Transforming Growth Factor β 1-mediated Activation of the Smooth Muscle α -Actin Gene in Human Pulmonary Myofibroblasts Is Inhibited by Tumor Necrosis Factor- α via Mitogen-activated Protein Kinase Kinase 1-dependent Induction of the Egr-1 Transcriptional Repressor

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Transforming growth factor (TGF) β 1 is a mediator of myofibroblast differentiation in healing wounds in which it activates transcription of the smooth muscle α -actin (SM α A) gene via dynamic interplay of nuclear activators and repressors. Targeting components of TGF β 1 signaling may be an effective strategy for controlling myofibroblasts in chronic fibrotic diseases. We examined the ability of proinflammatory tumor necrosis factor (TNF)- α to antagonize TGF β 1-mediated human pulmonary myofibroblast differentiation. TNF- α abrogated TGF β 1-induced SM α A gene expression at the level of transcription without disrupting phosphorylation of regulatory Smads. Intact mitogen-activated protein kinase kinase (Mek)–extracellular signal-regulated kinase (Erk) kinase signaling was required for myofibroblast repression by TNF- α via induction of the early growth response factor-1 (Egr-1) DNA-binding protein. Egr-1 bound to the GC-rich SPUR activation element in the SM α A promoter and potently suppressed Smad3- and TGF β 1-mediated transcription. Reduction in Smad binding to the SM α A promoter in TNF- α -treated myofibroblasts was accompanied by an increase in Egr-1 and YB-1 repressor binding, suggesting that the molecular mechanism underlying repression may involve competitive interplay between Egr-1, YB-1, and Smads. The ability of TNF- α to attenuate myofibroblast differentiation via modulation of a Mek1/Erk/Egr-1 regulatory axis may be useful in designing new therapeutic targets to offset destructive tissue remodeling in chronic fibrotic disease.

INTRODUCTION

Myofibroblasts are specialized stromal cells that synthesize interstitial collagens and contain a smooth muscle α -actin (SM α A)-enriched contractile apparatus designed to generate tensile force needed for wound closure and tissue healing (Skalli and Gabbiani, 1988; Ronnov-Jessen and Petersen, 1996; Zaleski and Shi, 1997; Gabbiani, 2003; Desmouliere *et al.*, 2005; Tomasek *et al.*, 2006). Transforming growth factor (TGF) β 1 is the principle mediator of myofibroblast differentiation in healing wounds where it accumulates in granulation tissue in activated form during leukocyte infiltration and potently stimulates transcription of the type I α 2 collagen subunit (COL1 α 2) and SM α A genes (Becker *et al.*, 2000; Massague and Wotton, 2000; Cogan *et al.*, 2002; Higashi *et al.*, 2003; Grotendorst *et al.*, 2004; Leask and Abraham, 2004; Subramanian *et al.*, 2004; Zhang *et al.*, 2005). Smad proteins 2 and 3 are activated by TGF β 1 receptor engagement and enter the nucleus in which they directly bind SM α A and

COL1 α 2 promoter DNA. Smad3 also reportedly forms additional off-promoter complexes with DNA-binding repressor proteins to govern transcriptional output of the COL1 α 2 gene in TGF β 1-activated myofibroblasts (Higashi *et al.*, 2003). Poor termination of TGF β 1-mediated myofibroblast differentiation could contribute to chronic fibroproliferative disease and excessive scar formation in the injured lung, heart, liver, kidney, and skin potentially causing pathobiologic phenomena referred to as “endless healing” (Tomasek *et al.*, 2002). In particular, pulmonary fibrosis typically is associated with chronic myofibroblast activation, deposition of interstitial collagen and SM α A, and dysfunctional parenchymal remodeling with a mortality of 50% at 5 y after diagnosis (Zhang *et al.*, 1996; Kunkel *et al.*, 2003; Noble, 2003; Phan, 2003; Selman and Pardo, 2003; Daniels *et al.*, 2004; Marshall *et al.*, 2004; Willis *et al.*, 2006; Wynn, 2007). Similarly, cardiac transplant chronic rejection is associated with pervasive interstitial myofibroblast accumulation and perivascular fibrosis that ultimately impairs heart graft perfusion, performance, and long-term survival (Weber, 1995; Armstrong *et al.*, 1997a,b,c; Subramanian *et al.*, 1998, 2002; Rosenkranz, 2004).

Our laboratory has identified several *cis*-regulatory elements in SM α A promoter that are highly conserved among vertebrate species and function as binding sites for multiple

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positive and negative *trans*-acting transcription factors (Foster *et al.*, 1992; Cogan *et al.*, 1995; Sun *et al.*, 1995). Among these factors, Pur α , Pur β , and YB-1 show preferential binding to single-stranded DNA and function as transcriptional repressors (Kelm *et al.*, 1999, 2003; Carlini *et al.*, 2002; Zhang *et al.*, 2005; Knapp *et al.*, 2006) whereas others such as transcription enhancer factor (TEF) 1; serum response factor (SRF); Smads 2, 3, and 4; and Sp1/Sp3 represent serum- or TGF β 1-responsive transcriptional activators (Kelm *et al.*, 1999; Cogan *et al.*, 2002; Subramanian *et al.*, 2004). A DNA element referred to as SPUR recently was identified in the mouse as a key component in both basal and TGF β 1-dependent transcriptional activation of the SM α A gene through its ability to bind heteromeric complexes of the Sp1/Sp3 activators and Pur α /Pur β protein repressors (Subramanian *et al.*, 2004; Knapp *et al.*, 2006). SPUR DNA also encompasses a TGF β 1 control element shown to be an essential mediator of myofibroblast differentiation and dermal wound healing in the mouse (Tomasek *et al.*, 2005). Dynamic physical interplay between transcriptional repressors and activators at SPUR is governed by Smad proteins that are rapidly imported into the nucleus of TGF β 1-activated myofibroblasts in which they seem to neutralize repressor:activator interactions and derepress the SM α A promoter during the early stages of the wound healing process.

Clinical therapeutic strategies for managing myofibroblast differentiation by specifically targeting TGF β 1 signal transduction may help alleviate dysfunctional tissue remodeling in chronic fibrotic disease (Daniels *et al.*, 2004; Yingling *et al.*, 2004; Wang *et al.*, 2005). Although comparatively little is known about mechanisms of myofibroblast attenuation, one approach under study is based on the ability of proinflammatory cytokines such as interferon (IFN) γ and TNF- α to antagonize profibrotic TGF β 1 signaling (Verrecchia and Mauviel, 2004; Breitkopf *et al.*, 2006; Dooley *et al.*, 2006). TNF- α is a 17.5-kDa polypeptide produced by activated macrophages as well as injured stromal and epithelial cells (Verrecchia and Mauviel, 2004; Saika *et al.*, 2006). TNF- α mediates host immune responses to microbial infection, tissue injury, and inflammation and has been implicated in etiology of chronic diseases such as rheumatoid arthritis, osteoarthritis, viral myocarditis, and heart failure (Tracey and Cerami, 1993). In these pathological contexts, TNF- α fosters degradation of the extracellular matrix by inducing the production of stromal collagenases or by direct inhibition of type I collagen biosynthesis. TNF- α binding to its two cognate receptors TNFR-1 and TNFR-2 variably activates the nuclear factor- κ B (NF- κ B), c-Jun NH $_2$ -terminal kinase (JNK), and the p38 or Ras-Raf-mitogen-activated protein kinase (Mek)-extracellular signal-regulated kinase (Erk) mitogen-activated protein (MAP) kinase signaling pathways (Verrecchia *et al.*, 2002; Wajant *et al.*, 2003). The antifibrotic property of TNF- α was evident in transgenic mice where overexpression of this inflammatory cytokine was observed to markedly diminish pulmonary fibrosis induced by either TGF β 1 or bleomycin instillation. TGF β 1 and TNF- α reportedly exhibit opposite effects on nitric oxide production in cardiac myocytes, cytotoxic T cell survival, and transcription of the type I collagen gene expression required for tissue repair and remodeling. Last, TNF- α augments fibroblast and smooth muscle cell migration (Goetze *et al.*, 1999), a process that has been definitively linked to reduced expression and deployment of SM α A-enriched microfilaments (Ronnov-Jessen and Petersen, 1996).

