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## Fibulin-5 gene expression in human lung fibroblasts is regulated by TGF- $\beta$ and phosphatidylinositol 3-kinase activity

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**Kuang, Ping-Ping, Martin Joyce-Brady, Xiao-Hui Zhang, Jyh-Chang Jean, and Ronald H. Goldstein.** Fibulin-5 gene expression in human lung fibroblasts is regulated by TGF- $\beta$  and phosphatidylinositol 3-kinase activity. *Am J Physiol Cell Physiol* 291: C1412–C1421, 2006. First published July 12, 2006; doi:10.1152/ajpcell.00087.2006.—Fibulin-5 (FBLN5), an extracellular matrix glycoprotein required for normal elastogenesis, is coordinately expressed with elastin during lung injury and repair. We found that treatment with transforming growth factor- $\beta$  (TGF- $\beta$ ) induced a rapid but transient increase in FBLN5 heterogeneous nuclear RNA (hnRNA) followed by a sustained increase in the steady-state level of FBLN5 mRNA. The transcription start site of the human FBLN5 gene was localized at 221 nucleotides upstream of the translation start site by using primer extension, Northern blots, and functional analysis of transcriptional activity in reporter plasmids containing 5'-flanking regions. TGF- $\beta$  markedly increased FBLN5 promoter activity in transient transfection assays. Two putative Smad-binding sites were identified within the proximal promoter and are required for this TGF- $\beta$  induction. Electrophoretic gel mobility shift assay revealed that TGF- $\beta$  strongly increased binding of Smad2 and Smad3 nuclear complexes to the proximal FBLN5 promoter and induced a Smad2/3-dependent binding of slow migrating nuclear protein complex. FBLN5 mRNA induction by TGF- $\beta$  was blocked by pretreatment with TGF- $\beta$  receptor inhibitor SB-431542, the phosphatidylinositol 3-kinase (PI3-kinase) inhibitor LY-294002, and actinomycin D. Basal and TGF- $\beta$ -induced FBLN5 hnRNA and mRNA were strongly and proportionally decreased by LY-294002, as was TGF- $\beta$ -induced phosphorylation of Akt, but not Smad3, as measured by Western blot analysis. In addition, LY-294002 markedly and proportionally decreased FBLN5 promoter activity in transient transfection analyses with TGF- $\beta$ -treated or untreated lung fibroblasts. These studies demonstrate that induction of FBLN5 gene expression in lung fibroblasts is mediated via canonical TGF- $\beta$ /Smad signaling and requires the PI3-kinase/Akt pathway.

elastogenesis; emphysema; transcription; Smads; Akt

FIBULIN-5 (FBLN5), originally known as EVEC and DANCE, is a 66-kDa glycoprotein within a newly recognized family of five extracellular matrix proteins that share an elongated structure of several calcium-binding epidermal growth factor (cbEGF)-like domains in tandem (39). FBLN5 is found mainly in elastic fiber enriched tissues including the aorta, the lung, the uterus, and the skin (10, 11, 25). FBLN5-deficient mice (FBLN5<sup>-/-</sup>) have a defect in the elastic fiber formation and exhibit loose skin, tortuous arteries, and emphysematous lungs, suggesting that FBLN5 is essential for elastogenesis (24, 41). FBLN5 mRNA is expressed in the embryo during the formation of the large blood vessels and the cardiac valves and also in the developing postnatal lung. Although the level of expression in adult tissues is low, FBLN5 mRNA

expression can be induced in conditions such as atherosclerotic plaque formation, development of pulmonary hypertension, and lung injury and repair (10, 11, 15, 21, 25). In humans, mutations of the FBLN5 gene cause the cutis laxa syndrome, a heritable elastic fiber-related disorder, and age-related macular degeneration (18, 38).

The role of FBLN5 in elastic fiber development is largely attributed to its modular structure and multifunctional domains, which enable FBLN5 to interact with microfibrils such as fibrillin-1 and integrins on cell surface (6, 24, 39). The six cbEGF-like domains enable interaction with elastin in a calcium-dependent manner, and the arginine-glycine-aspartate (RGD) motif, located in the first cbEGF-like domain, mediates FBLN5 binding to  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins on the cell surface (24, 39, 41). More recently, FBLN5 has been found to bind fibrillin-1, lysyl oxidase-like protein 1 (LOXL-1), and extracellular superoxide dismutase (6, 17, 28). These unique biological binding properties may impart FBLN5 not only a central role in the formation of the microfibrillar scaffold, deposition of soluble tropoelastin, and assembly of elastic fibers but also a critical function in maintaining mechanical properties, signaling transduction, and control of cell function and in regulating the local redox state of elastic tissues (6, 22, 28, 35, 37).

TGF- $\beta$  is a member of a superfamily of extracellular polypeptides with multifunctional effects. It is synthesized, secreted, and stored as an inactive form bound to latent TGF- $\beta$ -binding proteins in the extracellular matrix (1). Active TGF- $\beta$  binds and sequentially activates its receptor complexes and downstream Smad2/3 and non-Smad signaling pathways to mediate target gene expression (2, 32). TGF- $\beta$  may play an essential role in maintaining homeostasis and functional integrity of matrix tissue in the lung. TGF- $\beta$  promotes production of extracellular matrix proteins such as type I collagen and tropoelastin in lung fibroblasts (20) but represses production of matrix-degrading metalloproteinases in alveolar macrophages (5). Dysregulation of TGF- $\beta$  signaling pathways in integrin  $\beta_6$ - and fibrillin-1-deficient mice promotes the development of lung emphysema (23, 26).

Employing an elastase-induced mouse model of human emphysema, we previously found that FBLN5 is coordinately expressed with elastin during repair of lung injury (15), suggesting a potential role of this protein in the repair process. FBLN5 was expressed predominantly by lung fibroblasts in association with extracellular matrix and was degraded by elastase treatment (15). The molecular mechanisms underlying

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the upregulation of FBLN5 are unclear. We hypothesized that during the development of emphysema, proteolysis of lung matrix increases the release and cleavage of inactive TGF- $\beta$ , which in turn stimulates production of FBLN5, elastin, collagen, and other extracellular proteins to initiate the repair process. In the present study, we have identified and characterized the FBLN5 promoter and demonstrated that upregulation of FBLN5 gene expression by TGF- $\beta$  in human lung fibroblast is mediated by two Smad-binding sites located in the proximal promoter and is phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway dependent. Our results suggest that TGF- $\beta$  may play an important role in the repair of damaged lung elastic fibers by promoting synthesis of FBLN5, elastin, and other key elastogenic components.

## EXPERIMENTAL PROCEDURES

**Cell culture and reagents.** Cycloheximide (CHX), actinomycin D (ActD), and TGF- $\beta$  type I receptor inhibitor SB-431542 were obtained from Sigma (St. Louis, MO), LY-294002 (LY), U0126, PD-98059, SB2035580, and SP600125 from Calbiochem (La Jolla, CA), and recombinant human TGF- $\beta$ 1 and IL-1 $\beta$  from R&D (Minneapolis, MN). Human lung fibroblasts (IMR-90) were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 0.37 g/100 ml sodium pyruvate, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin in a humidified 5% CO<sub>2</sub>-95% air incubator at 37°C as previously described (13). Confluent cultures were rendered quiescent by refeeding with serum-free medium for 24 h before experimentation.

