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Cell Injury, Repair, Aging, and Apoptosis

The Critical Role of TAK1 in Accentuated Epithelial to Mesenchymal Transition in Obliterative Bronchiolitis after Lung Transplantation

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Therapies to limit or reverse fibrosis have proven unsuccessful, highlighting the need for a greater understanding of basic mechanisms that drive fibrosis and, in particular, the link between fibrosis and inflammation. It has been shown that pro-fibrotic transforming growth factor β1 (TGF-β1)–driven epithelial-to-mesenchymal transition (EMT) can be accentuated by tumor necrosis factor α (TNF-α). TGF-β–activated kinase 1 (TAK1) is activated by both TGF-β1 and TNF-α, activating both nuclear factor kappa-light-chain-enhancer of activated B cells and mitogen-activated protein kinase signaling pathways. In this study, we evaluated the potential for TAK1 to modulate the synergistic effect between TGF-β1 and TNF-α in driving EMT. Co-stimulation with TGF-β1 and TNF-α induced an accentuated and extended phosphorylation of TAK1 compared to either alone. TAK1 signaled downstream via nuclear factor kappa-light-chain-enhancer of activated B cells, and Jun N-terminal kinase-2, but independent of Jun N-terminal kinase-1, extracellular signal-regulated kinase-1/2, or p38 mitogen-activated protein kinase signaling to drive EMT in bronchial epithelial cells. Blocking either TAK1 or Jun N-terminal kinase-2 inhibited EMT. TAK1 phosphorylation was increased in the airway epithelium of patients with fibrotic airway disease. These data identify factors leading to and affected by accentuated and extended TAK1 phosphorylations potential novel therapeutic targets in inflammation-driven fibrotic diseases. *(Am J Pathol 2012, 180:2293–2308; http://dx.doi.org/10.1016/j.ajpath.2012.02.022)*

Fibrosis can affect multiple organs, including the lung, kidney, and liver, despite obvious etiological differences; it is hypothesized that the mechanisms leading to fibrosis may share a common cellular pathway. Our group has previously demonstrated *in vivo* and *in vitro* evidence that epithelial cells can undergo transforming growth factor-β1 (TGF-β1)-driven epithelial to mesenchymal transition (EMT) and that this phenomenon is involved in the expansion of the myofibroblast population during the development of obliterative bronchiolitis (OB),[1,2] a progressive disease of the small and medium airways resulting in airway obstruction and respiratory failure in the transplant airway.[3]

EMT is a process whereby epithelial cells lose their epithelial phenotype and functionality, including cell-to-cell adherence and polarity, whereas gaining characteristics typically associated with mesenchymal cells, such as an invasive phenotype and the ability to secrete extracellular matrix components.[4] This transition is associated with a decrease in E-cadherin, cytokeratin, and zona occludens-1 expression concurrent with an increase in vimentin, fibronectin, and N-cadherin expression. Furthermore, a change in the secretory profile of the cells is observed, including an increased secretion of matrix metalloproteinases (MMP) and collagen.

TGF-β1 is a pleiotropic molecule involved in numerous cell functions, such as modulation of the cell cycle and apoptosis, and has been shown to be elevated in the airways of patients with OB.[1] In addition, TGF-β1 contin-

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ues to be regarded as the master switch regulating fibrosis in multiple organs.[5–7] On binding to its receptor, TGF-β1 initiates the phosphorylation of a SMAD protein, which can then form a transcriptionally active heterodimer or trimer with other SMAD family proteins,[8,9] a process which has been shown to be important in driving fibrosis and EMT.[10,11]

However, TGF-β1 can also signal via activation of the mitogen-activated protein kinase cascade.[12,13] The mitogen-activated protein kinase cascade consists of several distinct signaling pathways, including extracellular signal-regulated kinase (ERK)-1/2, c-Jun N-terminal kinase (JNK)-1/2, and p38.[12,13] The mitogen-activated protein kinase-3 TGF-β activated kinase 1 (TAK1) is thought to play a key role in the initiation of noncanonical TGF-β signaling.