In this report, we examined the hypothesis that proinflammatory TNF- α elicits antifibrotic responses that specifically block TGF β 1-dependent SM α A gene expression in differen-

tiated human pulmonary myofibroblasts. We discovered that TNF- α inhibits TGF β 1 signaling by activating Mek1 and Erk1/2 kinases needed for induction of the DNA-binding protein early growth response factor-1 (Egr-1). TNF- α -inducible Egr-1 was observed to bind the GC-rich SPUR element in the SM α A promoter and potently suppressed Smad-mediated transcription of this gene in TGF β 1-activated myofibroblasts. The data suggest that Egr-1 functionally resembles the Pur protein repressors that also target SPUR sequences in quiescent stromal fibroblasts, rendering them less efficient in the expression of SM α A protein needed for myofibroblast contractility. TNF- α also offsets SM α A gene activation in myofibroblasts by enhancing interaction of the YB-1 transcriptional repressor with promoter DNA resulting in partial displacement of profibrotic Smad activators. A better understanding of dynamic interplay between transcriptional activators and repressors that control myofibroblast differentiation may facilitate development of novel antifibrosis therapeutic agents.

MATERIALS AND METHODS

Human Fibroblast Culture and Protein Extraction Methods

Human pulmonary fibroblasts (hPFBs) were established in primary culture from enzyme-dispersed tissue fragments of human neonatal lung tissue obtained at autopsy and were the kind gift of Dr. Daren L. Knochel (Departments of Pharmacy and Internal Medicine, The Ohio State University, Columbus, OH). Fibroblasts were maintained up to passage 7 with little observed change in growth rate, morphology, or growth factor responsiveness in a 1:1 mixture of Ham's F-12 and DMEM (1.0 g/l D-glucose) supplemented with penicillin-streptomycin-antimycotic gentamicin (50 μ g/ml), and 10% heat-inactivated fetal bovine serum (Zhang *et al.*, 2005). All culture medium reagents were obtained from Invitrogen (Carlsbad, CA). Recombinant human TGF β 1 and human TNF- α (R&D Systems, Minneapolis, MN) were added to cell cultures for varying periods and doses as noted in the text. The metabolic inhibitors U0126, PD98059 (Cell Signaling Technology, Danvers, MA), SP600125 (A.G. Scientific, San Diego, CA), and SB203580 (Calbiochem, San Diego, CA) were used as noted in the text. To prepare whole cell protein extracts we followed our previously published methods (Cogan *et al.*, 2002; Subramanian *et al.*, 2004; Zhang *et al.*, 2005). In brief, cell monolayers were rinsed twice with Dulbecco's phosphate-buffered saline (PBS) and then scraped into 0.5 ml of radioimmunoprecipitation assay buffer (PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], and 0.5 mM dithiothreitol [DTT]). After gentle rocking for 15 min at 4°C, the cell lysate was collected at 10,000 $\times$ g for 10 min at 4°C. To prepare nuclear protein extracts, cell monolayers were washed twice with PBS, scraped into fresh PBS, sedimented at 3000 rpm, washed once more in PBS, and resuspended in 8 packed cell volumes of a hypotonic buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl $_2$, 10 mM KCl, 0.2 mM PMSF, and 0.5 mM DTT. Cells were allowed to swell in hypotonic buffer for 10 min on ice before transfer to a Dounce homogenizer for processing with a type B pestle. After centrifugation for 15 min at 4000 rpm, supernatants were removed and retained as the cytosolic protein fraction. The nuclei pellet was resuspended in 1/2 packed pellet volume of ice-cold, low-salt buffer containing 20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl $_2$, 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT. An equal volume of high salt buffer containing 20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl $_2$, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT was added, and the preparation was gently mixed for 30 min at 4°C. Nuclear remnants were removed by centrifugation at 14,500 rpm for 30 min, and the supernatant was dialyzed at 4°C for 3 h against 50 volumes of dialysis buffer containing 20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT. Dialyzed nuclear protein was clarified by centrifugation at 14,500 rpm for 20 min, divided into aliquots, and stored at -80°C for use in biochemical assays.

DNA Binding Assay

Synthetic oligonucleotide probes used in this report were derived from selected sequences present in the human SM α A promoter (Cogan *et al.*, 1995; Sun *et al.*, 1995; Kelm *et al.*, 1996; Subramanian *et al.*, 2004; Zhang *et al.*, 2005). Reaction mixtures containing protein extract (100 μ g of protein) and 3'-biotinylated oligonucleotides (100 pmol; Integrated DNA Technologies, Coralville, IA) were incubated in a buffer containing poly(dI-dC), 10 mM Tris, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 0.12 mM PMSF, and 4% glycerol. Biotinylated DNA:protein complexes were incubated for 30 min

with a 0.6-ml aliquot of streptavidin-coated paramagnetic particles (Promega, Madison, WI). After washing four times with buffer containing 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 100 mM NaCl, the bound protein was eluted using 2× SDS protein denaturing buffer, size fractionated on 10% SDS-polyacrylamide gels, and then electrophoretically transferred to nitrocellulose membranes (Whatman Schleicher and Schuell, Keene, NH) for immunoblot evaluation. Protein blots were blocked at 4°C in Tris-buffered saline (TBS) containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 3% (wt/vol) nonfat dry milk, and 0.5% bovine serum albumin and incubated with the indicated antibodies for 90 min at room temperature with gentle rocking. For each of the primary antibodies used in our analysis, preimmune serum or antibodies presaturated with blocking peptides (in the case of peptide epitope antibodies for YB-1) were used as negative controls and protein extracts prepared from mouse heart or AKR-2B mouse embryonic fibroblasts known to contain SMαA gene repressors and activators (Cogan *et al.*, 1995; Sun *et al.*, 1995; Kelm *et al.*, 1999) were used as positive controls and to monitor antibody binding specificity. Blots were washed four times at room temperature over a 20-min period in TBS containing Tween 20 (0.05% vol/vol) and then incubated for 45 min with a species-appropriate, horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000; GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kingdom). The blots were washed as described above, processed for antibody visualization by chemiluminescence (GE Healthcare), and imaged onto Biomax film (Eastman Kodak, Rochester, NY). Commercial antibodies used in this study were specific for Smad2/3 (rabbit polyclonal antibody, 1:1000; Cell Signaling Technology), Egr-1 (clone 44D5 rabbit monoclonal antibody [mAb], 1:1000; Cell Signaling Technology; or C-19 rabbit polyclonal antibody, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), Smad7 (goat polyclonal antibody, 1:250; Abcam, Cambridge, MA), phosphorylated Smad2 (Ser 465/467-specific, rabbit polyclonal antibody, 1:1000; Cell Signaling Technology), SMαA (EPOS anti-human smooth muscle α-actin/HRP, clone 1A4, 1:200; Dako North America, Carpinteria, CA), Erk1/2 (anti-MAP kinase p44/p42, rabbit polyclonal antibody, 1:1000; Cell Signaling Technology), phosphorylated Erk1/2 (anti-MAP kinase phospho-p44/p42, T202/Y204-specific, clone E10 mouse mAb, 1:1000; Cell Signaling Technology), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (anti-GAPDH/HRP, 1:200; Santa Cruz Biotechnology). Rabbit polyclonal antibodies specific for YB-1 (anti-YB1 M85-110 and anti-YB1 M276-302, 1:2000) were described in our previous reports (Kelm *et al.*, 1999). The two Egr-1-specific antibodies gave slightly different results on immunoblots owing to Egr-1 size variation, possibly because of protein phosphorylation. However, antibodies from both vendors reacted with a dominant protein band that migrated on SDS gels with a molecular size expected for authentic Egr-1.