**RNA isolation, Northern blot analysis, and TaqMan real-time quantitative RT-PCR analysis.** Serum-starved confluent IMR-90 cells were untreated or treated with TGF- $\beta$  (1 ng/ml) for various time periods. For inhibitor studies, the pretreatments were performed with CHX (10  $\mu$ g/ml), ActD (10  $\mu$ g/ml), SB-431542 (20  $\mu$ M), LY-294002 (10  $\mu$ M), U0126 (10  $\mu$ M), PD-98059 (25  $\mu$ M), SB2035580 (25  $\mu$ M), and SP600125 (20  $\mu$ M) for 1 h before treatment with TGF- $\beta$  (1 ng/ml) for 16 h. Total RNA was prepared with RNeasy mini kit (Qiagen, CA) according to the manufacturer's protocol, followed by Northern analysis using <sup>32</sup>P-labeled cDNA probes for FBLN5 (GenBank accession no. NM\_006329) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as previously described (15, 16). The same total RNA samples were also treated with RNase-free DNase I (Qiagen) and assessed for FBLN5 mRNA expression using TaqMan reagents in the TaqMan real-time quantitative PCR assay.

To analyze gene expression using TaqMan real-time quantitative RT-PCR analysis, DNase-treated total RNA (2  $\mu$ g) was first reversely transcribed in a 50- $\mu$ l volume using random primers and the high-capacity cDNA Achieve kit (Applied Biosystems) according to the manufacturer's protocol. TaqMan reagents for detecting mRNA expression of FBLN5 or GAPDH were purchased from Applied Biosystems (Foster City, CA). To assess FBLN5 hnRNA expression, a set of sequence-specific 6-FAM dye-labeled probes plus forward and reverse primers flanking the first exon/intron boundaries of FBLN5 gene were designed and synthesized using PrimerExpress software (Applied Biosystems). The TaqMan probe contained the reporter dye 6-FAM linked to the 5'-end of the probe and a minor groove binder (MGB) and a nonfluorescent quencher (NFQ) at the 3'-end of the probe. Primers and probe used for human FBLN5 hnRNA analysis were as follows: forward primer, 5'-CCTCGCGCTCTTGA; reverse primer, 5'-CAGCGCCGAGAACCAC; and TaqMan probe, 5'-6-FAM-ATGCCAGGAATAAAA-MGB-NFQ. GAPDH mRNA expression was used as endogenous control for internal standardization and normalization of data (31). TaqMan PCR reactions were performed in triplicates using the ABI PRISM 7700 sequence detection system (Applied Biosystems) according to the manufacturer protocol. The data are means  $\pm$  SE of relative fold changes of three independent experiments

normalized to corresponding GAPDH mRNA expression. The significance was analyzed using two-tailed Student's *t*-test.

**Plasmid constructs, mutagenesis, transient transfection, and luciferase assay.** The sequences of human FBLN5 gene were obtained by searching www.genome.ucsc.edu. A fragment of the 5'-flanking region of human FBLN5 gene (-2150 to -1, related to the first translation start codon ATG) was obtained by PCR using human genomic DNA as template (Clontech, Palo Alto, CA) and the forward primer with *Kpn*I site (underlined) linked to its 5'-end (5'-TAAGGTACCACCACCCAGTC-TATGGTATTCTGTTATAGC-AGCC-3') and the reverse primer with *Xho*I linked to its 5'-end (5'-TAACTCGAGGTCCAAGACGCGGAG-GAGGAGATGCGAA-3'). With the use of these fragments as templates, a series of FBLN5 5'-flanking regions were similarly generated by PCR with further deletion from either its 5'- or 3'- $\phi$ -end. After digestion with *Kpn*I and *Xho*I and purification, these fragments were inserted into the pGL2 basic luciferase reporter (Promega, Madison, WI) as previously described (14). The constructs were numbered relative to the FBLN5 ATG translation start site: pGL2/-2150 to -1, pGL2/-2150 to -355, pGL2/-2150 to -236, pGL2/-2150 to -216, pGL2/-675 to -1, pGL2/-675 to -188, pGL2/-392 to -1, and pGL2/-315 to -1.

Mutagenesis of the FBLN5 promoter was performed to replace Smad-binding element AGAC-box with the sequence TTAA using the QuikChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. Complimentary mutant sense and antisense primers were synthesized as follows: for the sequence GTCT located at -360 to -363 (underlined): sense strand, 5'-CAAGATT-GTTGTGAGGATTTAAAGCCAGTTGGTGAGCGCT-3' and antisense strand, 5'-AGCGCTCACCAACTGGCTTTAATCCTCA-CAACAATCTTG-3', corresponding to bases -380 to -342; for the sequence AGAC located at -316 to -319 (underlined): sense strand, 5'-TCTGAACCAGCTGTGTCCTTAATGAGGCCCATTTGCA-TTG-3' and antisense strand, 5'-CAATGCAAATGGGG-CCTCAT-TAAGGACACAGCTGGTTCAGA-3', corresponding to bases -337 to -297. These primers, along with 125 ng of pGL2/-392 to -1 as a template, were used for site-directed mutagenesis to generate single and double mutant pGL2/-392 to -1 constructs. All constructs were verified by DNA sequencing.

Transient transfection with FBLN5 promoter luciferase constructs was performed as previously described (16). Briefly, transient transfection was performed with FuGENE 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. Transfection efficiency was monitored by cotransfection of pRLTK *Renilla* luciferase vector (0.25  $\mu$ g/well; Promega). At 24 h after transfection, cells were serum-starved for 6 h before conditioned with TGF- $\beta$ 1 (1 ng/ml), LY-294002 (10  $\mu$ M), or both for 16 h, before being harvested for luciferase assay. Firefly and *Renilla* luciferase activity were determined with the Dual-Luciferase Reporter system (Promega) and a luminometer (TD20/20; Turner Designs, Sunnyvale, CA). Firefly luciferase values were normalized to *Renilla* luciferase values and expressed as means  $\pm$  SE of relative firefly/*Renilla* luciferase activities of three or more independent experiments. The significance was analyzed using a two-tailed Student's *t*-test.

**Preparation of nuclear extracts and electrophoretic mobility shift assay.** Nuclear extracts were prepared on ice, and electrophoretic mobility shift assay (EMSA) was performed as previously described (14). FBLN5 promoter DNA fragments (-374 to -302, related to translational start site) were generated by PCR using a forward primer with 5'-end-linked restriction enzyme *Xba*I sequence (underlined; see below) and a reverse primer with 5'-end-linked restriction enzyme *Nco*I sequence (underlined; see below). PCR products were column-purified with Qiagen PCR clean-up kit followed by restriction enzyme digestion and gel purification. These purified FBLN5 promoter DNA fragments were end-labeled with [ $\alpha$ -<sup>32</sup>P]dCTP and Klenow (New England Biolabs). Nuclear extracts (15  $\mu$ g) were incubated with 1  $\times$  10<sup>5</sup> cpm of labeled elastin promoter fragments (1 ng). Competition study and supershift experiments were performed in the presence of 100 $\times$  unlabeled promoter

