β-Catenin signaling is required for TGF-β1-induced extracellular matrix production by airway smooth muscle cells

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Chronic obstructive pulmonary disease (COPD) and asthma are obstructive airway diseases characterized by structural changes and thickening of the airway wall (3, 58). These pathological features, referred to as airway remodeling, include increased airway smooth muscle mass and altered extracellular matrix (ECM) profile in the airways, which may contribute cooperatively to airway hyperresponsiveness and the airflow obstruction observed in both diseases (3, 26, 38, 58). Increased airway smooth muscle mass is associated with decreased lung function in severe asthma and may contribute to COPD pathogenesis, particularly in more severe states of disease (35, 41, 55). Altered expression of ECM proteins within and surrounding the smooth muscle bundle has also been observed in asthma, which contributes to disease pathogenesis (1, 21, 57). The mechanisms leading to the development and progression of airway remodeling are not well understood, but airway smooth muscle cells may contribute to the process through cell proliferation and by producing and releasing various inflammatory mediators, growth factors, and ECM proteins (39, 46, 57).

The ECM is an intricate structure of macromolecules that acts as mechanical support for maintenance of airway function and is produced by a variety of mesenchymal cells in the airways, including fibroblasts and airway smooth muscle cells (25, 51). The matrix is a dynamic network that has the potential to influence cellular functions such as proliferation, migration, cytokine secretion, and differentiation of various resident cell types in the airways (21, 22, 38). In the airway wall of patients with asthma, the ECM profile is altered with increased expression of specific collagens, fibronectin, tenascin, hyaluronan, versican, and laminin, whereas deposition of other components such as decorin are decreased (1, 38). COPD patients exhibit increased bronchial deposition of ECM proteins, including fibronectin, laminin, and collagens I, III and IV, and diminished decorin expression peribronchially, which collectively contribute to deteriorated lung function and airway remodeling (45, 65). Growth factors play an important role in the development of airway remodeling and are released during the chronic airway inflammation. In particular, transforming growth factor-β (TGF-β) is a multifunctional cytokine that is increased in airways of asthmatic and COPD patients and is stored in the ECM in its latent, inactive form (12, 16, 25). Cleaving of latent TGF-β results in its activation, thereby allowing it to stimulate various structural and inflammatory cells in the lung resulting in ECM production and airway remodeling (12, 25, 42, 44).

Accumulating evidence indicates that activation of β-catenin signaling is associated with various fibroproliferative diseases, which implies a functional role for β-catenin in tissue remodeling (5, 10, 13). β-Catenin is a member of the Armadillo family of proteins and is associated with the cadherin/catenin complexes at adherens junctions, where it stabilizes cell-cell contacts (13). We have previously shown that β-catenin regulates active tension development by airway smooth muscle by its capacity to stabilize these adherens junctions (37). In
addition, β-catenin serves a role in the Wnt signaling pathway by regulating T-cell factor (TCF)/Lymphoid enhancer factor (LEF)-mediated gene transcription (13). Cellular β-catenin levels are tightly regulated by the constitutively active enzyme glycogen synthase kinase-3 (GSK-3), which phosphorylates and thereby targets cytosolic β-catenin for proteosomal degradation (13, 40). Recently, various growth factors, including TGF-β, have been demonstrated to activate β-catenin signaling through GSK-3 inhibition, which cooperates with small signaling to induce gene transcription (9, 33, 53). Stabilized (non-phosphorylated) β-catenin activates several target genes, including matrix metalloproteinases, growth factors, ECM proteins as well as proinflammatory mediators and enzymes (6, 14, 17, 24, 32, 36, 49, 60, 67). Accordingly, we previously demonstrated a role for β-catenin in airway smooth muscle cell proliferation (28, 53). However, the role of β-catenin in ECM protein production by airway smooth muscle is still unclear. Insight in β-catenin signaling in airway smooth muscle may be of benefit to better understand development and progression of airway remodeling. Therefore, in the present study, we investigated the activation of β-catenin signaling and its contribution to ECM production by airway smooth muscle cells in response to TGF-β1.