Mammalian Protein Overexpression Plasmids, Cell Transfection, and Reporter Gene Assay

Monolayers of hPFBs maintained at 40–50% confluence were transfected as described previously (Zhang *et al.*, 2005). Optimized mixtures of the SMαA promoter: reporter fusion plasmids (VSMP4) with or without mammalian expression plasmids encoding various transcriptional regulatory proteins were transfected using the Mirus (Invitrogen) transfection reagent and protocol provided by the manufacturer. Transfection efficiencies using these methods and reagents typically were in the range of 40–50% (Zhang *et al.*, 2008). All plasmids used in this study were purified using QIAGEN preparative resin and a protocol provided by the manufacturer (QIAGEN, Valencia, CA). The Mek1-SSED and Mek1-K97M expression plasmids were kindly provided by Dr. Natalie Ahn (University of Colorado, Boulder, CO), the Egr-1 expression plasmid was a gift from Dr. Vikas P. Sukhatme (Harvard Medical School, Boston, MA), and the Smad2 and Smad3 expression plasmids were kindly provided by Dr. J. Horowitz (North Carolina State University, Raleigh, NC). Forty-eight hours after transfection, cells were washed three times with cold PBS and then lysed using chloramphenicol acetyltransferase (CAT) enzyme-linked immunosorbent assay (ELISA) lysis buffer (Roche Applied Science, Indianapolis, IN). Lysates were clarified by centrifugation at 14,000 × g for 10 min at 4°C, and the protein concentration of CAT protein-enriched supernatants determined by the bicinchoninic acid assay (Pierce Chemical, Rockford, IL). Equivalent amounts of supernatants were analyzed on immunoblot to verify overexpression of the various plasmid-encoded transcriptional regulatory proteins in transfected cells and expression of the SMαA promoter-driven CAT reporter gene was quantified using a commercial CAT-ELISA kit (Roche Applied Science). Reporter gene expression was normalized with respect to total cell protein and transfections were performed in triplicate and repeated three to five times. We did not observe discernible differences in cell size, growth rate, or yield of protein extract between cell preparations transfected with the various overexpression plasmids used in the study. Data sets were subjected to analysis of variance to assess statistical significance set at $p < 0.05$.

RNA Extraction, Real-Time Quantitative Polymerase Chain Reaction (PCR), and Chromatin Immunoprecipitation (ChIP)

Total RNA was extracted from hPFBs by using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, and 1-μg aliquots were reverse-

transcribed using SuperScript First Strand Synthesis System for reverse transcription (RT)-PCR kit (Invitrogen). PCR amplification was performed in a total volume of 50 μl by using TaqMan PCR core reagent kit (Applied Biosystems, Foster City, CA) with primers and probes specific to the human SMαA promoter as follows: forward primer, 5'-CCCTGCTCTGCTCTAGC-3'; reverse primer, 5'-GAATGGAGGCGCTGATCC-3'; and TaqMan probe, 5'-(FAM)-ACAACGTGAACGTTTTC-(MGBNFQ)-3'. A probe specific for 18S rRNA also was used as a control. Samples were processed for real-time quantitative PCR by using the ABI PRISM 7700 sequence detection system (Applied Biosystems). ChIP was performed using a commercial ChIP kit (Millipore, Billerica, MA) following the manufacturer's protocol. Briefly, monolayers of subconfluent hPFBs were exposed to 1% formaldehyde in PBS for 10 min, rinsed in PBS, and the fixed cells were collected into 200 μl of SDS lysis buffer supplemented with proteases inhibitors. After incubation on ice for 10 min, the lysates were sonicated using optimized conditions to obtain sufficient DNA shearing, diluted 10-fold into ChIP dilution buffer, pre-cleared with salmon sperm DNA:protein A beads for 30 min at 4°C, and incubated overnight at 4°C with anti-Egr-1 antibody. The material immunoprecipitated with anti-Egr-1 antibody was then collected on salmon sperm DNA:protein A beads during a 1-h incubation, washed with low-salt buffer followed by high-salt buffer, LiCl buffer, and TE. Protein:DNA complexes were eluted from the protein A beads by exposure to 5 M NaCl for 4 h at 65°C and treated with protease K, EDTA, and Tris-HCl for 1 h at 45°C. DNA was extracted using phenol:chloroform and precipitated with isopropanol. PCR was performed using primers specific for the SMαA promoter (forward strand, 5'-ACAGGCTCAAGTCTGTCTTTGCTC-3' and reverse strand, 5'-CCAAATCCCTGCAGATGGTGTCTT-3') by using the following conditions: 95°C for 4 min, 40 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 30 s, and a final extension phase of 72°C for 2 min. The appearance of an expected 230 base pairs PCR product in the various ChIP preparations was assessed by gel electrophoresis on 1.5% agarose.

RESULTS

TNF-α Inhibits TGFβ1-inducible SMαA Gene Activity in Human Pulmonary Fibroblasts without Inducing Smad7 or Blocking Smad2 Phosphorylation

We previously showed by immunoblot and immunofluorescence microscopy that TGFβ1 substantially increased SMαA mRNA and protein expression in early passage human pulmonary fibroblasts (Zhang *et al.*, 2005). As shown in Figure 1a, TNF-α (10 ng/ml) provided to hPFBs 2 h before treatment with TGFβ1 (5 ng/ml) markedly reduced the accumulation of SMαA protein relative to the level of expression noted in cells treated with TGFβ1 alone. Inhibition also was observed if TNF-α was added concurrently with exposure of the cells to TGFβ1 (data not shown). Immunofluorescence microscopic assessments of untreated control preparations of hPFBs incubated with the 1A4 SMαA-specific antibody revealed that only ~3% of these cells exhibited discernible expression of SMαA protein above that seen in control preparations that omitted the primary antibody. Supportive of immunoblot data shown in Figure 1a, exposure of the cells to a 48-h treatment with TGFβ1 resulted in strong induction of SMαA in ~90% of the cells, whereas dual treatment with both TGFβ1 and TNF-α reduced SMαA expression in these cells to background levels comparable to that observed in untreated control preparations (data not shown). Moreover, evaluation of cell number by using 4,6-diamidino-2-phenylindole nuclear staining showed that neither TGFβ1 nor TNF-α had substantial effect on cell abundance or survival at the doses used in this study over the course of a 48-h observation period. RT-PCR analysis revealed that accumulation of SMαA mRNA in TGFβ1-treated hPFBs was significantly blunted by TNF-α (Figure 1b). TGFβ1 also stimulated a fivefold increase in transcription from a VSMP4 SMαA promoter:CAT reporter fusion gene construct in transfected hPFBs, but this increase was significantly attenuated in the presence of TNF-α (Figure 1c). This observation suggested that the DNA sequence determinants for TNF-α-mediated repression of TGFβ1 response in hPFBs were contained within the same region of the SMαA core promoter previously shown to contain two TGFβ1-inducible elements referred to as

Figure 1. TNF- α suppresses TGF β 1-dependent production of SM α A protein and mRNA in human pulmonary myofibroblasts. (a) hPFBs were pretreated with TNF- α (10 ng/ml) for 2 h followed by exposure to TGF β 1 (5 ng/ml) for another 48 h after which SM α A expression in cell extracts was examined by immunoblot using an anti-SM α A antibody. Blots were reprobed with anti-GAPDH antibody to verify equivalent protein loading. (b) hPFBs were similarly pretreated with TNF- α but harvested after a 22-h exposure to TGF β 1 for isolation of total RNA. SM α A mRNA levels were quantitated by real-time PCR analysis using primers and TaqMan probe specific to the SM α A sequence. (c) CAT ELISA was performed on hPFBs transfected with VSMP4 after a 24-h exposure to TGF β 1 alone or TGF β 1 plus a 2-h pretreatment with TNF- α . Asterisks denote statistically significant differences between TGF β 1 treatments in the absence and presence of TNF- α ($p < 0.05$).

