THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 10, pp. 7026–7038, March 2, 2012

Interactions Between β-Catenin and Transforming Growth Factor-β Signaling Pathways Mediate Epithelial-Mesenchymal Transition and Are Dependent on the Transcriptional Co-activator cAMP-response Element-binding Protein (CREB)-binding Protein (CBP)*[S]

Received for publication, June 29, 2011, and in revised form, December 13, 2011 Published, JBC Papers in Press, January 12, 2012, DOI 10.1074/jbc.M111.276311

Beiyun Zhou[‡], Yixin Liu[‡], Michael Kahn[§], David K. Ann[¶], Arum Han[‖], Hongjun Wang[‡], Cu Nguyen[§], Per Flodby[‡], Qian Zhong[‡], Manda S. Krishnaveni[‡], Janice M. Liebler[‡], Parviz Minoo[1], Edward D. Crandall[‡2], and Zea Borok[‡§3]**

*From the [‡]Will Rogers Institute Pulmonary Research Center, Division of Pulmonary and Critical Care Medicine, Department of Medicine, [§]Department of Biochemistry and Molecular Biology, **Department of Pediatrics, [‖]Department of Dermatology and Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, California 90033, and the [¶]Department of Molecular Pharmacology, City of Hope Beckman Research Institute, Duarte, California 91010*

Background: Direct evidence for molecular interdependence between transforming growth factor-β (TGF-β) and Wnt pathways in mesenchymal gene regulation during epithelial-mesenchymal transition (EMT) is limited.
Results: TGF-β induction of α-smooth muscle actin (α-SMA) involves ternary complex formation among Smad3, β-catenin, and CBP.
Conclusion: TGF-β and β-catenin/CBP-dependent pathways coordinately regulate α-SMA induction.
Significance: Inhibition of β-catenin/CBP-dependent effects of TGF-β suggests a novel therapeutic approach to EMT/fibrosis.

Interactions between transforming growth factor-β (TGF-β) and Wnt are crucial to many biological processes, although specific targets, rationale for divergent outcomes (differentiation *versus* block of epithelial proliferation *versus* epithelial-mesenchymal transition (EMT)) and precise mechanisms in many cases remain unknown. We investigated β-catenin-dependent and transforming growth factor-β1 (TGF-β1) interactions in pulmonary alveolar epithelial cells (AEC) in the context of EMT and pulmonary fibrosis. We previously demonstrated that ICG-001, a small molecule specific inhibitor of the β-catenin/CBP (but not β-catenin/p300) interaction, ameliorates and reverses pulmonary fibrosis and inhibits TGF-β1-mediated α-smooth muscle actin (α-SMA) and collagen induction in AEC. We now demonstrate that TGF-β1 induces LEF/TCF TOPFLASH reporter activation and nuclear β-catenin accumulation, while LiCl augments TGF-β-induced α-SMA expression, further confirming co-operation between β-catenin- and TGF-β-dependent signaling pathways. Inhibition and knockdown of Smad3, knockdown of β-catenin and overexpression of ICAT abrogated effects of TGF-β1 on α-*SMA* transcription/expression, indicating a requirement for β-catenin in these Smad3-dependent effects. Following TGF-β treatment, co-immunoprecipitation demonstrated direct interaction between endogenous Smad3 and β-catenin, while chromatin immunoprecipitation (ChIP)-re-ChIP identified spatial and temporal regulation of α-*SMA* via complex formation among Smad3, β-catenin, and CBP. ICG-001 inhibited α-SMA expression/transcription in response to TGF-β as well as α-*SMA* promoter occupancy by β-catenin and CBP, demonstrating a previously unknown requisite TGF-β1/β-catenin/CBP-mediated pro-EMT signaling pathway. Clinical relevance was shown by β-catenin/Smad3 co-localization and CBP expression in AEC of IPF patients. These findings suggest a new therapeutic approach to pulmonary fibrosis by specifically uncoupling CBP/catenin-dependent signaling downstream of TGF-β.

Transforming growth factor (TGF)[4]-β and Wingless/int (Wnt) signaling pathways play critical roles in cell fate determination during development and in the adult (1, 2). While there is evidence for crosstalk between these two pathways in the context of development and tumorigenesis, effects of these interactions in non-transformed adult cells are not well defined. The divergent functional outcomes of these interactions are frequently context-specific, and precise targets and mechanisms in many cases remain unknown (3–7). Crosstalk between TGF-β and Wnt pathways has been thought of as either reciprocal transregulation in which one pathway regulates components of the other, or interactive, which refers to

* This work was supported, in whole or in part, by Grants DE014183 (to D. K. A.), DE010742 (to D. K. A.), ES017034 (to E. D. C.), ES018782 (to E. D. C.), HL038578 (to Z. B.), HL038621 (to E. D. C.), HL056590 (to P. M.), HL062569 (to Z. B.), HL089445 (to Z. B.), HL073722 (to M. K.), and HL095349 (to P. M.) from the National Institutes of Health. This work was also supported by the Hastings Foundation and the Whittier Foundation.
[S] This article contains supplemental Figs. S1–S4 and materials.
[1] Hastings Professor of Pediatrics.
[2] Hastings Professor and Kenneth T. Norris, Jr., Chair of Medicine.
[3] Edgington Chair in Medicine. To whom correspondence should be addressed: IRD 620, 2020 Zonal Avenue, Los Angeles, CA 90033. Tel.: 323-226-7923; Fax: 323-442-2611; E-mail: zborok@usc.edu.

[4] The abbreviations used are: TGF, transforming growth factor; EMT, epithelial-mesenchymal transition; AEC, alveolar epithelial cells; α-SMA, α-smooth muscle actin; ChIP, chromatin immunoprecipitation; CBP, cyclic AMP-responsive element-binding protein (CREB)-binding protein; ZO-1, zonula occludens-1; SBE, Smad-binding element; Abs, antibodies.

cross-communication between molecular components of the pathways (8). As an example of the former, stability of Smads in the TGF-β pathway can also be regulated by axin, which is a major regulator of Wnt signaling (5). In the latter case, LEF-1 and Smads have been shown to synergistically regulate the X-twin promoter (4, 9). However, evidence for interdependence between components of these pathways in transcriptional regulation of specific target genes in mammalian systems particularly in the context of EMT is limited (8, 10–12).

Epithelial-mesenchymal transition (EMT) is a process whereby fully differentiated epithelial cells undergo transition to mesenchymal phenotype and is well-recognized during development and tumor metastasis (13, 14). More recently, it has been recognized that EMT may also serve as a source of (myo)fibroblasts in response to epithelial injury/stress in the setting of fibrosis, including in the lung (15). In this regard, we previously co-localized epithelial and mesenchymal markers in >80% of hyperplastic alveolar epithelial cells (AEC) in idiopathic pulmonary fibrosis/usual interstitial pneumonia (IPF/UIP) tissue and demonstrated that AEC undergo EMT following TGF-β1 treatment *in vitro* (16), while lineage tracing studies demonstrated that up to 30% of cells of epithelial origin express mesenchymal markers following bleomycin or TGF-β1 administration (17, 18). TGF-β1, a master switch in induction of fibrosis in many tissues (including lung), is regarded as a central mediator of EMT (14, 19–23). TGF-β1 induces EMT of normal adult lung AEC *in vitro* and *in vivo* (16, 18). TGF-β signaling is mediated through both Smad-dependent and -independent pathways although many of its profibrotic actions (including EMT) appear to be mediated via Smads, especially Smad3 (24–26). While Smad3 is generally thought to activate the myogenic program during EMT, there is some controversy as indicated by a recent report demonstrating that Smad3 may have time-dependent stimulatory and inhibitory effects on expression of the myofibroblast marker α-smooth muscle actin (α-SMA) depending on sequential interactions with specific binding partners (27). Ample evidence also links Wnt/β-catenin-dependent signaling with induction of EMT during development and tumor progression, but its role in the context of fibrotic EMT is less well-defined (28–30). Evidence of nuclear accumulation of β-catenin, along with increased β-catenin target genes, Wnt ligands, Dickkopf proteins and phosphorylation of glycogen synthase kinase-3 (GSK-3β) in hyperplastic type II (AT2) cells in IPF lung tissue (31–36) suggest a role for aberrant Wnt/β-catenin pathway activation in the pathogenesis of IPF, perhaps through EMT.

Although both TGF-β and Wnt signaling pathways are known to participate in regulation of EMT, evidence for interactions between these pathways in the context of EMT is limited and mechanistic studies have largely focused on regulation of components of one pathway by the other (*i.e.* reciprocal transregulation). For example, SNAI1 and SNAI2, transcription factors that mediate EMT through effects on E-cadherin transcription, induce formation of β-catenin-TCF complexes that up-regulate TGF-β, which in turn induces EMT (37). Direct interactions between components of these pathways in transcriptional regulation of EMT target genes has focused largely on regulation of epithelial genes and little is known of direct effects on regulation of mesenchymal genes. In this regard, TGF-β3 stimulates transcription of LEF-1, which interacts with Smad2 and Smad4 to inhibit E-cadherin and induce EMT (38). Kim *et al.* (39) demonstrated formation of a cytoplasmic complex between tyrosine-phosphorylated β-catenin and Smad2 in the context of EMT but did not directly demonstrate transcriptional regulation of target genes by this complex.

We recently demonstrated that ICG-001, a novel small molecule inhibitor of TCF/β-catenin-dependent transcription that acts by specifically inhibiting β-catenin/cyclic AMP-responsive element-binding protein (CREB)-binding protein (CBP) but not β-catenin/p300 interactions, ameliorates fibrosis in a mouse model of bleomycin-induced lung injury while concurrently decreasing expression of α-SMA (40). Furthermore, ICG-001 prevented induction of α-SMA and collagen 1 in lung epithelial cells in response to TGF-β, suggesting that β-catenin/CBP interaction is absolutely required for regulation of at least a subset of mesenchymal genes by TGF-β and may be critically important in the pathogenesis of EMT and fibrosis.

In the current study, we utilized AEC to investigate TGF-β/β-catenin pathway convergence and specifically its consequences on transcription of *α-SMA,* a gene target associated with EMT. We demonstrate that β-catenin is necessary for induction of *α-SMA* transcription by TGF-β and furthermore that α-SMA induction by TGF-β is CBP-dependent suggesting that modulation of Smad/β-catenin/CBP-dependent signaling via specific small molecule inhibitors such as ICG-001 may provide a novel approach to therapeutic intervention in fibrosis and EMT.

EXPERIMENTAL PROCEDURES

Maintenance of Cell Lines and Induction of EMT—RLE-6TN cells (ATCC, Manassas, VA) were cultivated as previously described (16). To evaluate synergy between TGF-β1 and Wnt/β-catenin pathways in mediating EMT in AEC, RLE-6TN cells were treated with TGF-β1 and/or LiCl and proteins harvested for Western analysis. To examine effects of ICG-001 on TGF-β induction of α-SMA, RLE-6TN cells were treated with TGF-β1 (0.5 ng/ml) ± ICG-001 and analyzed by Western blotting or immunofluorescence. To assess the role of Smad3 activation in TGF-β induction of α-SMA protein, RLE-6TN cells were treated from the time of plating with TGF-β1 (2.5 ng/ml) together with SIS3 (0.5–6 μM), a specific inhibitor of Smad3 phosphorylation, and harvested for Western analysis. Details of cell treatments for induction or prevention of EMT are provided under supplemental materials.