MATERIALS AND METHODS

Cell culture. Human bronchial smooth muscle cell lines, immortalized by stable expression of human telomerase reverse transcriptase (hTERT), from three different donors were used for all experiments. The primary cultured human bronchial smooth muscle cells used to generate each cell line were prepared, as we have previously described (31), from macroscopically healthy segments of 2nd- to 4th-genera-
tion main bronchus obtained after lung resection surgery from patients with a diagnosis of adenocarcinoma (Dr. H. Unruh, Section of Thoracic Surgery, University of Manitoba). All procedures were approved by the Human Research Ethics Board of the University of Manitoba. As previously described in detail (31), each cell line was thoroughly characterized to passage 10 and higher and was shown to express a number of smooth muscle (sm) contractile phenotype marker proteins (e.g., sm-myosin heavy chain, smα-actin, and desmin). For all experiments, myocytes were grown on uncoated plastic dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with antibiotics (50 μg/ml streptomycin, 50 μg/ml penicillin, 1.5 μg/ml amphotericin B) and 10% (vol/vol) fetal bovine serum (FBS). Unless differently specified, cells were serum starved for 1 day in DMEM supplemented with antibiotics and ITS (5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenium). For TGF-β1 stimulation, cells were washed twice with warm (37°C) phosphate-buffered saline (PBS; composition: 140.0 mM NaCl, 2.6 mM KCl, 1.4 mM KH2PO4, 8.1 mM Na2HPO4·2H2O, pH 7.4) and subsequently subjected to stimulation with TGF-β1 in serum-free DMEM supplemented with antibiotics. When applied, the pharmacological inhibitor PKF115-584 was added 30 min before the addition of TGF-β1.

TOP/FOP-flash assay. For the TOP/FOP-flash luciferase assay, cells were grown to ~95% confluence on 100-mm dishes and then transfected with TOP/FOP plasmid DNA (Upstate Biotechnology, Charlottesville, VA) by using Lipofectamine 2000 in serum- and antibiotics-free DMEM. After 6 h, medium was changed to DMEM supplemented with antibiotics and 10% FBS, in which cells were grown for another 18 h. Cells were then trypsinized and replated in 96-well plate format and grown in DMEM supplemented with antibiotics and 10% FBS for another 8 h, after which cells were serum-deprived for 24 h. Cells were then subjected to TGF-β1 (2 ng/ml) stimulation in DMEM supplemented with antibiotics for 16 h, and luciferase activity was assayed via the Promega luciferase assay system (Madison, WI). TOP-flash activity was normalized to FOP-flash activity. The transfection efficiencies of the TOP-flash plasmid and FOP-flash plasmid were similar, as determined by cotransfection with enhanced green fluorescent protein (eGFP) and subsequent measurement of eGFP-positive airway smooth muscle cells by fluorescence microscopy (data not shown).

β-Catenin siRNA transfection. Airway smooth muscle cells were grown to ~90% confluence in six-well cluster plates and transfected with a 21-bp, double-stranded small interfering RNA (siRNA) tar-
gested against the β-catenin transcript (Qiagen, Venlo, The Netherlands). Cells were transfected in serum-free DMEM without any supplements by using 200 pmol of siRNA in combination with Lipofectamine 2000 transfection reagent. Control transfections were performed with use of a nonsilencing control siRNA (Qiagen). After 6 h of transfection, cells were washed once with warm (37°C) PBS followed by a period of 48 h in DMEM supplemented with antibiotics. Consecutively, medium was refreshed and cells were stimulated with TGF-β1 (2 ng/ml) for 24 or 48 h.

Mutant S33Y β-catenin plasmid transfection. Airway smooth muscle cells were transfected to ~90% confluence in six-well cluster plates and transfected with β-catenin-S33Y plasmid DNA (AddGene plasmid 19286, AddGene public repository, Cambridge, MA) (43). Cells were transfected in serum-free DMEM without any supplements by using 0.1–1 μg of plasmid in combination with Lipofectamine 2000 transfection reagent. Control transfections were performed using 2 μg green fluorescent protein (GFP) expression vector. After 6 h of transfection, cells were washed once with warm (37°C) PBS followed by a period of 48 h in DMEM supplemented with 10% FBS and antibiotics.