SPUR and TGF β 1-hypersensitive region (THR) that are located between -59 and -28 and -170 and -150, respectively. Together, the observed effects of TNF- α on hPFBs was suggestive of a rapid and dominantly acting inhibitory mechanism capable of neutralizing de novo transcriptional activation of the SM α A promoter by TGF β 1.

In some types of murine embryonic fibroblasts, TNF- α reportedly inhibits TGF β 1 signaling through up-regulation of Smad7 via activation of the NF- κ B signaling pathway (Bitzer *et al.*, 2000; Verrecchia *et al.*, 2002). Smad7 is an inhibitory Smad protein that forms a stable complex with

Figure 2. TNF- α neither augments expression of Smad7 nor blocks accumulation of phosphorylated Smad2 in human pulmonary myofibroblasts. (a) Immunoblot showing unaltered expression of Smad7 in TNF- α -treated hPFBs for periods up to 8 h. Similarly, b shows that Smad2 phosphorylation in TGF β 1-activated myofibroblasts was unaffected by TNF- α . Cells were pretreated with TNF- α (10 ng/ml) for 2 h before the addition of TGF β 1 (5 ng/ml) for an additional 15 min. GAPDH immunoblots are presented to indicate equivalent sample protein loadings on the gels.

the type I TGF β 1 receptor, thus interfering with the phosphorylation and subsequent nuclear import of Smad2: Smad4, and Smad3:Smad4 transcriptional activation complexes (Massague and Wotton, 2000; Shi and Massague, 2003). However, induction of Smad7 by TNF- α may not occur in all cell types. For example, TNF- α -dependent activation of NF- κ B actually inhibited Smad7 expression in human embryonic kidney 293 cells (Nagarajan *et al.*, 2000), whereas Smad7 levels did not noticeably increase in human dermal fibroblasts or chondrocytes after treatment with TNF- α (Verrecchia *et al.*, 2000; Huang *et al.*, 2002; Roman-Blas *et al.*, 2007). To examine whether Smad7 expression in human pulmonary fibroblasts was altered by TNF- α , quiescent cells in low-serum medium were treated with 10 ng/ml TNF- α and then evaluated for Smad7 expression by immunoblot analysis. As shown in Figure 2a, no significant increase in Smad7 expression was detected over a 8-h observation period relative to the amount of GAPDH housekeeping protein. To determine whether the kinase function of the type I TGF β 1 receptor was impaired in TNF- α -treated hPFBs, we examined the kinetics of Smad2 phosphorylation in the presence and absence of TNF- α . Smad2 phosphorylation was detected in nuclear extracts prepared from hPFBs as early as 5 min and sustained for 2 h after exposure of cells to TGF β 1 (Figure 2b). However, the ability of the TGF β 1 receptor to mediate phosphorylation of receptor-regulated Smad2 was not significantly impaired by TNF- α (Figure 2b). Collectively, these observations suggest that TNF- α does not disrupt TGF β 1-mediated activation of the SM α A gene in hPFBs by altering positive or negative Smad signaling.

Erk1/2 MAP Kinase Is Required for TNF- α -mediated SM α A Gene Repression

TNF- α might indirectly block Smad-mediated responses by activating inhibitory MAP kinases that reduce Smad protein effectiveness as transcriptional activators in myofibroblasts. For example, TNF- α has been reported to inhibit TGF β 1 activation of collagen expression via the JNK pathway through the induction of *c-jun* that forms a complex with Smad3 and prevents its binding to certain *cis*-regulatory promoter controls elements as well as interaction with the p300 transcriptional activating protein (reviewed in Verrec-

Figure 3. Intact Mek/Erk signaling was required for TNF- α to repress activation of the SM α A gene by TGF β 1 in human pulmonary myofibroblasts. As shown in a, Quiescent hPFBs were pretreated with DMSO (vehicle), SP600125 (10 μ M, JNK inhibitor), or SB203580 (20 μ M, p38 inhibitor). (b) Cells were pretreated with vehicle, UO126 (10 μ M, Mek1/2 inhibitor), or PD98059 (50 μ M, Mek1 inhibitor). After 1 h, some preparations were pretreated with TNF- α (10 ng/ml) for another 2 h before adding TGF β 1 (5 ng/ml) to all preparations followed by a final 48-h incubation period. SM α A expression in whole cell lysates was examined by immunoblot analysis. GAPDH immunoblots are provided to indicate equivalent sample protein loadings on the gels.

chia and Mauviel, 2004). To examine this issue with specific regard to SM α A gene expression in neonatal human pulmonary fibroblasts, we used the small molecule inhibitor SP600125 to inhibit JNK in hPFBs before their exposure to TGF β 1 and TNF- α . The ability of TNF- α to block TGF β 1-mediated accumulation of SM α A protein was not significantly altered by SP600125, indicating that the JNK pathway may not be required for TNF- α -mediated repression in this particular human fibroblast lineage (Figure 3a). Small molecule inhibitors of the p38- and Mek1/2-mediated MAP kinase signaling cascades also were examined for their ability to influence the action of TNF- α on SM α A gene expression in TGF β 1-activated in pulmonary myofibroblasts. As shown in Figure 3a, the p38 inhibitor SB203580 did not abolish TNF- α -mediated repression. Interestingly, both SP600125 and SB203580 were able to restrict the overall magnitude of SM α A protein induction by TGF β 1 alone, suggesting that both JNK- and p38-dependent signaling may supplement Smads in mediating de novo activation of the SM α A gene and/or the general process of human pulmonary myofibroblast differentiation (Shi and Massague, 2003). In contrast, use of either the Mek1/2 inhibitor U0126 or the Mek1 inhibitor PD98059 blocked the ability of TNF- α to repress induction of SM α A by TGF β 1 (Figure 3b). These results suggested that intact Mek1/2-to-Erk1/2 signaling was required for full repression of the SM α A gene by TNF- α . To verify actual Erk kinase activation by TNF- α , we examined phosphorylated Erk1 and Erk2 intermediates in whole cell extracts prepared from TNF- α -treated hPFBs. As shown in Figure 4, Erk phosphorylation was observed within 5 min of exposure to TNF- α with peak accumulation of phosphorylated Erk1/2 occurring after 15 min.

To further validate the potential role of Mek1/2-to-Erk1/2 signaling in myofibroblast differentiation, hPFBs were co-transfected with the TGF β 1-responsive VSMP4 SM α A promoter:CAT reporter gene construct together with a mammalian expression plasmid encoding a constitutively active form of Mek1 kinase (SSED). SSED-Mek1 has been reported

Figure 4. Immunoblot analysis showing TNF- α -mediated activation of Erk 1 and 2 in human pulmonary fibroblasts. Quiescent hPFBs were treated with TNF- α (10 ng/ml) for up to 90 min, lysed, and examined for Erk1/2 phosphorylation by using a phospho-epitope-specific anti-Erk1/2 antibody. Total Erk1/2 protein levels in lysed cell preparations also was examined to verify that the increase in Erk1/2 phosphorylation was not simply the result of an increase in Erk1/2 protein availability which remain constant for the duration of the experiment (0 and 15 min time points shown in the bottom panel).

to increase the level of phosphorylated Erk1/2 (Mansour *et al.*, 1994) and thus potentially useful for mimicking the downstream actions of TNF- α in hPFBs. Results presented in Figure 5 show that overexpression of SSED-Mek1 in hPFBs potentially repressed both baseline and TGF β 1-inducible transcription from the VSMP4 SM α A promoter construct. In comparison, overexpression of a dominant-negative Mek1 mutant (K97M) did not alter the ability of TGF β 1 to activate the VSMP4. These results strongly implicated a central role for the Mek1 kinase in negative regulation of the SM α A gene during myofibroblast differentiation.