Western Analysis—Western analysis was performed as previously described (41) and further detailed in supplemental materials. Primary antibodies (Abs) used for Western analysis include: mouse anti-α-SMA (A5228, Sigma), anti-β-catenin (#C7207, Sigma), anti-β-catenin (pTyr654) (#ab24925, Abcam, Cambridge, MA), anti-E-cadherin (#610181, BD Biosciences Pharmingen, San Diego, CA), anti-eIF-4E (#610270, BD Biosciences Pharmingen), anti-β-actin (#ab6276, Abcam), and anti-GAPDH (#AM4300, Applied Biosystems, Austin, TX), and rabbit anti-lamin A/C (#SC20681, Santa Cruz Biotechnology, Santa Cruz, CA), anti-active-β-catenin (anti-ABC clone 8E7) (#05665, Millipore, Billerica, MA), anti-CBP (#SC-369, Santa

Cruz Biotechnology), anti-zonula occludens-1 (ZO-1) (#40–2200, Zymed Laboratories Inc., South San Francisco, CA), anti-phospho-GSK-3β (#9336, Cell Signaling, Danvers, MA), anti-phospho-Smad3 (#9514, Cell Signaling), and anti-Smad3 (#28379, Abcam).

Human Tissues—Lung tissues were obtained from the Lung Tissue Research Consortium (LTRC), a multicenter resource of the National Heart, Lung, and Blood Institute (NHLBI) under a standardized protocol that integrates clinical and pathological information to establish the diagnosis of IPF based on currently accepted international consensus guidelines (42). Prior to specimen procurement, all LTRC subjects undergo an extensive, standardized, pre-operative phenotypic evaluation which includes demographics, detailed occupational history, complete pulmonary function testing, and a standardized chest CT scan. Samples from a total of 17 IPF patients (14 male, 3 female; average age 55.47 ± 6.18 years) were used in co-immunostaining for β-catenin/Smad3 and CBP staining. Since there were only a limited number of slides available for each patient, 11 were used for β-catenin/Smad co-localization, of which 7 were adequate for quantitation. 7 were used for CBP staining.

Immunofluorescence Microscopy—For α-SMA staining, RLE-6TN cells were fixed with 4% paraformaldehyde for 10 min at room temperature, followed by incubation with 0.3% Triton X-100 for 10 min at room temperature. Cells were then incubated with anti-α-SMA Ab at room temperature for 1 h, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary Ab (Vector Laboratories, Burlingame, CA). F-actin staining was carried out as previously described (16). Images were viewed with an Olympus BX60 microscope equipped with epifluorescence optics (Olympus, Melville, NY) and captured with a cooled charge-coupled device camera (Magnafire; Olympus). Co-localization of β-catenin and Smad3 was performed using formalin-fixed, paraffin-embedded lung sections from IPF patients. Lung tissue sections were blocked with CAS block and incubated with anti-β-catenin (Sigma) and anti-Smad3 (Abcam) Abs at 4 °C overnight following microwave antigen retrieval (Antigen Unmasking Solution, Vector Laboratories). Control slides were incubated with non-immune rabbit or mouse IgG alone or together with either anti-β-catenin or anti-Smad3 Ab. Co-localization of β-catenin and surfactant protein-C (SP-C) or Smad3 and Nkx2.1 was performed by incubating sections with anti-β-catenin and anti-pro-SP-C (#AB3786, Millipore) Abs or anti-Smad3 and anti-Nkx2.1 (#MS-699-P1, Thermo Scientific) Abs. Images were captured using a ZEISS LSM 510 confocal system (Carl Zeiss, Jena, Germany) or Perkin Elmer Spinning Disc Laser Scan Microscope with UltraVIEW ERS software, equipped with 3 lasers (green fluorescence was detected using the 488-nm laser line of an Argon laser, red fluorescence was detected using the 543-nm laser line of the HeNe laser and DAPI staining was detected using the 405-nm laser line of a 405–30 Diode laser).

Immunohistochemistry—Following antigen retrieval, IPF lung sections were incubated with anti-CBP Ab using a standard alkaline phosphatase/avidin-biotin complex protocol (Vectastain ABC-AP (#AK5000)). CBP was visualized with Vector Red Alkaline Phosphatase Substrate Kit (Vector). IgG was used as negative control. Slides were imaged using Aperio Scan Scope CS and pictures were taken on an Aperio ImageScope (Vista, CA).

Transient Transfection Assays—RLE-6TN cells were co-transfected with a LEF/TCF TOPFLASH reporter or FOPFLASH control (from R.T. Moon, Howard Hughes Medical Institute, Seattle, WA) using Lipofectamine 2000 (Invitrogen) followed by treatment with TGF-β1 (2.5 ng/ml). To establish that TGF-β1-induced activation of TOPFLASH involved activation of β-catenin, the TOPFLASH reporter was co-transfected with ICAT expression vector pCS2/ICAT (0.25 μg) followed by TGF-β1 treatment. To confirm a role for activation of β-catenin in TGF-β1-induced *α-SMA* expression, RLE-6TN cells transduced with a lentiviral expression vector containing Myc-tagged ICAT cDNA, pRRL.hCMV.Sin.mycICAT.IRES.GFP, or control vector, pRRL.hCMV.Sin.IRES GFP, were co-transfected with 0.75 μg of *α-SMA*-luciferase (*α-SMA*-Luc) reporter followed by treatment with TGF-β1 (2.5 ng/ml) for 48 h. To evaluate the effect of Smad3 on *α-SMA* promoter activity, RLE-6TN cells were co-transfected with *α-SMA*-Luc and Smad3 expression vector pRK-5F/Smad3. To evaluate the functional importance of two putative Smad3-binding elements (SBE) for *α-SMA* promoter activity in epithelial cells, two Smad binding site (SBE1 and SBE2) mutants, *α-SMA*p-Luc-SBEm1 and *α-SMA*p-Luc-SBEm2, as well as wild type *α-SMA*p-Luc (from S. Phan, University of Michigan, Ann Arbor, MI), were transfected into RLE-6TN cells, followed by treatment with TGF-β1. To evaluate the effects of ICG-001 and SIS3 on *α-SMA* promoter activity, RLE-6TN cells were transfected with *α-SMA*-Luc, followed by TGF-β1 treatment with or without ICG-001 or SIS3 for 24 h. Co-transfection of a *Renilla* luciferase reporter was used to normalize for transfection efficiency. Firefly and *Renilla* luciferase activity were determined with the Dual-Luciferase reporter assay system (Promega, San Luis Obispo, CA). For details see supplemental materials.

Knockdown of β-Catenin and Smad3—For β-catenin knockdown, RLE-6TN cells were transfected with small-interfering RNA (siRNA) targeting the rat β-catenin gene or control non-targeting siRNA using Lipofectamine 2000. After 6 h, medium was replaced with fresh medium supplemented with TGF-β1 (0.5 ng/ml) for an additional 48 h. Proteins were harvested for detection of β-catenin and α-SMA expression by Western analysis. For Smad3 knockdown, RLE-6TN cells were transduced with lentivirus expressing Smad3 short hairpin RNA (shRNA) or control non-silencing shRNA using polybrene (final concentration 8 μg/ml). The following day, cells were treated with TGF-β1 and proteins were harvested for Western analysis after 4 days of treatment. Additional details are provided in supplemental materials.

Preparation of Nuclear Extracts and Co-immunoprecipitation—Nuclear extracts were harvested from RLE-6TN cells ± TGF-β1 (2.5 ng/ml) ± SIS3 (3 μM) (Calbiochem, La Jolla, CA) or RLE-6TN cells ± TGF-β1 (0.5 ng/ml) ± ICG-001 for 24 h using the ProteoExtract subcellular protein extraction kit (Calbiochem). Nuclear extracts (100 μg) were diluted in protein binding buffer (PBB) to a final volume of 1 ml, followed by addition of 2 μg of rabbit or mouse IgG and 20 μl of protein-A/G plus-agarose (Santa Cruz Biotechnology). After incuba-

tion, samples were centrifuged and supernatants collected, followed by addition of 2 μg of rabbit polyclonal anti-CBP or anti-Smad3 Abs, or normal rabbit IgG. Proteins were precipitated with protein A/G Plus-agarose equilibrated in PBB and eluted by addition of 30 μl of 2× Laemmli buffer containing 2-mercaptoethanol. Samples were analyzed by Western blotting for β-catenin and Smad3, using Abs mentioned above. For detecting β-catenin dephosphorylated at Ser-37 and Thr-41 or phosphorylated at Tyr-654 (pTyr654), RLE-6TN cells treated with TGF-β1 for 6 h were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris (pH 7.2), 0.1% Triton X-100, 0.1% SDS, 1% deoxylcholate, 5 mM EDTA) supplemented with 30 μl phosphatase inhibitors (Affymetrix, Santa Clara, CA). Active-β-catenin or p-Tyr654-β-catenin was detected by Western blot following precipitation by incubation of protein lysate (500 μg) with 0.5 μg of anti-ABC or anti-β-catenin pTyr654 Ab at 4 °C overnight. For additional details see supplemental materials.

Production of Lentivirus in 293T Cells—A Myc-tagged ICAT cDNA (from B. M. Gumbiner, University of Virginia, Charlottesville, VA) from pCS2/ICAT was cloned into the lentivirus backbone vector pRRL.hCMV.Sin.IRES.GFP. Infectious lentivirus was created by cotransfection of pRRL.hCMV.Sin.mycICAT.IRES.GFP plasmid with pCMVΔR8.91 and pMD.G into human 293T cells. The infection mixture was added dropwise to 293T cells plated on 100-mm culture dishes and incubated at 37 °C overnight. Virus was harvested after 48 h and concentrated by centrifugation. Titers of virus stocks were determined by p24 Elisa Assay Kit (Cell Biolabs, San Diego, CA). For additional details see supplemental materials.

