroblasts. In I-Smad7-infected cells, we also observed the ablation of TGF-βR1-induced R-Smad2 phosphorylation vs. LacZ controls. Overdriven I-Smad7 was associated with significantly increased expression of intermolecular activative 65-kDa matrix metalloproteinase-2 (MMP-2) protein in culture medium of myofibroblasts compared with LacZ-infected cells. Expression of the 72-kDa MMP-2 variant, e.g., the inactive form, was not altered by exogenous I-Smad7 transfection/overexpression. Furthermore, I-Smad7 overexpression was associated with a significant increase and decrease in expression of p27 and phospho-Rb protein, respectively, as well as reduced [3H]thymidine incorporation vs. Ad-LacZ-infected controls. We suggest that negative modulation of R-Smad phosphorylation by ectopic I-Smad7 may contribute to the downregulation of collagen in cardiac myofibroblasts and may suppress the proliferation of these cells. Thus treatments targeting the collagen deposition by overexpression of I-Smad7 may provide a new therapeutic strategy for cardiac fibrosis.

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ment of cardiac fibrosis in post-MI heart (58) wherein cardiac myofibroblasts contribute to cardiac repair. Furthermore, bleo-
mycin-induced lung fibrosis, which is mediated by consistent activation of TGF-β1, is prevented by the administration of exogenous (adenoviral delivery) I-Smad7 (36). Nevertheless, surprising little is known of control of matrix turnover by I-Smad7 in cardiac myofibroblasts. Thus we sought to define I-Smad7 modulation of collagen in cultured cardiac myofib-
blasts by addressing collagen synthesis and MMP expression in cells overexpressing I-Smad7.

MATERIALS AND METHODS

Primary adult cardiac myofibroblast culture. Adult cardiac myo-
fibroblast cultures were established from ventricular tissue of male Sprague-Dawley rats according to the methods of Brilla et al. (5) with minor modifications (22). Adult rats (175–200 g body mass) were killed, and hearts were subjected to Langendorff perfusion with a flow of 5 ml/min at 37°C with recirculating Joklik’s medium containing 0.1% collagenase (chaotropic agent) and 2% BSA for 25–35 min. Liberated cells were collected by centrifugation at 2,000 rpm for 10 min. Cells were resuspended in DMEM-F-12 and plated on a 100-mm noncoated culture flask at 37°C with 5% CO2 for 2 h. Cardiac fibroblasts attached to the bottom of the culture flask during a 2-h incubation, whereas nonadherent myocytes were removed by chang-
ing the culture medium. The cells were maintained in DMEM-F-12 incubation, whereas nonadherent myocytes were removed by chang-
ing condition, and separated on 10% SDS-PAGE (for MMP-2 and TGF-β1 and I-Smad7 adenovirus. After 72 h treat-
ment, cells were scraped on the cell culture medium of 100-mm dishes. Both the cell lysate and culture medium were removed and placed in test tubes, and net collagen production was determined with a hydroxyproline-based assay as described previously (56). The result from these experiments is expressed as degree of increase against values from control samples was separated on 10 or 12% SDS gels by SDS-PAGE. Separated protein was transferred on a 0.45 μM polyvinylidene difluoride (PVDF) membrane that was blocked at room temperature for 1 h or overnight at 4°C in Tris-buffered saline with 0.2% Tween 20 (TBS-T) containing 5% skim milk and probed with primary antibodies for 1 h at room temperature. The primary antibody against phosphorilated R-Smad2 was diluted 1:250 in 0.2% TBS-T with 5% skim milk, whereas the total R-Smad2 primary antibody was diluted 1:200 in 0.2% TBS-T with 5% skim milk. The primary antibody for actin was diluted 1:500 in 0.2% TBS-T with 5% skim milk. Phos-
phorylated (P) extracellular signal-regulated kinase (ERK) and P-c-Jun NH2-terminal kinase (JNK) antibodies were diluted 1:250 in 0.2% TBS-T with 5% skim milk, and p27 primary antibodies were diluted 1:1,000; phospho-Rb primary antibodies were diluted 1:500. Second-
ary antibodies included horseradish peroxidase (HRP)-labeled anti-
rabbit for phosphorylated R-Smad2 and anti-mouse for total R-Smad2, actin, P-ERK, and P-JNK. All secondary antibodies were diluted 1:10,000 with 0.2% TBS-T with 1% skim milk and incubated for 1 h at room temperature. Protein bands on Western blots were visualized by ECL Plus detection reagents (Amersham-Pharmacia Baie d’Urfe, Canada). Relatively even protein loading was confirmed by immunoblotting against actin.