Egr-1 Is Induced by TNF- α -dependent Mek1 and Binds to the SM α A Promoter

In considering possible candidates that could modulate transcriptional activity downstream from Mek1, activation of Erk1 and -2 reportedly has been linked to enhanced expression of Egr-1, a proinflammatory transcriptional regulatory protein that contributes to smooth muscle phenotypic mod-

Figure 5. Effects of exogenous Mek1 signaling on SM α A promoter activity in TGF β 1-treated human pulmonary myofibroblasts. hPFBs were transfected with the SM α A promoter:CAT reporter fusion gene (VSMP4) together with expression plasmids encoding either a constitutively active Mek1 mutant (SSED) or dominant-negative Mek1 mutant (K97M). Control cells were transfected with same amount of empty pcDNA3 expression vector. After exposure to TGF β 1 for 24 h (gray-shaded bars), CAT protein levels were analyzed in cell lysates. The black bars denote CAT reporter gene expression in transfected cells treated with vehicle alone. Asterisks denote statistically significant differences between transfections performed using the empty plasmid and Mek1-SSED ($p < 0.05$).

Figure 6. Egr-1 expression in human pulmonary fibroblasts and interaction with a TGF β 1-response element in the SM α A promoter. (a) Transiently elevated Egr-1 protein expression in nuclear preparations from TNF- α -treated hPFBs. Quiescent hPFBs were exposed to TNF- α (10 ng/ml) for various periods up to 180 min, and resulting nuclear protein extracts were analyzed by Egr-1 immunoblot. (b) Egr-1 binds to SPUR DNA but not the binding sites for Smad proteins in either the PAI1 promoter (SBE) or the SM α A promoter (THR) nor does it bind the MCAT element in the SM α A promoter needed for gene induction by the TEF1 muscle-specific *trans*-activating protein. The lane labeled ns denotes Egr-1 binding to a nonspecific DNA probe and serves as a negative control. (c) ChIP analysis revealing formation of a complex between the Egr-1 protein and SPUR DNA in native chromatin context. Quiescent hPFBs were treated with TNF- α (10 ng/ml) or vehicle for 1 h and then processed for ChIP using either nonspecific (control) or anti-Egr-1 (anti-Egr1) antibodies in combination with SPUR-specific primers as described in *Materials and Methods*. (d) Overexpression of constitutively active Mek1 (Mek-SSED) in hPFBs enhanced expression of Egr-1 nuclear protein and that inhibition of Mek1 kinase activity in transfected cells by using U0126 abolished both basal and Mek1-inducible expression of Egr-1. hPFBs were transfected with either the Mek1-SSED expression plasmid (3 μ g) or identical amounts of empty pcDNA3 vector, and the resulting nuclear extracts were processed 24 h later for evaluation by Egr-1 immunoblot. GAPDH immunoblots in a and d are provided to indicate equivalent sample protein loadings on the gels.

ulation and neointimal formation during arteriosclerosis (Silverman *et al.*, 1997; Srivastava *et al.*, 1998; Santiago *et al.*, 1999; McCaffrey *et al.*, 2000; Yan *et al.*, 2000; Tan *et al.*, 2003; Khachigian, 2006; Harada *et al.*, 2007). Egr-1 is an inducible, zinc-finger type protein that binds to GC-rich regulatory sequences such as those contained in the COL1 α 2 promoter in which it reportedly competes with binding of the ubiquitous Sp1 gene-activating protein and inhibits transcription during inflammation, wound healing, and vascular injury (Silverman *et al.*, 1997; Tan *et al.*, 2003). Egr-1 expression was transiently elevated in the nuclear protein fraction of hPFBs within 60 min after exposure to TNF- α (Figure 6a). To examine whether TNF- α -inducible Egr-1 expression also was capable of binding to the SM α A promoter, both *in vitro* DNA binding and *in vivo* chromatin-immunoprecipitation assays were performed using nuclear preparations from TNF- α -treated hPFBs. As shown in Figure 6b, Egr-1 bound exclusively to SPUR, a GC-rich segment of the SM α A promoter that encompasses a TGF β 1-responsive element consisting of tandem binding sites for the Sp1/Sp3 activators

and the Pur α and Pur β repressors. We and others previously showed that the GC-rich core of SPUR was required for myofibroblast differentiation and efficient wound healing in the mouse dermis and heart. Compared with untreated control cells, TNF- α -treated hPFBs also showed an enhanced SPUR:Egr-1 complex formation in native chromatin preparations within 60 min after treatment (Figure 6c), consistent with our observation of Egr-1 accumulation in the cell nucleus and enhanced SPUR binding activity during this same time interval (Figures 6, a and b). Additionally, overexpression of constitutively active Mek1-SSED in transfected hPFBs was associated with a threefold increase in Egr-1 protein level (Figure 6d). Inclusion of the U0126 inhibitor in the culture medium of transfected cells completely blocked both basal and Egr-1 induction, confirming that this was a Mek1-mediated process.

Egr-1 Inhibits TGF β 1- and Smad-mediated Activation of the SM α A Promoter

To determine whether the observed binding of Egr-1 to the SPUR *cis*-regulatory element mediated functional responsiveness of the SM α A promoter, we examined how Egr-1 overexpression in hPFBs influenced activity of the VSMP4 SM α A promoter:CAT reporter fusion gene in the presence of TGF β 1. In addition, expression plasmids encoding either Smad3, Smad2, or Egr-1 were combined and transfected along with the VSMP4 reporter gene into other preparations of hPFBs to determine whether exogenously supplied Smad proteins would respond to Egr-1 in the same manner as endogenous Smads in TGF β 1-treated cells. This approach was used to minimize complications because of possible interference from Smad-independent MAP kinase signaling when cells are exposed to TGF β 1 (Hanafusa *et al.*, 1999; Yue *et al.*, 1999; Massague and Wotton, 2000; Hayashida *et al.*, 2003; Wilkes *et al.*, 2005; Ramirez *et al.*, 2006). We observed that overexpression of Egr-1 in transfected hPFBs significantly blocked both TGF β 1-mediated and Smad3-dependent activation of the SM α A promoter (Figure 7). Egr-1 also similarly repressed promoter activation in cells cotransfected with Smad2 alone or Smad2 plus Smad3, although, as we reported previously (Cogan *et al.*, 2002), Smad2 was slightly less potent than Smad3 as a SM α A gene activator (data not shown). Notably, overexpression of the Smad3 TGF β 1 signaling agonist stimulated more than a 10-fold increase in SM α A promoter activity in transfected hPFBs but was nearly completely attenuated in a dose-dependent manner by overexpression of Egr-1 protein (Figure 7). These data are consistent with the notion that TNF- α -inducible Egr-1 may block SM α A transcriptional activation in TGF β 1-activated myofibroblasts by interfering with the ability of Smad3 to mediate transcriptional activation of the SM α A promoter.