The role of inflammation in the development of fibrosis remains poorly understood. Recently it has been demonstrated by our group and others that the inflammatory cytokine tumor necrosis factor α (TNF-α) can accentuate TGF-β1–driven EMT.[2,14,15] TNF-α signals through two distinct transmembrane receptors, tumor necrosis factor receptor 1 (TNFR1) and tumor necrosis factor receptor 2 (TNFR2).[16] TNFR1 is ubiquitously expressed and can respond to both membrane-bound and soluble (TNF_{sol}) forms of TNF-α, whereas TNFR2 is highly regulated in its expression, mainly on immune cells, and responds efficiently, only to membrane-bound TNF-α.[17]

The canonical signaling pathway activated by TNF-α has been extensively investigated in response to TNFR1 activation which induces a signaling cascade via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). TNFR1 recruits TNF-receptor–associated factor 2 (TRAF2) along with E3 ubiquitin ligases forming a complex that activates TAK1 via polyubiquitination. Activated TAK1 recruits the IκB kinase (IKK) complex composed of three subunits, the regulatory IKKγ and the catalytic IKKα and IKKβ, which are responsible for the degradation of Iκ-Bα and subsequent activation of NF-κB signaling.[18–20]

In this study, we comprehensively investigate the role of TAK1 in TGF-β1–driven, TNF-α–accentuated EMT, and investigated the downstream signaling mechanisms involved in this synergy. We, furthermore, investigated TAK1 phosphorylation and localization in epithelial cells in lung tissue from patients with fibrotic airway disease (OB) and normal controls.

Materials and Methods

Antibodies

TNFR1 (HM2020; Hycult, Uden, The Netherlands) and TNFR2 (HM2007; Hycult)[21] primary antibodies were used to detect receptor expression by flow cytometry. E-cadherin (610181; Becton, Dickinson and Company, Oxford, UK), fibronectin (F3648; Sigma-Aldrich, Poole, UK), cytokeratin-19 (sc-6278; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), vimentin (sc-6260; Santa Cruz Biotechnology) and β-actin (a2228; Sigma-Aldrich) primary antibodies were used throughout this study for the detection of changes in EMT marker expression. TAK1 (4505; Cell Signaling, Danvers, MA), phospho-TAK1 (4536; Cell Signaling), SMAD3 (ab29379; Abcam, Cambridge, UK), phospho-SMAD3 (ab52903; Abcam), IKKβ (2370; Cell Signaling), phospho-IKKα/β (2681; Cell Signaling), JNK-1/2 (9252; Cell Signaling), phospho-JNK-1/2 (4668; Cell Signaling), p38 (sc-7149; Santa Cruz Biotechnology), phospho-p38 (ab4822; Abcam), ERK-1/2 (4695; Cell Signaling), phospho-ERK-1/2 (4370; Cell Signaling), phospho-Iκ-Bα (9246; Cell Signaling), Iκ-Bα (sc-1643; Santa Cruz Biotechnology), phospho-c-Jun (2361; Cell Signaling), c-Jun (9165; Cell Signaling), TAB1 (3226; Cell Signaling), TAB2 (3745; Cell Signaling), and TAB3 (ab85655; Abcam) primary antibodies were used throughout this study for the detection of changes in protein phosphorylation, localization, and association. Anti-rabbit IgG (ab37415; Abcam) was used to pre-clear cell lysate before TAK1 or JNK-1/2 immunoprecipitation. RAN (4462; Cell Signaling) primary antibody was used as a control for protein localization Western blots.

Cell Isolation and Culture

Primary bronchial epithelial cells (PBECs) were isolated from stable lung transplant patients via airway brushings, as previously described.[22] PBECs were cultured at ~3500 cells/cm^2 in small airway growth media (CC-3118; Lonza, Basel, Switzerland) on either collagen (5005-B; Nutacon, Leimuiden, The Netherlands) (0.5% collagens I and III) coated glass cover slips or collagen-coated tissue culture flasks. This study was performed in accordance with approval from the Newcastle and North Tyneside Local Regional Ethics Committee and informed written consent from all study patients.