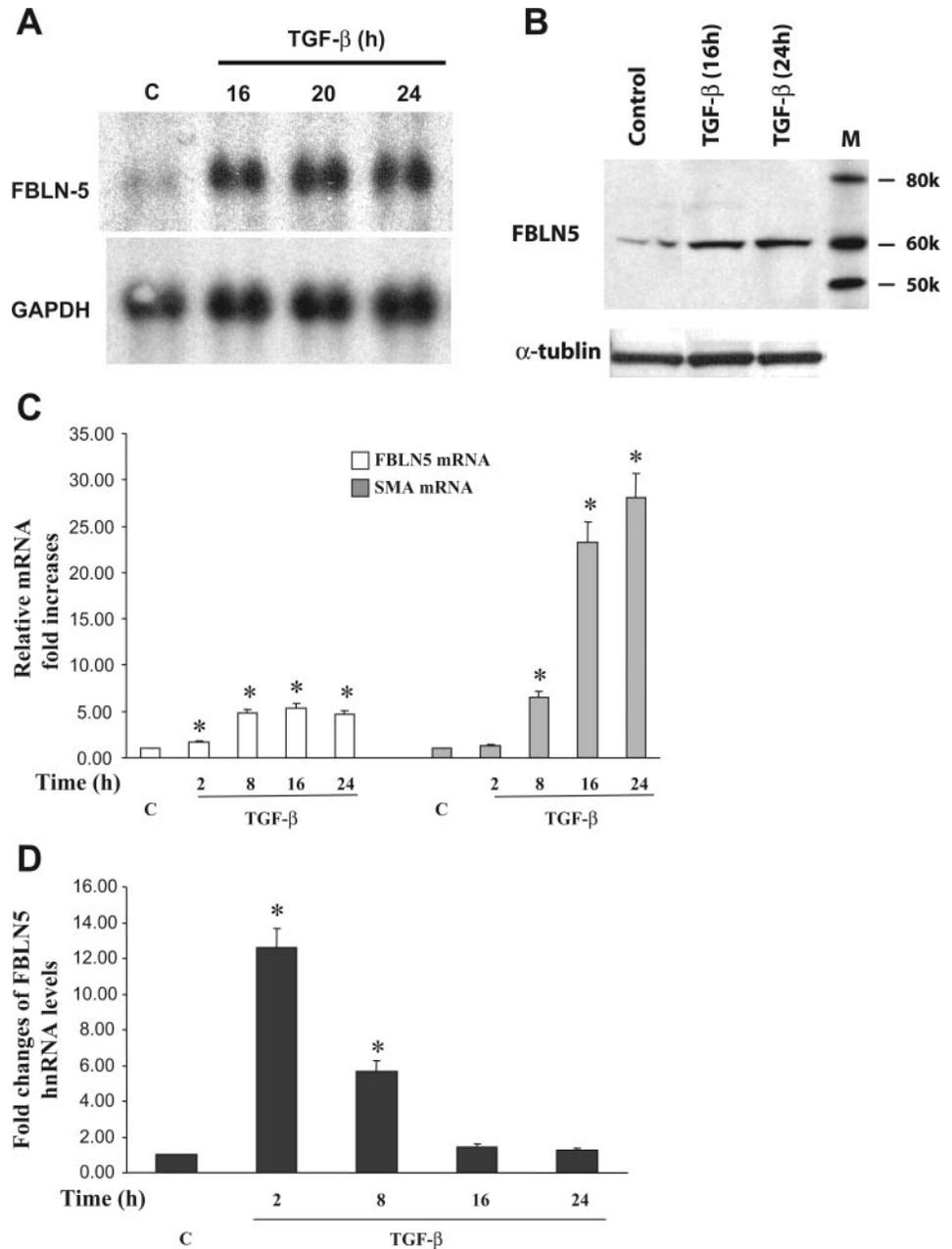


Fig. 1. Effect of TGF- $\beta$  on the steady-state levels of fibulin-5 (FBLN5) heterogeneous nuclear RNA (hnRNA) and mRNA. *A*: confluent fibroblast cultures were serum-starved for 24 h before untreated (C) or treated with TGF- $\beta$ 1 (1 ng/ml) for 16, 20, and 24 h. Cells were harvested and total RNA was isolated, followed by sequential Northern blot analysis using  $^{32}$ P-labeled cDNA probes for human FBLN5,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively. Data are representative of 3 similar experiments. *B*: effect of TGF- $\beta$  on FBLN5 protein expression. Whole cell lysates were isolated from fibroblasts either untreated (control) or treated with TGF- $\beta$  (1 ng/ml) for 16 and 24 h. Equal protein extracts were analyzed by SDS-PAGE, followed by Western blot analysis with anti-FBLN5 and anti- $\alpha$ -tubulin antibodies. *C* and *D*: kinetic regulation of FBLN5 mRNA, SMA mRNA, and FBLN5 hnRNA expression by TGF- $\beta$ . Total RNA was isolated from serum-starved cells untreated (C) or treated with TGF- $\beta$  for various time periods as indicated. Levels of FBLN5 mRNA and SMA mRNA (Fig. 1C) were assessed using TaqMan real-time PCR. Similarly, the level of FBLN5 hnRNA (Fig. 1D) was determined using TaqMan real-time PCR with a set of specific probe and primers complementary to the first exon/intron boundary. \* $P$  < 0.05 compared with untreated cells (C).

DNA fragments and anti-Smad3 or Smad2 antibodies (Santa Cruz Biotechnology), respectively. Reactions were resolved on a preelectrophoresed 4% nondenaturing polyacrylamide gel (40:1 acrylamide-bisacrylamide) in  $1\times$  Tris-borate-EDTA at 150 V for 2.5 h. The forward and reverse primers were synthesized by IDT (Coralville, IA), and their sequences (5' to 3'; sense strand) are 5'-AATTCTAGATTGTGAG-GAG-TCTAGCCAGTTG-3' and 5'-TAAGGTACCAAATGGGGCCT-CAGTCTGGACAC-3', respectively.

**Whole cell extract preparation and Western blot analysis.** Whole cell extracts were prepared and Western analysis was performed as previously described (16). Briefly, confluent lung fibroblasts were serum-starved for 24 h before left untreated or treated with TGF- $\beta$ 1 (1 ng/ml) or they were pretreated with inhibitors as described above for various time periods. After being washed twice with cold PBS, cells were harvested in cold RIPA lysis buffer containing aprotinin (1  $\mu$ g/ml), pepstatin (1  $\mu$ g/ml), leupeptin (1  $\mu$ g/ml), and PMSF (0.1  $\mu$ M). Total protein (50  $\mu$ g) was resolved in SDS-PAGE, followed by Western analysis using FBLN5

antibody (Santa Cruz Biotechnology) and anti-phospho antibodies against Akt, Smad2 and 3, c-Jun, and p38 at the recommended dilution ratio (Cell Signaling, Beverly, MA). After stripping, the same blot was probed with either anti- $\alpha$ -tubulin antibody or non-phospho antibody against Akt (Cell Signaling) to monitor the loading control.

**Statistical analysis.** Data are presented as means  $\pm$  SE of three independent experiments performed in triplicate and normalized to *Renilla* luciferase activities for transfection of FBLN5 promoter luciferase reporters or to levels of GAPDH mRNA for real-time quantitative PCR. Data were analyzed using a two-tailed Student's *t*-test, and  $P$  < 0.05 was considered statistically significant.

## RESULTS

To assess the effect of TGF- $\beta$  on FBLN5 gene expression, human lung fibroblasts were grown to confluence and rendered serum-starved for 24 h before treatment with TGF- $\beta$ 1 (1