Binding of Egr-1 to the SM α A Promoter Has Opposite Effects on the Binding of Smad Activator and YB-1 Repressor to Their Cognate Transcriptional Regulatory Sites

Smads and Egr-1 regulate transcription activity in myofibroblasts through use of spatially distinct binding sites within the SM α A promoter. As shown above, Egr-1 bound to the SPUR element (positioned between -59 and -28 base pairs from the transcriptional start site), whereas regulatory Smads2 and -3 interact exclusively with a THR between positions -170 and -150 located ~100 base pairs upstream from SPUR (Becker *et al.*, 2000; Subramanian *et al.*, 2004). This eliminated the possibility that Egr-1 and Smads might compete for the same or overlapping DNA binding sites in the promoter. From a theoretical standpoint, however, it was

Figure 7. Egr-1 overexpression abrogates TGFβ1- and Smad-activated SMαA promoter activity in human pulmonary myofibroblasts. hPFBs were transiently cotransfected with the VSMP4 reporter gene plus various amounts (0.1–1 μg) of expression plasmid encoding full-length Egr-1. After 16 h, transfected cells were placed in serum-free medium for 48 h, and TGFβ1 (5 ng/ml) was added to cells for another 24 h. Top, CAT ELISA values for cells transfected with varying amounts of the Egr-1 expression plasmid and then subjected to treatment with TGFβ1. Bottom, CAT ELISA was performed on hPFBs cotransfected with VSMP4 reporter (0.5 μg), a fixed amount of expression plasmid encoding for Smad3 (0.5 μg), and varying amounts of Egr-1 expression plasmid (0.3, 0.6, or 1.0 μg). After 48 h in serum-free medium, cell lysates were prepared and analyzed for CAT activity. In all cases, pcDNA control vector was used to ensure mole equivalent amounts of DNA in each transfection mixture. Transfections were performed in triplicate and asterisks denote statistically significant differences between extracts prepared from cells after TGFβ1 treatment (top) or after Smad3 transfection (bottom) in the absence or presence of Egr-1 expression plasmids ($p < 0.05$).

possible that binding of Egr-1 repressor at its downstream SPUR site could alter the affinity of Smad activators for their more upstream THR binding site. To examine this idea, we first prepared a double-stranded DNA probe encompassing a 164-base pair portion of the SMαA promoter containing the THR and SPUR *cis*-regulatory elements located between positions –192 and –28 in the 5'-flanking region. On incubating this probe with nuclear extracts prepared from hPFBs that had been exposed to combinations of TNF-α and TGFβ1, we observed that the amount of Smad2 and -3 binding to the THR site (Figure 8b) decreased 28 and 24%,

Figure 8. Enhanced binding of Egr-1 to the SMαA promoter in TNF-α-treated human pulmonary myofibroblasts is accompanied by decreased Smad interaction with the promoter but increased binding of YB-1. (a) Although nuclear levels of Smad2 and -3 did not change appreciably when hPFBs were treated with both TGFβ1 and TNF-α, the amount of Smad binding to a 191-base pair segment of the SMαA promoter containing the TGFβ1-regulatable THR control element decreased significantly (SPUR+THR double-stranded DNA probe; b). Egr-1 protein levels in nuclear extracts increased substantially after treatment with TNF-α (a) as did Egr-1 interaction with the double-stranded SPUR+THR DNA probe (b), which was notably higher than binding observed in extracts from cells treated with TGFβ1 alone (b). SMαA protein level increased after exposure to TGFβ1 but decreased if cells also were exposed to TNF-α (a). The level of GAPDH protein in cytosolic extracts (a) indicates equivalent sample protein loading on the gel. In nuclear extracts prepared from hPFBs treated with TNF-α alone (c), binding of YB-1 repressor protein to the reverse strand of DNA encompassing the THR region of the SMαA promoter increased by ~60% over baseline levels ($p < 0.05$).

respectively, over a 3-h exposure period to TNF-α compared with Smads from cells treated with TGFβ1 alone ($p < 0.001$), with no apparent decrease in Smad protein availability in the nuclear compartment (Figure 8a). The data indicated that although TNF-α did not impair nuclear translocation of TGFβ1-regulated Smads, Egr-1 binding at the SPUR component of the double-stranded DNA probe (Figure 8b) seemed to significantly reduce Smad2 and Smad3 binding at the THR despite the physical distance spanning these regulatory sites in the SMαA promoter. Together, the data suggested that Egr-1 might act as a THR binding antagonist thus providing one explanation for its observed behavior in blocking Smad-dependent SMαA gene expression during myofibroblast differentiation. The ability of Egr-1 to reduce Smad-mediated promoter activation did not seem to require the formation of off-DNA Egr-1:Smad sequestering complexes because we were unable to detect anti-Egr-1 antibody-immunoprecipitable Smad2, Smad3, or Smad4 in nuclear extracts prepared from TNF-α-treated myofibroblasts (data not shown).

We also examined whether TNF-α was able to alter interaction of the YB-1 repressor with the SMαA promoter. Previous work showed that YB-1 attenuated activation of the

type I collagen promoter by TGF β 1 through the formation of a sequestering complex with Smad3 (Higashi *et al.*, 2003). In addition, our previous studies on hPFBs showed that TGF β 1 displaced YB-1 from the reverse strand of the THR site in the SM α A promoter (Zhang *et al.*, 2005). However, as depicted in Figure 8c, TNF- α enhanced YB-1 interaction with the reverse strand of the THR element in nuclear extracts prepared from these cells by ~60% over the course of a 30-min exposure period ($p < 0.05$). Collectively, the results suggest that TNF- α might antagonize TGF β 1-mediated activation of the SM α A promoter in human pulmonary myofibroblasts by 1) stimulating Egr-1 and YB-1 repressor interaction with the downstream SPUR and upstream THR sites, respectively; and 2) restricting access of Smad2 and -3 to the THR activation site.

DISCUSSION

Recent studies indicate that inflammation and fibrosis are distinctly different, perhaps dichotomous phases of the wound healing process (Kaminski *et al.*, 2000; Henson, 2003; Kunkel *et al.*, 2003; Verrecchia and Mauviel, 2004). Profibrotic TGF β 1 is proteolytically released from a latent state during cellular injury and functions as the chief mediator of myofibroblast differentiation and extracellular matrix biosynthesis needed for tissue repair, regeneration, and remodeling (Gabbiani, 2003; Shi and Massague, 2003; Tomasek *et al.*, 2005). The wound provisional matrix also helps organize neovascular elements to restore oxygen and nutrient perfusion to the damaged tissue bed. Secretion of the proinflammatory cytokine TNF- α also is elevated after tissue injury to assist in neovasculogenesis but persists during formation of the provisional matrix when it may help balance TGF β 1-mediated matrix biosynthesis in myofibroblasts and prevent progression to hypertrophic scarring (Verrecchia and Mauviel, 2004). The process of matrix equilibration seems quite important in tissue homeostasis since TGF β 1-mediated fibrosis after corneal injury is much more severe in TNF- α null mice that lack robust inflammatory responses compared with wild-type mice (Saika *et al.*, 2006). Several pulmonary fibroproliferative diseases seem to involve dysfunctional signal transduction interplay between profibrotic TGF β 1 activated by alveolar phagocytic cells and proinflammatory TNF- α released by polarized T cells. If unchecked, faulty signaling could promote cycles of matrix deposition and destruction that could eventually compromise parenchymal cell function (Moller, 2003). Similarly, mononuclear cell release of IFN γ , a T-helper 1 cytokine that often functions in tandem with TNF- α , suppresses fibroblast proliferation and collagen deposition and has long been associated with classic type I, innate-immune responses (Kunkel *et al.*, 2003). Notably, the antifibrotic activity of IFN γ seems to be partly based on its ability to functionally sequester TGF β 1 receptor-regulated Smad3 protein as a heteromeric complex with the YB-1 transcriptional repressor protein thus preventing binding of activated Smads to their cognate CAGA *cis*-activation sequence motifs in the type I collagen promoter (Higashi *et al.*, 2003; Dooley *et al.*, 2006). Aside from this one mechanistic detail, the molecular pathways governing termination of TGF β 1-mediated myofibroblast differentiation are not well understood but theoretically important in the search for new therapeutic targets to prevent chronic fibroproliferative diseases of the heart, lung, liver, and kidney. Accordingly, the objectives of this study were to 1) assess the ability of proinflammatory TNF- α to block activation of the SM α A promoter, the leading early indicator of myofibroblast differentiation and fibrogenesis in healing wounds; and

2) identify the specific molecular mechanism(s) used by antifibrotic TNF- α to intercept profibrotic TGF β 1 signaling in differentiated pulmonary myofibroblasts.