Cell Treatment

PBECs were cultured with or without TGF-β1 (10 ng/mL) (100–21; Peprotech, London, UK) or TNF-α (20 ng/mL) (PHC3015; Invitrogen, Paisley, UK) for 72 hours for the assessment of EMT marker expression. The times used for phosphorylation assays were protein dependent and are described for each result. Cells were treated with TNF-α, wild-type TNF-α (TNF_{cys}), TNFR1-specific mutant (Cys-TNF32W/86T) TNF (TNF_{cys}R1), or TNFR2-specific mutant (Cys-TNF143N/145R) TNF (TNF_{cys}R2) that oligomerize due to cystine residues at the N-terminal, allowing the molecules to mimic transmembrane surface expressed TNF-α (all 20 ng/mL).[23,24] SMAD3 (SMAD*i*–specific inhibitor of SMAD3; 10 μmol/L) (566405; Calbiochem, Nottingham, UK), p38 (p38*i*–SB 203580; 10 μmol/L) (559389; Calbiochem), ERK-1/2 (ERK*i*)(FR180204; 5 μmol/L) (328007; Calbiochem), JNK-1/2 (JNK*i*)(SAPK Inhibitor II; 5 μmol/L) (420119; Calbiochem), IKKβ (IKK*i*)(IKK-2 Inhibitor IV; 5 μmol/L) (401481; Calbiochem), or TAK1 chemical inhibitors (TAK*i*)[(5Z)-7-Oxozeaenol, *Curvularia* sp; 1 μmol/L] (499610; Calbiochem); were added to cells 1 hour before stimulation with TGF-β1 or TNF-α, as previously described. Dose response curves for each inhibitor looking at effect on EMT and cell viability were used to select appropriate inhibitor concentrations. SMAD3 (SMAD*si*; 5 nmol/L) (SI00082481; Qiagen, Crawley, UK) (5′-ATCAAGGGATTTCCTATGGAA-3′), IKKβ

(IKK*si*; 0.1 nmol/L) (SI02777376; Qiagen) (5′-CTG-GAGAAGTACAGCGAGCAA-3′), TAK1 (TAK*si*; 3 nmol/L) (SI02758763; Qiagen) (5′-AAGATGGTATATACCAAGTTA-3′), JNK-1 (JNK-1*si*; 5 nmol/L) (SI02757209; Qiagen) (5′-GTG-GAAAGAATTGATATATAA-3′), or JNK-2 siRNA (JNK-2*si*; 10 nmol/L) (SI00300797; Qiagen) (5′-GCCGUCCUUUUCA-GAACCAT-3′) si-RNAs; were delivered via lipid transfection (301705) 24 hours before stimulation with TGF-β1 or TNF-α. A sequence scrambled negative control was used in all experiments (3 nmol/L) (SI03650318; Qiagen).

Flow Cytometry

Following treatment, cells were harvested, re-suspended in phosphate buffered albumin (PBA) [0.25% (w/v) bovine serum albumin, 0.02% (w/v) NaN_3 in PBS] containing TNFR1-specific (5 μg/mL) and TNFR2-specific (2.5 μg/mL) antibodies and incubated on ice for 1 hour. Cells were washed with PBA and incubated with PBA containing fluorescein isothiocyanate-conjugated secondary antibody for 1 hour on ice. Cells were washed with PBA, samples were supplemented with 1 μg/mL propidium iodide and analyzed on a BD FACSCanto II flow cytometer (Becton Dickinson Company).

Cellular Fractioning

Nuclear and non-nuclear fractions from PBECs were isolated using the NE-PER Nuclear and Cytoplasmic Extraction Kit (78833; Pierce, Rockford, IL), according to the manufacturer's instructions.

SDS-PAGE and Western Blot

Protein concentration was determined using the BCA Protein Assay kit (23225; Pierce), according to the manufacturer's instructions. Total cell lysates (20 to 50 μg of protein) were separated on 4% to 12% Bis-Tris gradient gels (NP0321; Invitrogen) and wet transferred onto HyBond-P polyvinylidene difluoride membranes (RPN203N; Amersham, Little Chalfont, UK). Membranes were blocked [5% (w/v) BSA in TBS-Tween20 (0.5% w/v)] before incubation with primary antibodies and subsequent detection with appropriate horseradish peroxidase conjugated secondary antibodies (ab6728 and ab16284; Abcam). Complexes were visualized using SuperSignal West Pico Chemiluminescent Substrate (34077; Pierce). Images were acquired using a Syngene G:Box Chemi (Syngene, Cambridge, UK). Band density was analyzed using ImageJ.[25] Results were normalized to β-actin for EMT markers, and total unphosphorylated protein for phosphorylation studies.