Immunodetection of MMP-2, MT1-MMP, and TIMP-2. Cardiac myofibroblasts were cultured until confluent, and the medium was changed to DMEM-F-12 in the absence of serum. After a 24-h incubation, I-Smad7 (100 MOI), LacZ (100 MOI) adenovirus, and TGF-β1 (10 ng/ml) for 4 h were added to the medium. The conditioned media was collected at 24 h TGF-β1 stimulation and/or I-Smad7 adenovirus administration. The expression of MMP-2, MT1-MMP, and TIMP-2 in the I-Smad7-infected cells was examined by Western blot analysis. Sample proteins (i.e., conditioned media) were prepared under reducing con-
dition, and separated on 10% SDS-PAGE (for MMP-2 and MT1-MMP) or 12% SDS-PAGE (for TIMP-2). Separated proteins (40 μg) were electrically transferred on PVDF membranes (Bio-Rad). After the nonspecific background was blocked with 5% milk in TBS-T, PVDF membranes were incubated with the antibodies for anti-rabbit polyclonal MMP-2 (AB809; 1:1,000), MT1-MMP (AB8221; 1:1,000), and TIMP-2 (AB8107; 1:1,000) for 1 h at room temperature. After the membrane was washed with Tween 20 buffer, the membranes were incubated with goat anti-rabbit HRP (1:10,000) for 1 h at room temperature. The immunoreactive bands were then visualized by ECL Plus detection (Amersham) according to the manufacturer’s instructions and were developed on film. The even loading was confirmed by incubating membranes in Ponceau S solution (0.1% Ponceau S in 5% acetic acid; Sigma).

Hydroxyproline assay. To test the total collagen production, pri-
mary cardiac myofibroblasts were seeded in equal numbers in
100-mm cell culture dishes and grown until confluent in DMEM-F-12 with 10% FBS and 50 μg/ml ascorbic acid. Cells were starved for 48 h before the addition of TGF-β1 and I-Smad7 adenovirus. After 72 h treatment, cells were scraped on the cell culture medium of 100-mm dishes. Both the cell lysate and culture medium were removed and placed in test tubes, and net collagen production was determined with a hydroxyproline-based assay as described previously (56). The result was expressed as degree of increase against values from control cardiac myofibroblasts.

[3H]Thymidine incorporation. Proliferation assays were done on cells cultured on 24-well plates at a density of 0.2 × 105 cells/well (counted with a hemocytometer) in DMEM-F-12 with 10% FBS. After the cells reached ~60% confluence, medium was replaced with DMEM-F-12 + 0% FBS, 10% FBS, or 2% FBS. Cells were infected with 100 MOI I-Smad7 adenovirus vector for 24 h. Other groups of cells were starved for 24 h and then 10 ng/ml of TGF-β1 were added to the media for 24 h. Cells were then pulsed for 4 h at room temperature with 2.0 μCi/ml [methyl-3H]thymidine (Amersham Phar-
Reagents. Primary antibodies against P-ERK, P-JNK, and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The primary antibody specific to phosphorylated Src465/467 R-Smad2 was obtained from Calbiochem (San Diego, CA). Total R-Smad2 primary antibody was purchased from Cell Signaling (Beverly, MA). The primary antibodies against MMP-2, MT1-MMP, and TIMP-2 were obtained from Chemicon (Temecula, CA). HRP-labeled anti-mouse and HRP-labeled anti-rabbit secondary antibodies were purchased from Bio-Rad. TGF-β1 peptide was purchased from R&D Systems (Minneapolis, MN).

Statistics. All values are expressed as means ± SE. One-way ANOVA followed by Student-Newman-Keuls post hoc analysis was used to compare the differences among multiple groups (SigmasStat, Point Richmond, CA). Significant differences among groups were defined by P < 0.05.