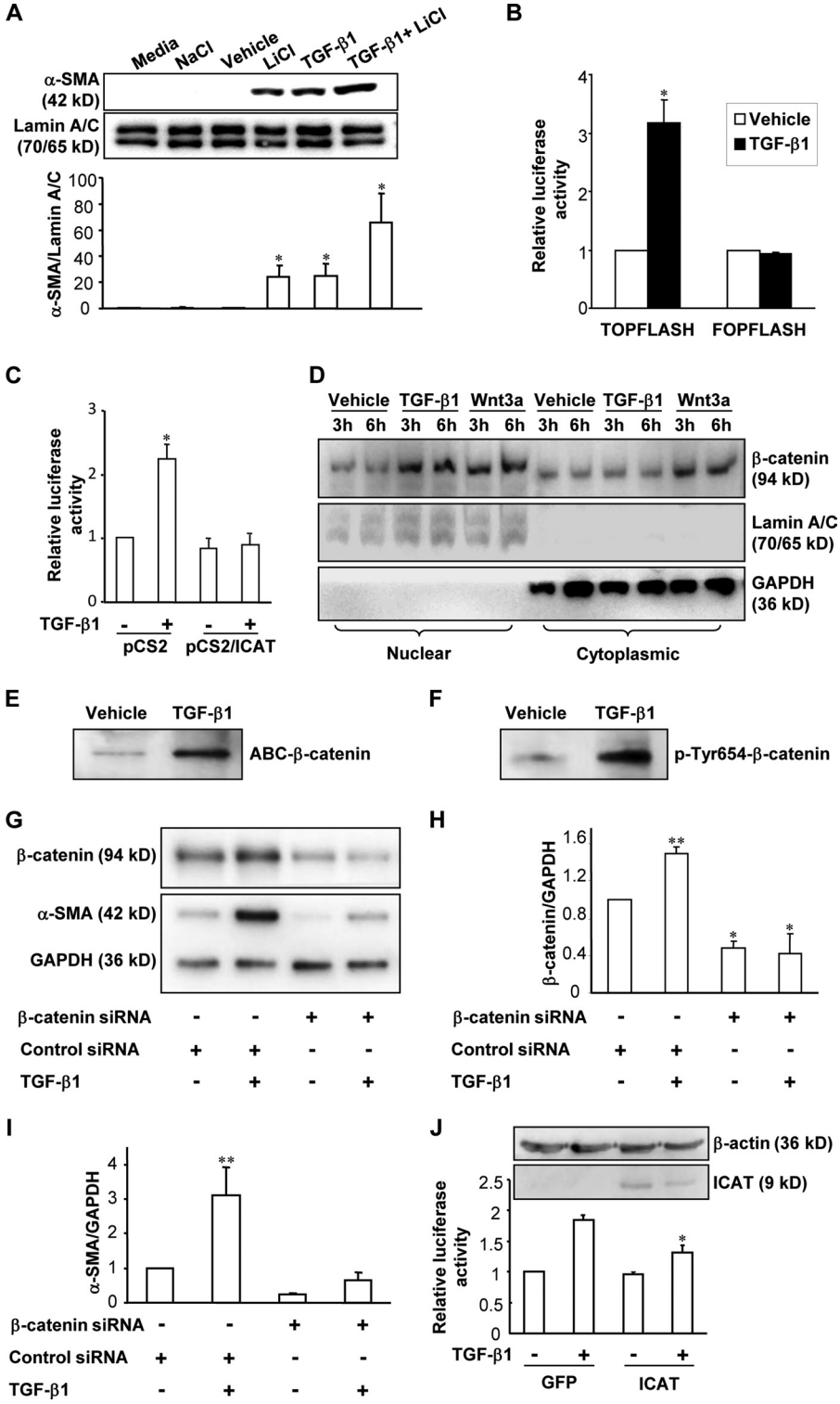
Chromatin Immunopreciptation (ChIP) and re-ChIP Assays—Chromatin was cross-linked with formaldehyde and ChIP was performed per manufacturer's instructions (ChIP-IT Express Kit, Active Motif, Carlsbad, CA). Briefly, 200 μl of ChIP reaction containing ~6 μg of soluble chromatin, 25 μl of protein G magnetic beads, 20 μl of ChIP buffer, 1 μl of protease inhibitors and 2 μg of anti-β-catenin, anti-Smad3, anti-CBP, or corresponding control Ab was incubated at 4 °C overnight. After washing with ChIP buffer I and II, chromatin was eluted, reverse cross-linked and treated with proteinase K. SBE1- or SBE2-containing DNA fragments of the rat *α-SMA* promoter were amplified by PCR or using SYBR-Green PCR with a 7900HT fast real time PCR system (Applied Biosystems, Foster City, CA). Primer pairs are as follows: SBE1 forward: 5′-GGCTGGTGAATCCTGATTAC-3′ and SBE1 reverse: 5′-CAAAAGATGCTTGGGTCACC-3′; SBE2 forward: 5′-TCTGGCCACCCAGATTAGAG-3′ and SBE2 reverse: 5′-CTTACCCTGATGGCGACTGG-3′. Size of PCR products containing SBE1 or SBE2 is 146 bp and 209 bp, respectively. The amplification protocol for qPCR was set as follows: 95 °C denaturation for 10 min followed by 40 cycles of 15-s denaturation at 95 °C, 1 min of annealing/extension, and data collection at 60 °C. For re-ChIP assays, the first eluate from the β-catenin-precipitated DNA-protein complex was desalted, followed by a second ChIP using 2 μg of anti-Smad3 (Abcam) or anti-CBP Ab according to instructions supplied with the Re-ChIP-IT kit (Active Motif). After elution, PCR was performed to amplify the 146 bp DNA fragment containing SBE1 at the rat *α-SMA* promoter using the primer pairs described above.

Statistical Analysis—Data are shown as means ± S.E.; *n* is the number of observations. We used z-tests to determine whether the ratiometric data (*i.e.* normalized against control) are different from control. $p < 0.05$ was considered significant.

RESULTS

TGF-β1 Activates β-Catenin-dependent Signaling and Synergizes with Wnt/β-catenin Signaling Pathway to Induce α-SMA Expression—To evaluate if TGF-β1-mediated EMT involves crosstalk with Wnt/β-catenin signaling, we first investigated synergistic interactions between TGF-β1 and Wnt/β-catenin pathways in induction of EMT. Rat lung epithelial cells (RLE-6TN) were treated with TGF-β1 and/or LiCl (an activator of Wnt signaling by inactivation of GSK-3β (43)) for 6 days. As shown in Fig. 1*A*, TGF-β1 or LiCl alone increased α-SMA expression by 24.0 ± 8.7- or 24.9 ± 9.3-fold, respectively, while treatment with TGF-β1 and LiCl together increased α-SMA expression by 65.2 ± 22.1-fold. Based on a calculated interaction response of 0.13 using the formula (log[(TGF-β1+LiCl)/(TGF-β1)+(LiCl)]), we conclude that TGF-β1 and LiCl synergistically induce α-SMA expression in AEC (44), suggesting that TGF-β1 and Wnt/β-catenin signaling pathways cooperate to induce α-SMA expression. To explore possible crosstalk between these two pathways, a TCF/LEF reporter, TOPFLASH (which contains a TCF/LEF response element), and its mutant FOPFLASH (which contains a mutated TCF/LEF response element), were transfected into RLE-6TN cells ± TGF-β1. TGF-β1 increased TOPFLASH reporter activity by ~3-fold (3.4 ± 0.4) but had no effect on FOPFLASH (Fig. 1*B*). Moreover, overexpression of ICAT, a 9-kDa polypeptide that specifically disrupts interaction of TCF and β-catenin (45), abrogated TGF-β1-induced TOPFLASH reporter activity (Fig. 1*C*), indicating that TGF-β1 activates β-catenin-dependent transcription. To confirm activation of β-catenin by TGF-β1, we assessed nuclear accumulation of β-catenin following treatment of RLE-6TN cells with TGF-β1. Treatment with Wnt3a-conditioned medium was used as control (Fig. 1*D*, *lanes 5* and *6 versus lanes 1* and *2*). TGF-β1 treatment induced accumulation of β-catenin in the nucleus (Fig. 1*D*, *lanes 3* and *4 versus lanes 1* and *2*). Quantitative analysis showed that treatment of RLE-6TN cells with TGF-β1 induced rapid phosphorylation of GSK-3β on serine-9 (supplemental Fig. S1, *lanes 2–4 versus lane 1*) indicating that TGF-β1 increases β-catenin nuclear accumulation at least in part by preventing β-catenin degradation via GSK-3β-dependent ubiquitin/proteasome machinery. Western analysis showed an increase in the signaling-competent form of β-catenin (dephosphorylated at Ser-37 and Thr-41), consistent with β-catenin activation by TGF-β1 (46, 47) (Fig. 1*E*). Because phosphorylation of β-catenin at tyrosine 654 has been reported to play a role in its release from E-cadherin and induction of EMT (39), p-Tyr654-β-catenin was also analyzed. As shown in Fig. 1*F*, p-Tyr654-β-catenin was increased after 6 h of TGF-β1 treatment. To further investigate the mechanism(s) whereby these two signaling pathways may interact in the context of EMT, we used siRNA to knock down β-catenin expression in TGF-β1-treated RLE-6TN cells and examined effects on α-SMA expression (Fig. 1, *G–I*). Quantitative analysis revealed that

TGF-β1 increased α-SMA expression by ~3-fold (Fig. 1*I*). Importantly, knockdown of β-catenin (reflected by a reduction in total β-catenin) (Fig. 1*H*) significantly abrogated α-SMA induction in TGF-β1-treated RLE-6TN cells and decreased levels in vehicle controls (Fig. 1*I*). In addition, transduction of RLE-6TN cells with ICAT lentiviral expression vector pRRL.hCMV.Sin.mycICAT.IRES.GFP or control vector pRRL.hCMV.Sin.IRES.GFP followed by transfection with the 764-bp-*α-SMA*-luciferase reporter demonstrated ~2-fold induction of *α-SMA*-luciferase reporter activity in response to TGF-β1 in the absence of ICAT (Fig. 1*J*, *lane 2 versus lane 1*), which was significantly reduced by overexpression of ICAT (Fig. 1*J*, *lane 4 versus lane 2*), supporting involvement of β-catenin-dependent signaling in the TGF-β1-mediated induction of α-SMA and suggesting that these effects are transcriptionally mediated.



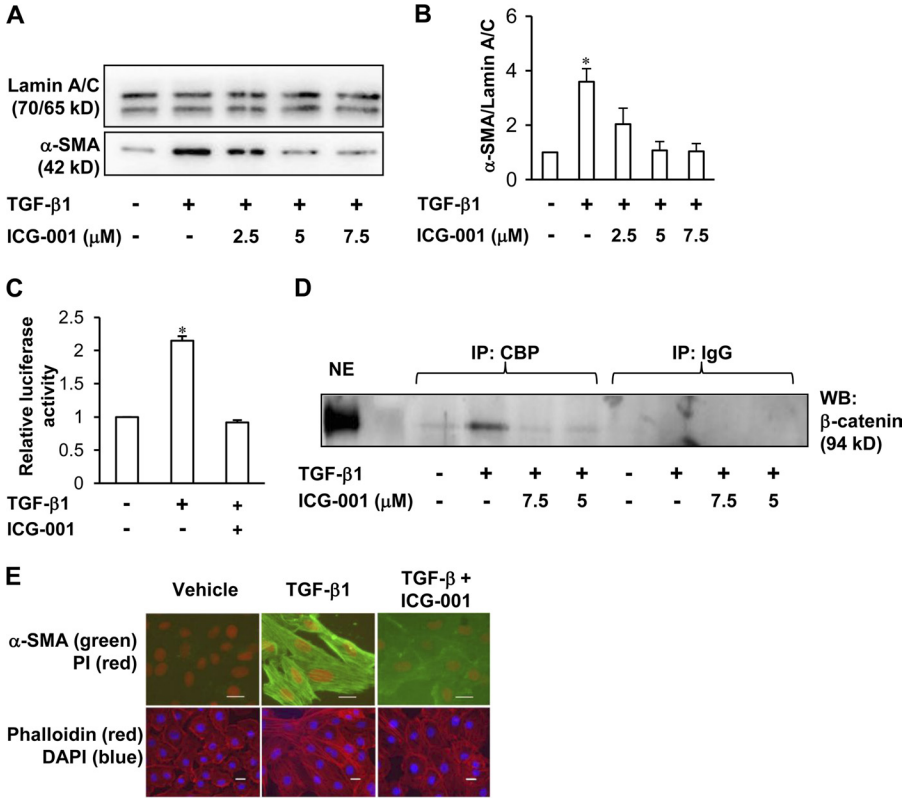


FIGURE 2. **ICG-001 inhibits TGF-β1-induced α-SMA induction and EMT.** Representative Western blot (*A*) and quantitative analysis (*B*) of α-SMA protein in RLE-6TN cells treated with TGF-β1 (0.5 ng/ml) ± ICG-001 for 2 days. Lamin A/C is used as loading control. *C*, RLE-6TN cells were transfected with 764-bp α-*SMA* reporter, followed by treatment with TGF-β1 (2.5 ng/ml) for 24 h in the presence and absence of ICG-001 (5 μM). Luciferase activity was normalized to *Renilla* luciferase activity ($n = 5$, *, $p < 0.05$ compared with other conditions). *D*, co-immunoprecipitation of β-catenin with CBP ($n = 3$) in RLE-6TN cells treated with TGF-β1 ± ICG-001 for 24 h. NE is RLE-6TN nuclear extract which was used as positive control for β-catenin. *E*, representative immunofluorescence image ($n = 3$) of staining for α-SMA and phalloidin in RLE-6TN cells treated with TGF-β1 ± ICG-001 for 4 days. RLE-6TN cells treated with TGF-β vehicle are shown as control. Scale bar = 20 μm.

