YB-1 Coordinates Vascular Smooth Muscle α -Actin Gene Activation by Transforming Growth Factor β 1 and Thrombin during Differentiation of Human Pulmonary Myofibroblasts

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Profibrotic regulatory mechanisms for tissue repair after traumatic injury have developed under strong evolutionary pressure to rapidly stanch blood loss and close open wounds. We have examined the roles played by two profibrotic mediators, transforming growth factor β 1 (TGF β 1) and thrombin, in directing expression of the vascular smooth muscle α -actin (SM α A) gene, an important determinant of myofibroblast differentiation and early protein marker for stromal cell response to tissue injury. TGF β 1 is a well known transcriptional activator of the SM α A gene in myofibroblasts. In contrast, thrombin independently elevates SM α A expression in human pulmonary myofibroblasts at the posttranscriptional level. A common feature of SM α A up-regulation mediated by thrombin and TGF β 1 is the involvement of the cold shock domain protein YB-1, a potent repressor of SM α A gene transcription in human fibroblasts that also binds mRNA and regulates translational efficiency. YB-1 dissociates from SM α A enhancer DNA in the presence of TGF β 1 or its Smad 2, 3, and 4 coregulatory mediators. Thrombin does not effect SM α A gene transcription but rather displaces YB-1 from SM α A exon 3 coding sequences previously shown to be required for mRNA translational silencing. The release of YB-1 from promoter DNA coupled with its ability to bind RNA and shuttle between the nucleus and cytoplasm is suggestive of a regulatory loop for coordinating SM α A gene output in human pulmonary myofibroblasts at both the transcriptional and translational levels. This loop may help restrict organ-destructive remodeling due to excessive myofibroblast differentiation.

INTRODUCTION

Fibrosis is a serious complication of chronic cardiopulmonary diseases and postsurgical complication of heart and lung transplant (Pickering and Boughner, 1990; Armstrong *et al.*, 1997; Howell *et al.*, 2002; Chapman, 2004). Although management of acute allograft rejection is accomplished through the use of immunosuppressive agents that can limit immune cell infiltration, the cause of chronic rejection and failure in accepted allografts is poorly understood thus offering no treatment solution short of retransplant. Leading the formation of scar tissue is a specialized stromal cell referred to as the myofibroblast that contains abundant microfilament networks composed of smooth muscle-specific contractile protein isoforms (Tomasek *et al.*, 2002; Hinz and Gabbiani, 2003; Grotendorst *et al.*, 2004). Chronic accumulation of myofibroblasts is associated with excessive extracel-

lular matrix protein biosynthesis, hypercontractility, and organ-destructive remodeling. Vascular smooth muscle α -actin (SM α A) is one of the major contractile proteins expressed by differentiated myofibroblasts (Darby *et al.*, 1990; Ronnov-Jessen and Petersen, 1996; Hinz *et al.*, 2001; Cogan *et al.*, 2002). Normally, SM α A-enriched myofibroblasts are early, transient participants in stromal wound healing processes. We reason that characterization of the initial molecular events associated with activation of the SM α A gene in stromal cells would help identify novel checkpoints for regulating the extent of myofibroblast involvement in wound healing. Therapeutic interventions aimed at managing potentially rate-limiting steps in myofibroblast differentiation may help protect against uncontrolled tissue remodeling and the so-called “endless healing” phenotype (Tomasek *et al.*, 2002) with attendant hypertrophic scarring and loss of parenchymal cell function.

In recent studies from our laboratory, we have observed that the SM α A gene is transcriptionally activated by the cytokine TGF β 1 via Smad-dependent signaling (Cogan *et al.*, 2002; Subramanian *et al.*, 2004). In the context of wound healing, this potent, profibrotic cytokine is released from a latent state at the site of tissue injury by activators intimately associated with local monocyte/macrophage infiltration and adhesion such as matrix metalloproteinase (MMP)-2 and -9, thrombospondin-1, and α V β 6 integrin (Leask and Abraham,

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2004). Less well understood, and especially relevant to the situation of organ transplantation in the immunosuppressed human patient population, is the role of nonimmune based surgical trauma and graft ischemic/reperfusion injury in promoting myofibroblast activation and chronic tissue remodeling. The serine protease thrombin, released during donor organ harvest and subsequent engraftment not only initiates the enzyme cascade responsible for coagulation but also has been reported to activate latent TGF β 1 (Howell *et al.*, 2002) and possess profibrotic and extravascular cellular signal transduction properties that lead to tissue remodeling in both the native and transplanted heart and lung (Erlich *et al.*, 2000; Holschermann *et al.*, 2000; Mackman, 2003). In this regard, our studies of syngeneic murine heart grafts has revealed a form of alloantigen-independent organ remodeling (Armstrong *et al.*, 1997) that evolves slowly but resembles in form the far more robust perivascular and interstitial fibrotic response observed in long-term cardiac allografts in immunosuppressed recipients (Subramanian *et al.*, 2002). In accepted allografts, we hypothesize that persistent, low-grade immune cell infiltration amplifies an underlying peritransplant wound healing response based on the profibrotic action of TGF β 1 and thrombin released as a consequence of mechanical and/or reperfusion injury during transplant surgery. Amplification of these nonimmune-based wound healing processes by alloantigen or alloantibody augments dysfunctional remodeling leading to premature graft failure.

To more fully understand the molecular mechanism of peri-transplant wound healing, we have examined the effect of thrombin on SM α A gene regulation in human pulmonary fibroblasts (hPFBs) and compared its action to TGF β 1, a well known transcriptional activator of the smooth muscle actin gene. In contrast to regulatory mechanism reported for TGF β 1, thrombin had a significant, potentially supplemental effect on SM α A translation. A common feature of SM α A up-regulation mediated by both thrombin and TGF β 1 was reliance on the cold shock domain protein YB-1 (Matsumoto and Wolffe, 1998; Wolffe, 1998) that represses SM α A gene transcription in stromal fibroblasts yet exhibits additional RNA binding properties that regulate mRNA translational efficiency. YB-1 dissociated from SM α A enhancer DNA and was exported from the nucleus in the presence of TGF β 1 or its Smad coregulatory mediators. On the other hand, thrombin displaced YB-1 from SM α A exon 3 mRNA coding sequences previously shown to be required for translational silencing (Kelm *et al.*, 1999b). Thrombin-facilitated RNA release and nuclear reentry of YB-1 provides compelling evidence for a regulatory loop to control SM α A gene output via coordinate control at both the transcriptional and translational levels. Published evidence indicates that thrombin activates several growth and inflammation-associated genes and that promoter regions flanking these genes contained binding sites for YB-1 (Minami *et al.*, 2004). Besides reducing SM α A gene expression, YB-1 also represses type I collagen α 1 and α 2 gene transcription (Norman *et al.*, 2001; Higashi *et al.*, 2003). Thus, nuclear YB-1 may temper TGF β 1-dependent, myofibroblast accumulation while stimulating inflammatory cytokines necessary for angiogenesis, restored microperfusion, and infiltration of organ-reconstructive circulating progenitor cells into the wound provisional matrix.