RESULTS

Net collagen content in cardiac myofibroblasts overexpressing ectopic I-Smad7. To determine if the protein level of the main component of extracellular matrix, i.e., collagen, is altered by overexpression of ectopic I-Smad7, total collagen content was determined in adult cardiac myofibroblasts using hydroxyproline assay. As shown in Fig. 1, data were expressed as degree of increase units against the mean control value for each experiment. Exposure of cardiac myofibroblasts to TGF-β1 (5 ng/ml) for 3 days was associated with marked increase of total collagen production compared with the control group. However, infection of the adult cardiac myofibroblasts with I-Smad7 adenovirus resulted in significant attenuation of net collagen production in the absence and presence of TGF-β1 treatment compared with LacZ-infected myofibroblasts.

Prolonged activation of P-Smad2 by TGF-β1 in adult primary cardiac myofibroblasts. Although it is known that TGF-β1 functions to activate R-Smads in a variety of cell types, we examined the specific activation pattern of phosphorylated Smad2 in primary cardiac myofibroblasts. The cells were cultured with TGF-β1 at indicated time points (5 min, 30 min, 1 h, 6 h, and 12 h) following starving in DMEM-F-12 containing 0% serum for 48 h. Phosphorylated Smad2 (P-Smad2) and total Smad2 expression were measured by Western blot analysis (Fig. 2A). We found that the expression level of P-Smad2 in quiescent cardiac fibroblasts is fairly low, and it was observed to increase at 5 min TGF-β1 stimulation. It peaked at 30 min TGF-β1 treatment and remained activated until 12 h treatment of TGF-β1. Dose-dependent Smad2 activation by TGF-β1 was also investigated in the current study (Fig. 2B); however, we found no significant alteration of P-Smad2 expression in cardiac myofibroblasts stimulated with different doses of TGF-β1 ranging from 0.5 to 10 ng/ml.

Blockade of expression of P-Smad2 by ectopic I-Smad7 in cultured cardiac myofibroblasts. As an intracellular negative regulator of TGF-β signal, I-Smad7 was reported to inhibit the phosphorylation of Smad2 in a variety of cell types. To determine the effect of ectopic I-Smad7 on the expression and activation of Smad2 in cultured cardiac myofibroblasts, protein was extracted from myofibroblasts following overexpression of I-Smad7 via an adenovirus system. X-Gal staining showed >95% cells were positive in the infection of 100 MOI (data not shown). Furthermore, high-level expression of the protein in cardiac myofibroblasts was also confirmed by Western blot analysis using anti-flag antibody (data not shown). After adding I-Smad7 adenovirus to cells 24 h before TGF-β1 stimulation, we observed that ectopic overexpression of I-Smad7 was associated with a decrease in the synthesis of TGF-β1-induced P-Smad2 compared with LacZ controls, and this inhibitory effect is TGF-β1 stimulation time-dependent. As shown in Fig. 3, P-Smad2 was significantly elevated following
TGF-β1 stimulation at 1, 2, and 6 h. Although ectopic I-Smad7 decreased the expression of Smad2 in the absence of TGF-β1 treatment, it failed to block the activation of P-Smad2 induced by TGF-β1 at 1 h. However, at 6 h TGF-β1 treatment, this TGF-β1-mediated R-Smad2 activation process was markedly attenuated by forced expression of I-Smad7 in cardiac myofibroblasts. Moreover, the expression level of total Smad2 was not altered across untreated or I-Smad7- and LacZ-infected cultures. We found the attenuation of Smad2 activation by ectopic I-Smad7 was achieved in a dose-dependent manner.

Effect of overdriven I-Smad7 on mitogen-activated protein kinases activation in cardiac myofibroblasts. It is well known that TGF-β can activate mitogen-activated protein kinases (MAPK) signaling pathway, including JNK and ERK (2, 18). As important downstream effectors of the TGF-β signal pathway, they have also been implicated in TGF-β-regulated collagen deposition (15, 60). We suggest that the inhibitory role of I-Smad7 on collagen accumulation in cardiac myofibroblasts may be partially mediated by MAPKs. As indicated in Fig. 5, we found markedly decreased expression of P-ERK in I-Smad7 adenovirus-infected cells compared with control LacZ-expressing cells. However, the activation of ERK induced by 1 h TGF-β1 stimulation was not completely blocked by ectopic I-Smad7 protein. Additionally, forced expression of I-Smad7 exerted no effect on activation of JNK in cardiac myofibroblasts in the presence or absence of 1 h TGF-β1 treatment.