Isolation of mRNA and real-time PCR analysis. Total mRNA was extracted by use of the RNeasy mini kit (Qiagen). Briefly, cells were harvested in RNAlater stabilization buffer and homogenized by passing the lysate 10 times through a 20-gauge needle. Lystase were then mixed with an equal volume of 70% ethanol, and total RNA was purified in RNeasy mini spin columns. The eluted mRNA was quantified using spectrophotometry (Nanodrop, Thermo Scientific, Wilmington, MA). Equal amounts of total mRNA (1 μg) were then reverse transcribed and stored at −20°C until further use. cDNA was subjected to real-time PCR, which was performed with an Illumina Eco Personal QPCR System (Westburg, Leusden, The Netherlands). In short, 5 μl absolute blue QPCR SYBR green mix, containing fluorescein to account for well-to-well variation, 0.1 μM of gene-specific forward and reverse primer (listed in Table 1) and 1 μl of 1:2 diluted cDNA sample were used in a total volume of 10 μl and added to a 48-well plate. Real-time PCR data were analyzed by the comparative cycle threshold (Cq: amplification cycle number) method. The amount of target gene was normalized to the endogenous reference gene 18S ribosomal RNA (designated as ΔCc). Relative differ-
ces were determined by using the equation 2−(ΔΔCq). Cycle parameters were denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s or 40 cycles followed by 5 min at 72°C.

Preparation of cell lysates. To obtain whole cell lysates, cells were washed once with ice-cold (4°C) PBS then lysed in ice-cold sodium dodecyl sulfate (SDS) buffer (composition: 62.5 mM Tris, 2% wt/vol SDS, 1 mM NaF, 1 mM Na3VO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 7 μg/ml pepstatin A, pH 6.8). Lysates were then sonicated and protein concentration was determined by using Pierce protein determination according to the manufacturer’s instructions. Lysates were stored at −20°C till further use.

Western blot analysis. Equal amounts of protein (10–20 μg/lane) were subjected to electrophoresis on polyacrylamide gels, transferred to nitrocellulose membranes, and analyzed for the proteins of interest by using specific primary and horseradish peroxidase (HRP)-conjugated secondary antibodies. By using enhanced chemiluminescence reagents, bands were recorded in the

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\[ \beta\text{-catenin mRNA abundance in airway smooth muscle cells} \] (Fig. 1C).

**TGF-\(\beta_1\)-induced \(\beta\text{-catenin} \) is transcriptionally active.** We next determined whether TGF-\(\beta_1\)-induced \(\beta\text{-catenin}\) was coupled to the activation of gene transcription. Stimulation of airway smooth muscle for up to 24 h with TGF-\(\beta_1\) (2 ng/ml) resulted in a marked, time-dependent increase in the expression of nonphosphorylated \(\beta\text{-catenin}\) (Fig. 2A). This increase was linked with activation of TCF/LEF-dependent gene transcription, as assayed using the \(\beta\text{-catenin}\) sensitive TOP-flash luciferase assay (Fig. 2B). Collectively, these findings indicate that TGF-\(\beta_1\) induces \(\beta\text{-catenin}\) mRNA and protein expression in airway smooth muscle, which subsequently activates TCF/LEF-dependent gene transcription.

**Functional role of \(\beta\text{-catenin} \) signaling in TGF-\(\beta_1\)-induced ECM expression.** Given the importance of TGF-\(\beta_1\) in regulation of ECM production by airway smooth muscle cells, we examined the role of \(\beta\text{-catenin}\) in this process. Stimulation of airway smooth muscle cells for 24 h with TGF-\(\beta_1\) (2 ng/ml) induced mRNA expression of the ECM proteins collagen I\(_\alpha_1\) (collagen, type 1, alpha 1), fibronectin, and versican (Fig. 3A). In contrast, mRNA abundance of collagen III, laminin \(\alpha_1\), and laminin \(\alpha_2\) did not change significantly, whereas decorin mRNA abundance was attenuated by TGF-\(\beta_1\) stimulation (Fig. 3A).

Next, we investigated the effect of \(\beta\text{-catenin}\) silencing on TGF-\(\beta_1\)-induced ECM gene expression. Initial experiments indicated that after transfection of the cells with \(\beta\text{-catenin}\) siRNA, optimal reduction in the expression of \(\beta\text{-catenin}\) was achieved at 48–72 h of treatment (53). Therefore, airway smooth muscle cells were transfected with specific \(\beta\text{-catenin}\) siRNA or nontargeting siRNA with concomitant serum deprivation and treated with TGF-\(\beta_1\) (2 ng/ml) for 24 h thereafter to ensure optimal \(\beta\text{-catenin}\) knockdown during the window of TGF-\(\beta_1\) treatment. In nontargeting siRNA-treated airway smooth muscle cells, TGF-\(\beta_1\) induced the expression of collagen I\(_\alpha_1\), fibronectin, and versican (Fig. 3B). Silencing of \(\beta\text{-catenin}\) by specific siRNA did not affect