MATERIALS AND METHODS

Cell Culture Methods and Preparation of Protein Extracts

Human pulmonary fibroblasts (hPFB) were established in primary culture from enzyme-dispersed tissue fragments of human neonatal lung tissue ob-

tained at autopsy and were the kind gift of Dr. Daren L. Kneoll (Departments of Pharmacy and Internal Medicine, The Ohio State University, Columbus, OH). Pulmonary fibroblasts were maintained in a 1:1 mixture of Ham's F-12 and DMEM (1.0 g/l D-glucose) supplemented with penicillin-streptomycin-Fungizone, Gentamicin (50 μ g/ml), and 10% heat-inactivated fetal bovine serum (hiFBS; all culture medium reagents from Invitrogen, Carlsbad, CA). Nonhuman primate COS7 kidney fibroblasts were maintained in DMEM (4.5 g/l D-glucose) supplemented with penicillin-streptomycin and 10% hiFBS. Cell lines were cultivated in a humidified incubator at 37°C at 5% CO₂. Fibroblasts were rendered quiescent by a 48-h exposure to HEPES-buffered DMEM (1.0 g/l D-glucose) containing 0.5% hiFBS and penicillin-streptomycin-Fungizone. Recombinant human TGF β 1 (5 ng/ml, final concentration; R&D Systems, Minneapolis, MN) was added to cultures for varying periods before preparation of protein extracts. Human plasma thrombin (1000 NIH U/mg protein) was obtained from Calbiochem (La Jolla, CA) and used between 1 U and 10 U/ml as noted in the text. The metabolic inhibitors actinomycin D and cycloheximide were used at 5 and 10 μ g/ml, respectively, in experiments on thrombin-activated myofibroblasts. Inhibitors of the TGF β 1 type I receptor serine/threonine kinase (SB431542; Sigma-Aldrich, St. Louis, MO) and thrombin serine protease (Thromstop, N- α -NAPAP; American Diagnostica, Stamford, CT) were administered at 1 and 0.1 μ M, respectively, for 1 h before exposing hPFBs to medium supplemented with TGF β 1 or thrombin plus fresh aliquots of inhibitors. For protein extract preparations, cell monolayers were washed twice with Dulbecco's phosphate-buffered saline (PBS), scraped into fresh PBS, sedimented at 3000 rpm, washed once more in PBS, and resuspended in eight packed-cell volumes of hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], and 0.5 mM dithiothreitol [DTT]). Cells were allowed to swell for 10 min on ice before transfer to a Dounce homogenizer for processing with a type B pestle. Nuclei were collected from ruptured cells by centrifugation for 15 min at 4000 rpm and suspended in one-half packed-pellet volume of ice-cold, low salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 0.02 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT). High salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT) equal to one-half packed-pellet volume was added, and the nuclei were further extracted with gentle rocking for 30 min at 4°C. Nuclei were collected by centrifugation for 30 min at 14,500 rpm and dialyzed 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. After dialysis, supernatants were collected by centrifugation at 14,500 rpm for 20 min for use in biochemical assays. Whole cell extracts were prepared from PBS-rinsed monolayers using radioimmunoprecipitation (RIPA) buffer (1 $\times$ PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail, 0.2 mM PMSF, and 0.5 mM DTT) at 0.6 ml/100-mm culture plate followed by gentle rocking for 15 min at 4°C. Adherent lysed cell remnants were scraped into 0.3 ml of RIPA buffer and combined with the original lysate into a single microcentrifuge tube and the supernatant fraction was collected at 10,000 $\times$ g for 10 min at 4°C.

DNA- and RNA-binding Assays

Several synthetic biotinylated oligonucleotide probes were used in this study as follows: 1) a pyrimidine-rich, 30-base segment of single-strand DNA encompassing the reverse strand of the 5'-flanking region of the mouse SM α A gene located between -195 and -164 (also referred to as D_R, 5'-ccctcgtctt-gtctctctacgtacacctctct-3') and previously shown to bind YB-1 with high affinity; Cogan *et al.*, 1995; Kelm *et al.*, 1999a); 2) the RNA coding element located in exon 3 of SM α A mRNA (CE-RNA, 5'-gggagaauggggauggggcacaaga-3'), previously shown to bind YB-1 and Pur proteins (Kelm *et al.*, 1999b); and 3) its mutant counterpart (CE-RNAmut, 5'-uugagaauggguuucgugggcacaacaga-3'). Underlined sequences denote regions changed by mutation. Reaction mixtures containing protein extract (100 μ g of protein) and 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, 4% glycerol. Protein-biotinylated DNA complexes were captured on streptavidin-immobilized paramagnetic particles (Promega, Madison, WI; 0.6 ml/reaction, 30-min incubation) as described previously (Cogan *et al.*, 2002; Subramanian *et al.*, 2004). After washing four times with buffer containing 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 100 mM NaCl, bound protein was eluted using 2 $\times$ protein denaturing buffer and analyzed by SDS-PAGE and immunoblotting procedures.

