SUPPLEMENTAL MATERIALS AND METHODS

β-CATENIN/CBP-DEPENDENT REGULATION OF TGF-β-MEDIATED EPITHELIAL-MESENCHYMAL TRANSITION (EMT) BY SMAD3

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Running head: β-catenin/CBP and Smad3 interactions in EMT

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EXPERIMENTAL PROCEDURES

Cell treatment. To evaluate synergy between TGF-β1 and Wnt/β-catenin pathways in mediating EMT in AEC, RLE-6TN cells were treated with TGF-β1 (2.5 ng/ml) and/or LiCl (7.5 mM) and proteins were harvested for Western analysis after 6 days. To examine effects of ICG-001 on TGF-β induction of α-SMA protein by Western blotting, RLE-6TN cells were seeded at 12,000 cells/well in 24-well plates and treated with TGF-β1 (0.5 ng/ml) ± ICG-001 at concentrations of 2.5-7.5 μM for 2 days. In this and all subsequent experiments, vehicle (4 mM HCl containing 0.1% BSA) and DMSO were used as TGF-β1 and ICG-001 controls, respectively. To evaluate effects of ICG-001 on TGF-β-induced morphological changes by immunostaining, RLE-6TN cells were seeded at 8,000 cells/well in 8-well chamber slides and treated with TGF-β1 (0.5 ng/ml) ± ICG-001 (7.5 μM) for 4 days. To assess the effects of SIS3 on TGF-β induction of α-SMA protein, RLE-6TN cells plated at a density of 12,000 cells/well in 24-well plates were treated from the time of plating with TGF-β1 (2.5 ng/ml) together with SIS3 (0.5-6 μM; DMSO used as control) and harvested for Western analysis after 4 days. To assess nuclear translocation of β-catenin, nuclear extracts were harvested from RLE-6TN cells treated with TGF-β1 for 3 or 6 hours. Wnt3a conditioned medium harvested from L-Wnt-3A cells (L-M(TK-) cells stably expressing Wnt-3A (ATCC) was used as positive control. Timing of treatments with TGF-β varied depending on specific endpoints being evaluated. For western analyses to evaluate α-SMA protein, cells were treated with TGF-β for at least 2 days since significant induction of α-SMA was only observed after 48 hours. For morphologic analyses, TGF-β treatment was for 4 days to allow full phenotypic transition. For evaluation of synergy between TGF-β and LiCl, treatment was for 6 days since synergistic effects were not observed at earlier timepoints, consistent with the observation that induction of α-SMA by LiCl alone was not seen until day 4 (not shown). Nuclear β-catenin induction in response to TGF-β was evaluated at 3 and 6 hours and was timed to precede induction of α-SMA.

Western analysis. AEC or RLE-6TN cells were lysed in 2% SDS buffer (62.5 mM Tris-HCl, 2% SDS and 10% glycerol). Samples were separated by SDS-PAGE and transferred to Immuno-Blot polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA). Membranes were blocked with 5% non-fat milk in 20 mM Tris (pH 7.5), 0.5 M NaCl and 0.01% Tween 20 overnight at 4°C, followed by incubation with primary Ab for 1 hour at room temperature (RT). After washing with PBS (pH 7.2), membranes were incubated with goat anti-rabbit or anti-mouse secondary Ab (Promega, Madison, MI). Peroxidase activity was detected with enhanced chemiluminesecence (ECL) or Super Signal (Pierce, Rockford, IL) and images were analyzed using a FluorChem imager (Alpha Innotech, San Leandro, CA).