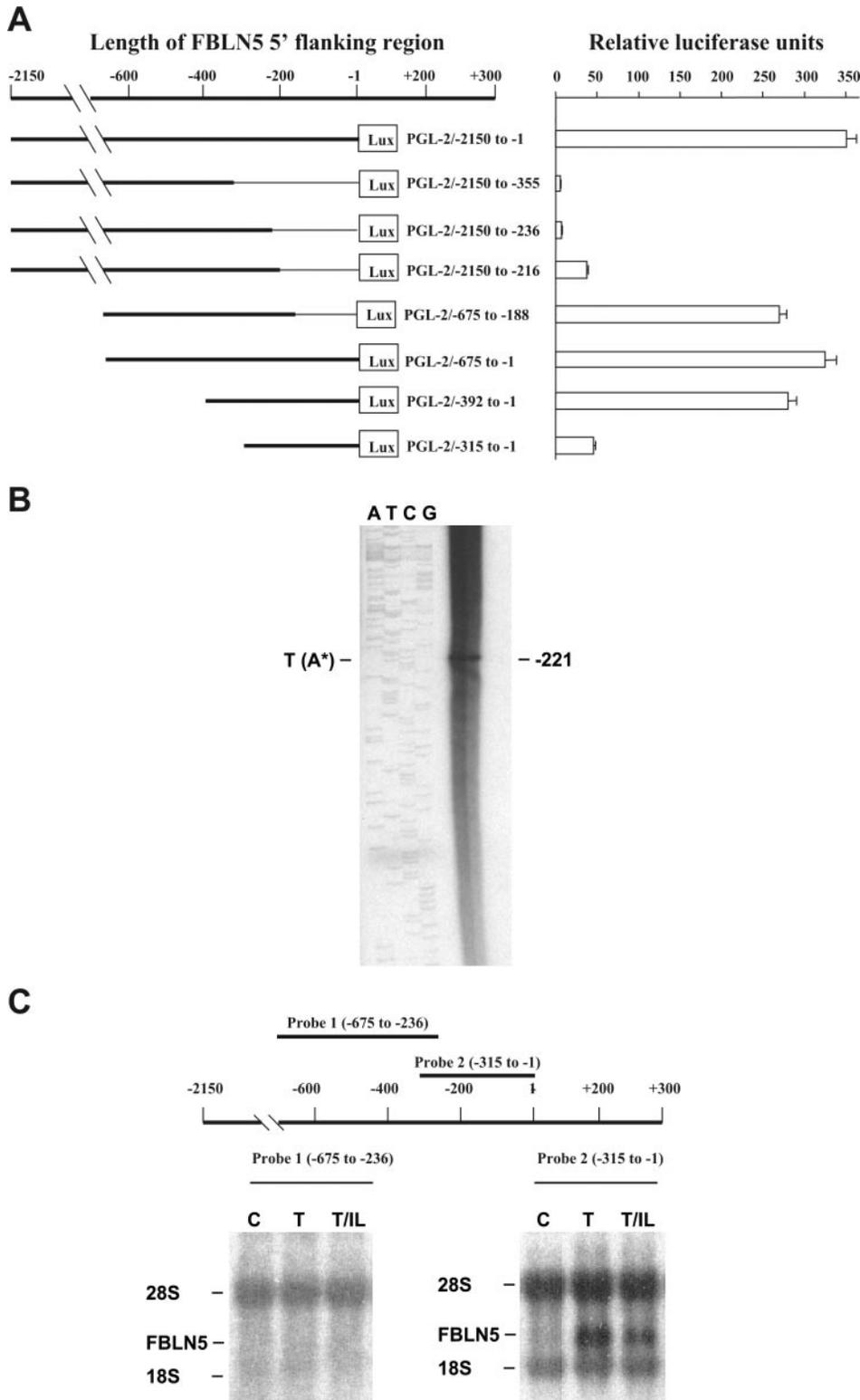


Fig. 2. Identification and characterization of FBLN5 promoter. *A*: deletion analysis of FBLN5 5'-flanking region. *Left*, sequences of constructs containing the 5'-flanking region of FBLN5 gene that were used in transfection studies; *right*, the luciferase activity of each of these constructs after transfection. *B*: primer extension analysis of the transcriptional initiation site of FBLN5. *Lanes A, T, C, and G* represent sequencing products of the pBlue-script plasmid DNA as size markers; *right* lane represents primer extension products when <sup>32</sup>S-labeled primer was annealed to the total RNA isolated from TGF- $\beta$ -treated cells. *C*: confirmation of transcriptional start site using Northern blot analysis. Total RNA was isolated from serum-starved confluent fibroblasts untreated (*C*) or treated with TGF- $\beta$  (*T*; 1 ng/ml) alone or plus IL-1 $\beta$  (250 pg/ml; *T/IL*) for 16 h. Northern blot analysis was performed using <sup>32</sup>P-labeled *probe 1* and *probe 2* of PCR fragments of FBLN5 5'-flanking regions as indicated.

ng/ml) for various time periods. Northern analysis showed that human lung fibroblasts expressed minimal basal level of FBLN5 mRNA but not  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) mRNA, a marker of the myofibroblast phenotype. The steady-state levels of both FBLN5 mRNA and  $\alpha$ -SMA mRNA were markedly increased by TGF- $\beta$  treatment (Fig. 1A). Western

blot analysis revealed that the basal level of FBLN5 protein was also increased by TGF- $\beta$  treatment (Fig. 1B). The data shown are representative of three experiments.

The kinetic expression profiles of FBLN5 and  $\alpha$ -SMA were determined using TaqMan real-time PCR (Fig. 1C). The level of FBLN5 mRNA was increased 1.6-fold by TGF- $\beta$  after 2 h

of treatment, became maximal at 5.2-fold after 8 h, and remained elevated up to 24 h (Fig. 1C, right). In contrast, the level of FBLN5 hnRNA, which reflects the rate of gene transcription, was rapidly increased (12.6-fold) by TGF- $\beta$  at 2 h of treatment (Fig. 1D). The transcriptional rate gradually declined toward baseline, which was 5.6-fold above controls at 8 h and decreased to the basal level at 16 h after treatment (Fig. 1D). The expression pattern for TGF- $\beta$ -induced  $\alpha$ -SMA mRNA expression was more delayed. An increase in  $\alpha$ -SMA mRNA was evident at 8 h following treatment and reached its maximal at 24 h (Fig. 1C, left). TGF- $\beta$  did not affect the rate of GAPDH transcription and mRNA expression as previously described and was used as the internal normalization control (31).

To explore *cis*- and *trans*-acting elements that may underlie the regulation of FBLN5 gene transcription by TGF- $\beta$ , the promoter region of the gene was cloned and deletion constructs were generated from the 5'-flanking region by being inserted into the pGL2 basic luciferase reporter. Transient transfection indicated that sequences in two regions, -392 to -315 and -236 to -188, were required for the basal transcriptional activity (Fig. 2A). Primer extension assay was carried out to map the transcription start site by using total RNA from TGF- $\beta$ -treated human lung fibroblasts and a  $^{35}$ S-labeled anti-sense oligonucleotide complementary to the sequence located at +1 to +25. A major single extension product was identified and located at an adenine residue 221 bases upstream from the translational start site (Fig. 2B). To further verify this result, we performed Northern analysis using probes with two overlapping DNA fragments around this potential site. The probes, synthesized by PCR, are indicated in Fig. 2C, top, as *probe 1* (-675 to -236) and *probe 2* (-1 to -315). No transcripts corresponding to the size of FBLN5 mRNA were detected in the hybridization using *probe 1*. A transcript the size of the 28S ribosome was present, but this signal was not affected by treatment with TGF- $\beta$  or IL-1 $\beta$  (Fig. 2C, bottom left). In contrast, a transcript the size of FBLN5 mRNA was detected in the hybridization with *probe 2*, and this signal was induced by TGF- $\beta$  and attenuated by addition of IL-1 $\beta$  (Fig. 2C, bottom right). Transcripts with sizes of the 18S and the 28S ribosomal RNA were also detected using *probe 2*, but these were not affected by treatment with TGF- $\beta$  or IL-1 $\beta$  (Fig. 2C, bottom right). These data support the initiation of FBLN5 transcription by TGF- $\beta$  in lung fibroblasts at the adenine located 221 bases upstream of the first ATG translation initiation codon and the requirement of sequences between -392 to -315 for promoter activity.