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

Fibroblasts at 40–50% confluence were transfected with mixtures of the previously described SM α A promoter:reporter fusion plasmids (VSMP4 and VSMP8) plus plasmids encoding various transcriptional regulatory proteins (Cogan *et al.*, 2002; Subramanian *et al.*, 2004). The Mirus (Invitrogen, San Diego, CA) transfection reagent was used with a protocol provided by the manufacturer. VSMP4 contains multiple SM α A promoter regulatory elements including two different transcriptional activation elements referred to as SPUR (Subramanian *et al.*, 2004) and THR (Cogan *et al.*, 2002). VSMP8 has

been widely used to generate transgenic mouse lines and contains a 3.6-kb 5'-flanking/first intron promoter fragment from mouse SM α A genomic DNA containing both smooth muscle-specific developmental control and tissue injury response elements (Wang *et al.*, 1997, 1998; Maeda *et al.*, 1999; Lopes *et al.*, 2003; Hassanain *et al.*, 2005). For translation assays, the P4/CE (3') construct was made by incorporating the exon 3 coding sequence of the mouse SM α A gene into VSMP4 as described previously (Kelm *et al.*, 1999b). The exon 3 coding element previously was shown to repress chloramphenicol acetyltransferase (CAT) reporter gene mRNA translation, but not transcription, when placed in the 5'-untranslated region of the CAT reporter gene at a position 3' to the transcriptional start site. Plasmids encoding the human Smad 2, 3, and 4 proteins were kindly provided by Drs. L. Choy and R. Derynck (University of California, San Francisco, San Francisco, CA). Plasmids were purified using QIAGEN preparative resin and a protocol provided by the manufacturer (QIAGEN, Valencia, CA). Forty-eight hours after transfection, cells were washed three times with ice-cold PBS and then lysed using CAT enzyme-linked immunosorbent assay (ELISA) lysis buffer (Roche Diagnostics, Indianapolis, IN). Whole cell extracts were clarified at 14,000 $\times$ g for 10 min at 4°C and briefly stored at -20°C before immunoassay. Total protein in extracts was determined by BCA colorimetric assay (Pierce Chemical, Rockford, IL), and reporter gene activity was determined using a commercial CAT ELISA kit (Roche Diagnostics) and expressed on a per microgram protein basis. Transfections were performed in triplicate and repeated three to five times. Data sets were subjected to analysis of variance to assess statistical significance set at $p < 0.05$.

Immunoblot, Northern Blot, and Immunofluorescence Microscopy Procedures

Proteins (10- μ g aliquots) were size-fractionated by SDS-PAGE using 10% polyacrylamide gels and then electrophoretically transferred to nitrocellulose membranes (Whatman Schleicher and Schuell, Keene, NH). After overnight blocking at 4°C in Tris-buffered saline (TBS) (25 mM Tris-HCl, pH 7.5, and 150 mM NaCl) containing 3% (wt/vol) nonfat dry milk and 0.5% bovine serum albumin (BSA), blots were incubated with selected rabbit polyclonal antibodies (1–2 μ g/ml) for 90 min at room temperature with gentle rocking. Antibodies specific for Smad proteins were obtained commercially (Santa Cruz Biotechnology, Santa Cruz, CA) and YB-1/MSY1- and Pur α / β -specific rabbit polyclonal antibodies (anti-MSY1 M85-110 or anti-MSY1 M276-302 and anti-Pur P42-69, respectively) have been described previously (Kelm *et al.*, 1999a). Antibodies for detection of p42/p44 Erk proteins and their phosphorylated derivatives were obtained from Upstate Biotechnology (Lake Placid, NY). Blots were washed four times at room temperature over a 20-min period in TBS containing Tween 20 [0.05% (vol/vol)]. Horseradish peroxidase-conjugated, goat anti-rabbit secondary antibody (1:1500) was then applied for 45 min after which time the blots were washed as described above and processed for antibody visualization by chemiluminescence (GE Healthcare, Piscataway, NJ) and imaged onto Biomax film (Eastman Kodak, Rochester, NY). For Northern blots, cells were extracted with TRIzol reagent (Invitrogen), and 10- μ g aliquots of total RNA were size fractionated by formaldehyde/agarose denaturing gel electrophoresis, diffusion blotted onto nitrocellulose membrane, and hybridized using a radiolabeled actin cDNA probe that recognizes all actin isoform mRNAs (Strauch and Reeser, 1989). For densitometric evaluation, blots were exposed to a phosphor screen for analysis in a PhosphorImager (GE Healthcare). To examine YB-1 and SM α A localization after thrombin activation (5 U/ml; 5 min), hPFBs were washed in PBS, fixed in 2% paraformaldehyde for 30 min, permeabilized in 0.1% Triton X-100 for 15 min, preblocked with 1.5% goat serum for 30 min, and then incubated for 1 h with primary antibodies diluted in 3% BSA (anti-MSY1 M276-302 at 7 μ g/ml or anti-human SM α A mouse monoclonal clone 1A4; [DakoCytomation California, Carpinteria, CA], at 1:100 dilution). After washing in PBS, samples were treated with goat anti-mouse secondary antibody conjugated with Alexa 594 (for SM α A detection) or goat anti-rabbit secondary antibody conjugated with Alexa 568 (for YB-1 detection). Secondary antibodies were obtained from Molecular Probes (San Diego, CA) and used at 1:1000 dilutions. Some samples also were briefly treated with 4,6-diamidino-2-phenylindole (DAPI) (1:1000; Molecular Probes) to visualize cell nuclei. Cells were viewed under epifluorescence illumination optics using an Axioscope microscope equipped with a 40 $\times$ Neofluar objective (Carl Zeiss, Thornwood, NY).