Inhibition of β-Catenin/CBP-dependent Signaling by ICG-001 Prevents TGF-β1-induced EMT and α-SMA Induction—ICG-001, a selective competitive inhibitor of interactions between β-catenin and CBP, has been shown to prevent activation of a subset of downstream β-catenin/TCF-responsive target genes such as survivin (48). We utilized ICG-001 to disrupt the β-catenin pathway in TGF-β1-treated RLE-6TN cells. Cells were treated with TGF-β1 in the presence or absence of increasing concentrations of ICG-001 for 2 days. As shown in Fig. 2, *A* and *B*, TGF-β-induced increases in α-SMA were prevented by ICG-001 in a dose-dependent fashion. Furthermore, transfection with an α-*SMA* reporter plasmid containing 764-bp 5′-flanking region and 5′-UTR of the rat α-*SMA* promoter, followed by treatment with TGF-β1 in the presence or absence of ICG-001, revealed that ICG-001 significantly inhibited TGF-β1-induced α-*SMA* promoter activity (Fig. 2*C*), consistent with a role for β-catenin/CBP-dependent signaling in induction of α-SMA transcriptional activity by TGF-β. Consistent with these findings, co-immunoprecipitation (co-IP) followed by Western analysis further revealed that TGF-β-induced association of β-catenin with CBP was reversed by ICG-001 (Fig. 2*D*). Multiple attempts at reciprocal co-IP experiments using an anti-β-catenin Ab for "pull-down" failed to detect the associated CBP in immunoprecipitated complexes, likely due to high abundance of β-catenin. Immunostaining with an anti-α-SMA Ab (Fig. 2*E*, *upper panel*) and labeling with

FIGURE 1. **TGF-β1 activates β-catenin-dependent signaling.** *A*, representative Western blots (*upper panel*) and quantitative analysis of α-SMA (*lower panel*) following treatment of RLE-6TN cells with TGF-β1 (2.5 ng/ml) and/or LiCl (7.5 mM) for 6 days. Controls include media only, NaCl (7.5 mM) and TGF-β vehicle. Lamin A/C is used as a loading control ($n = 4$, *, $p < 0.05$ compared with vehicle). *B*, RLE-6TN cells were transfected with a LEF/TCF TOPFLASH reporter or its mutant, FOPFLASH, followed by treatment with TGF-β1 (2.5 ng/ml) for 24 h. Luciferase activity was normalized to *Renilla* luciferase activity ($n = 3$, * = $p < 0.05$ compared with vehicle). *C*, ICAT expression plasmid pCS2/ICAT and TOPFLASH reporter were co-transfected in RLE-6TN cells followed by TGF-β1 treatment. Reporter activity was determined 48 h after transfection and normalized to *Renilla* luciferase activity ($n = 3$, * = $p < 0.05$ (significantly different from pCS2 in the absence of TGF-β1)). *D*, representative Western blot ($n = 3$) for β-catenin in nuclear and cytoplasmic fractions harvested from RLE-6TN cells treated with TGF-β1 for indicated times. Lamin A/C and GAPDH are used to verify purity of nuclear and cytosolic fractions, respectively. Treatment with Wnt3a is used as a positive control. Cell lysates from RLE-6TN cells treated with TGF-β1 for 6 h were immunoprecipitated using anti-active-β-catenin (*E*) or anti-p-Tyr654-β-catenin (*F*) Abs. Immunoprecipitated active-β-catenin, dephosphorylated on Ser-37 and Thr-41 (*E*) or p-Tyr654-β-catenin (*F*), were detected by Western blot ($n = 2$). Representative Western blot (*G*) and quantitative analysis of total β-catenin (*H*) and α-SMA (*I*) in lysate from RLE-6TN cells transfected with β-catenin or control siRNA followed by TGF- β treatment for 48 h. GAPDH is used as a loading control ($n = 3$; *, $p < 0.05$ compared with control siRNA; **, $p < 0.05$ compared with control siRNA in the absence of TGF-β1). *J*, α-*SMA* reporter was transfected into RLE-6TN cells transduced with lentiviral vector expressing ICAT or control vector expressing GFP. Firefly luciferase activity was measured following treatment with TGF-β1 and normalized to *Renilla* luciferase activity. Expression of Myc-tagged ICAT was detected by Western blotting using anti-myc Ab (*inset*). β-Actin is used as loading control ($n = 3$, *, $p < 0.05$ compared with cells transduced with GFP control vector treated with TGF-β1).

fluorescently tagged phalloidin (which labels F-actin; Fig. 2*E*, *lower panel*) further demonstrated that TGF-β1 increased α-SMA expression in a fibril-associated pattern (Fig. 2*E*, *middle panels*), whereas treatment with ICG-001 decreased α-SMA and prevented morphological changes induced by TGF-β1 (Fig. 2*E*, *right panels*), consistent with inhibition of EMT. Observed inhibitory effects of ICG-001 strongly suggest that TGF-β1 induction of EMT in AEC involves activation of β-catenin/CBP-dependent signaling.

Transcriptional Regulation of α-SMA Expression by TGF-β1 is Smad-dependent—To establish a basis for specific molecular interactions between components of the TGF-β and Wnt/β-catenin-dependent signaling pathways, we examined the potential role of Smad3 in TGF-β-dependent induction of α-SMA. Smad3 is known to be expressed in RLE-6TN cells and to be phosphorylated after stimulation by TGF-β (49). As expected, Western analysis revealed that Smad3 was rapidly phosphorylated in RLE-6TN cells after TGF-β1 treatment (supplemental Fig. S2). Moreover, treatment with *SIS*3, an inhibitor of Smad3 phosphorylation (50), blocked α-SMA protein induction in a dose-dependent manner (Fig. 3, *A* and *B*), indicating a critical role for Smad3 in TGF-β1 induction of α-SMA. To further assess a role for Smad3 in mediating TGF-β1 induction of *α-SMA*, RLE-6TN cells were transduced with lentivirus harboring shRNA for Smad3. As shown in Fig. 3, *C–E*, knockdown of Smad3 inhibited induction of α-SMA by TGF-β1 by 57.4 ± 13.7%. Previous studies demonstrated that the 764-bp 5′-flanking region and 5′-UTR of the rat *α-SMA* promoter harbor two putative SBEs, located at −552 to −513 (SBE1) and −5 to +28 (SBE2) relative to the transcription start site at +1, respectively (51). To evaluate the role of Smad3 specifically in transcriptional regulation of the *α-SMA* promoter, RLE-6TN cells were transfected with the 764-bp *α-SMA*-reporter followed by treatment with TGF-β1 ± SIS3. As shown in Fig. 3*F*, SIS3 inhibited TGF-β1-induced *α-SMA* promoter activity. In addition, overexpression of Smad3 activated *α-SMA* promoter activity (Fig. 3*G*), further suggesting that transcriptional regulation of *α-SMA* promoter in epithelial cells (as in fibroblasts) is Smad3-dependent. Reporter assays using two Smad binding site (SBE1 and SBE2) mutants, *α-SMA*p-Luc-SBEm1 and *α-SMA*p-Luc-SBEm2, as well as wild type *α-SMA*p-Luc (51), in RLE-6TN cells showed that mutation of SBE1, but not SBE2, significantly decreased both basal and TGF-β-induced transcriptional activity of *α-SMA* (Fig. 3*H*). These findings confirm a role for Smad3 acting through SBE1 in transcriptional regulation of α-SMA expression by TGF-β in epithelial cells.

Interaction of Endogenous β-Catenin and Smad3 at the α-SMA Promoter Is CBP-dependent—Having established a role for Smad3 in induction and transcriptional regulation of α-SMA by TGF-β and that TGF-β effects on α-SMA and EMT are β-catenin-dependent, we sought to determine if these effects of TGF-β involved direct interactions between Smad3 and β-catenin. Co-IP experiments performed with anti-Smad3 Ab for pull-down using nuclear extracts from RLE-6TN cells treated with a combination of TGF-β1 and SIS3 revealed that association between endogenous β-catenin and Smad3 was increased (*lane 2 versus lane 1*) in TGF-β1-treated cells (Fig. 4*A*). Furthermore, TGF-β1-induced interaction of endogenous β-catenin and Smad3 was attenuated by concomitant treatment with SIS3 (Fig. 4*A*, *lane 3 versus lane 2*). Similar to results in Fig. 2*D*, despite multiple attempts, reciprocal experiments using an anti-β-catenin Ab for "pull-down", however, failed to detect the associated Smad3 in immunoprecipitated complexes. These results led us to postulate that TGF-β1 induces formation of a protein complex between β-catenin and Smad3 that interacts through SBE1 with the *α-SMA* promoter and that phosphorylation of Smad3 is important for this association. ChIP assays in RLE-6TN cells treated with TGF-β1 showed occupancy of β-catenin at the SBE1- but not SBE2-containing region of the *α-SMA* promoter (supplemental Fig. S3). Furthermore, TGF-β1 increased occupancy of β-catenin at the SBE1-containing region (Fig. 4*B*, *column 2 versus column 1*) of the *α-SMA* promoter, which was decreased by treatment with SIS3 (Fig. 4*B*, *column 3 versus column 2*). In addition, ChIP-re-ChIP assay indicated concurrent occupancy by both β-catenin and Smad3 at the SBE1-containing region of the *α-SMA* promoter (Fig. 4*C*). These observations suggest that Smad3 and β-catenin function in concert in response to TGF-β1 signaling to activate *α-SMA* gene transcription during EMT. Because ICG-001 inhibits TGF-β1-induced EMT and α-SMA reporter activity by specifically interfering with the interaction between β-catenin and CBP, we further investigated whether this interaction modulates interaction of β-catenin with the SBE1-containing region of the *α-SMA* promoter. Treatment of cells with ICG-001 inhibited TGF-β1-induced β-catenin occupancy of the *α-SMA* promoter, suggesting that in addition, interactions between Smad3 and β-catenin are CBP-dependent (Fig. 4*D*). Consistent with this, ChIP assay with pull-down using anti-CBP Ab revealed increased occupancy by CBP at the SBE1-containing region of the *α-SMA* promoter in response to TGF-β (Fig. 5*A*), which was inhibited by ICG-001. Furthermore, ChIP-re-ChIP assay (Fig. 5*B*) indicated concurrent occupancy by both β-catenin and CBP of the SBE1-containing region of the *α-SMA*.