Transient transfection assays. RLE-6TN cells were plated at 6×10^4 cells/well in 24-well plates 24 hours before transfection. Cells were co-transfected with 0.5 μg of a LEF/TCF TOPFLASH reporter or FOPFLASH control (from R.T. Moon, Howard Hughes Medical Institute, Seattle, WA) using Lipofectamine 2000 (Invitrogen). 24 hours following transfection, cells were treated with TGF-β1 (2.5 ng/ml) for an additional 24 hours. To establish that TGF-β1-induced activation of TOPFLASH involved activation of β-catenin, the TOPFLASH reporter was co-transfected with 0.25 μg of ICAT expression vector pCS2/ICAT using Lipofectamine 2000, followed by treatment with TGF-β1 for 24 hours. 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 encompassing 764 bp of the proximal rat *α-SMA* promoter, which was amplified from rat genomic DNA with primers 5′-ACGGTCCTTAAGCATGATAT-3′ and 5′-CTTACCCTGATGGCGACTGGCTGG-3′ according to the published sequence (GenBank, S76011) and cloned into pGL3-Basic vector, 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 0.4 μg of *α-SMA*-Luc and 0.25 μg of Smad3 expression vector pRK-5F/Smad3 using Lipofectamine 2000. 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. H. Phan, University of Michigan, Ann Arbor, MI) were transfected into RLE-6TN cells, followed by treatment with TGF-β1 (2.5 ng/ml) for 48 hours.

Knockdown of β-catenin and Smad3. To knockdown β-catenin, RLE-6TN cells were plated at 4×10^4 cells/well in 24-well plates one day before transfection in 500 μl of growth medium without antibiotics. 40 pmoles of small-interfering RNA (siRNA) targeting the rat *β*-catenin gene (sequence: 5′-CCACGCUGCAUAAUCUCCUGCUACA-′) (Invitrogen) or control nontargeting siRNA were complexed with 1.5 μl Lipofectamine 2000 in 100 μl of Opti-MEM (Invitrogen) and added to each well. After 6 hours, medium was replaced with fresh medium supplemented with TGF-β1 (0.5 ng/ml) for an additional 48 hours. To knockdown Smad3, RLE-6TN cells were transduced with lentivirus expressing Smad3 short hairpin RNA (shRNA) (#V3LMM-486080, Open Biosystems, Huntsville, AL) or control pGIPZ nonsilencing shRNA (Open Biosystems) using polybrene (final concentration 8 μg/ml).

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). Protein concentration of nuclear extracts was determined using the Bio-Rad Protein Assay Kit. Nuclear extracts (100 μg) were diluted in protein binding buffer (PBB; 20 mM HEPES, 75 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40 and protease inhibitor cocktail III (5 μL/ml, Calbiochem)) to a final volume of 1000 μl in a 1.5 mL eppendorf tube, followed by addition of 2 μg of rabbit IgG and 20 μl of protein-A/G plus-agarose (Santa Cruz Biotechnology) equilibrated in PBB. After incubation on a rotator for 2 hours at 4 ^{o}C, samples were centrifuged and supernatants collected, followed by addition of 2 μg of rabbit polyclonal anti-CBP (sc-369, Santa Cruz Biotechnology), or anti-Smad3 (ab28379, Abcam) Ab, or normal rabbit IgG (sc-2027, Santa Cruz Biotechnology). Following incubation on a rotator overnight at 4 ^{o}C, 30 μl of 50% slurry of protein-A/G plus-agarose equilibrated in PBB was added and incubated for 1 hour at 4^{o}C. Protein-A/G plus-agarose beads were washed 5 times by addition of 500 μl of PBB and incubation for 5 min at 4 ^{o}C. Precipitated complexes were eluted by addition of 30 μL of

2×Laemmli buffer containing 2-mercaptoethanol and boiled for 5 min before loading for SDS-PAGE, and analyzed for β-catenin, Smad3, and CBP using Abs mentioned above.