RESULTS

Differential Action of TGF β 1 and Thrombin on SM α A Expression in Human Pulmonary Myofibroblasts

Thrombosis and fibrosis, mediated by the actions of thrombin and TGF β 1, respectively, are tissue repair processes that minimize blood loss and facilitate construction of a myofibroblast-enriched, provisional extracellular matrix needed to close open wounds. Of particular interest were possible similarities between TGF β 1- and thrombin-based SM α A

Figure 1. (A) Immunoblot depicting SM α A protein accumulation in hPFBs over a 48-h period after exposure to TGF β 1 (0–5.0 ng/ml). (B) Northern blot showing actin mRNA levels (nonmuscle β -actin and γ -actin and SM α A) over a 16-h period after treatment of quiescent hPFBs with either TGF β 1 (5 ng/ml) or thrombin (2 or 10 U/ml). RNA sample from mouse AKR-2B embryonic fibroblasts after treatment with TGF β 1 (5 ng/ml, 16 h) is shown in the lane marked "+" to serve as a positive control for TGF β 1 response and to denote positions of actin mRNAs. An ethidium bromide-stained agarose gel showing 28 and 18S rRNAs is shown at the bottom to illustrate the relative amount of total RNA applied to each lane. (C) Immunoblots showing SM α A and β -actin (β A) protein levels in thrombin-treated (Thr, 5 U/ml) hPFBs (left). Thrombin-induced accumulation of SM α A was inhibited by cycloheximide (+ cycl, 10 μ g/ml) but not actinomycin D (+ actD, 5 μ g/ml) relative to thrombin alone (contl) as shown on the right.

gene induction required for conversion of stromal fibroblasts into contractile smooth muscle-like myofibroblasts. As a first approach, we compared the effects of TGF β 1 and thrombin on the behavior of single strand-specific, nucleic acid-binding proteins first identified by our laboratories as essential negative regulators of SM α A gene transcription in rodent myofibroblasts and smooth muscle cells (Cogan *et al.*, 1995; Sun *et al.*, 1995; Kelm *et al.*, 1999a). SM α A gene expression in primary cultures of hPFBs exhibited a robust, 9-fold increase in response to TGF β 1 over the same concentration range previously demonstrated to be effective for gene stimulation in mouse embryonic fibroblast and neonatal rat aortic smooth muscle cell lines (Figure 1A). TGF β 1 also stimulated more than a 10-fold increase in transcription from reporter genes containing a 191-base pair mouse SM α A enhancer element harboring TGF β 1-activating elements (VSMP4) as well as a larger 3.6-kb 5'-flanking/first intron promoter fragment (VSMP8) from mouse SM α A genomic DNA (Min *et al.*, 1990) that contains additional smooth muscle-specific developmental control elements (our unpublished data). Similarly, transcription of SM α A mRNA from the native human SM α A gene was markedly enhanced in hPFBs by TGF β 1 administered over a 16-h period (Figure 1B). In contrast, Northern blots prepared from hPFBs exposed to either 2 or 10 U/ml thrombin showed only a very modest increase in nonmuscle β - and γ -actin mRNA levels between 2 and 8 h before settling back to a low baseline level of expression after 16 h (Figure 1B). Despite its rather unre-

Figure 2. DNA-binding assay showing diminished binding of YB-1 to its cognate pyrimidine-rich, single-stranded DNA binding site in the SM α A MCAT enhancer within 60 min after exposure of hPFBs to TGF β 1 (5 ng/ml). Changes in the size of the YB-1 protein pool in unfractionated hPFB nuclei were relatively modest over the first 60 min of exposure to TGF β 1 (right lanes).

markable effect on net SM α A mRNA accumulation, however, thrombin administered to hPFB at doses between 2–10 U/ml caused a rapid and significant increase in SM α A protein level (Figure 1C). Evaluation of Western blots prepared from hPFBs treated with 5 U/ml thrombin plus the metabolic inhibitors actinomycin D or cycloheximide indicated a requirement for active SM α A protein biosynthesis rather than *de novo* SM α A mRNA transcription (Figure 1C). Together, the data indicated that rapidly elevated SM α A protein synthesis in hPFBs after exposure to thrombin seemed to be based largely on a posttranscriptional control mechanism involving highly efficient utilization of a small pool of available SM α A mRNA.

TGF β 1 Dissociates YB-1 Repressor from the Activated SM α A Promoter

The recent literature contains several reports implicating the YB-1 single strand-specific, nucleic acid-binding protein, or its murine counterpart MSY-1, in control of both gene transcription and mRNA translation (Capowski *et al.*, 2001; Esnault and Malter, 2003; Kohno *et al.*, 2003; Skabkin *et al.*, 2004; Matsumoto *et al.*, 2005). With regard to elevated SM α A gene expression observed in human myofibroblasts, we discovered that the transcription- and translation-promoting activities of TGF β 1 and thrombin, respectively, were associated with a marked change in YB-1 subcellular distribution and nucleic acid binding activity. As shown in Figure 2, the physical interaction of hPFB YB-1 with its cognate pyrimidine (Pyr)-rich, single-stranded DNA (ssDNA) binding element from the SM α A enhancer decreased markedly within 30 min after exposure to 5 ng/ml TGF β 1. Further reduction of YB-1 interaction with DNA was evident during the next 3 h followed by a very slight recovery at 6 h. Reduced ssDNA binding occurred during the 60-min interval when little change was noted in the level of available YB-1 protein in unfractionated hPFB nuclear protein extracts, suggesting that loss of YB-1–ssDNA interaction in TGF β 1-activated hPFBs was not due to simple dilution of the available protein pool.

Reduced interaction between YB-1 protein and SM α A enhancer DNA in TGF β 1-activated human myofibroblasts supported our previous findings showing that cold-shock domain proteins function to repress SM α A gene transcription (Kelm *et al.*, 1999a; Carlini *et al.*, 2002). However, our previous studies on mouse embryonic fibroblasts containing the MSY-1 homologue of human YB-1 provided only indirect evidence for repressor function because the experimental approach was designed simply to reveal a general increase in transcriptional activity when point mutations known to block MSY-1 binding to Pyr-rich, ssDNA were placed into the context of SM α A enhancer:reporter fusion

Figure 3. (A) Nonhuman primate COS7 fibroblasts were transfected with VSMP4 plus either an empty mammalian expression vector (pCDNA3), an expression plasmid encoding the MSY-1 mouse homologue of YB-1 (MSY1), equal molar amounts of three expression plasmids encoding Smad 2, Smad 3, and Smad 4 (Smads) or a combination of all four expression plasmids (MSY1 + Smads). CAT ELISA performed 48 h after transfection revealed that MSY1 inhibited constitutive VSMP4 expression, whereas Smads were capable of activating VSMP4 as well as partly neutralizing the inhibitory effect of MSY-1 overexpression on VSMP4 activity. (B) Immunoblots showing native YB-1 levels in the nuclear, DNA-bound, and cytosolic fractions of fibroblasts after transfection with equal molar amounts of Smad 2, Smad 3, and Smad 4 expression plasmids. YB-1 interaction with a single-strand oligonucleotide probe containing its cognate, pyrimidine-rich binding site (YB-1:ssDNA-bound) decreased substantially by 6 h after transfection. Over the 48-h observation period, nuclear levels of YB-1 diminished (YB-1:nucleus) but increased in the cytosol (YB-1:cytosol). Smad 2 and Smad 3 proteins accumulated in the nuclear fraction within 24 h after transfection (Smad 2,3:nucleus).