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\text{Table 1. Primers used for determination of ECM genes and 18S ribosomal mRNA by qRT-PCR analysis}
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<table>
<thead>
<tr>
<th>Primer</th>
<th>NCBI Accession Number</th>
<th>Primer Sequence</th>
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<tr>
<td>(\beta\text{-catenin} ) Forward</td>
<td>NM_001904</td>
<td>5' CCCCCTAAATGCTGAGCCTTT</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
<td>3' AATCCACTGCTGAGCAGCC</td>
</tr>
<tr>
<td>Collagen I (\alpha_1) Forward</td>
<td>NM_000888</td>
<td>5' AGGCAAGGAGCTGAGAAAT</td>
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<tr>
<td>Reverse</td>
<td></td>
<td>3' CCTGCGCTGGGCTGAGTT</td>
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<tr>
<td>Collagen III (\alpha_1) Forward</td>
<td>NM_000909</td>
<td>5' GGGGACATGCTGAGAGTT</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
<td>3' CTCCTCTCAACAGCCTG</td>
</tr>
<tr>
<td>Decorin Forward</td>
<td>NM_001920</td>
<td>5' AATTGAGATGCTGAGTT</td>
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<tr>
<td>Reverse</td>
<td></td>
<td>3' CAGCTGCAAGGCTGAGTT</td>
</tr>
<tr>
<td>Fibronectin Forward</td>
<td>NM_212482</td>
<td>5' GGGGACATGCTGAGAGTT</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
<td>3' CTCCTCTCAACAGCCTG</td>
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<tr>
<td>Laminin (\alpha_1) Forward</td>
<td>NM_005559</td>
<td>5' ACGGTAATGAGCTGAGTT</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
<td>3' CTCCTCTCAACAGCCTG</td>
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<tr>
<td>Laminin (\alpha_2) Forward</td>
<td>NM_000426</td>
<td>5' GGGGACATGCTGAGAGTT</td>
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<tr>
<td>Reverse</td>
<td></td>
<td>3' CTCCTCTCAACAGCCTG</td>
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<tr>
<td>Versican Forward</td>
<td>NM_004385</td>
<td>5' ACGGTAATGAGCTGAGTT</td>
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<tr>
<td>Reverse</td>
<td></td>
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<td>PAI-1 Forward</td>
<td>NG_013213.1</td>
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</tr>
<tr>
<td>18S rRNA Forward</td>
<td>NR_003286.2</td>
<td>5' TTGGGAAATGCTGAGTT</td>
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<tr>
<td>Reverse</td>
<td></td>
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basal mRNA abundance of collagen Iα1, fibronectin, and versican (Fig. 3B). However, the TGF-β1-induced expression of collagen Iα1 and fibronectin was attenuated when β-catenin was downregulated (Fig. 3B). Though silencing β-catenin expression appeared to decrease in TGF-β1-induced versican expression, differences were not statistically significant (Fig. 3B). β-Catenin has a dual function in cellular signaling, first regulating TCF/LEF-dependent gene transcription and second stabilizing cell-cell contacts by being a component of cadherin-based adherens junctions (13, 37). Downregulation of β-catenin by siRNA disrupts cell-cell contacts and this possibly affects TGF-β receptor signaling. Therefore, we investigated the effect of β-catenin siRNA on the mRNA expression of the smad-dependent gene plasminogen activator inhibitor-1 (PAI-1) (15, 56). Stimulation of airway smooth muscle cells with TGF-β1