Co-localization of β-Catenin with Smads and Expression of CBP in Lungs of Patients with IPF—We previously reported that α-SMA co-localizes with AT2 cell markers Nkx2.1 and pro-SP-C in more than 80% of hyperplastic AT2 cells in IPF lung, suggesting epithelial cells may serve as a novel source of myofibroblasts through EMT in IPF (16). To establish *in vivo* relevance of crosstalk between TGF-β1 and β-catenin signaling pathways in EMT and IPF pathogenesis, paraffin-embedded lung samples from IPF patients were analyzed for co-localization of β-catenin with Smad3 in hyperplastic AT2 cells. Immunoreactive β-catenin and Smad3 were identified in both nuclear and perinuclear regions of hyperplastic AT2 cells adjacent to fibroblast foci (Fig. 6*A*). Membrane and cytoplasmic β-catenin and cytoplasmic Smad3 were also observed. Counting of 28 high-power fields in lung sections from 7 patients, revealed co-localization of nuclear β-catenin with Smad3 in 51.02 ± 3.51% of cells. Control slides using mouse or rabbit IgG in place of either or both β-catenin and Smad3 Abs showed no nonspecific cross-reactivity of secondary Abs (supplemental Fig. S4). To confirm that cells expressing β-catenin or Smad3 were AT2 cells, we further performed co-immunostaining with

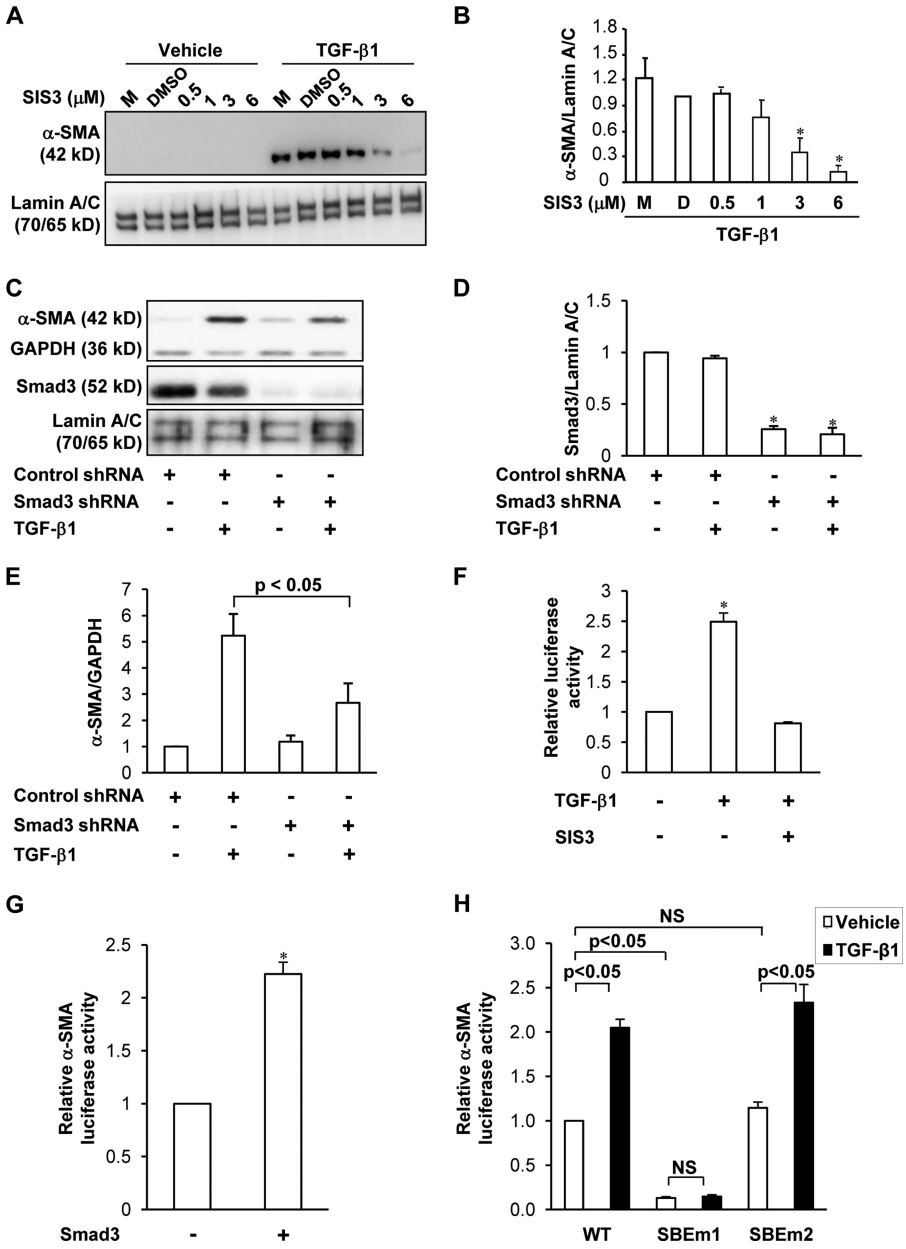


FIGURE 3. **TGF-β-induced α-SMA induction and transcription in RLE-6TN cells is Smad-dependent.** Representative Western blot (*A*) and quantitative analysis (*B*) of α-SMA protein in RLE-6TN cells treated with TGF-β1 (or vehicle DMSO) ± SIS3. *M* denotes medium. Lamin A/C is used as a loading control ($n = 3$, *, $p < 0.05$ (significantly different from DMSO). Western blot (*C*) and quantitative analysis of Smad3 (*D*) and α-SMA (*E*) protein using cell lysate from RLE-6TN cells transduced with lentivirus expressing shRNA for Smad3 or control pGIPZ nonsilencing shRNA followed by TGF-β1 treatment. Lamin A/C and GAPDH were used as loading controls ($n = 3$, *, $p < 0.05$, significantly different from control shRNA). *F*, RLE-6TN cells were transfected with 764-bp α-*SMA* reporter, followed by treatment with TGF-β1 (2.5 ng/ml) for 24 h in the presence and absence of SIS3 (6 μM). Luciferase activity was normalized to *Renilla* luciferase activity ($n = 5$, *, $p < 0.05$ compared with TGF-β1 vehicle and TGF-β1/SIS3 together). *G*, co-transfection of RLE-6TN cells with rat α-*SMA* promoter reporter, together with Smad3 expression plasmid pRK-5F/Smad3 or empty vector pRK-5F, followed by TGF-β1 treatment for 48 h. Firefly luciferase activity is normalized to *Renilla* luciferase activity ($n = 3$, *, $p < 0.05$, significantly different from the pRK-5F). *H*, co-transfection of RLE-6TN cells with wild type α-*SMA*p-Luc and SBE1 and SBE2 mutants α-*SMA*p-Luc-SBEm1 and α-*SMA*p-Luc-SBEm2, followed by TGF-β1 treatment for 15 h. Firefly luciferase activity is normalized to *Renilla* luciferase activity ($n = 8$, *, $p < 0.05$; *NS*, not significantly different).

anti-β-catenin/anti-pro-SP-C or anti-Smad3/anti-Nkx2.1 Abs. Fig. 6*B* demonstrates that cells expressing nuclear β-catenin also express cytoplasmic pro-SP-C, while Smad3-expressing cells express nuclear Nkx2.1 (Fig. 6*C*). In addition, using immunohistochemistry to stain for CBP in lung sections from 7 patients, CBP was detected in the nucleus (*pink*, Fig. 7, *A* and *B*) in hyperplastic AT2 cells in IPF lung. Control slides using rabbit IgG showed no detectable signals (Fig. 7*C*). These results further support a role for interaction between Wnt/β-catenin and TGF-β/Smad pathways in mediating epithelial abnormalities, including EMT, in IPF.

DISCUSSION

We report here that TGF-β-induced convergence of β-catenin-dependent and canonical Smad3 signaling is critical during TGF-β-induced effects on EMT. We describe the functional implications of molecular interactions between Smad3 and specifically the β-catenin/CBP complex in orchestrating

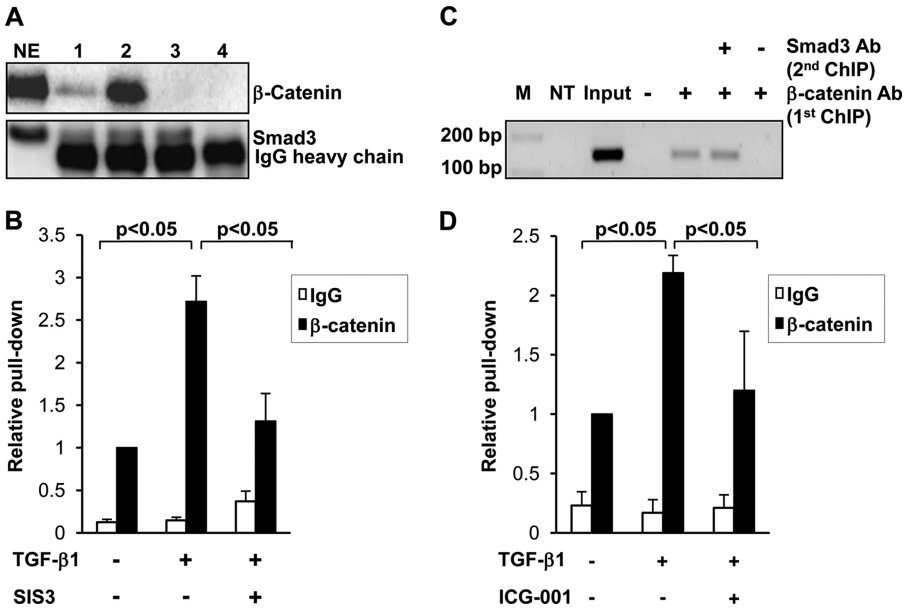


FIGURE 4. **TGF-β1 induces CBP-dependent interaction between Smad3 and β-catenin and occupancy of the SBE1 region of the α-SMA promoter.** *A*, co-IP was performed with anti-Smad3 Ab using nuclear extracts (*NE*) harvested from RLE-6TN cells treated with TGF-β1 vehicle and DMSO (*lane 1*), TGF-β1, and DMSO (*lane 2*) and TGF-β1 together with SIS3 (3 μM) (*lane 3*). IgG is used as control (*lane 4*). Associated β-catenin was analyzed by Western blotting. NE was used as WB control. *B*, ChIP assay was performed with anti-β-catenin Ab for pull-down using chromatin harvested from RLE-6TN cells in the presence or absence of TGF-β1 (0.5 ng/ml) ± SIS3 (3 μM), followed by amplification of the SBE1-containing region at the α-*SMA* promoter by qPCR ($n = 3$). IgG pull-down is used as control. *C*, ChIP-re-ChIP assay was performed first with mouse IgG (lane 4) or anti-β-catenin Ab (*lanes 5*, *6*, and *7*) and then with anti-Smad3 (*lane 6*) or rabbit IgG (*lane 7*) for pull-down using chromatin harvested from RLE-6TN cells treated with TGF-β1. Enrichment of SBE1-containing region at the α-*SMA* promoter was identified by PCR ($n = 3$). *M* and *NT* denote marker and no template, respectively. *D*, ChIP assay was performed with anti-β-catenin Ab for pull-down using chromatin harvested from RLE-6TN cells in the presence or absence of TGF-β1 (0.5 ng/ml) ± ICG-001 (7.5 μM), followed by amplification of SBE1-containing region at the α-*SMA* promoter by qPCR ($n = 3$). ChIP efficiency was calculated relative to untreated cells precipitated with anti-β-catenin Ab, which was set as 1. IgG pull-down is used as control.