Gelatin Zymography

Culture medium was separated under nonreducing conditions on an 8% SDS-PAGE gel containing 1% gelatin. Gels were incubated with 2.5% Triton X-100 for 30 minutes and then overnight in substrate buffer (50 mmol/L Tris-HCl, 200 mmol/L NaCl, and 5 mmol/L $CaCl_2$). Bands were visualized by staining with Coomassie stain (40% methanol, 10% acetic acid, and 0.05% Coomassie Brilliant Blue R250).

Immunoprecipitation

Starting protein concentration was assayed via bicinchoninic acid protein assay. Immunoprecipitation of key signaling proteins was performed using the Catch and Release kit (17–500; Millipore, Watford, UK), according to the manufacturer's instructions with incubation and washing steps optimized for each antibody. Beads were precleared with pan-IgG antibody and efficacy and specificity of isolation determined via SDS-PAGE and Western blot.

Indirect ELISA

Immunoprecipitates were coated onto 96-well enzyme-linked immunosorbent assay (ELISA) plates overnight. Phospho targeting primary antibodies were incubated overnight before detection with appropriate horseradish peroxidase-conjugated secondary antibodies with tetramethylbenzidine peroxide (DY999; R&D Systems, Abingdon, UK) as the substrate. Selected samples were analyzed by Western blot to demonstrate selectivity compared to IgG controls.

Sircol Collagen Assay

Secreted collagen (collagens I-IV) was detected in culture medium using the Sircol Collagen Assay (S1000; Biocolor, Carrickfergus, UK), according to the manufacturer's instructions.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde and incubated with primary antibodies overnight before detection with appropriate fluorochrome conjugate secondary antibodies (fluorescein-isothiocyanate F2012 and tetramethylrhodamine-5-isothiocyanate T6778; Sigma-Aldrich) with DAPI (H-1200; Vector Labs, Peterborough, UK) used as a nuclear counterstain. Images were acquired using a Leica TCS-SP-2UV laser scanning confocal microscope (Leica, Mannheim, Germany) at $\times$63 magnification, with consistent acquisition settings used throughout. Intensity of expression of nuclear expression was analyzed by isolating nuclei in the blue channel, then recording the mean pixel intensity in the red channel using Adobe Photoshop Creative Suite 5 (Adobe Systems Incorporated, San Jose, CA). Analysis was performed on 20 randomly selected cells from multiple fields.

Immunohistochemistry

Formalin fixed tissue sections from patients with OB and unused donor lung were de-waxed in Xylenes and rehydrated in sequentially decreasing concentrations of ethanol. Slides were boiled in 0.1 M EDTA (pH 8.0) for 10

minutes to facilitate antigen retrieval, the staining process was then performed as previously described.

Statistical Analysis

Differences in EMT marker expression (normalized to β-actin as a loading control), phosphorylation of key signaling proteins (normalized to the unphosphorylated protein as a loading control), and collagen secretion were assessed using a Mann-Whitney *U*-test. Data are expressed as mean ± SEM. Differences with P values of <0.05 were considered statistically significant.

Results

TNF-α Accentuates EMT via TNFR1

TNF-α, an inflammatory cytokine capable of accentuating but not driving EMT, signals via two distinct transmembrane receptors (TNFR1 and TNFR2), which play independent roles in cellular responses.[16,26] Untreated PBECs express TNFR1 on their cell surface, but express little to no TNFR2 (Figure 1A). Stimulation with TGF-β1 ≤10 ng/mL had no effect on basal TNFR1 or TNFR2 expression on the cell surface in PBECs (data not shown). To investigate the relative contribution of TNFR1 and TNFR2 ligation on the accentuation of TGF-β1–driven EMT, we used two distinct isoforms of TNF-α; the 26 kDa membrane expressed form (TNF_{cys}) and the 17 kDa soluble form (TNF_{sol}). TNF_{cys} can signal via both TNFR1 and TNFR2, however, TNF_{sol} signals preferentially via TNFR1.[17] Co-stimulation of PBECs with both TGF-β1 and TNF_{sol}, or TNF_{cys} strongly accentuates TGF-β1–driven EMT compared to TGF-β1 alone, suggesting signaling is occurring via TNFR1. To confirm this, PBECs were co-treated with TGF-β1 and TNFR1 (TNF_{cys}R1), and TNFR2 (TNF_{cys}R2)-specific mutant TNF_{cys} and EMT assessed. Only TNF_{cys}R1 was able to accentuate TGF-β1–driven EMT confirming the accentuation of TGF-β1–driven EMT occurs via TNFR1 through both TNF_{sol} and TNF_{cys}. None of the TNF isoforms alone had an effect on EMT protein marker expression alone, but TNF_{sol}, TNF_{cys}, and TNF_{cys}R1 alone induced the secretion of pro-MMP-9 (Figure 1, B and C).