Fig. 1. Induction of β-catenin expression by TGF-β1 in airway smooth muscle cells. A: airway smooth muscle cells were stimulated with increasing concentrations of TGF-β1 (0.1–10 ng/ml) for 24 h. Expression of total β-catenin was evaluated by immunoblotting. Equal protein loading was verified by the analysis of β-actin. Responses of TGF-β1 (2 ng/ml) on total β-catenin expression were quantified by densitometry and normalized to β-actin expression, representing means ± SE of 3 independent experiments. B: airway smooth muscle cells were stimulated with TGF-β1 (2 ng/ml) for up to 2 h. Induction of ser9/21 phosphorylation of glycosgen synthase kinase-3 (GSK-3) was evaluated by immunoblotting using a phospho-specific antibody. Equal protein loading was verified by the analysis of total GSK-3. Responses of TGF-β1 on GSK-3 phosphorylation were quantified by densitometry and normalized to total GSK-3 expression, representing means ± SE of 3 independent experiments. C: quantitative RT-PCR (qRT-PCR) analysis of β-catenin mRNA expression after 24 h of TGF-β1 (2 ng/ml) stimulation. Expression of β-catenin by TGF-β1 is expressed relative to untreated airway smooth muscle cells. Data represent means ± SE of 4 independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with untreated airway smooth muscle cells. Statistical significance determined by 2-tailed Student’s t-test for paired observations.

Fig. 2. Induction of nonphosphorylated β-catenin in human airway smooth muscle in response to TGF-β1. A: airway smooth muscle cells were stimulated with TGF-β1 (2 ng/ml) for up to 24 h. Expression of nonphosphorylated β-catenin was evaluated by immunoblotting. Equal protein loading was verified by the analysis of GAPDH. Nonphosphorylated β-catenin expression was quantified by densitometry and normalized to GAPDH, representing means ± SE of 5 independent experiments. *P < 0.05 and ***P < 0.001 compared with untreated airway smooth muscle cells. Statistical significance determined by 1-way ANOVA followed by a Newman-Keuls multiple-comparison test. B: TGF-β1 (2 ng/ml; 16 h.) induces β-catenin-dependent gene transcription, determined by the TOP-flash luciferase assay. TOP-flash luciferase activity was normalized to FOP-flash luciferase activity and expressed as a percentage of control representing means ± SE of 4 independent experiments. **P < 0.01, 2-tailed Student’s t-test for paired observations.
resulted in a fivefold induction of PAI-1 mRNA expression. Silencing of β-catenin expression did not affect the basal or TGF-β1-induced expression of PAI-1 (Fig. 3C). These findings implicate that the responsiveness of smooth muscle cells to TGF-β1 is not altered by downregulation of β-catenin expression.

To further verify the functional role of β-catenin/TCF interactions in TGF-β1-induced ECM gene expression, we pharmacologically inhibited β-catenin signaling by PKF115-584 a compound that disrupts the interaction of the transcriptionally active (nonphosphorylated) β-catenin/T-cell factor-4 (TCF-4) complex (4, 47, 52). In line with the findings obtained with β-catenin siRNA, PKF115-584 did not significantly affect basal expression of collagen Iα1 (P = 0.432), fibronectin (P = 0.693), or versican (P = 0.394) but largely attenuated the TGF-β1-induced expression of these ECM genes (Fig. 3D).

Although pharmacological inhibitors may be less specific than siRNA, these data show that β-catenin signaling can be directly targeted by using a small molecule. Taken together, these data indicate that β-catenin signaling activated by TGF-β1 regulates the expression of specific ECM genes in airway smooth muscle.

To confirm the significance of β-catenin signaling in regulating ECM protein expression by airway smooth muscle cells, we assessed the role of β-catenin in TGF-β1-induced fibronectin protein expression. Stimulation of airway smooth muscle cells with TGF-β1 induced fibronectin protein expression in a time-dependent manner (Fig. 4A). Silencing of β-catenin markedly reduced basal and TGF-β1-induced expression of total and nonphosphorylated β-catenin (Fig. 4B). Importantly, downregulation of β-catenin also attenuated TGF-β1-induced fibronectin protein expression in the airway smooth muscle cells (Fig. 4B). To corroborate these findings we used PKF115-584 to inhibit β-catenin signaling pharmacologically. In agreement with the findings using β-catenin siRNA, PKF115-584 prevented the increased fibronectin expression induced by TGF-β1 (Fig. 4C). Collectively, these data indicate that induction of transcriptionally active β-catenin by TGF-β1 is required for ECM mRNA and protein expression by airway smooth muscle cells.

