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. TGF-β induction of α-SMA (α-smooth muscle actin) involves ternary complex formation among Smad3, β-catenin and CBP.

Results: TGF-β and β-catenin/CBP-dependent pathways coordinately regulate α-SMA induction.

Conclusion: 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 vs block of epithelial proliferation vs epithelial-mesenchymal transition (EMT)) and precise mechanisms in many cases remain unknown. We investigated β-catenin-dependent and transforming growth factor-β (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.

TGF-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 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-twix 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 upregulate 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 in 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, St. Louis, MO), 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, 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/Smad3 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 RT, followed by incubation with 0.3% Triton X-100 for 10 min at RT. Cells were then incubated with anti-α-SMA Ab at RT for 1 hour, 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). For β-catenin staining, filters were incubated with anti-β-catenin Ab followed by biotinylated anti-mouse secondary Ab (Vector Laboratories). Signal was amplified with avidin conjugated to Texas-Red (Vector). 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 were 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 hours. 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-SMAp-Luc-SBEm1 and α-SMAp-Luc-SBEm2, as well as wild type α-SMAp-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 hours. 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 hours, medium was replaced with fresh medium supplemented with TGF-β1 (0.5 ng/ml) for an additional 48 hours. 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 four 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-β (2.5 ng/ml) ± SIS3 (3 μM) (Calbiochem, La Jolla, CA) or RLE-6TN cells ± TGF-β (0.5 ng/ml) ± ICG-001 for 24 hours 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 incubation, samples were centrifuged and supernatants collected, followed by addition of 2 μg of rabbit polyclonal anti-CBP or anti-Smad3 Ab, 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×Laemmlli 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 Ser37 and Thr41 or phosphorylated at Tyr654 (pTyr654), RLE-6TN cells treated with TGF-β for 6 hrs were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris (pH 7.2), 0.1% Triton X-100, 0.1% SDS, 1% deoxycholate, 5 mM EDTA) supplemented with 30 μl phosphatase inhibitors (Affymaxtrix, Santa Clara, CA). Active-β-catenin or p-Tyr654β-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 cocktail was added dropwise to 293T cells plated on 100 mm culture dishes and incubated at 37°C overnight. Virus was harvested after 48 hours 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 immunoprecipitation (ChIP) and re-ChIP assays-Chromatin was cross-linked with formaldehyde and ChIP was performed per manufacturer 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‘-CTTACCCTGATGGCGACTGG-3’; SBE2 forward: 5‘-CTTACCCTGATGGCGACTGG-3’ and SBE2 reverse: 5‘-CACAAGATCGTTGGTGCAACC-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 ± sem.; 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) for 6 days. As shown in Fig. 1A, 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 \((\log((\text{TGF-β1+LiCl}))/\log(\text{TGF-β1)+(LiCl))))\), 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. 1B). 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. 1C), 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. 1D, lanes 5 and 6 versus lanes 1 and 2). TGF-β1 treatment induced accumulation of β-catenin in the nucleus (Fig. 1D, 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 (Fig. S1, lanes 2 to 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 Ser37 and Thr41), consistent with β-catenin activation by TGF-β1 (46,47) (Fig. 1E). Since 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. 1F, p-Tyr654-β-catenin was increased after 6 hrs 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. 1G-I). Quantitative analysis revealed that TGF-β1 increased α-SMA expression by ~3-fold (Fig. 1I). Importantly, knockdown of β-catenin (reflected by a reduction in total β-catenin) (Fig. 1H) significantly abrogated α-SMA induction in TGF-β1-treated RLE-6TN cells and decreased levels in vehicle controls (Fig. 1I). 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-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. 1J, lane 2 versus lane 1), which was significantly reduced by overexpression of ICAT (Fig. 1J, 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.