Fig. 3. Representative Western blot analysis of P-Smad 2 expression in primary cardiac myofibroblasts infected with adenoviral I-Smad7 [S7, 100 multiplicity of infection (MOI)] in the presence and absence of TGF-β1 treatment (T; 10 ng/ml). A: expression of P-Smad 2 in adenovirus-infected myofibroblasts treated with TGF-β1 at 1 h. B: expression of P-Smad 2 in adenovirus-infected myofibroblasts treated with TGF-β1 at 2 h. C: expression of P-Smad 2 in adenovirus-infected myofibroblasts treated with TGF-β1 at 6 h. Constitutive expression P-Smad 2 is dramatically decreased in ectopic I-Smad7-infected cells compared with cells infected with control LacZ adenovirus. However, the TGF-β1-induced expression of P-Smad 2 was attenuated in myofibroblasts stimulated with TGF-β1 at 6 h.

Fig. 4. Dose-dependent effect of I-Smad7 on the activation of receptor-regulated (R)-Smad 2 in cardiac primary myofibroblasts.

Fig. 5. Representative Western blot analysis of c-Jun NH2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) activation in cardiac myofibroblasts infected with adenoviral I-Smad7 (S7, 100 MOI) in the presence and absence of TGF-β1 treatment (10 ng/ml).
TIMP-2, immunodetection of these proteins was performed on the same samples to further investigate the possibility that alterations in these protein expressions may lead to the accelerated processing of pro-MMP-2 into MMP-2. We observed a significant increase of pro-MMP-2 in the culture medium of cardiac myofibroblasts infected with I-Smad7 adenovirus compared with LacZ-infected controls (Fig. 6). However, no difference regarding synthesis of TIMP-2 was defined in cells overexpressing I-Smad7.

**Influence of ectopic I-Smad7 on expression of p27 and phospho-Rb.** Overexpressed I-Smad7 was associated with a significant increase of the p27-to-β-tubulin ratio in myofibroblasts in 2% FBS-DMEM vs. values derived from Lac-Z-infected controls, noninfected 10% serum-treated, and 2% noninfected serum-treated cells (Fig. 7). Conversely, ectopic I-Smad7 was associated with a significant decrease of the ratio phospho-Rb/β-tubulin protein band intensity in myofibroblasts (in 2% FBS-DMEM media) vs. values of Lac-Z-infected cells in 2% serum as well as noninfected 2 and 10% serum cultured cells (Fig. 7). Thus I-Smad7 overexpression was associated with a decreased level of phospho-Rb protein under these current culture conditions.

**Ectopic I-Smad7 is associated with decreased cardiac myofibroblast proliferation.** The effect of ectopic I-Smad7 on primary myofibroblast [3H]thymidine incorporation in low-serum conditions (FBS-DMEM-F-12) was tested. Thymidine incorporation was significantly inhibited by I-Smad7 vs. LacZ-infected controls in 2% FBS-DMEM-F-12 as well as noninfected 2% serum-treated and 10% serum-treated cells (Fig. 8).

**DISCUSSION**

Excessive collagen deposition or pathological fibrosis is an important contributor to LV dysfunction. The abnormal deposition of collagen in various etiologies of heart disease has a dual effect. Although its tensile strength protects the injured heart from dilatation and rupture of the infarcted region, excessive matrix deposition leads to cardiac fibrosis, which impairs the already attenuated cardiac function. Therefore, antifibrotic agents that target steps in collagen synthesis and degradation pathways represent promising strategies for these diseases. TGF-β is an important player in the fibrosis of a variety of tissues, including cardiac tissues. Previous evidence of blockade of TGF-β signal with antisense, neutralizing antibody or decorin has highlighted the therapeutic potential by targeting this molecule in pathological fibrosis (1, 19, 27). Following the binding of the TGF-β ligand to TβRII, TβRI is activated, which leads to the phosphorylation of R-Smads, including Smad2 and Smad3, and subsequently initiates the transduction of TGF-β signal. On the other hand, activated TβRI receptor also functions to induce the expression of I-Smad7, which antagonizes TGF-β signaling either by competing for binding of R-Smads to TβRI, thereby blocking R-Smads from interacting with the receptor (14, 52), or by increasing ubiquitin-mediated degradation of TGF-β receptor (58). Although touted as a putative means to block canonical TGF-β signaling (58), information of the effects of I-Smad7 expression is incomplete.