The sequence analysis of the proximal FBLN5 promoter from -1 to -665 bp using MatInspector (16) revealed a palindromic CCAAT/enhancer binding sequence located at -243 to -250 (CCAATTGG) and two AGAC-boxes of Smad3/4 binding element located at -360 to -363 (5'-GTCT-3') and at -316 to -319 (5'-AGAC-3') (4) (Fig. 3). To determine the biological function of these two Smad-binding sites, we performed transfection assays to examine the TGF- $\beta$  responsiveness in FBLN5 promoter constructs with either the AGAC-box deletion or mutations (Fig. 4, A and B). Further deletion of the sequence including Smad-binding sites markedly decreased basal transcriptional activity of the FBLN5 promoter and abolished induction by TGF- $\beta$  (Fig. 4A). Mutation of each Smad-binding sequence alone partially attenuated

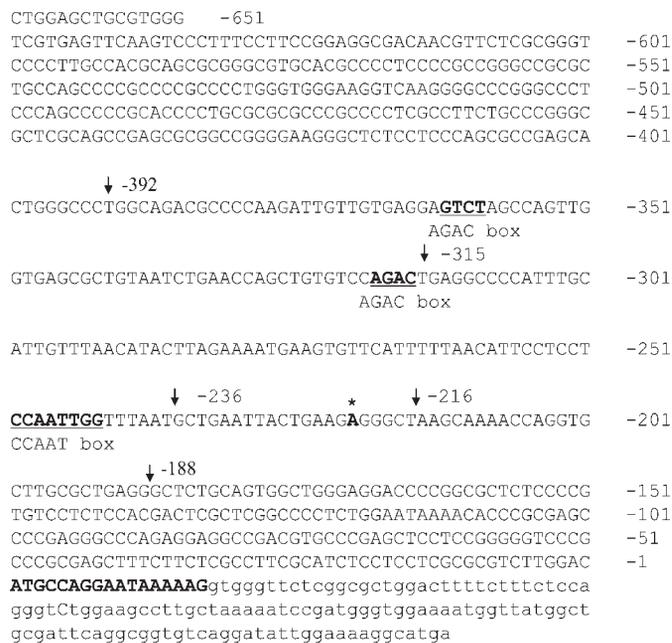


Fig. 3. The 5'-flanking sequence alignment of human FBLN5 gene. The transcription initiation site is in bold type and indicated with an asterisk. The inverted CCAAT sequence and two TGF- $\beta$  responsive binding sites are in bold, underlined type and marked as CCAAT box and AGAC box, respectively. The coding region sequence of FBLN5 first exon is shown in bold type, and the partial sequence of the first intron is shown in lowercase letters.

the transcriptional activation of the FBLN5 promoter by TGF- $\beta$  (Fig. 4B). However, mutations at both AGAC-boxes completely blocked activation and induced a significant increase of basal FBLN5 promoter activity (Fig. 4B). These data suggested that these two potential Smad-binding elements are required for upregulation of FBLN5 promoter by TGF- $\beta$ .

EMSA was performed to examine the binding of nuclear proteins to the putative Smad-binding sequence in the proximal FBLN5 promoter. Two nuclear protein complexes were detected that bound to this domain. The binding was strongly increased upon treatment with TGF- $\beta$ . In addition, a high-molecular-weight complex was detected following TGF- $\beta$  treatment (Fig. 4C, 2nd and 3rd lanes). The binding specificity of these complexes was assessed by competition studies: the formation of these three complexes was abrogated by the presence of 100 $\times$  unlabeled promoter DNA fragments (Fig. 4C, 4th and 5th lanes). To determine whether these complexes are related to Smad2/3, we performed supershift assays using anti-Smad3 or Smad2 antibodies (Santa Cruz Biotechnology). The addition of the Smad3 antibody abrogated formation of *complexes 1* and *3* and dramatically decreased formation of *complex 2* (Fig. 4C, 5th and 6th lanes). The addition of the Smad2 antibody partially decreased binding of *complex 1*, strongly decreased formation of *complex 2*, and also abolished formation of *complex 3* (Fig. 4C, 7th and 8th lanes). These results suggest that *complex 1* contains only Smad3, whereas *complex 2* contains both Smad2 and Smad3. The slow migrating band (*complex 3*) induced by TGF- $\beta$  likely represents a complex composed of Smad2/3 and other unidentified DNA-binding proteins (Fig. 4C, 3rd lane) (19).

The effect of TGF- $\beta$  is mediated through canonical TGF- $\beta$  receptors by activation of Smad-dependent and Smad-indepen-