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 (500 μl), consisting of 12 μg pRRL.hCMV.Sin.mycICAT.IRES.GFP or control pRRL.hCMV.Sin.IRES.GFP, 8 μg pCMVΔR8.91, 3.5 μg pMD.G, 69 μl 2 M $CaCl_2$ and H_2O, was mixed with 500 μl warm HBS (220 mM NaCl, 60 mM HEPES and 1.5 mM Na_2HPO_4; pH 7.2), added dropwise to 293T cells plated on 100 mm culture dishes and incubated at 37°C overnight. Virus was harvested after 48 hours, filtered through 0.45 μm filters, and concentrated by centrifugation at 20,000 g at 4°C overnight. Virus was then resuspended in medium, aliquoted and stored at -80°C.

FIGURE LEGENDS

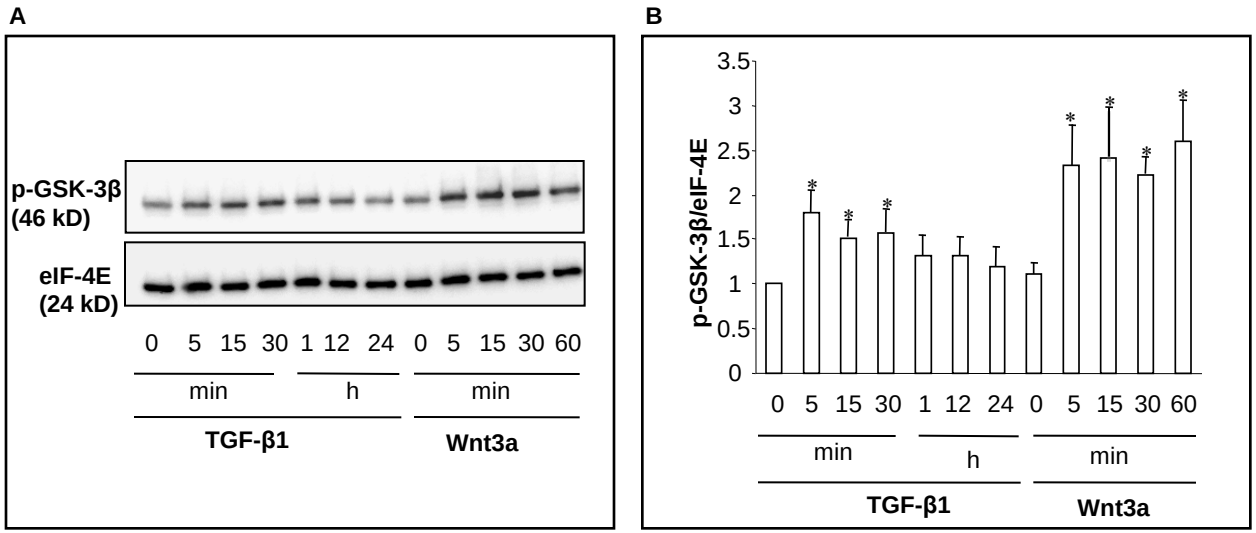
Fig. S1. Effect of TGF-β on GSK-3β phosphorylation in RLE-6TN cells. Representative Western blot (A) and quantitative analysis (B) of phospho-GSK-3β following treatment of RLE-6TN cells with TGF-β1 (2.5 ng/ml) for indicated times (n=3, * = $p<0.05$ compared to t=0). Treatment with Wnt3a conditioned medium is used as a positive control. eIF-4E is used as loading control.

Fig. S2. Effect of TGF-β on Smad3 phosphorylation in RLE-6TN cells. Representative Western blot (A) and quantitative analysis (B) of phospho-Smad3 (p-Smad3) protein in lysates from RLE-6TN cells treated with TGF-β 1 (2.5 ng/ml). eIF-4E is used as loading control (n=3, * = $p<0.05$ (significantly different from TGF-β vehicle)).