Some lines of evidence have pointed to the negative regulatory role of I-Smad7 in the activation of R-Smads in a variety of tissues. For example, overexpression of I-Smad7 was shown to attenuate Smad2/3-mediated inhibition of embryonic morphogenesis (63, 64). In bleomycin-induced lung fibrosis, ectopic I-Smad7 was also associated with inhibition of Smad2 phosphorylation, decreased type I procollagen mRNA, and net collagen production (36). Moreover, blockade of activation of Smad2 and downregulation of collagen type I, III, and IV was observed in renal tubular epithelial cells (50) and smooth muscle cells (24) overexpressing I-Smad7. In hepatic stellate cells, ectopic expression of I-Smad7 also leads to abrogation of Smad2 activation (8). Data from our laboratory have shown that I-Smad7 expression was decreased in both scar tissue and remnant tissue in post-MI rat hearts, accompanied by decreased cytosolic expression of phosphorylated Smad2 (58). Although this in vivo study implies that abnormal expression of I-Smad7 may play a role in cardiac fibrosis, the underlying mechanisms remain unclear.

In this study, we found that the marked prolonged activation of Smad2 by TGF-β1 was effectively blocked by ectopic expression of I-Smad7 in both a dose-dependent and time-dependent manner, suggesting I-Smad7 prevented TGF-β1-mediated phosphorylation of Smad2 in cardiac myofibroblasts. However, this inhibitory effect was specifically on the activation process, given the fact that adenovirus-mediated I-Smad7 overexpression did not alter total Smad2 expression. Moreover, consistent with our published in situ immunostaining analysis showing decreased expression of both collagen type I and type III in primary cardiac myofibroblasts overexpressing ectopic I-Smad7 (58), we now demonstrate that overdriven I-Smad7 significantly attenuated total collagen synthesis induced by
Fig. 7. Representative Western blot of p27 expression (A) and pRb expression (B). Histogramic representation of band absorbance ratio in adult primary cultured cardiac myofibroblasts (P1) infected with 100 MOI I-Smad7 adenovirus for 24 h in the presence or absence of 2% FBS-DMEM-F-12 and LacZ control virus (in 2% FBS-DMEM-F-12). P ≤ 0.05 vs. 2% FBS-DMEM-F-12 control (*), vs. LacZ (#), and vs. 10% FBS-DMEM-F-12 (γ). Data are expressed as means ± SE for a pool of 5 experiments. Data are quantified by densitometric scanning. C: p27/β-tubulin relative band absorbance. D: pRb/β-tubulin relative band absorbance.
Together the data indicate that blockade of Smad2 activation by overexpression of exogenous I-Smad7 may result in inhibition of TGF-β1-induced collagen deposition. This association between Smad2 activation and synthesis of collagen also supports that phosphorylated Smad2 may play a major role in the transcriptional regulation of collagen promoter, a process requiring nuclear translocation of P-Smad2. To further investigate whether nuclear translocation of P-Smad2 was also involved in the regulation of collagen production by I-Smad7, we performed immunofluorescence analysis of Smad2 localization following I-Smad7 adenovirus administration. However, no significant inhibition of the nuclear translocation of P-Smad2 by overexpression of I-Smad7 was found in adult cardiac myofibroblasts (data not shown). Therefore, attenuation of collagen synthesis in cardiac myofibroblasts caused by overdriven I-Smad7 may be mediated by decreased expression of P-Smad2 and not by blockade of nuclear translocation of P-Smad2.

In addition to traditional Smad-mediated transcriptional activation, TGF-β-induced activation of the MAPK pathways has also been shown to play a role in TGF-β-responsive biological consequences, including collagen deposition (40, 55). Although cross talk between Smad proteins and MAPKs has been implicated in the modulation of Smad phosphorylation and translocation, MAPK pathway activation may precede and be independent from Smad protein activation (10). To address the possible effect of I-Smad7 on the activation of JNK and ERK kinases, we performed Western blot analysis of these MAPKs in cardiac myofibroblasts overexpressing I-Smad7 protein. Although we observed attenuation of P-ERK expression by overdriven I-Smad7, the 1-h-TGF-β1-induced activation of ERK was not ablated by I-Smad7 protein, suggesting that blockade of ERK activation via I-Smad7 may be achieved in a TGF-β-independent manner. In addition to the induction of JNK by TGF-β1, ectopic expression of I-Smad7 has been indicated to synergistically cause a significant stimulation of JNK activity in Mv1Lu cells (32). However, in cardiac myofibroblasts overexpressing I-Smad7 proteins, we did not observe induction of JNK activity either by TGF-β1 or overdriven I-Smad7 protein. Thus the data indicate that ERK may play a more important role than JNK in I-Smad7-mediated downregulation of collagen production in cardiac myofibroblasts.