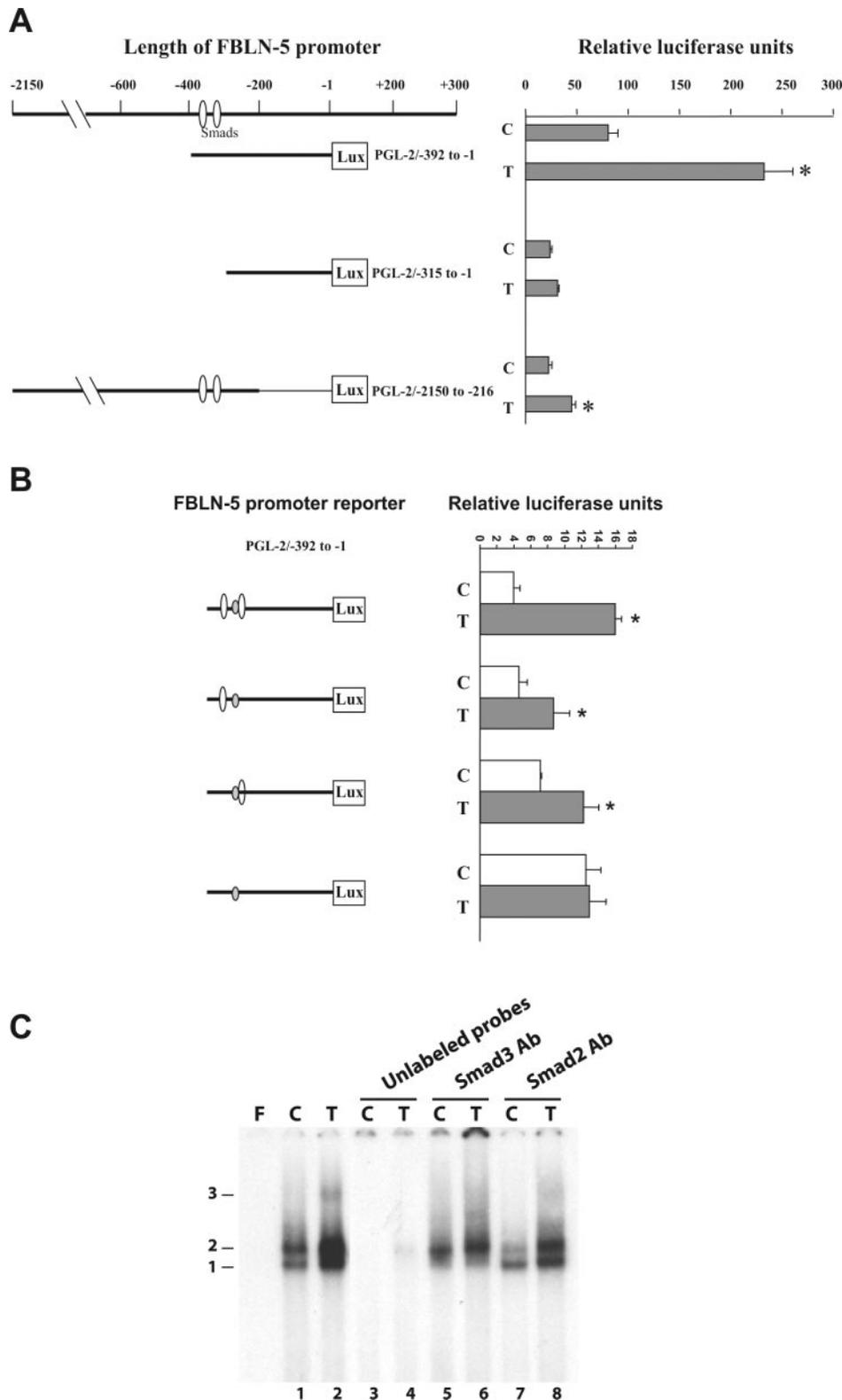


Fig. 4. Functional analysis of TGF- $\beta$  responsive elements. **A:** deletion analysis of Smad-binding sites in proximal FBLN5 promoter. *Left*, a schematic diagram of deletion constructs used; *right*, the relative luciferase activity of these constructs after transfection without (C) or with TGF- $\beta$ 1 (T; 1 ng/ml). \* $P < 0.05$  compared with control. **B:** site-directed mutagenesis of Smad-binding elements in the FBLN5 promoter. *Left*, a schematic diagram of wild-type and mutant constructs, with which the core sequence GTCT or AGAC in the proximal FBLN5 promoter was replaced; *right*, the relative luciferase activity of these constructs after transient transfection without (C) or with TGF- $\beta$ 1 (T; 1 ng/ml). \* $P < 0.05$  compared with control. **C:** binding of nuclear proteins to FBLN5 promoter. EMSA was performed in the presence of 2  $\mu$ g of poly(dI-dC) by incubation of the  $^{32}$ P-end-labeled FBLN5 promoter fragments (-374 to -302) without (F) or with nuclear extracts isolated from untreated (C) or TGF- $\beta$ -treated fibroblasts (T, 1 ng/ml, 2 h). Additional reactions contained either 100 $\times$  unlabeled DNA fragments or antibodies against Smad2 or 3. Reactions were resolved using 4% native polyacrylamide gel, and unbound probes were run off the gel to separate the complexes 1 and 2.

dent signal transduction pathways (2, 32). Recent studies have demonstrated that a variety of non-Smad signaling cascades activated by TGF- $\beta$  play a critical role in the determining ultimate biological outcomes (32). To determine the signaling pathways that may be activated by TGF- $\beta$  in human lung fibroblasts, we isolated and analyzed whole cell lysates by Western blot analysis

using anti-phospho antibodies against the corresponding kinases. Treatment with TGF- $\beta$  increased phosphorylation of Smad2, Akt, c-Jun, and p38, which was reversed by the TGF- $\beta$  receptor I inhibitor SB-431542 (Fig. 5A). TGF- $\beta$  treatment increased the level of basal p42/44 phosphorylation, which was blocked by pretreatments with U0126 or PD-98059 (data not shown).

To determine whether these signaling pathways play a role in the upregulation of FBLN5 mRNA by TGF- $\beta$ , we pretreated fibroblasts for 1 h with CHX (10  $\mu$ g/ml), PI3-kinase inhibitor LY-294002 (10  $\mu$ M), p44/42 inhibitor U0126 (10  $\mu$ M), PD-98059 (25  $\mu$ M), ActD (10  $\mu$ g/ml), or p38 MAPK inhibitor SB2035580 (25  $\mu$ M) before treatment with TGF- $\beta$  for 16 h, followed by total RNA isolation and Northern blot analysis (Fig. 5B). SB-431542 (10  $\mu$ M) (8) was also used to selectively

inhibit TGF- $\beta$  type I receptor, followed by RNA isolation and analysis using TaqMan real-time PCR (Fig. 5C). Pretreatment with LY-294002, ActD, or SB-431542 reversed TGF- $\beta$  induced increase in FBLN5 mRNA, whereas pretreatment with CHX (10  $\mu$ g/ml), U0126, SB2035580, or PD-98059 had no such effect (Fig. 5, B and C).

We further examined the role of PI3-kinase activity on FBLN5 gene expression. Pretreatment with LY-294002 strongly decreased levels of basal FBLN5 hnRNA and significantly blocked its induction by TGF- $\beta$  (Fig. 6A, left). Similarly, LY-294002 decreased the basal expression of FBLN5 mRNA in a time-dependent fashion and completely abolished its increase by TGF- $\beta$  (Fig. 6A, right). However, LY-294002 did not decrease the ratio of induction of FBLN5 hnRNA and mRNA by TGF- $\beta$  (Fig. 6A). These data indicate that inhibition of PI3-kinase activity decreased the basal levels of FBLN5 but did not block TGF- $\beta$  activity. This result was verified in transient transfection studies employing the FBLN5 promoter, demonstrating that LY-294002 markedly inhibited FBLN5 promoter activity but did not block the effect of TGF- $\beta$  (Fig. 6B). In contrast, TGF- $\beta$ -stimulated expression pattern of FBLN5 hnRNA and mRNA was not altered by pretreatment with U0126 or SB2035580 (data not shown).

The effect of LY-294002 on PI3-kinase pathway was verified by examining the inhibition of its downstream target, Akt phosphorylation. Western analysis showed that LY-294002 blocked levels of basal and TGF- $\beta$ -induced phosphorylation of Akt (Fig. 6C) but did not affect TGF- $\beta$ -induced phosphorylation of Smad3 (Fig. 6C). Pretreatment with SB2035580 and U0126 blocked phosphorylation of p38 by TGF- $\beta$  and p42/42 phosphorylation, respectively (data not shown). These results suggest that the induction of FBLN5 gene expression by TGF- $\beta$  requires the PI3-kinase/Akt activity.

DISCUSSION

Perturbation of lung matrix tissue releases active TGF- $\beta$ , initiating a signal transduction cascade that stimulates extracellular matrix production and deposition. Our data demonstrate that addition of exogenous TGF- $\beta$  to human lung fibroblasts induces a marked increase of FBLN5 gene expression by