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. 2A-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. 2C), 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. 2D). 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. 2E, upper panel) and labeling with fluorescently-tagged phalloidin (which labels F-actin; Fig.
Using two Smad binding site (SBE1 and SBE2) mutants, cells (as in fibroblasts) is Smad3-dependent. Reporter assays addition, overexpression of Smad3 activated transcriptional regulation of promoter activity (Fig. 3G), further suggesting that inhibited TGF-β1 and Smad3 was increased (lane 2 versus lane 1) in TGF-β1-treated cells (Fig. 4A). Furthermore, TGF-β1-induced interaction of endogenous β-catenin and Smad3 was attenuated by concomitant treatment with SIS3 (Fig. 4A, lane 3 versus lane 2). Similar to results in Fig. 2D, 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 (Fig. S3). Furthermore, TGF-β1 increased occupancy of β-catenin at the SBE1-containing region (Fig. 4B, column 2 versus column 1) of the α-SMA promoter, which was decreased by treatment with SIS3 (Fig. 4B, 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. 4C). 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. Since 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. 4D). 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. 5A), which was inhibited by ICG-001. Furthermore, ChIP-re-ChIP assay (Fig. 5B) 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. 6A). 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 non-specific cross-reactivity of secondary Abs (Fig. S4). To confirm that cells expressing β-catenin or Smad3 were AT2 cells, we further performed co-immunostaining with anti-β-catenin/anti-pro-SP-C or anti-Smad3/anti-Nkx2.1 Abs. Fig. 6B demonstrates that cells expressing nuclear β-catenin also express cytoplasmic pro-SP-C, while Smad3-expressing cells express nuclear Nkx2.1 (Fig. 6C). In addition, using immunohistochemistry to stain for CBP in lung sections from 7 patients, CBP was detected in the nucleus (pink, Fig. 7A and 7B) in hyperplastic AT2 cells in IPF lung. Control slides using rabbit IgG showed no detectable signals (Fig. 7C). 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 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 synergetic 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 Ser37 and Thr41 were detected after 3-6 hours 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 hours of treatment. Thus, for Western analysis to evaluate α-SMA protein levels, we treated the cells for 48 hours, while to assess morphological changes, cells were treated 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 co-activator 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. 4C-D, Fig. S4 and Fig. 5) showing that TGF-β1 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. 4B and 4D) 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 TGF-β1 induced TOPFLASH (which encompasses a TCF/LEF response element) but not FOPFLASH (which includes a mutated TCF/LEF response element) (Fig. 1B), and ICAT (which specifically disrupts interaction between β-catenin and TCF) inhibited both TOPFLASH (Fig. 1C) and α-SMA reporter activities in response to TGF-β1 (Fig. 1J), 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 downregulation 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. 2D), 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. 4C) and β-catenin/CBP (Fig. 5B) 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 modification 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), (39). Specifically, Kim et al. (2009a) reported that β-catenin located at adherens junctions becomes phosphorylated at Tyr654 in an αβ 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 (Figs. 3F and 3G). Importantly, knockdown of Smad3 (Figs. 3C-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 coordinating regulation of 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 knockout 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.
REFERENCES


**FOOTNOTES**

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Fig. 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 to 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 hours. Luciferase activity was normalized to Renilla luciferase activity (n=3, * = p<0.05 compared to 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 hours 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 hrs were immunoprecipitated using anti-active-β-catenin (E) or anti-p-Tyr654-β-catenin (F) Abs. Immunoprecipitated active-β-catenin, dephosphorylated on Ser37 and Thr41 (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 hrs. GAPDH is used as a loading control (n=3; * = p<0.05 compared to control siRNA; ** = p<0.05 compared to 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 to cells transfused with GFP control vector treated with TGF-β1).

Fig. 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 hours in the presence and absence of ICG-001 (5 µM). Luciferase activity was normalized to Renilla luciferase activity (n=5, * = p<0.05 compared to other conditions). (D) Co-immunoprecipitation of β-catenin with CBP (n=3) in RLE-6TN cells treated with TGF-β1 ± ICG-001 for 24 hours. 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.

Fig. 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 hours in the presence and absence of SIS3 (6 µM). Luciferase activity was normalized to Renilla luciferase activity (n=5, * = p<0.05 compared to 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 hours. 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-Luc and SBE1 and SBE2 mutants α-SMA-Luc-SBE1 and α-SMA-Luc-SBE2, followed by TGF-β1 treatment for 15 hours. Firefly luciferase activity is normalized to Renilla luciferase activity (n=8, * = p<0.05; NS = not significantly different).
Fig. 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.