genes (Cogan *et al.*, 1995; Sun *et al.*, 1995). The binding of other SM α A gene repressors, Pura and Pur β , also was inhibited in these mutant constructs thus potentially obscuring the specific contribution of MSY-1 to gene repression. To examine the regulatory properties of MSY-1 more directly, nonhuman primate COS7 kidney fibroblasts were transfected with the TGF β 1-responsive mouse SM α A enhancer construct VSMP4 plus a mammalian expression plasmid encoding full-length mouse MSY-1 cDNA. As shown in Figure 3A, overexpression of mouse MSY-1 protein in simian COS7 fibroblasts was sufficient to block baseline transcription from the VSMP4 enhancer consistent with its action as a SM α A gene repressor. Additional results presented in Figure 3A indicate that combined overexpression of Smad proteins 2, 3, and 4 caused more than a twofold increase in SM α A enhancer activity in COS7 fibroblasts as well as significantly offset the inhibitory effect of mouse MSY-1 protein overexpression on enhancer transcriptional activity. These observations were an important extension of our recent studies showing that Smads also can partly relieve repression of the SM α A enhancer by the Pura protein (Subramanian *et al.*, 2004). To examine how Smad protein overexpression influenced the distribution and DNA-binding properties of endogenous YB-1 protein, we examined nuclear, cytosolic, and DNA-bound protein fractions by Western blot analysis. Activation of the SM α A enhancer by overexpression of Smads 2, 3, and 4 during a 48-h period was accompanied by a decrease in the level of YB-1 protein in COS7 nuclei with concomitant accumulation in the cytoplasm.

mic fraction (Figure 3B). Depletion of YB-1 from the nucleus also was accompanied by decreased interaction with its cognate, Pyr-rich ssDNA enhancer-binding element. Interestingly, ssDNA–YB-1 interaction decreased rapidly subsequent to Smad protein overexpression and was substantially reduced in parallel with depletion of YB-1 from the nucleus. Nuclear uptake of Smads 2 and 3 exhibited a notable lag phase and was not significantly increased until 24–48 h after transfection (Figure 3B) when a significant increase in VSMP4 enhancer activity was noted (our unpublished data). The results imply that interruption of YB-1 interaction with single-strand SM α A enhancer DNA and nuclear export were early responses to Smad protein overexpression in transfected fibroblasts.

Thrombin Disrupts Interaction of YB-1 and the Pur α/β Corepressor Proteins with Cytoplasmic SM α A mRNA

Smad 2-, 3-, and 4-dependent activation of SM α A enhancer transcription in fibroblasts was associated with redistribution of YB-1 between the nuclear and cytosolic protein pools. We next examined whether the ability of thrombin to activate SM α A gene expression and myofibroblast differentiation (Bogatkevich *et al.*, 2001) also involved redeployment of YB-1 protein but in a manner that would be functionally consistent with translational rather than transcriptional control. YB-1 and related cold-shock domain family members are known to influence RNA-based processes in several ways, including unfolding secondary structural elements in mRNA as well as recruiting other proteins required for ribonucleoprotein (RNP) packaging, transport, turnover, and/or translation (Kohn *et al.*, 2003). Of initial interest was how thrombin influenced YB-1 subcellular distribution during SM α A gene activation and whether modulation of YB-1 pools by thrombin was mechanistically related to the reduction in nuclear YB-1 level observed in TGF β 1-activated hPFBs. We also were interested in examining how the single strand-specific nucleic acid binding proteins, Pur α and Pur β , behaved in the presence of thrombin because not only do these proteins collaborate with YB-1/MSY-1 to repress SM α A gene expression (Kelm *et al.*, 1999a; Carlini *et al.*, 2002), they also have been identified as important linker proteins for tethering mRNA to microtubules and motor proteins as well as transport of 1000S mRNA:protein granules in other cell types (Ohashi *et al.*, 2002; Kanai *et al.*, 2004). For specifically analyzing how thrombin might influence translational control in myofibroblasts, we used a novel VSMP4:CAT reporter fusion construct containing a segment of the SM α A mRNA coding element (CE) located in exon 3 that has structural similarity to the MCAT-THR enhancer within the SM α A promoter including a high degree of purine and pyrimidine asymmetry. The salient feature of this construct is that the CE blocks translation when placed 3' to the transcriptional start site of the CAT reporter gene (Kelm *et al.*, 1999b). Constitutive transcriptional activity of this construct in fibroblasts is mediated by the VSMP4 component and unaffected by the inserted CE(3') module. However, translation of the CAT-CE(3') mRNA transcript is markedly impaired due to high-affinity association of available YB-1/MSY-1, Pur α , and Pur β with the CE(3') motif. In this capacity, the CAT-CE(3') fusion represents a useful tool for assessing how agents such as thrombin alter mRNA translational efficiency in a YB-1/MSY-1/Pur α /Pur β binding-dependent manner. More than a 3.5-fold increase in CAT protein synthesis was observed when CAT-CE(3')-transfected hPFBs were exposed to 5 U/ml thrombin over a 10-min period (Figure 4A). Posttransfection treatments with thrombin were designed to be of short duration to specifi-

Figure 4. (A) ELISA depicting CAT protein in transfected hPFBs that accumulated during a 10-min exposure to vehicle (solid bar) or thrombin (5 U/ml, shaded bar). hPFBs were transfected 48 h before treatment with the P4/CE(3') reporter construct that contains the coding element (CE) from exon 3 of SM α A mRNA previously shown to bind YB-1 and Pur proteins required for translational repression of CAT mRNA (Kelm *et al.*, 1999b). (B) Binding of native YB-1, Pur α , and Pur β in hPFB nuclear and cytosol extracts to the exon 3 RNA coding element (3CE). Thrombin (5 U/ml, 10-min exposure) reduced binding of cytosolic YB-1 and Pur proteins to the 3CE RNA probe while enhancing these interactions in the nuclear compartment.