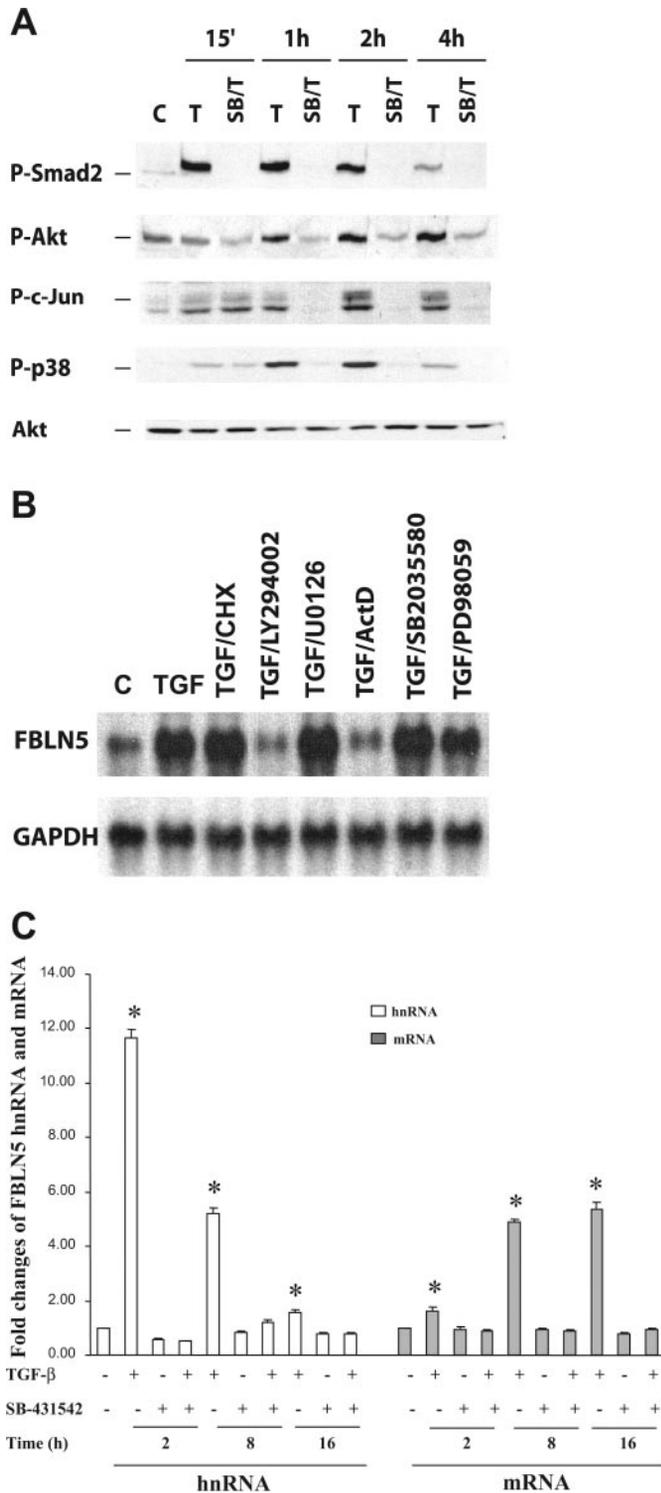


Fig. 5. Characterization signaling pathways involved in TGF- $\beta$ -induced upregulation of FBLN5 gene expression. A: determining signaling pathways activated by TGF- $\beta$ . Serum-starved confluent cells were untreated (C) or treated with TGF- $\beta$ 1 (T; 1 ng/ml) or pretreated with TGF- $\beta$  type I receptor inhibitor SB-431542 (SB; 10  $\mu$ M) for 1 h before incubation with TGF- $\beta$ 1 for various time periods as indicated. Whole cell lysates were isolated and subjected to sequential Western blot analysis using antibodies against phospho-Akt (Ser473), phospho-Smad2 (Ser465/467), phospho-c-Jun (Ser65), and phospho-p38 MAPK (Thr180/Tyr182). The Akt antibody against total Akt protein was used as a loading control. B: effect of specific signaling inhibitors on the upregulation of FBLN5 mRNA by TGF- $\beta$ . Serum-starved confluent fibroblasts were untreated (C) or treated with TGF- $\beta$ 1 (TGF; 1 ng/ml) or pretreated with cycloheximide (CHX; 10  $\mu$ g/ml), LY-294002 (10  $\mu$ M), U0126 (10  $\mu$ M), actinomycin D (ActD, 10  $\mu$ g/ml), SB2035580 (25  $\mu$ M), or PD98059 (25  $\mu$ M) for 1 h before addition of TGF- $\beta$ 1 for 16 h. Total RNA was isolated and followed by Northern blot analysis using  $^{32}$ P-labeled human cDNA probes for FBLN5 and GAPDH. C: effect of TGF- $\beta$  type I receptor inhibitor SB-431542 on FBLN5 hnRNA and mRNA expression. Serum-starved confluent IMR-90 cells were untreated or treated with TGF- $\beta$ 1 (1 ng/ml) or pretreated with TGF- $\beta$  receptor I inhibitor SB-431542 (10  $\mu$ M) for 1 h before addition of TGF- $\beta$ 1 for various time periods as indicated. The expression profiles of FBLN5 hnRNA and mRNA were assessed using TaqMan real-time PCR. \* $P$  < 0.05 compared with control.

increasing the level of transcription. To further evaluate the regulation of FBLN5 gene transcription, we identified and characterized the FBLN5 promoter using promoter deletion analysis, primer extension, Northern analysis, and site-directed mutagenesis assays. A major transcription start site was mapped at the adenine base located at 221 nucleotides upstream of the translational start site (Fig. 2). Sequence analysis with the MatInspector program revealed that the FBLN5 promoter lacks a typical TGF- $\beta$ -binding protein-binding TATAA box, as does the elastin promoter (33). The palindromic sequence CCAATTGG is located at -243 to -250 and functions

as a potential CCAAT/enhancer binding element (Fig. 3). TATA-less promoters commonly exhibit multiple transcriptional start sites, but we did not observe this in the FBLN5 promoter.

In other systems, exposure to TGF- $\beta$  induced formation of a heterologous complex of Smad proteins that bind to promoter regions and activate gene expression (36). The DNA sequence 5'-AGAC-3' functioned as the Smad-binding element (SBE) for both Smad3 and Smad4 (4). The FBLN5 proximal promoter contains two such TGF- $\beta$  responsive elements (underlined) in the sequences GTCTAGCCA, located at -355 to -363, and the sequence TGTCCAGAC, located at -316 to -324 (Fig. 3). These two SBEs are located within a distance of 40 base pairs and are required for basal FBLN5 promoter activity and its maximal induction by TGF- $\beta$  (Figs. 3 and 4). Deletion of promoter sequences containing these elements resulted in loss of basal promoter activity and TGF- $\beta$  responsiveness, indicating that recruitment and assembly of the transcriptional initiation complex on the proximal FBLN5 promoter requires these sequence motifs. Functional analysis of these sites using site-directed mutagenesis revealed that mutation of the individual sites induced an increase of basal promoter activity and partial blockade of TGF- $\beta$  responsiveness. Mutation of both sites led to a further increase in basal promoter activity and complete inhibition of the TGF- $\beta$  effect (Fig. 4). The mutations may have increased or introduced binding to an as yet uncharacterized activation element, induced a conformational change on FBLN5 promoter that facilitated transcriptional initiation, or blocked binding of a transcriptional inhibitor. These data suggest that a specific FBLN5 transcriptional module involving two Smad-binding sites regulates FBLN5 gene expression in response to TGF- $\beta$ .

EMSA studies showed that Smad2 and Smad3 nuclear complexes specifically bound to these two SBEs containing FBLN5 proximal promoter fragments and were markedly increased upon treatment with TGF- $\beta$ . A slow migrating nuclear protein complex was detected in the nuclear extract isolated from TGF- $\beta$ -treated cells (Fig. 4C). The binding of this TGF- $\beta$ -induced high-molecular-weight complex to FBLN5 promoter was disrupted in the presence of antibodies against Smad3 or Smad2 (Fig. 4C), suggesting that the complex contains Smad2/3 and other unknown nuclear proteins. Smad