Fig. 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.

Fig. 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.

Fig. 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.

Fig. 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 Tyr654 and dephosphorylation at Ser37 and Thr41. 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.
**FIGURE 1**

**A**

Electrophoresis of α-SMA (42 kD) and Lamin A/C (70/65 kD) proteins under different conditions: Media, NaCl, Vehicle, LiCl, TGF-β, TGF-β+ LiCl. The graph shows the relative levels of α-SMA/Lamin A/C.

**B**

Bar graph comparing relative luciferase activity for TOPFLASH and FOPFLASH. The graph indicates a significant increase in luciferase activity with TGF-β1 treatment.

**C**

Graph showing relative luciferase activity with TGF-β1 treatment in the presence or absence of pCS2/pCS2/ICAT.

**D**

Western blot analysis of β-catenin, Lamin A/C, and GAPDH (36 kD) in nuclear and cytoplasmic fractions under different conditions: Vehicle, TGF-β1, Wnt3a, Vehicle, TGF-β1, Wnt3a. The graph shows the protein levels at 3h and 6h.

**E**

Western blot for ABC-β-catenin under Vehicle and TGF-β conditions.

**F**

Western blot for p-Tyr654-β-catenin under Vehicle and TGF-β conditions.

**G**

Western blots for β-catenin (94 kD), α-SMA (42 kD), and GAPDH (36 kD) with β-catenin siRNA, Control siRNA, and TGF-β treatments.

**H**

Graph showing the ratio of β-catenin/GAPDH with β-catenin siRNA, Control siRNA, and TGF-β treatments.
**I**

- **α-SMA/GAPDH**
  - β-catenin siRNA: - - + +
  - Control siRNA: + + - -
  - TGF-β: - + - +

**J**

- Relative luciferase activity
  - TGF-β1: - + - +
  - GFP: - + - +
  - ICAT: - + - +

**β-actin (36 kD)**

**ICAT (9 kD)**
**A**

Vehicle | TGF-β1
---|---
SIS3 (µM) | M DMSO 0.5 1 3 6 M DMSO 0.5 1 3 6
α-SMA (42 kD) | [image]
Lamin A/C (70/65 kD) | [image]

**B**

SIS3 (µM) | M D 0.5 1 3 6
---|---
α-SMA/Lamin A/C | [image]

**C**

α-SMA (42 kD) | [image]
GAPDH (36 kD) | [image]
Smad3 (52 kD) | [image]
Lamin A/C (70/65 kD) | [image]

Control shRNA | + + - -
Smad3 shRNA | - - + +
TGF-β | - + - +

**D**

Smad3/Lamin A/C | [image]

Control shRNA | + + - -
Smad3 shRNA | - - + +
TGF-β | - + - +

**E**

α-SMA/GAPDH | [image]

Control shRNA | + + - -
Smad3 shRNA | - - + +
TGF-β | - + - +

**F**

TGF-β1 | - + +

**G**

Relative α-SMA luciferase activity | [image]

Smad3 | - +

**H**

Relative α-SMA luciferase activity | [image]

WT | SBEm1 SBEm2

p < 0.05

p<0.05

p<0.05

NS
Figure 4

A

Relative pull-down

\[ \text{Relative pull-down} = \frac{\text{Experimental Value}}{\text{Control Value}} \]

B

![Graph A](image)

C

![Graph B](image)

D

![Graph C](image)
FIGURE 5

A

![Graph showing relative pull-down with TGF-β1 and ICG-001](image)

B

![ChIP assay with CBP Ab and β-catenin Ab](image)
FIGURE 6

A

DAPI Smad3
β-catenin Smad3/β-catenin

B

β-catenin (red)
Pro-SP-C (green)

C

DAPI Smad3 Nkx2.1 Smad3/Nkx2.1
EPITHELIAL-MESENCHYMAL TRANSITION (EMT) BY SMAD3

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