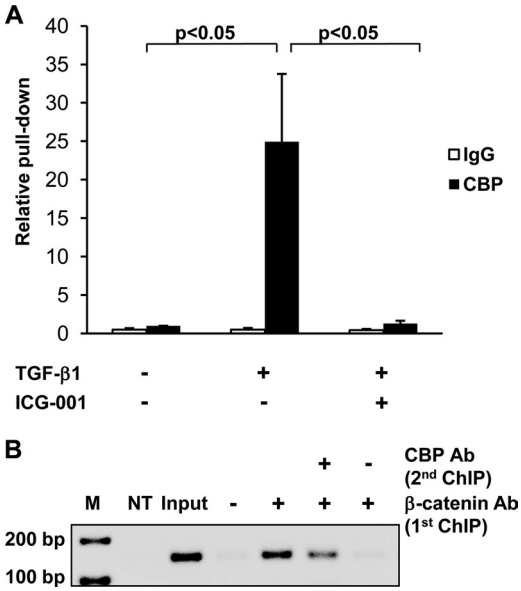


FIGURE 5. **TGF-β1 induces β-catenin and CBP occupancy of SBE1 region of the α-*SMA* promoter.** *A*, ChIP assay was performed with anti-CBP Ab for pull-down using chromatin harvested from RLE-6TN cells treated with TGF-β1 (0.5 ng/ml) ± ICG-001 (7.5 μM), followed by amplification of SBE1-containing region of the α-*SMA* promoter by qPCR. Data were processed from two pull-downs with qPCR performed in quadruplicate. ChIP efficiency was calculated relative to untreated cells precipitated with anti-CBP Ab, which was set as 1. IgG pull-down is used as control. *B*, ChIP-re-ChIP assay was performed first with mouse IgG (*lane 4*) or anti-β-catenin Abs (*lanes 5*, *6*, and *7*) and then with anti-CBP (*lane 6*) or rabbit IgG (*lane 7*) Abs for pull-down using chromatin harvested from RLE-6TN cells treated with TGF-β1. Enrichment of SBE1-containing region at the α-*SMA* promoter was identified by PCR ($n = 3$). *M* and *NT* denote marker and no template, respectively.

aspects of EMT. ICG-001, a specific inhibitor of β-catenin/CBP interactions, prevents TGF-β1-induced EMT and α-SMA induction, demonstrating a novel requirement for CBP-dependent β-catenin signaling in regulation of the EMT target gene α-*SMA* by TGF-β. These data linking TGF-β/Smad3 to pleiotropic β-catenin/CBP-dependent signaling pathways in the context of EMT add to the growing list of TGF-β molecular interactions with other transcriptional networks and suggest a potential new therapeutic approach to pulmonary fibrosis.

In the current study, we demonstrate that TGF-β and Wnt/β-catenin signaling synergize to increase expression of α-*SMA*, a hallmark of EMT, in non-malignant adult AEC. While transcriptional regulation of α-SMA has been extensively investigated during fibroblast to myofibroblast differentiation (51), its regulation in epithelial cells in the context of EMT is not well defined. We utilized α-*SMA* gene regulation as a reliable readout with which to investigate specific molecular interactions between TGF-β and β-catenin-dependent signaling intermediates during EMT. Duration of treatment with TGF-β varied depending on the specific endpoints evaluated. β-Catenin nuclear translocation, phosphorylation of β-catenin at tyrosine 654 and dephosphorylation of β-catenin at Ser-37 and Thr-41 were detected after 3–6 h of TGF-β treatment, suggesting the importance of β-catenin as an early signaling molecule in TGF-β-mediated EMT. Time course studies (data not shown) demonstrated that significant induction of α-SMA by TGF-β was only observed after 48 h of treatment. Thus, for Western analysis to evaluate α-*SMA* protein levels, we treated the cells for 48 h, while to assess morphological changes, cells were treated

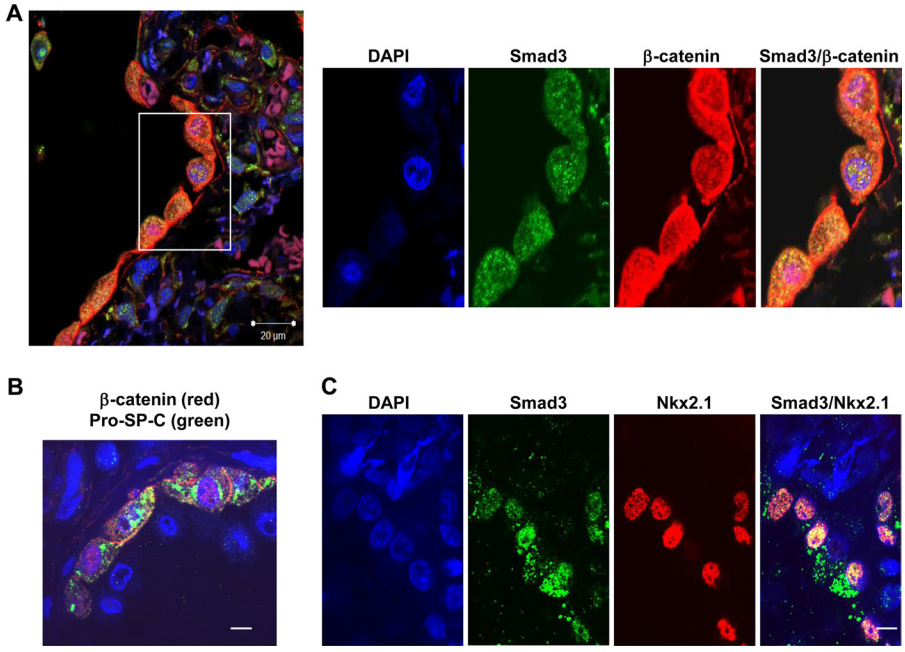


FIGURE 6. **Co-localization of β-catenin with Smad3 in hyperplastic AT2 cells of IPF lung.** *A*, representative immunofluorescence staining for β-catenin (*red*) and Smad3 (*green*) in hyperplastic AT2 cells of IPF lung tissue with anti-β-catenin and anti-Smad3 Abs. Nuclei (*blue*) are stained with DAPI. *B*, immunofluorescence staining for β-catenin (*red*) and pro-SP-C (*green*, cytoplasmic) in hyperplastic AT2 cells of IPF lung tissue. Nuclei (*blue*) are stained with DAPI. Scale bar = 20 μm. *C*, immunofluorescence staining for Smad3 (*green*) and Nkx2.1 (*red*, nuclear) in hyperplastic AT2 cells of IPF lung tissue. Nuclei (*blue*) are stained with DAPI. Scale bar = 20 μm.

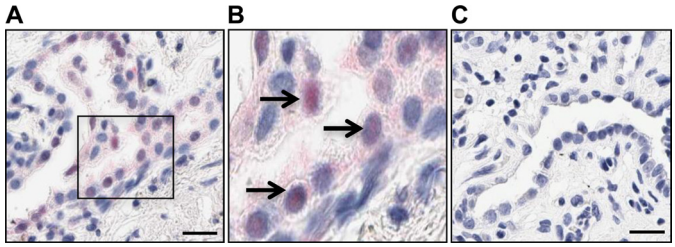


FIGURE 7. **Expression of CBP in IPF lung.** *A*, representative immunohistochemistry for staining of CBP (*pink*) in hyperplastic AT2 cells of IPF lung tissue using anti-CBP Ab. Nuclei (*blue*) are stained with hematoxylin. Scale bar = 20 μm. *B*, magnified views of the *rectangles* shown in *A*. *C*, negative control using IgG.

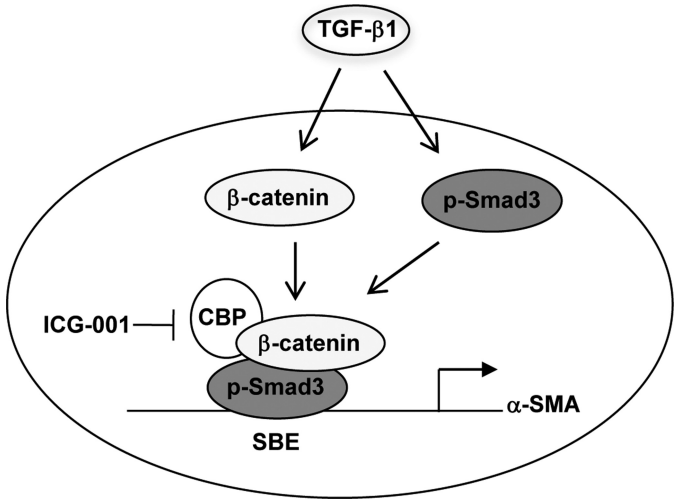


FIGURE 8. **Model for transcriptional regulation of α-SMA by TGF-β1.** In addition to initiating phosphorylation of Smad3 and nuclear translocation of p-Smad3, TGF-β1 induces nuclear accumulation of active β-catenin through phosphorylation at Tyr-654 and dephosphorylation at Ser-37 and Thr-41. In the nucleus, a multi-protein complex among p-Smad3, β-catenin, and CBP is formed which interacts with the Smad binding element (*SBE*) at the α-SMA promoter to regulate α-SMA expression during EMT.

with TGF-β for 4 days to allow full phenotypic transition. 6 days of treatment was required for synergy between TGF-β and LiCl to become evident, likely due to a requirement for at least 4 days for LiCl alone to induce α-SMA expression (data not shown).

Our results indicate that, in epithelial cells, SBE1 plays a critical role in regulation of α-*SMA* expression. We further demonstrate that α-*SMA* is a direct downstream target of conjoint signaling specifically under the auspices of the coactivator CBP by β-catenin and TGF-β pathways and provide evidence by co-IP for interaction between endogenous Smad3 and β-catenin following TGF-β1 treatment, which is dependent on Smad3 as well as interactions with CBP. Based on these results, we propose that TGF-β regulation of the α-*SMA* promoter is β-catenin-dependent, involving interactions of β-catenin as a bridge between CBP and Smad3 that in turn interacts with SBE1 of the α-*SMA* promoter (Fig. 8). This model is supported by ChIP data (Fig. 4, *C* and *D*, supplemental Fig. S4 and Fig. 5) showing that TGF-β increases concurrent occupancy of Smad3, β-catenin, and CBP at the distal (but not proximal) SBE (SBE1) of the α-*SMA* promoter. Furthermore, reduction in β-catenin occupancy of the α-*SMA* promoter by SIS3 and ICG-001 (Fig. 4, *B* and *D*) indicates both Smad- and CBP-dependence of β-catenin interaction with the α-*SMA* promoter. These findings are evidence for a role for β-catenin/CBP-dependent signaling in TGF-β1-regulated α-*SMA* transcription, providing novel mechanistic insights into induction of EMT by TGF-β1. Since TFG-β1 induced TOPFLASH (which encompasses a TCF/LEF response element) but not FOPFLASH (which includes a mutated TCF/LEF response element) (Fig. 1*B*), and ICAT (which specifically disrupts interaction between

β-catenin and TCF) inhibited both TOPFLASH (Fig. 1*C*) and α-*SMA* reporter activities in response to TFG-β1 (Fig. 1*J*), these results suggest that TCF/LEF may be also involved in the formation of a multi-protein complex with β-catenin, Smad3, and CBP.