cally assess de novo translation of preexisting CAT mRNA. Pulmonary fibroblasts transfected with the CE(3') reporter gene construct were maintained for 48 h before the 10-min treatment with thrombin to allow for steady-state accumulation of CAT-CE(3') mRNA. Direct assessment of YB-1 and Pur protein interaction with CE-RNA also was evaluated in both the hPFB nuclear and cytosolic protein pools after short-term exposure to thrombin. As shown in Figure 4B, thrombin specifically disrupted binding between CE-RNA and the YB-1 and Pur proteins in the cytosolic pool. Interestingly, interaction of the CE-RNA with these proteins in the nuclear compartment was markedly enhanced by thrombin over this same time period. Protein interaction with SM α A RNA was sequence specific because transversion mutations introduced into the CE-RNA at positions held in common with SM α A enhancer sites required for ssDNA-specific binding of YB-1 and Pur proteins eliminated all binding in both the nuclear and cytosolic compartments (our unpublished data).

Rapid Deployment of SM α A Thin Filaments and Nuclear Uptake of YB-1 in Myofibroblasts Is Mediated by Thrombin-specific Signaling

Net redistribution of YB-1 from the cytosol to the nucleus occurred rapidly in thrombin-treated hPFBs. Immunofluorescence microscopy was used to examine spatial relationships between the subcellular distribution of YB-1 and assembly of SM α A thin filaments in thrombin-treated hPFBs. Within 5 min after exposure to thrombin, diffusely distributed cytosolic YB-1 was consolidated within myofibroblast nuclei (Figure 5). Brief exposure to thrombin also resulted in a striking increase in deployment of SM α A thin filament networks in hPFBs. Together with the results of biochemical fractionation and SM α A mRNA binding studies shown in Figure 4, the morphological analysis of thrombin-activated myofibroblasts implied that relocation of YB-1 from the cytosol to nuclear compartment was temporally linked to rapid assembly of the SM α A cytoskeleton in myofibroblasts. Because thrombin has been implicated as one of the several

Figure 5. Immunofluorescence microscopic evaluation of YB-1 and SM α A distribution in thrombin-activated hPFBs. Nuclear compartmentalization of YB-1 and deployment of SM α A microfilaments were evident within 5 min of exposure to 5 U/ml thrombin (Th) compared with vehicle-treated controls (C). Nuclei were identified in YB-1 antibody-treated hPFBs by staining with DAPI (PI). Magnification, 40 $\times$ objective.

cellular protease capable of activating latent TGF β 1 stores in stromal cells, we performed additional studies to evaluate the relative contribution of TGF β 1- and thrombin-specific receptor signaling in myofibroblast SM α A protein accumulation. As shown in Figure 6, inclusion of an inhibitor of the type I TGF β 1 receptor serine/threonine kinase in the culture medium (SB431542) substantially reduced SM α A protein accumulation in TGF β 1-treated hPFBs, but it had no grossly restrictive effect on induction by thrombin. Further supporting an independent molecular mechanism of action, a thrombin serine protease inhibitor attenuated SM α A accumulation in thrombin-treated hPFBs but did not prevent induction in the presence of TGF β 1 (Figure 6). The ability of thrombin to rapidly augment SM α A expression in hPFBs does not seem to involve amplification of an underlying TGF β 1-dependent process such as activation of latent TGF β 1 or increased mRNA transcription but instead seems to have its basis in a thrombin-specific action such as enhanced efficiency of SM α A protein synthesis and/or thin

Figure 6. Immunoblots depicting SM α A levels in hPFBs in the presence of TGF β 1 or thrombin with or without their cognate inhibitors. Lanes 1 and 6, vehicle-treated controls; lane 2, TGF β 1 alone; lane 3, TGF β 1 + SB431542 kinase inhibitor; lane 4, thrombin alone; lane 5, thrombin + SB431542 kinase inhibitor; lane 7, TGF β 1 alone; lane 8, TGF β 1 + thrombin protease inhibitor; lane 9, thrombin alone; lane 10, thrombin + thrombin protease inhibitor. Each inhibitor attenuated SM α A expression in an independent, ligand-specific manner with little effect on GAPDH expression.

Figure 7. (A) Thrombin-dependent changes in YB-1 subcellular compartmentalization required the Ras-Raf-MEK-ERK mitogen-activated protein (MAP) kinase signaling pathway. Immunoblot analysis showed that YB-1 levels decreased in the cytosol and markedly increased in the nucleus of hPFBs within 1 min after treatment with thrombin (1 U/ml). The MAP kinase inhibitors U0126 and PD98059 both blocked thrombin-dependent nuclear import of YB-1. GAPDH expression in the two protein fractions indicated equivalent protein loading for each sample and was largely unaffected by the various treatments. The nomenclature 1/1' and 1/5' denotes 1 U/ml thrombin for either 1 or 5 min, respectively. (B) Enhanced SM α A synthesis in thrombin-treated hPFBs required ERK protein phosphorylation. Immunoblot analysis was performed using antibodies specific for SM α A and phosphorylated forms of p44 and p42 ERK signaling intermediaries. A 5-min exposure to thrombin caused rapid phosphorylation of p42 and p44 and enhanced SM α A synthesis but was without effect in hPFBs that were treated with the U0126 MEK1 inhibitor.

filament stability. In this regard, phosphorylated extracellular signal-regulated kinase (ERK) proteins have been shown to mediate thrombin PAR-1 receptor-based signal transduction and promote rapid elevation of smooth muscle-specific myosin gene expression in thrombin-treated vascular smooth muscle cells (Reusch *et al.*, 2001). As shown in Figure 7A, the Ras-Raf-(mitogen-activated protein kinase kinase (MEK)-ERK pathway inhibitors U0126 and PD98059 both blocked nuclear uptake of YB-1 and increased the size of the cytosolic YB-1 pool relative to baseline levels seen in control hPFB preparations. MEK1 inhibition by U0126 also partly blocked accumulation of SM α A protein in thrombin-treated hPFBs while causing a notable reduction in the level of phosphorylated p44/p42 ERK signaling intermediary proteins in hPFBs (Figure 7B).