The induction of Egr-1 through the Ras-Raf-Mek-Erk MAP kinase signaling cascade has been reported in a variety of cell types, including kidney and intestinal epithelial cells, vascular cells, and astrocytes in the CNS (Morawietz *et al.*, 1999; McCaffrey *et al.*, 2000; Tan *et al.*, 2003; Autieri *et al.*, 2004; Cho *et al.*, 2006; Khachigian, 2006; Harada *et al.*, 2007). In human lung fibroblasts, we observed that overexpression of a constitutively active Mek1 variant was accompanied by increased expression of Egr-1 protein. Accumulation of Egr-1 protein was significantly attenuated by the U0126 Mek1 inhibitor and was observed in human pulmonary fibroblasts that were transfected with the Mek1 variant or exposed directly to TNF- α . Data presented in this report also indicated that suppression was dominant over TGF β 1-mediated activation of the SM α A promoter in human pulmonary myofibroblasts and dependent on intact Mek1-Erk1/2 MAP kinase signaling. Biochemical inhibition of p38 and JNK signal transduction in neonatal human pulmonary myofibroblasts had no discernible effect on SM α A repression by TNF- α nor was termination of TGF β 1 signaling by TNF- α associated with increased expression of the Smad7 antagonist or reduced efficiency of Smad2 and -3 agonist phosphorylation by the type I receptor kinase (Bitzer *et al.*, 2000; Nagarajan *et al.*, 2000; Verrecchia *et al.*, 2000). In contrast, TNF- α reportedly interferes with type I collagen subunit gene expression in various types of fibroblasts via a more combinatorial strategy involving alteration of Smad7 expression (Bitzer *et al.*, 2000; Nagarajan *et al.*, 2000) as well as modulation of Jun NH $_2$ -terminal kinase activity (Verrecchia *et al.*, 2003; Verrecchia and Mauviel, 2004). From a mechanistic standpoint, the regulation of collagen matrix deposition in mammalian fibroblasts may be more complicated than the scheme used to regulate expression of the SM α A contractile protein gene and conceivably could involve more than one signal transduction cascade. Indeed, multiple-level control of Smad7 expression as well as JNK and perhaps p38 signaling might be needed to coordinate progression along the collagen fiber assembly arm of matrix biosynthesis with the remodeling arm that drives accumulation of matrix metalloproteinases required for collagen fiber degradation and remodeling. Superimposed on these functional needs may be additional differences between dermal, renal, pulmonary, hepatic, and cardiac fibroblast lineages that could specify how signal transduction operates based on cell fate determination rules laid down during early embryogenesis. Notably, SM α A and type I collagen gene transcriptional control mechanisms share a need for ubiquitous Sp1 and TGF β 1-activated Smads, but it is important to realize that the SM α A gene additionally is activated by the SRF, myocardin, and TEF1 DNA-binding proteins that are widely viewed as important *trans*-determinants of smooth and cardiac myogenesis. Conversely, activator protein-1 seems relatively important for activation of the COL1 α 2 gene promoter but does not seem to play a significant role in activation of SM α A gene transcription. Comparative analysis of SM α A and type I collagen gene transcriptional control mechanisms used by various fibroblast subtypes from fetal, neonatal, and adult tissues may be quite informative in this regard and provide much needed insight into the molecular basis of myofibroblast functional plasticity in normal development and chronic fibrotic disease.

We discovered that expression of the Egr-1 transcription factor downstream from Mek-Erk1/2 activation could be a factor in down-regulation of myofibroblast differentiation

based on the ability of this DNA-binding protein to block both TGF β 1- and Smad2/3/4-inducible SM α A gene transcription. In TNF- α -treated human pulmonary fibroblasts, Egr-1 was able to bind the tissue injury-responsive SPUR sequence motif in the SM α A promoter. This was an important observation because SPUR was shown previously to be the binding site for a Smad-regulated Sp1/3:Pur α transcriptional control complex in a mouse myofibroblast-like cell line (Cogan *et al.*, 2002; Subramanian *et al.*, 2004) as well as an essential determinant in the SM α A promoter required for dermal wound healing in the mouse (Tomasek *et al.*, 2005). Importantly, the myofibroblast suppression property of TNF- α also could be mediated by activation of Mek1-Erk1/2 signaling because earlier reports indicate that Erk-dependent phosphorylation of the Smad2 or -3 linker regions prevents their nuclear translocation, thus providing a significant impediment to TGF β 1-mediated myofibroblast differentiation (Kretschmar *et al.*, 1999). Analysis of Smad nuclear translocation in TGF β 1-stimulated fibroblasts overexpressing the constitutively active form of Mek1 may clarify the relative contributions of Erk- and Egr-1-directed forms of myofibroblast repression. The TGF β 1/TNF- α /Erk/Egr-1 regulatory axis may provide a physiological rheostat that allows rapid emergence of SM α A-positive myofibroblasts yet prevents unchecked expansion of these contractile cells in healing wounds.

Egr-1 is not expressed in normal tissue but rapidly induced by inflammatory cytokines released into damaged heart, lung, and kidney tissue beds as a result of mechanical trauma and hypoxia (McCaffrey *et al.*, 2000; Yan *et al.*, 2000; Okada *et al.*, 2002; Autieri *et al.*, 2004; Khachigian, 2006; Harada *et al.*, 2007). Hypoxia induces Egr-1 expression in the microvascular endothelium and adventitial fibroblasts via protein kinase α -mediated Ras/Raf/Mek/Erk signaling (Khachigian, 2006). Repression of both myofibroblast differentiation and connective tissue biosynthesis by Egr-1 coupled with increased transcription of Egr-1-dependent genes such as interleukin (IL)-1 β , monocyte chemoattractant protein-1, intercellular adhesion molecule-1, macrophage inflammatory protein-2, 10-kDa interferon-inducible protein, and (RANTES) protein may improve wound healing efficiency because these proinflammatory cytokines and chemokines seem to collaborate with hypoxia-induced hypoxia-inducible factor α and vascular endothelial growth factor in the repair and expansion of microvascular networks needed to restore blood flow to damaged tissues. We recently reported that hypoxia (3% molecular oxygen) significantly repressed SM α A gene expression in primary culture preparations of mouse cardiac fibroblasts (Roy *et al.*, 2007). Although the role of Egr-1 in mediating transcriptional response of the SM α A gene to hypoxia was not specifically examined in that study, we did report a positive correlation between hypoxia, SM α A gene repression, and elevated levels of YB-1 protein in cardiac fibroblasts. As we now show, YB-1 and Egr-1 seem to function as corepressors in antagonizing the human pulmonary fibroblast response to TGF β 1. We also recently proposed that dysfunctional remodeling of syngeneic murine heart grafts was initiated by cardiac myofibroblast differentiation arising as a consequence of mechanical trauma and ischemia-reperfusion injury inflicted by repeated transplant surgery (Zhang *et al.*, 2008). In this alloantigen-independent model of cardiac injury, induction of Egr-1 expression was noted in hearts subjected to one or two rounds of transplant surgery (David and Strauch, unpublished data).