The transcriptional co-activators CBP and p300 play key roles in diverse cellular processes, including proliferation, differentiation, cell cycle regulation, and development (52, 53), and modulate Wnt signaling through interactions with β-catenin. Although highly homologous and frequently viewed as functionally interchangeable, evidence from both *in vitro* and *in vivo* studies indicates that in fact these co-activators play unique and distinct roles in regulation of these cellular processes. Consistent with their non-redundant functions, recruitment of either p300 or CBP by β-catenin may differentially regulate subsets of target genes *in vitro* and *in vivo* and differentially acetylate substrates (54). ICG-001 is a novel small molecule inhibitor that specifically disrupts the interaction between β-catenin and CBP but not p300, enabling selective down-regulation of a subset of β-catenin/TCF responsive genes (48, 55). We found that ICG-001 abrogated TGF-β-induced α-*SMA* expression and morphological changes of EMT in RLE-6TN cells (Fig. 2), consistent with the notion that this process is β-catenin/CBP-dependent. Furthermore, treatment of RLE-6TN cells with TGF-β increased interaction between β-catenin and CBP that was disrupted by ICG-001 (Fig. 2*D*), suggesting potential formation of a ternary complex of β-catenin, Smad3, and CBP at SBE1 of the α-*SMA* promoter. ChIP-reChIP assay showed co-occupancy by both β-catenin/Smad3 (Fig. 4*C*) and β-catenin/CBP (Fig. 5*B*) of SBE1 of the α-*SMA* promoter, while treatment with ICG-001 showed decreased β-catenin occupancy of the α-*SMA* promoter in response to TGF-β, further supporting CBP-dependence of the interaction between β-catenin and α-SMA. We recently demonstrated that ICG-001 ameliorates pulmonary fibrosis and reduces α-*SMA* expression in a model of bleomycin-induced lung injury (40), suggesting selective activation of targets downstream of β-catenin/CBP-dependent transcription in this process. These results implicate β-catenin/CBP-dependent transcriptional regulation in TGF-β-induced EMT/fibrosis and further suggest that selective modulation of the β-catenin/CBP interaction may be useful as a therapeutic strategy for fibrosis in general and pulmonary fibrosis in particular.

Smad2 and Smad3 are direct mediators of transcriptional effects of TGF-β signaling (24). Despite significant homology, Smad2 and Smad3 are differentially regulated in a cell-specific fashion, and have both overlapping and distinct roles in development and the adult (56). Studies in animal models support a central role for Smad3 in TGF-β-induced EMT and fibrosis (57), although some studies have also suggested a role for Smad2 (58). Specifically, Kim *et al.* (39) reported that β-catenin located at adherens junctions becomes phosphorylated at Tyr-654 in an α3β1 integrin-dependent manner and forms a complex with p-Smad2 that translocates to the nucleus in mouse primary AEC in a Wnt ligand-independent manner. The extent to which these processes contribute to TGF-β-promoted alveolar EMT remains to be determined, although our data strongly argue for a role for Smad3 in regulation of the α-*SMA* promoter during transition to mesenchymal phenotype. SIS3 decreased α-*SMA* transcription induced by TGF-β1, while the α-*SMA* promoter was activated by overexpression of Smad3 (Fig. 3, *F* and *G*). Importantly, knockdown of Smad3 (Figs. 3, *C–E*) significantly decreased TGF-β1-induced α-SMA expression, while knockdown of Smad2 had only minor effects (data not shown). Overall, these results suggest a key role for Smad3 in coordinately regulating α-*SMA* gene expression through requisite interactions with β-catenin/CBP in TGF-β1-induced EMT in AEC. Together with our recent demonstration that lung epithelial-specific knock-out of TGF-β receptor type II reduced phosphorylation of Smad3 in response to TGF-β and increased survival in and resistance to bleomycin-induced pulmonary fibrosis (59), our data suggest that Smad3 is a key factor in TGF-β-mediated EMT/fibrosis.

In summary, we propose a model in which TGF-β1 initiates direct interaction between phosphorylated Smad3, β-catenin, and CBP to regulate α-*SMA* transcription during EMT through formation of a multiprotein complex at the distal SBE of the α-*SMA* promoter (Fig. 8). Induction of interaction between β-catenin and CBP by TGF-β and inhibition of this interaction and of EMT by ICG-001 and SIS3, suggest that CBP is required for regulation of α-*SMA* by Smad3 and β-catenin. Demonstration of a role for activation of α-*SMA* gene expression through association of Smad3, β-catenin, and CBP has important implications for regulation of the myofibroblast phenotype in human disease. Together with our present findings of co-localization of β-catenin with Smad3, CBP nuclear staining, and our previous finding of co-localization of epithelial and mesenchymal markers in hyperplastic AT2 cells (16) in the lungs of IPF patients, our results suggest that 1) β-catenin, and in particular β-catenin through its interaction with CBP, is a key mediator of TGF-β-induced EMT, 2) conjoint activation of TGF-β and β-catenin pathways coordinates induction of fibrosis in IPF, and 3) the effects of ICG-001 may point the way to development of new therapeutic approaches to pulmonary fibrosis.

Acknowledgments—Confocal microscopy was performed in the Cell and Tissue Imaging Core of the USC Research Center for Liver Disease (National Institutes of Health P30 DK048522) and USC Cell and Tissue Imaging Core of the USC Norris Comprehensive Cancer Center (NCI P30 CA014089 and NEI03040). Human lung tissue was obtained from the Lung Tissue Research Consortium, a resource of the National Heart, Lung and Blood Institute.

REFERENCES

1. Whitman, M. (1998) Smads and early developmental signaling by the TGFβ superfamily. *Genes Dev.* **12,** 2445–2462
2. Labbé, E., Lock, L., Letamendia, A., Gorska, A. E., Gryfe, R., Gallinger, S., Moses, H. L., and Attisano, L. (2007) Transcriptional cooperation between the transforming growth factor-β and Wnt pathways in mammary and intestinal tumorigenesis. *Cancer Res.* **67,** 75–84
3. Nishita, M., Hashimoto, M. K., Ogata, S., Laurent, M. N., Ueno, N., Shibuya, H., and Cho, K. W. (2000) Interaction between Wnt and TGF-β signaling pathways during formation of Spemann's organizer. *Nature* **403,** 781–785
4. Labbé, E., Letamendia, A., and Attisano, L. (2000) Association of Smads with lymphoid enhancer binding factor 1/T cell-specific factor mediates cooperative signaling by the transforming growth factor-β and wnt path-