DISCUSSION

In this report, we described a novel role for the YB-1 nucleic acid-binding protein in coordinating SM α A gene expression in human pulmonary myofibroblasts. YB-1 contains a highly conserved cold-shock domain that mediates nucleic acid

binding in response to cellular stress signals, including low temperature, drug toxicity, reactive oxygen signaling, and UV damage (Matsumoto and Wolffe, 1998) (Kohn *et al.*, 2003). In addition to SM α A, several other genes important in wound healing and cellular proliferation and survival also are regulated by YB-1, including those encoding α 1 and α 2 (I) collagen (Norman *et al.*, 2001; Higashi *et al.*, 2003), the B chain isoform of platelet-derived growth factor (PDGF) (Steinina *et al.*, 2000), MMP-2 (Mertens *et al.*, 1997), vascular endothelial growth factor (Coles *et al.*, 2004), granulocyte macrophage-colony stimulating factor (Coles *et al.*, 2000), p21 and p53 (Kohn *et al.*, 2003), and the Fas death receptor (Lasham *et al.*, 2000). In our recent studies of myofibroblasts and arterial smooth muscle cells, we discovered that MSY-1, the YB-1 homologue in rodents, was able to repress SM α A gene transcription by binding the pyrimidine-rich strand of the MCAT enhancer located in an inverted repeat between positions -190 and -150 of the SM α A promoter (Kelm *et al.*, 1999a; Carlini *et al.*, 2002). Alternative base pairing within the inverted repeat produces a thermodynamically stable stem-loop structure. The pyrimidine-rich, reverse-strand component of the loop binds YB-1 thus potentially disrupting promoter interaction with transcriptional activators such as Smad 2, Smad 3, Sp1, and TEF-1 that require duplex DNA proximal to the MCAT enhancer (Cogan *et al.*, 2002; Subramanian *et al.*, 2004). The MCAT enhancer region also exhibits substantial chromatin conformational change in response to TGF β 1 (Becker *et al.*, 2000). Relationships between YB-1, RNA binding, and thrombin signaling also were noted and of interest because thrombin activation of PDGF-B gene transcription in human endothelial cells has been shown to require YB-1 proteolytic processing and nuclear translocation (Steinina *et al.*, 2000).

Results reported here extend findings of TGF β 1-regulated interplay between Sp1, a GC-rich SM α A promoter element called SPUR, and the Pur family of single-strand nucleic acid binding proteins in myofibroblasts (Subramanian *et al.*, 2004). Although YB-1 also exhibits TGF β 1-dependent interplay with the SM α A promoter, its action is centered on the MCAT enhancer located ~120 base pairs upstream from SPUR. However, the ability of YB-1 and Pur repressor proteins to self-associate and bind diverse transcriptional activators, including Sp1, serum response factor, TEF-1, and Smad proteins (Kelm *et al.*, 1999a, 2003; Subramanian *et al.*, 2004) implies that they do not function in complete physical isolation. Although originally identified as repressors of SM α A gene transcription, YB-1 and Pur proteins also have roles in RNA processing and transport of RNPs along cytoskeletal filaments in the cytoplasm (Ruzanov *et al.*, 1999) (Ohashi *et al.*, 2000, 2002; Stickeler *et al.*, 2001; Kohn *et al.*, 2003; Kanai *et al.*, 2004). The YB-1 protein family member p50 is one of the most abundant components of ribonucleoprotein particles in eukaryotes and has a notable mRNA-dependent affinity for muscle and nonmuscle actin filaments (Ruzanov *et al.*, 1999). Excessive amounts of p50 in RNPs effectively blocked both translation and F-actin interaction, whereas high mRNA:p50 ratios were associated with active polyribosomal RNPs and RNP-actin filament complexes. Titration studies using human recombinant YB-1 and atomic force microscopy revealed that RNPs with a low YB-1:mRNA ratio were unfolded and better configured for efficient polyribosomal binding and translation (Skabkin *et al.*, 2004). Published reports also indicated that C-terminal proteolytic processing (Steinina *et al.*, 2000) may influence YB-1 shuttling dynamics between the nuclear and cytoplasmic compartments thus providing another control point for governing protein function. Pur proteins also have been identi-

fied in RNPs where they form links with motor proteins such as kinesin and myosin Va and IIB in neuronal cells (Ohashi *et al.*, 2000, 2002; Kanai *et al.*, 2004). Pur protein-RNPs are thought to couple microtubules with kinesin motor proteins to transport mRNAs from their sites of synthesis in cell bodies to distal polyribosomes located near synaptic terminals.

As RNA-binding proteins, YB-1 and Pur proteins perform unique tasks in the cytosol that complements their more conventional roles as nuclear transcriptional regulatory proteins. In this regard, myofibroblasts rapidly assemble a polarized SM α A thin filament system capable of producing directional forces necessary for reshaping the provisional extracellular matrix and guiding wound contraction. We speculate that myofibroblasts use YB-1 and Pur proteins to coordinate the transcription, transport, and compartmentalization of SM α A mRNA in a manner that facilitates efficient construction and deployment of a polarized smooth muscle-like contractile apparatus at sites of tissue injury. In this scheme, nuclear YB-1 and Pur proteins that become released from SM α A enhancer DNA in TGF β 1-activated myofibroblasts may no longer function as transcriptional repressors but instead assist as chaperones in the packaging, transport, and translation of SM α A mRNA. RNA-based actions of YB-1 and Pur proteins seem to be directed by thrombin signaling that likely coexists with TGF β 1 signaling within the complex microenvironment of the healing wound. Transience is a key feature of SM α A gene expression and thin filament deployment in myofibroblasts and we speculate that thrombin-directed release of YB-1 and Pur proteins from cytosolic RNPs and subsequent nuclear reentry may serve this requirement by rerepressing the SM α A gene and terminating myofibroblast differentiation. Although requiring additional experiments, this hypothetical scheme constitutes a regulatory loop whereby SM α A gene output in TGF β 1-activated myofibroblasts initially is amplified at the translational control level, then quenched by thrombin (Figure 8). The magnitude of SM α A protein output in myofibroblasts subsequent to transcriptional activation by TGF β 1 may be rate-limited by thrombin-dependent shuttling of YB-1 and Pur proteins off cytosolic mRNA and onto enhancer DNA in the nucleus. The Ras-Raf-MEK-ERK signaling pathway stimulated by thrombin may be especially relevant in attenuation of TGF β 1 signaling because ERK-mediated phosphorylation of the Smad 2 or 3 linker region has been shown to prevent nuclear accumulation of these proteins (Kretzschmar *et al.*, 1999). ERK signaling in pulmonary myofibroblasts might also influence ribosomal protein phosphorylation and increase the net rate of protein synthesis during wound healing similar to its role in mechanical force-injured cardiac fibroblasts (MacKenna *et al.*, 1998). On the other hand, thrombin may employ ERK to counteract TGF β 1 action on myofibroblast differentiation via induction of the Egr-1 DNA-binding protein known to activate promoters for proinflammatory tumor necrosis factor- α (Verrecchia and Mauviel, 2004) and interleukin-1 β (Tan *et al.*, 2003) that both have well characterized anti-fibrotic, matrix-destructive properties. Related studies on endothelial cells showed that thrombin in some cases can counteract TGF β 1 signals because a PAR-1 peptide agonist caused TGF β 1 receptor internalization and suppressed Smad 2 and 3 nuclear translocation (Tang *et al.*, 2005). Together, the available evidence suggests that thrombin may provide an important physiological rheostat designed to promote rapid construction of the contractile provisional matrix in healing wounds in parallel with hemostasis while preventing maladaptive tissue fibrosis due to chronically unchecked TGF β 1 signaling. A