MMPs play a crucial role in the remodeling process of myocardium following MI (47, 51). MMP-2, also known as gelatinase A, is important in maintenance of cardiac matrix, since this metalloproteinase may process denatured collagen and gelatin. The mechanism underlying activation of pro-MMP-2 is known to involve other MMPs such as MMP-1 (7), autoactivation (3), and the urokinase plasminogen system (33). MMP-2 activation is also mediated by MT-MMP1 (41), which requires the assistance of TIMP-2, which functions as a link between MT1-MMP and pro-MMP-2 to form a ternary complex at the cell surface (48). Few studies have revealed the importance of I-Smad7 in the activation process of MMPs, and we found that ectopic I-Smad7 protein was associated with accelerated activation of pro-MMP-2 in cardiac myofibroblasts. The concomitant increase of MT1-MMP expression is notable, since it may contribute to the proteolytic cleavage of pro-MMP-2 to MMP-2. Although TIMP-2 is also necessary for the proteolysis of MMP-2 (4), the role of TIMP-2 in cardiac myofibroblasts may be secondary, since its expression is unchanged in the presence of overdriven ectopic I-Smad7 when compared with controls. Nevertheless, because the binding of TIMP-2 to MMP-2 as well as MT1-MMP affects the enzymatic activity of MMP-2 (6), further study may be needed to clarify the precise role of TIMP-2 in the regulation of MMP-2 activation by I-Smad7.

MMP expression can be modified at the transcriptional level by a variety of physiological signals, including growth factors and cytokines. Despite the induction of MMP-2 by I-Smad7, exposure of the quiescent cardiac myofibroblasts to TGF-β1 was not associated with any marked alteration of MMP-2 activity as observed in other cell types (9, 57). As mentioned above, I-Smad7 has been shown to be able to exert a TGF-β-independent regulatory effect on gene transcription (38), and thus whether activation of MMP-2 by I-Smad7 acts via the TGF-β signal remains unclear. Because we found that the treatment of myofibroblasts with TGF-β led to upregulation of MT1-MMP, the effect of overexpression of I-Smad7 parallels that of the ligand. This could be caused either by complete disruption of the TGF-β-I-Smad7 regulatory loop, or more
likely, by a TGF-β-independent I-Smad7 signaling path. The latter possibility seems plausible in view of the recent suggestion that I-Smad7 is complex, and possibly pleuripotent (12). We suggest that overdriven I-Smad7 may induce the disruption of MT1-MMP-dependent processing of pro-MMP2, partially contributing to downregulated collagen synthesis.

The application of agents that target myofibroblast cell cycle progression and thus proliferation represent promising strategies for managing global cardiac fibrosis. The cyclin-dependent kinase inhibitors are important in cell cycle progression in normal cells (42, 53). In cultured myofibroblasts, we found that ectopic I-Smad7 is associated with a significant increase in p27 expression. Because p27 inhibits the formation of the cdk2/cyclin E complex, it is a salient marker for cell cycle inhibition. We also found that the phospho-Rb expression was significantly decreased by I-Smad7 overexpression in myofibroblasts, thus potentially limiting cell cycle progression at the G1 phase as previously suggested (17, 28). In combination with data showing reduced proliferation in transfected cells, we suggest that altered p27 and phospho-Rb expression may rate-limiting factors in regulating the cell cycle in myofibroblasts when influenced by ectopic I-Smad7.

In conclusion, we provide evidence that ectopic expression of I-Smad7 appears to inhibit TGF-β-induced collagen production in primary cardiac myofibroblasts. This antifibrotic effect of I-Smad7 involved both TGF-β-dependent activation of Smad2 and TGF-β-independent activation of MMP-2. Finally, overdriven I-Smad7 is also associated with inhibition of proliferation of myofibroblasts. Modification of TGF-β signaling by ectopic I-Smad7 may allow for the development of therapeutical strategies to attenuate post-MI cardiac fibrosis.

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