β-Catenin activation is sufficient for fibronectin expression by airway smooth muscle. Next we hypothesized that increased expression of β-catenin was sufficient to induce fibronectin expression by airway smooth muscle cells. To test this hypothesis, airway smooth muscle cells were transiently transfected with a constitutively active β-catenin mutant (S33Y-β-catenin). This S33Y-β-catenin mutant has a serine-to-tyrosine substitution at position 33 and is therefore insensitive to GSK-3-mediated phosphorylation and proteasomal degradation (43). Airway smooth muscle cells were transfected with increasing amounts of the S33Y-β-catenin mutant (0.1–1 μg), whereas control cultures were transfected with GFP (Fig. 5A). The transfection with the S33Y-β-catenin mutant resulted in an increased expression of total β-catenin, which was optimal when cells were transfected with 1.0 μg of plasmid DNA (Fig. 5, A and B). The transfection with the constitutively active S33Y-β-catenin mutant (1 μg) also resulted in a strong increase in fibronectin expression by airway smooth muscle cells (Fig. 5, A and C), whereas the expression of the smad regulated gene PAI-1 was not affected (data not shown). These data indicate that an increase in transcriptionally active β-catenin is...
sufficient to increase the expression of fibronectin by airway smooth muscle cells.

**DISCUSSION**

In the present study, we demonstrate that β-catenin signaling plays an important role in the regulation of ECM production by airway smooth muscle. We show that in response to TGF-β stimulation β-catenin protein abundance is stabilized, which subsequently results in increased TCF/LEF-dependent gene transcription. Downregulation of β-catenin expression or pharmacological inhibition of the nuclear β-catenin/TCF-4 complex attenuates TGF-β-induced expression of collagen I and fibronectin, indicating a requirement of β-catenin signaling in this process. Furthermore, expression of a degradation-resistant β-catenin mutant (S33Y-β-catenin) strongly activates the production of fibronectin, implying that increased expression of β-catenin is sufficient to activate ECM protein production in airway smooth muscle cells. Collectively, these findings indicate that β-catenin signaling is both required and sufficient for the regulation of ECM production by airway smooth muscle.

Our present and previously published data indicate a key role for β-catenin in smooth muscle biology. In its role as an adherens junction-associated protein that links to the actin cytoskeleton, β-catenin is important for contractile force generation of airway smooth muscle cells, presumably by stabilizing cell-cell contacts, allowing force transmission between neighboring cells (37). Moreover, growth factors that stimulate airway smooth muscle growth induce nuclear accumulation of β-catenin, which activates TCF/LEF-dependent gene transcription and subsequent induction of cell proliferation (28, 30, 53). Our present findings indicate that matrix protein expression by airway smooth muscle is also regulated by β-catenin dependent gene transcription. These data are consistent with findings in vascular smooth muscle cells, in which growth factor-induced β-catenin activation and subsequent induction of TCF/LEF-dependent gene transcription, regulates smooth muscle cell proliferation, vascular endothelial growth factor (VEGF) se-
β-Catenin is required for ECM production by ASM cells

Cytosolic β-catenin levels are tightly regulated by the constitutively active enzyme GSK-3β. A fraction of cellular GSK-3β forms a so-called destruction complex with axin, casein kinase I, and adenomatous polyposis coli; this complex phosphorylates and subsequently targets cytosolic β-catenin for proteasomal degradation (13). This tight regulation explains the relatively low abundance of active (nonphosphorylated) β-catenin at baseline, even though total β-catenin protein expression is abundant at the plasma membrane. The activity of GSK-3β is negatively regulated by serine phosphorylation, which can be induced by numerous stimuli, including growth factors (23, 28, 29, 53). Growth factors are believed to increase β-catenin stabilization in part via this inactivation of GSK-3. In line with this contention, we demonstrate that TGF-β1 induces a strong and sustained phosphorylation of GSK-3 in airway smooth muscle cells, followed by increased expression of active, nonphosphorylated β-catenin. Accumulation of stabilized β-catenin contributes to the cellular expression of total β-catenin. Therefore, the overall cellular expression of β-catenin is also augmented by TGF-β1 stimulation; however, this increase distinct from the rise in active β-catenin expression as the plasma membrane associated pool of β-catenin is not induced in response to TGF-β1 (28). We demonstrate that the expression of the stable S33Y-β-catenin mutant, which has a serine-to-tyrosine substitution at position 33 and therefore is insensitive to GSK-3-mediated phosphorylation and proteasomal degradation, is sufficient to activate fibronectin deposition by airway smooth muscle cells. This demonstrates the importance of rigorous control of β-catenin expression by GSK-3 in regulating cellular responses of the airway smooth muscle. In addition to GSK-3-dependent regulation of β-catenin, we found that TGF-β1 induced an increased β-catenin mRNA expression, indicating that the de novo synthesis of the protein is also regulated by this growth factor. Indeed, we have previously shown that expression of a dominant negative H-RAS as well as pharmacological inhibition of ERK1/2 kinase (e.g., MK) attenuates growth factor-induced β-catenin mRNA and protein expression (28). Taken together, these findings demonstrate that TGF-β1 regulates β-catenin expression in part by increasing protein stability and in part by ERK1/2-dependent de novo synthesis of the protein in airway smooth muscle cells.