Figure 8. Hypothetical scheme for coordination of SM α A transcription and translation by YB-1 in TGF β 1- and thrombin-activated hPFBS. Rapid SM α A translation and thin filament deployment by thrombin may commence from an available pool of preexisting, cytosolic SM α A mRNPs. Replenishment and amplification of this pool may require de novo activation of the SM α A gene via a slower, TGF β 1-dependent transcriptional process requiring both nuclear uptake of Smads plus dissociation of YB-1 from the SM α A promoter. Displaced YB-1 may be sequestered from the nucleus within cytosolic SM α A mRNPs. As ERK-dependent translation of SM α A messenger RNA proceeds, YB-1 nuclear reentry may terminate TGF β 1-dependent transcription and myofibroblast differentiation.

key question to be addressed by future experimentation is to determine whether the thrombin-mediated nuclear reentry of YB-1 and Pur proteins amplifies myofibroblast TGF β 1-dependent differentiation by facilitating the transport and translation of SM α A mRNA, terminates the TGF β 1 signal by enabling YB-1 and Pur proteins to reaccess their cognate repressor sites in the SM α A enhancer, or both. Chronic diseases associated with excessive accumulation of myofibroblasts, faulty SM α A gene expression, and hypertrophic scarring such as idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, kidney epithelial mesenchymal transition, and transplant arteriosclerosis all may exhibit dysregulation in the coordination of thrombin and TGF β 1 signaling as a common predisposing condition.

Although the mechanistic details will require further study, disruption of YB-1 binding to the MCAT enhancer in the SM α A promoter seems to be an early response to TGF β 1 treatments. SM α A gene activation required Smad binding to multiple CAGA sites distributed within the large 40-base pair segment of DNA that contains the seven-base pair MCAT enhancer (Subramanian *et al.*, 2004). There are three CAGA motifs between -190 and -150, but only two base pairs of one CAGA overlaps with the MCAT site. Thus, although loss of YB-1 binding could improve Smad access to one of its cognate sites in the promoter, the positions of the other two sites may render them less susceptible to YB-1 interference. Indeed, Smad binding is observed in a different region of the -190/-150 segment called the THR (TGF β 1 hyperreactivity region) but not at the MCAT where YB-1 binds (Subramanian *et al.*, 2004). Chromatin conformation analysis before and after TGF β 1 also revealed that the MCAT-THR region unfolds during myofibroblast differentiation (Becker *et al.*, 2000). Although the molecular basis for unfolding is not known, competitive interplay between YB-1 and Smads does not necessarily have to occur on promoter DNA but instead may involve protein complexes assembled in the cytosol that indirectly influence protein subunit availability or function in the nucleus. Data presented in Figure 3 imply that changes in YB-1 interaction with promoter DNA as well as the reduction in nuclear level of YB-1 both precede nuclear accumulation of Smad 2 and 3. In this regard, there is evidence that the anti-TGF β 1 properties of interferon- γ derive in part from its ability to foster binding of YB-1 to

Smad 3 (Higashi *et al.*, 2003). Within the complex, YB-1 prevents Smad 3 from linking with its p300 histone acetyltransferase coactivator thus blocking transcription of the type α 2(I) collagen gene in fibroblasts. Understanding dynamic interplay between YB-1, Smads, and MCAT-THR DNA will require more detailed examination of protein complex formation in the cytosol as well as protein-DNA interaction and assessment of DNA secondary structure in the nucleus. SM α A gene activation may involve more sophisticated control than can be provided by simple replacements between repressors and activators on promoter DNA.

In summary, YB-1 represses SM α A gene transcription and binds the MCAT region of the SM α A promoter previously shown to undergo chromatin unfolding in TGF β 1-activated myofibroblasts. Transcriptional activation of the SM α A gene by TGF β 1 was accompanied by loss of YB-1 binding to the MCAT enhancer and nuclear export. In contrast, thrombin stimulated SM α A gene activation at the translational level and caused YB-1 and Pur proteins to dissociate from translational repression sites on mRNA in parallel with rapid SM α A protein synthesis. Because thrombin by itself had no significant activating effect on SM α A mRNA transcription, it will be important in future studies to establish whether thrombin-dependent nuclear reentry of YB-1 is designed to rerepress the SM α A gene and/or destabilize preexisting SM α A mRNA thus contributing to termination of both the TGF β 1 activation signal and myofibroblast differentiation. In a normally healing wound, myofibroblasts are transient participants and active forms of TGF β 1 and thrombin are likely to coexist. The ability of TGF β 1 and thrombin to independently exploit the DNA- and RNA-binding properties of YB-1, respectively, adds a new dynamic perspective to control of gene expression during myofibroblast differentiation that may reveal strategies for therapeutic management of these cells in chronic fibrotic diseases that affect the heart, lung, liver, and kidney.

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