From the standpoint of therapeutic intervention, approaches that target production of individual cytokines re-

leased after tissue injury may not be very effective in managing wound healing outcomes because proinflammatory TNF- α , IFN γ , and IL-1 β often exhibit redundant behavior broadly related to their common ability to stimulate NF- κ B-mediated gene expression (Kunkel *et al.*, 2003). In contrast, identifying downstream negative effectors of myofibroblast differentiation may be a more useful strategy because of their possible rate-limiting roles in the etiology of chronic fibrotic disease. Egr-1 is activated early after tissue injury and directs a family of target genes whose expression is likely to be essential for various aspects of the wound repair process (Yan *et al.*, 2000; Cho *et al.*, 2006; Khachigian, 2006). The development of therapeutic agents that augment Egr-1 expression or mimic its ability to neutralize Smad-based transcription could offer clinical benefits by minimizing unregulated myofibroblast differentiation without the complications presented by targeting functionally redundant and possibly beneficial immune cell-derived cytokines (Harada *et al.*, 2007). Of particular interest would be the characterization of Egr-1-activated gene products such as signaling kinases and phosphatases or identifying peptide domains in the Egr-1 molecule that inactivate receptor-regulated Smads or prevent them from forming transcriptional activation complexes with Sp1/3 or SRF within the SM α A promoter. An earlier report also mentions that Egr-1 interferes with Sp1 binding to GC-rich sites in the type I collagen collagen promoter leading us to speculate that Sp1 interaction with SPUR might be similarly disturbed by Egr-1 resulting in loss of SM α A gene transcription (Tan *et al.*, 2003). However, preliminary assessment of nuclear extracts prepared from TNF- α -treated human pulmonary fibroblasts showed little stoichiometric change in Sp1:SPUR interaction relative to extracts prepared from untreated control cells (Liu and Strauch, unpublished data). Conceivably, Egr-1 may alter Sp3 interaction with SPUR. We previously showed that Sp3 was weaker than Sp1 in the ability to transactivate the SM α A promoter in transfected fibroblasts, a finding that was consistent with earlier reports indicating that Sp1 and Sp3 have different, possibly opposite effects on the expression of certain mammalian genes (Cogan *et al.*, 2002). Moreover, Sp3 in fibroblasts and vascular smooth muscle cells exhibits a higher capacity to bind Pur repressor proteins compared with Sp1 (Knapp *et al.*, 2006). Collectively, these data suggest that Egr-1 could attenuate transcriptional output from the SM α A promoter in the presence of TNF- α by altering dynamic interplay of Sp1 and Sp3 with SPUR DNA, perhaps shifting the balance toward preferential binding of Sp3. Investigation of possible competitive displacement of transcriptional activators mediated by Egr-1 might be accomplished by characterizing the relative DNA binding activity of Sp1 and Sp3 in titration studies using recombinant Egr-1 or by monitoring the level of each DNA-binding protein by chromatin immunoprecipitation using TGF β 1- and TNF- α -treated pulmonary myofibroblasts.

Concurrent with altered protein:DNA interactions at SPUR, the observed enhanced loading of YB-1 at the THR element in TNF- α -treated fibroblasts expressing Egr-1 may cause widespread changes in DNA structure within the 200-base pair segment of the SM α A promoter that directs TGF β 1 responsiveness in myofibroblasts (Figure 9). We reported previously that de novo transcriptional activation of the SM α A gene in human pulmonary myofibroblasts by TGF β 1 required nuclear export of the YB-1 repressor (Zhang *et al.*, 2005). However, TNF- α -mediated blockade of TGF β 1 activation did not simply reverse nuclear export of YB-1 even though binding of this repressor to SM α A promoter DNA was gradually and significantly enhanced by this

Figure 9. Hypothetical model for dynamic interplay of Egr-1 and YB-1 transcriptional repressors with Smad activators in human pulmonary myofibroblasts. The top illustration depicts a state of SM α A gene transcriptional activation in myofibroblasts exposed to profibrotic TGF β 1. Termination of myofibroblast activity by antifibrotic TNF- α is shown in the bottom illustration and may involve functional displacement of Smad activators via concerted binding of Egr-1 and YB-1 repressors to their spatially distinct binding sites at SPUR and THR, respectively. Unwinding of duplex DNA at the THR may be fostered by collaboration between the YB-1 and Pura/ β repressors that bind to opposite strands of this essential Smad activation site in the SM α A promoter.

proinflammatory cytokine. Moreover, as YB-1 binding to the reverse strand of the MCAT/THR region increased after exposure to TNF- α , Smad binding to the THR was diminished. The observed displacement of Smads 2 and 3 may occur independently from loading of Egr-1 onto SPUR DNA, given the physical distance separating the SPUR and THR regions, although this remains to be examined experimentally. Currently we cannot rule out the existence of hypothetical "super-repression" protein complex consisting of Egr-1:YB-1 multimers spanning the SPUR-THR segment within the context of native chromatin. However, immunoprecipitation analysis performed on nuclear and cytosolic extracts prepared from human pulmonary myofibroblasts treated with either TGF β 1 or TNF- α alone or in various combinations revealed little evidence for the existence of stable Egr-1:YB-1 protein complexes. There is a theoretical possibility that formation and/or biochemical stabilization of this hypothetical super-repressor complex may require promoter DNA substrate to anchor Egr-1:YB-1 protein:protein interactions. This idea currently is under investigation using a DNA-based, solid-phase ELISA analytical approach. The model presented in Figure 9 also predicts that Egr-1 need not bind and sequester Smads to mediate transcriptional repression since direct physical disruption of the Smad binding site at the THR by YB-1 may serve this purpose. YB-1 has well documented duplex-DNA unwinding activity (Gaudreault *et al.*, 2004), and we previously presented evidence showing that YB-1 and Smads compete for binding to the region of the SM α A promoter harboring the THR element (Subramanian *et al.*, 2004). YB-1 binds with high-affinity to the reverse strand of the MCAT/THR motif and may maintain this segment of DNA in an unwound state with assistance from the Pura and Pur β corepressor proteins that bind the forward strand of this same region (Strauch *et al.*, 1997; Kelm *et al.*, 1999; Carlini *et al.*, 2002). We are examining duplex MCAT/THR melting in the presence and absence of recombinant YB-1 and Smads to validate various aspects of the working hypothesis presented in Figure 9. Egr-1 could also augment expression of other factors needed to facilitate YB-1-mediated binding and unwinding at THR such as regulatory kinases or phosphatases. We have emerging data showing that Erk1/2 activation is necessary for YB-1 nuclear uptake, oligomerization, and binding to

single-strand nucleic acids in human pulmonary fibroblasts (Willis and Strauch, unpublished data). YB-1 is a phosphoprotein whose nuclear translocation in cancer cells was shown to be dependent on serine phosphorylation (Sutherland *et al.*, 2005). Given its central role in controlling both SM α A and type I collagen gene expression, exploring ways to stabilize compartmentalization of YB-1 in the cell nucleus or developing cell-permeable peptide derivatives that mimic the localized duplex DNA-unwinding and/or Smad3-sequestering activities of YB-1 (Higashi *et al.*, 2003; Dooley *et al.*, 2006) may lead to the development of myofibroblast-targeted therapy for controlling excessive scar formation.

In summary, we have discovered that Mek1-dependent induction of Egr-1 represents a key event in attenuating myofibroblast differentiation during wound healing stimulated by the release of activated TGF β 1. We presented several models to explain how Egr-1 might neutralize Smad action, including hypothetical mechanisms based on dynamic interplay between transcriptional activator and repressor proteins at SPUR DNA as well alteration of DNA structure in a segment of the SM α A promoter referred to as the THR that is known to harbor tandemly arrayed binding sites for the single-strand specific gene repressor YB-1 and TGF β 1 receptor-regulated Smad transcriptional activators. In particular, the finding that binding of Egr-1 at SPUR DNA and YB-1 at THR DNA was associated with reduced binding of Smad activator at THR DNA provides new insight into the molecular dynamics of SM α A promoter regulation in myofibroblasts. Further characterization of these mechanisms may help in identifying new targets for the possible prevention of chronic fibroproliferative diseases based on dysfunctional regulation of myofibroblasts after tissue injury.

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