In the airways, TGF-β1 is sequestered in inactive complex in the ECM; upon activation it stimulates cell surface serine/threonine receptor kinases, leading to phosphorylation of smad (small phenotype and mothers against decapentaplegic-related protein) family of intracellular signaling proteins. The activated smads translocate to the nucleus and associate with DNA binding partners and various transcriptional coactivators, thereby regulating gene transcription (2, 44). Smad phosphorylation is critical for TGF-β1 signaling, yet simultaneously a variety of other pathways, including ERK1/2 and GSK-3/β-catenin signaling, are activated by TGF-β1 that in turn support functional TGF-β1-driven responses (2, 33). Biochemical studies have shown that smad and β-catenin may cooperatively regulate TCF/LEF transcription factors, resulting in synergistic activation of gene transcription (14, 48). The TCF/LEF family of transcription factors are the downstream effectors of the canonical Wnt/β-catenin signaling pathway and consists of four members, i.e., TCF-1, LEF, TCF-3 and TCF-4, which all share homology in their DNA binding domain. Stabilized
β-catenin is required for ECM production by ASM cells

Increased airway smooth muscle mass in concert with aberrant ECM deposition in the airways is thought to contribute to the pathogenesis of chronic inflammatory lung diseases, like asthma and COPD (35, 58). The expression of various ECM components in the airways is altered in these chronic inflammatory lung diseases (1, 3, 38, 45). More specifically, in asthma the expression of fibronectin, hyaluronan, versican, biglycan, lumican, and collagen I are increased within as well as surrounding the airway smooth muscle (1, 57, 61). In COPD, changes in airway smooth muscle mass and altered ECM deposition are less pronounced compared with asthma but may become more important in more severe stages of disease (35). In addition to increased bronchial ECM deposition, the expression of laminin β2 is increased in the airway smooth muscle bundle of COPD patients and inversely correlates to the forced expiratory volume in 1 s (45). The expression and composition of ECM in the airways of patients with chronic inflammatory lung diseases may also strongly influence airway smooth muscle function and thereby contribute to disease pathogenesis. For instance, fibronectin and collagen I have been demonstrated to enhance airway smooth muscle proliferation, migration, cell survival, and negatively affect the contractile capacity of airway smooth muscle cells (22, 27, 34, 54). The significance of the interaction between the ECM and airway smooth muscle in vivo has recently been established in an animal model of chronic allergic asthma. In this study it was demonstrated that inhibition of the interaction between the ECM proteins and their integrins attenuated allergen-induced airway smooth muscle remodeling (20). Corticosteroids used for control of asthma and COPD symptoms do not seem to have a beneficial effect on ECM production by airway smooth muscle and may under certain circumstances even increase the matrix production (19, 39, 50). Matrix protein production can be induced by growth factors that are upregulated in asthma and COPD, like TGF-β1, connective tissue growth factor (CTGF), and VEGF (8, 12, 66). The underlying mechanisms by which these growth factors, in particular TGF-β1, induce matrix production is therefore of major interest. We demonstrate that β-catenin signaling is activated in response to TGF-β1 and plays an important regulatory role in ECM production by airway smooth muscle. This suggests that targeting β-catenin-dependent gene transcription is a strategy worth pursuing in future studies, particularly since corticosteroids used for the control of asthma and COPD symptoms do not seem to have a beneficial effect on ECM production by airway smooth muscle (19, 39, 50).

Collectively, these findings indicate that β-catenin signaling is activated in response to TGF-β1, which is required and sufficient for the regulation of ECM production by airway smooth muscle cells. The present data adds to the increasing evidence indicating the importance of β-catenin in airway smooth muscle function. The regulatory role of β-catenin in airway smooth muscle may be of importance in TGF-β1-driven airway wall remodeling. Targeting β-catenin-dependent gene transcription may therefore hold promise as a therapeutic intervention.

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DISCLOSURES

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

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