Fig. S3. β-catenin associates with SBE1, not SBE2 at α-SMA promoter. ChIP assay was performed with anti-β-catenin Ab for pull-down using chromatin harvested from RLE-6TN cells treated with TGF-β1. Enrichment of SBE1-containing (lane 3), but not SBE2-containing region (lane 7) at the α-SMA promoter was identified. IgG was used as ChIP negative control (lane 4 and 8). Lane 2 and 6 are input. Lane 5 and 9 are no template for PCR negative control, and M denotes molecular weight marker (bp).

Fig. S4. Controls for immunofluorescence staining for β-catenin and Smad3 colocalization in hyperplastic type II cells of IPF lung tissue with IgG, β-catenin (red) or Smad3 (green) alone. Nuclei (blue) are stained with DAPI. Scale bar = 20 μm.

Fig. S1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eIF-4E
(24 kD)
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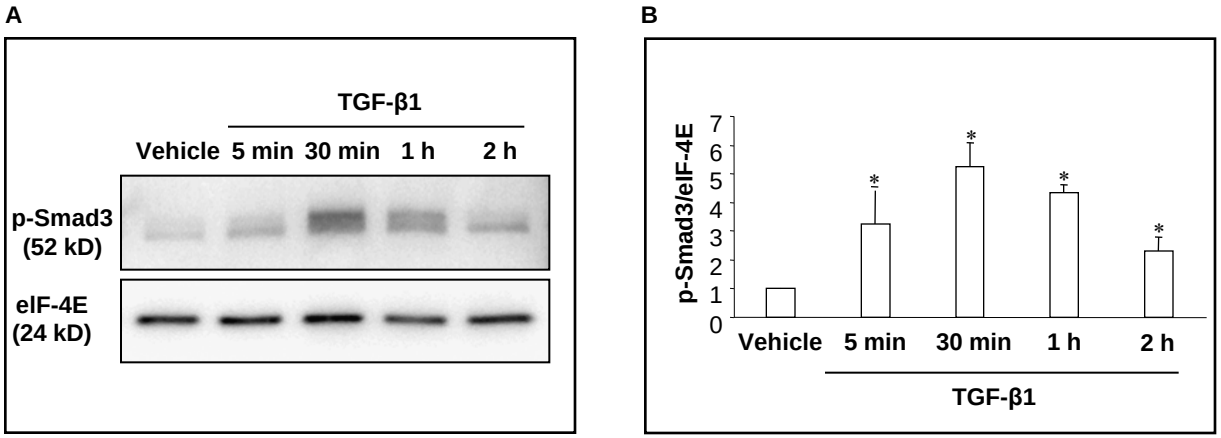
min
h
min
TGF-β1
Wnt3a
B
p-GSK-3β/eIF-4E
3.5
3
2.5
2
1.5
1
0.5
0
0 5 15 30 1 12 24 0 5 15 30 60
min
h
min
TGF-β1
Wnt3a

Fig. S2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Vehicle
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30 min
1 h
2 h
p-Smad3
(52 kD)
eIF-4E
(24 kD)
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p-Smad3/eIF-4E
0
1
2
3
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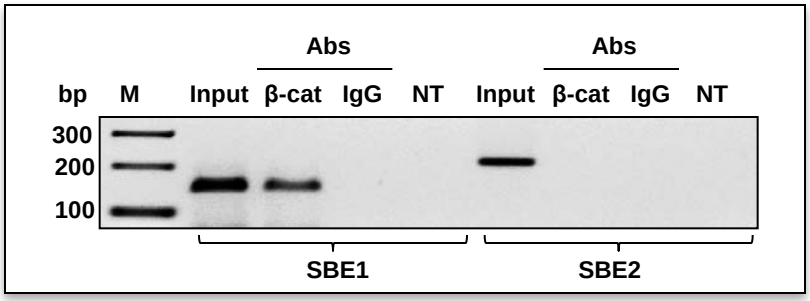
5 min
30 min
1 h
2 h
TGF-β1

Fig. S3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Abs
bp
M
Input
β-cat
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Input
β-cat
IgG
NT
300
200
100
SBE1
SBE2

Fig. S4