ways. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 8358–8363
5. Furuhashi, M., Yagi, K., Yamamoto, H., Furukawa, Y., Shimada, S., Nakamura, Y., Kikuchi, A., Miyazono, K., and Kato, M. (2001) Axin facilitates Smad3 activation in the transforming growth factor β signaling pathway. *Mol. Cell. Biol.* **21,** 5132–5141
6. Tian, X., Du, H., Fu, X., Li, K., Li, A., and Zhang, Y. (2009) Smad4 restoration leads to a suppression of Wnt/β-catenin signaling activity and migration capacity in human colon carcinoma cells. *Biochem. Biophys. Res. Commun.* **380,** 478–483
7. Dong, Y., Drissi, H., Chen, M., Chen, D., Zuscik, M. J., Schwarz, E. M., and O'Keefe, R. J. (2005) Wnt-mediated regulation of chondrocyte maturation: modulation by TGF-β. *J. Cell. Biochem.* **95,** 1057–1068
8. Minoo, P., and Li, C. (2010) Cross-talk between transforming growth factor-β and Wingless/Int pathways in lung development and disease. *Int J Biochem. Cell Biol.* **42,** 809–812
9. Lei, S., Dubeykovskiy, A., Chakladar, A., Wojtukiewicz, L., and Wang, T. C. (2004) The murine gastrin promoter is synergistically activated by transforming growth factor-β/Smad and Wnt signaling pathways. *J. Biol. Chem.* **279,** 42492–42502
10. Masszi, A., Fan, L., Rosivall, L., McCulloch, C. A., Rotstein, O. D., Mucsi, I., and Kapus, A. (2004) Integrity of cell-cell contacts is a critical regulator of TGF-β1-induced epithelial-to-myofibroblast transition: role for β-catenin. *Am. J. Pathol.* **165,** 1955–1967
11. Tian, Y. C., and Phillips, A. O. (2002) Interaction between the transforming growth factor-β type II receptor/Smad pathway and β-catenin during transforming growth factor-β1-mediated adherens junction disassembly. *Am. J. Pathol.* **160,** 1619–1628
12. Kim, Y., Kugler, M. C., Wei, Y., Kim, K. K., Li, X., Brumwell, A. N., and Chapman, H. A. (2009) Integrin α3β1-dependent β-catenin phosphorylation links epithelial Smad signaling to cell contacts. *J. Cell Biol.* **184,** 309–322
13. Thiery, J. P., and Sleeman, J. P. (2006) Complex networks orchestrate epithelial-mesenchymal transitions. *Nat. Rev. Mol. Cell Biol.* **7,** 131–142
14. Zavadil, J., and Böttinger, E. P. (2005) TGF-β and epithelial-to-mesenchymal transitions. *Oncogene* **24,** 5764–5774
15. Thiery, J. P., Acloque, H., Huang, R. Y., and Nieto, M. A. (2009) Epithelial-mesenchymal transitions in development and disease. *Cell* **139,** 871–890
16. Willis, B. C., Liebler, J. M., Luby-Phelps, K., Nicholson, A. G., Crandall, E. D., du Bois, R. M., and Borok, Z. (2005) Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-β1: potential role in idiopathic pulmonary fibrosis. *Am. J. Pathol.* **166,** 1321–1332
17. Tanjore, H., Xu, X. C., Polosukhin, V. V., Degryse, A. L., Li, B., Han, W., Sherrill, T. P., Plieth, D., Neilson, E. G., Blackwell, T. S., and Lawson, W. E. (2009) Contribution of epithelial-derived fibroblasts to bleomycin-induced lung fibrosis. *Am. J. Respir. Crit. Care Med.* **180,** 657–665
18. Kim, K. K., Kugler, M. C., Wolters, P. J., Robillard, L., Galvez, M. G., Brumwell, A. N., Sheppard, D., and Chapman, H. A. (2006) Alveolar epithelial cell mesenchymal transition develops *in vivo* during pulmonary fibrosis and is regulated by the extracellular matrix. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 13180–13185
19. Sime, P. J., Xing, Z., Graham, F. L., Csaky, K. G., and Gauldie, J. (1997) Adenovector-mediated gene transfer of active transforming growth factor-β1 induces prolonged severe fibrosis in rat lung. *J. Clin. Invest.* **100,** 768–776
20. Roberts, A. B., Tian, F., Byfield, S. D., Stuelten, C., Ooshima, A., Saika, S., and Flanders, K. C. (2006) Smad3 is key to TGF-β-mediated epithelial-to-mesenchymal transition, fibrosis, tumor suppression, and metastasis. *Cytokine Growth Factor Rev.* **17,** 19–27
21. Willis, B. C., and Borok, Z. (2007) TGF-β-induced EMT: mechanisms and implications for fibrotic lung disease. *Am. J. Physiol. Lung Cell Mol. Physiol.* **293,** L525–L534
22. Bergeron, A., Soler, P., Kambouchner, M., Loiseau, P., Milleron, B., Valeyre, D., Hance, A. J., and Tazi, A. (2003) Cytokine profiles in idiopathic pulmonary fibrosis suggest an important role for TGF-beta and IL-10. *Eur. Respir. J.* **22,** 69–76
23. Khalil, N., Parekh, T. V., O'Connor, R., Antman, N., Kepron, W., Yehaualaeshet, T., Xu, Y. D., and Gold, L. I. (2001) Regulation of the effects of TGF-β1 by activation of latent TGF-β1 and differential expression of TGF-β receptors (TβR-I and TβR-II) in idiopathic pulmonary fibrosis. *Thorax* **56,** 907–915
24. Shi, Y., and Massagué, J. (2003) Mechanisms of TGF-β signaling from cell membrane to the nucleus. *Cell* **113,** 685–700
25. Flanders, K. C. (2004) Smad3 as a mediator of the fibrotic response. *Int. J. Exp. Pathol.* **85,** 47–64
26. Valcourt, U., Kowanetz, M., Niimi, H., Heldin, C. H., and Moustakas, A. (2005) TGF-β and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition. *Mol. Biol. Cell* **16,** 1987–2002
27. Masszi, A., Speight, P., Charbonney, E., Lodyga, M., Nakano, H., Szászi, K., and Kapus, A. (2010) Fate-determining mechanisms in epithelial-myofibroblast transition: major inhibitory role for Smad3. *J. Cell Biol.* **188,** 383–399
28. Kim, K., Lu, Z., and Hay, E. D. (2002) Direct evidence for a role of β-catenin/LEF-1 signaling pathway in induction of EMT. *Cell Biol. Int* **26,** 463–476
29. LaGamba, D., Nawshad, A., and Hay, E. D. (2005) Microarray analysis of gene expression during epithelial-mesenchymal transformation. *Dev Dyn* **234,** 132–142
30. Moustakas, A., and Heldin, C. H. (2007) Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. *Cancer Sci* **98,** 1512–1520
31. Chilosi, M., Poletti, V., Zamò, A., Lestani, M., Montagna, L., Piccoli, P., Pedron, S., Bertaso, M., Scarpa, A., Murer, B., Cancellieri, A., Maestro, R., Semenzato, G., and Doglioni, C. (2003) Aberrant Wnt/β-catenin pathway activation in idiopathic pulmonary fibrosis. *Am. J. Pathol.* **162,** 1495–1502
32. Königshoff, M., Balsara, N., Pfaff, E. M., Kramer, M., Chrobak, I., Seeger, W., and Eickelberg, O. (2008) Functional Wnt signaling is increased in idiopathic pulmonary fibrosis. *PLoS One* **3,** e2142
33. Königshoff, M., Kramer, M., Balsara, N., Wilhelm, J., Amarie, O. V., Jahn, A., Rose, F., Fink, L., Seeger, W., Schaefer, L., Günther, A., and Eickelberg, O. (2009) WNT1-inducible signaling protein-1 mediates pulmonary fibrosis in mice and is upregulated in humans with idiopathic pulmonary fibrosis. *J. Clin. Invest.* **119,** 772–787
34. Yang, I. V., Burch, L. H., Steele, M. P., Savov, J. D., Hollingsworth, J. W., McElvania-Tekippe, E., Berman, K. G., Speer, M. C., Sporn, T. A., Brown, K. K., Schwarz, M. I., and Schwartz, D. A. (2007) Gene expression profiling of familial and sporadic interstitial pneumonia. *Am. J. Respir. Crit. Care Med.* **175,** 45–54
35. Selman, M., Pardo, A., Barrera, L., Estrada, A., Watson, S. R., Wilson, K., Aziz, N., Kaminski, N., and Zlotnik, A. (2006) Gene expression profiles distinguish idiopathic pulmonary fibrosis from hypersensitivity pneumonitis. *Am. J. Respir. Crit. Care Med.* **173,** 188–198
36. Königshoff, M., and Eickelberg, O. (2010) WNT signaling in lung disease: a failure or a regeneration signal? *Am. J. Respir. Cell Mol. Biol.* **42,** 21–31
37. Medici, D., Hay, E. D., and Olsen, B. R. (2008) Snail and Slug promote epithelial-mesenchymal transition through β-catenin-T-cell factor-4-dependent expression of transforming growth factor-β3. *Mol. Biol. Cell* **19,** 4875–4887
38. Nawshad, A., Medici, D., Liu, C. C., and Hay, E. D. (2007) TGFβ3 inhibits E-cadherin gene expression in palate medial-edge epithelial cells through a Smad2-Smad4-LEF1 transcription complex. *J. Cell Sci.* **120,** 1646–1653
39. Kim, K. K., Wei, Y., Szekeres, C., Kugler, M. C., Wolters, P. J., Hill, M. L., Frank, J. A., Brumwell, A. N., Wheeler, S. E., Kreidberg, J. A., and Chapman, H. A. (2009) Epithelial cell α3β1 integrin links β-catenin and Smad signaling to promote myofibroblast formation and pulmonary fibrosis. *J. Clin. Invest.* **119,** 213–224
40. Henderson, W. R., Jr., Chi, E. Y., Ye, X., Nguyen, C., Tien, Y. T., Zhou, B., Borok, Z., Knight, D. A., and Kahn, M. (2010) Inhibition of Wnt/β-catenin/CREB-binding protein (CBP) signaling reverses pulmonary fibrosis. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 14309–14314
41. Zhou, B., Zhong, Q., Minoo, P., Li, C., Ann, D. K., Frenkel, B., Morrisey, E. E., Crandall, E. D., and Borok, Z. (2008) Foxp2 inhibits Nkx2.1-mediated transcription of SP-C via interactions with the Nkx2.1 homeodomain. *Am. J. Respir. Cell Mol. Biol.* **38,** 750–758
42. Han, M. K., Swigris, J., Liu, L., Bartholmai, B., Murray, S., Giardino, N.,

Thompson, B., Frederick, M., Li, D., Schwarz, M., Limper, A., Flaherty, K., and Martinez, F. J. (2010) Gender influences health-related quality of life in IPF. *Respiratory Med.* **104,** 724–730
43. Klein, P. S., and Melton, D. A. (1996) A molecular mechanism for the effect of lithium on development. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 8455–8459
44. Kiela, P. R., LeSueur, J., Collins, J. F., and Ghishan, F. K. (2003) Transcriptional regulation of the rat NHE3 gene. Functional interactions between GATA-5 and Sp family transcription factors. *J. Biol. Chem.* **278,** 5659–5668
45. Gottardi, C. J., and Gumbiner, B. M. (2004) Role for ICAT in β-catenin-dependent nuclear signaling and cadherin functions. *Am. J. Physiol. Cell Physiol.* **286,** C747–C756
46. Staal, F. J., Noort Mv, M., Strous, G. J., and Clevers, H. C. (2002) Wnt signals are transmitted through N-terminally dephosphorylated β-catenin. *EMBO Reports* **3,** 63–68
47. van Noort, M., Weerkamp, F., Clevers, H. C., and Staal, F. J. (2007) Wnt signaling and phosphorylation status of β-catenin: importance of the correct antibody tools. *Blood* **110,** 2778–2779
48. Ma, H., Nguyen, C., Lee, K. S., and Kahn, M. (2005) Differential roles for the coactivators CBP and p300 on TCF/β-catenin-mediated survivin gene expression. *Oncogene* **24,** 3619–3631
49. Felton, V. M., Borok, Z., and Willis, B. C. (2009) *N*-acetylcysteine inhibits alveolar epithelial-mesenchymal transition. *Am. J. Physiol. Lung Cell Mol. Physiol.* **297,** L805–L812
50. Jinnin, M., Ihn, H., and Tamaki, K. (2006) Characterization of SIS3, a novel specific inhibitor of Smad3, and its effect on transforming growth factor-β1-induced extracellular matrix expression. *Mol. Pharmacol.* **69,** 597–607
51. Hu, B., Wu, Z., and Phan, S. H. (2003) Smad3 mediates transforming growth factor-β-induced α-smooth muscle actin expression. *Am. J. Respir. Cell Mol. Biol.* **29,** 397–404
52. Kawasaki, H., Eckner, R., Yao, T. P., Taira, K., Chiu, R., Livingston, D. M., and Yokoyama, K. K. (1998) Distinct roles of the co-activators p300 and CBP in retinoic-acid-induced F9-cell differentiation. *Nature* **393,** 284–289
53. Roth, J. F., Shikama, N., Henzen, C., Desbaillets, I., Lutz, W., Marino, S., Wittwer, J., Schorle, H., Gassmann, M., and Eckner, R. (2003) Differential role of p300 and CBP acetyltransferase during myogenesis: p300 acts upstream of MyoD and Myf5. *EMBO J.* **22,** 5186–5196
54. Kalkhoven, E. (2004) CBP and p300: HATs for different occasions. *Biochem. Pharmacol.* **68,** 1145–1155
55. Emami, K. H., Nguyen, C., Ma, H., Kim, D. H., Jeong, K. W., Eguchi, M., Moon, R. T., Teo, J. L., Oh, S. W., Kim, H. Y., Moon, S. H., Ha, J. R., and Kahn, M. (2004) A small molecule inhibitor of β-catenin/CREB-binding protein transcription. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 12682–12687
56. Brown, K. A., Pietenpol, J. A., and Moses, H. L. (2007) A tale of two proteins: differential roles and regulation of Smad2 and Smad3 in TGF-β signaling. *J. Cell. Biochem.* **101,** 9–33
57. Flanders, K. C., Major, C. D., Arabshahi, A., Aburime, E. E., Okada, M. H., Fujii, M., Blalock, T. D., Schultz, G. S., Sowers, A., Anzano, M. A., Mitchell, J. B., Russo, A., and Roberts, A. B. (2003) Interference with transforming growth factor-β/ Smad3 signaling results in accelerated healing of wounds in previously irradiated skin. *Am. J. Pathol.* **163,** 2247–2257
58. Kasai, H., Allen, J. T., Mason, R. M., Kamimura, T., and Zhang, Z. (2005) TGF-β1 induces human alveolar epithelial to mesenchymal cell transition (EMT). *Respir Res* **6,** 56
59. Li, M., Krishnaveni, M. S., Li, C., Zhou, B., Xing, Y., Banfalvi, A., Li, A., Lombardi, V., Akbari, O., Borok, Z., and Minoo, P. (2011) Epithelium-specific deletion of TGF-β receptor type II protects mice from bleomycin-induced pulmonary fibrosis. *J. Clin. Invest.* **121,** 277–287