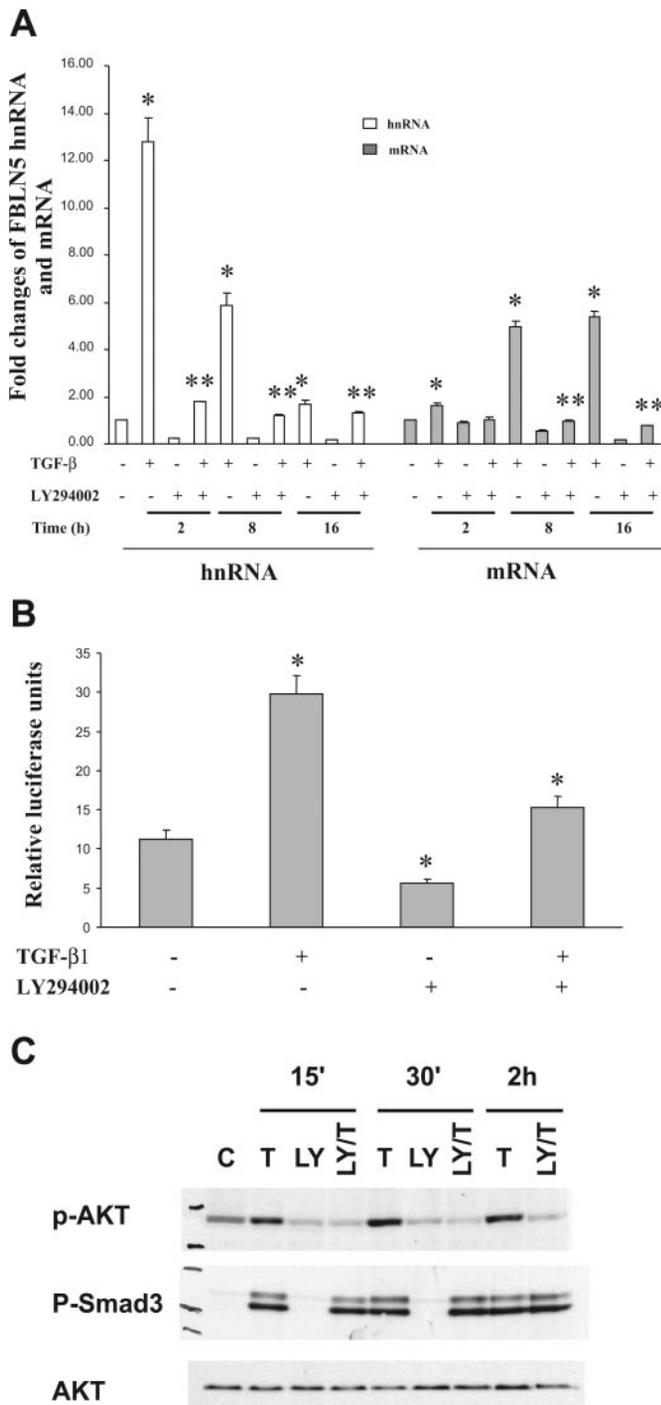


Fig. 6. Effect of phosphatidylinositol 3-kinase inhibitor LY-294002 on FBLN5 gene expression. **A**: serum-starved confluent fibroblasts were untreated or treated with TGF- $\beta$ 1 (1 ng/ml) or pretreated with LY-294002 (10  $\mu$ M) for 1 h before addition of TGF- $\beta$ 1 for various time periods as indicated. TaqMan real-time PCR was performed on isolated mRNA to assess the expression profiles of FBLN5 hnRNA and mRNA. \* $P$  < 0.5 compared with control. \*\* $P$  < 0.05 compared with LY-294002-treated cells. **B**: effect of LY-294002 on FBLN5 promoter activity. Proximal FBLN5 promoter pGL2/-392 to -1 was cotransfected with pRLTK *Renilla* luciferase vector using FuGENE-6. At 24 h after transfection, fibroblasts were serum-starved for 6 h before treatment with TGF- $\beta$ 1 (1 ng/ml), LY-294002 (10  $\mu$ M), or both for 16 h followed by harvesting for luciferase assay. Data are normalized to *Renilla* luciferase activity and expressed as relative luciferase units. \* $P$  < 0.05 compared with control. **C**: effect of LY-294002 on the phosphorylation of Akt and Smad3 by TGF- $\beta$ . Confluent serum-starved fibroblasts were untreated (C) or treated with TGF- $\beta$ 1 (T; 1 ng/ml) or pretreated with LY-294002 (LY; 10  $\mu$ M) for 1 h before incubation with TGF- $\beta$ 1 for various time periods as indicated. Whole cell lysates were isolated and subjected to sequential Western blot analysis using antibodies against phospho-Akt (Ser473), phospho-Smad3 (Ser433/435), and total Akt protein, respectively. Data are representative of 3 similar experiments.

proteins require multiple AGAC binding sites and other DNA-binding cofactors to specifically activate target genes. The ultimate biological consequences of TGF- $\beta$  exposure are determined by Smads-specific interactions with a large repertoire of DNA binding factors, coactivators, and corepressors (19). The high-molecular-weight complex may contain one or more cofactors needed to achieve optimal activation of the FBLN5 promoter. Taken together, our results indicate that upregulation of FBLN5 transcription by TGF- $\beta$  is mediated via the binding of Smad2/3 nuclear complexes to these two putative Smad-binding sites in the proximal FBLN5 promoter. In contrast, a previous study (35) in murine 3T3-L1 cells reported that induction of FBLN5 gene expression by TGF- $\beta$  is regulated by the Smad3-independent mechanisms as assessed using a dominant negative Smad3 retroviral system. The failure of dominant negative Smad3 to inhibit TGF- $\beta$ -induced FBLN mRNA in 3T3-L1 likely reflects the dramatic reduction in the responsiveness of virally transfected fibroblasts to TGF- $\beta$  stimulation, because this also occurred in virally transfected green fluorescent protein expression fibroblasts (35). Another possibility is that TGF- $\beta$  signaling is differentially regulated in the cellular context of virally transfected murine 3T3-L1 cells compared with primary embryonic human lung fibroblasts.

Our results indicate that blockade of the basal level of Akt phosphorylation by LY-294002 markedly decreased the basal levels of FBLN5 hnRNA and mRNA (Fig. 6A) and promoter activity (Fig. 6B), indicating an enhancer role for PI3-kinase/Akt activity in normal FBLN5 expression in human lung fibroblasts. Akt-mediated phosphorylation of Smad3 on a novel serine/threonine site was also found to increase total serine phosphorylation of Smad3 and enhance the TGF- $\beta$  effect in mesangial cells, which is decreased upon treatment with LY-294002 (34). However, our results showed that increases in Smad phosphorylation and ratios of induction of FBLN5 hnRNA, mRNA, and promoter activity by TGF- $\beta$  were not affected by LY-294002, whereas TGF- $\beta$ -induced Akt phosphorylation, FBLN5 hnRNA and mRNA expression, and promoter activity were strongly decreased (Fig. 6, A–C). These results suggest a requirement for Akt activity in the transcriptional activation of the FBLN5 promoter.

We demonstrate that exposure to exogenous TGF- $\beta$  induced a rapid but transient increased the rate of transcription of the FBLN5 gene, as assessed by determination of FBLN5 hnRNA levels (Fig. 1D). Activation of MAP kinase results in the phosphorylation of Smad2/3 at multiple phosphorylation sites in the linker region between the MH1 and MH2 domains to decrease TGF- $\beta$ -induced nuclear accumulation of these Smads and negatively regulate Smad-dependent transcription (12). The basal level of p44/42 phosphorylation in human lung fibroblasts was increased by TGF- $\beta$  treatment (data not shown). However, suppression of the p44/42 phosphorylation with U0126 or PD-98059 did not alter the TGF- $\beta$ -induced expression pattern of FBLN5 mRNA. These results indicate that activity of MAP kinase does not regulate FBLN5 gene expression.

Our results and those of others demonstrate that prolonged exogenous TGF- $\beta$  also induces a remarkable increase in  $\alpha$ -SMA mRNA expression in human lung fibroblasts. It is well known that exposure of fibroblasts to TGF- $\beta$  stimulates the differentiation of fibroblasts into myofibroblasts with expression of  $\alpha$ -SMA (29, 40). FBLN5 is an integrin-binding protein

and plays an important role in the regulation of cell and matrix function (6, 28, 35, 37). Whether the increase of FBLN5 expression by TGF- $\beta$  promotes transition to the myofibroblast phenotype in human lung fibroblasts is not clear.

Recent studies have revealed that emphysema, and other elastic fiber-related disorders, appears in adult mice deficient in FBLN5 or LOXL-1, one of the lysyl oxidase family enzymes that initiate elastin cross-linking (17, 24, 41). These studies have shown that LOXL-1 binds to FBLN5 and localizes to elastic fibers in a FBLN5-dependent fashion, suggesting that FBLN5 may directly recruit LOXL-1 to the microfibrillar scaffold to cross-link tropoelastin and assemble elastic fibers. Our previous work showed that FBLN5 and elastin are coordinately expressed during the lung repair process in the elastase-induced emphysema model (15). TGF- $\beta$  may play an important role in initiating lung injury repair process by promoting resynthesis of key elastogenic molecules like FBLN5 and LOXL-1. These studies indicate that development of emphysema may result from mutation or disruption of gene expression of key molecules other than elastin, implicating an additional layer of molecular complexity in elastogenesis and related disorders.

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