Identifying Key Downstream Signaling Mediators

To identify the key signaling events involved in the synergistic action of TNF-α on TGF-β1–driven EMT, the phosphorylation of key signaling molecules in response to with TGF-β1 or TNF-α was investigated. Indirect ELISA for SMAD3, IKKβ, TAK1, and JNK-1/2 phosphorylation were performed on immunoprecipitates isolated from PBECs whole cell lysates using relevant total antibodies. TGF-β1 induced significant SMAD3 phosphorylation from 30 minutes, which lasted beyond 60 minutes. Stimulation with TNF-α did not induce significant phosphorylation of SMAD3 at any time point. Co-stimulation of PBECs with TGF-β1 and TNF-α induced significant phosphorylation of SMAD3 in a similar fashion to that of TGF-β1 alone, although in both instances phosphorylation was nonsignificantly lower (Figure 2A). TGF-β1 did not induce significant IKKβ phosphorylation at any time point, whereas TNF-α induced significant phosphorylation of IKKβ from 1 minute, peaking at 10 minutes and lasting for 30 minutes. Co-stimulation with TGF-β1 and TNF-α induced significant phosphorylation of IKKβ in a fashion similar to that of TNF-α alone, although phosphorylation was nonsignificantly increased in all instances (Figure 2B).

TGF-β1 induced significant phosphorylation of TAK1 from 1 minute onward, peaking at 10 minutes and remaining phosphorylated for at least 60 minutes. TNF-α alone induced significant phosphorylation of TAK1 from 10 minutes onward, peaking at 30 minutes and again lasting beyond 60 minutes, although the level of phosphorylation was lower compared to TGF-β1 stimulations. Co-stimulation induced significant phosphorylation of TAK1 from 1 minute onward, peaking at approximately 10 minutes, and again lasting beyond 60 minutes with an accentuated, as opposed to additive, effect observed from 5 minutes onward (Figure 2C). TGF-β1 stimulation also induced a significant increase in JNK-1/2 phosphorylation at 5 and 10 minutes, with significance lost beyond 30 minutes. TNF-α alone induced significant phosphorylation of JNK-1/2 from 5 minutes onward, peaking at 10 minutes with significance lost beyond 30 minutes. The degree of phosphorylation with TNF-α treatment was greater than that seen with TGF-β1. Co-stimulation induced significant phosphorylation of JNK-1/2 from 1 minute onward, peaking at 10 minutes and lasting beyond 60 minutes with an accentuated effect observed at 1, 30, and 60 minute in co-stimulated cells compared to those stimulated with TGF-β1 or TNF-α alone (Figure 2D).

In all instances, selected samples were analyzed by Western blot, alongside IgG controls to demonstrate efficacy and specificity of pull down (Figure 2E). Total lysate from PBECs stimulated with TGF-β1 or TNF-α for 30 minutes were analyzed by Western blot for total and phosphorylated forms of key signaling proteins. For signaling molecules already discussed, the effect was similar to that observed by indirect ELISA. In addition, neither p38 nor ERK-1/2 displayed any change in phosphorylation (Figure 2F).

Unpicking the Signaling Cascade

We proceeded to investigate the effects of inhibiting SMAD3, IKKβ, TAK1, and JNK-1/2 on TGF-β1–driven EMT, and its accentuation by TNF-α alongside the concomitant effect on phosphorylation of other key signaling molecules.

Pretreatment with SMAD*i* strongly reduced SMAD3 phosphorylation 30 minutes poststimulation with TGF-β1 or TNF-α, with no detected change in the phosphorylation of other signaling molecules (Figure 3A; see also Supplemental Figure S1A at *http://ajp.amjpathol.org*). Pretreatment of PBECs with SMAD*i* prevented the change in cell morphology, the down-regulation of E-cadherin expression, the increase in fibronectin and vimentin expression, and the increase in pro-MMP-9 and collagen I-IV secretion in response to TGF-β1, returning levels to near baseline.

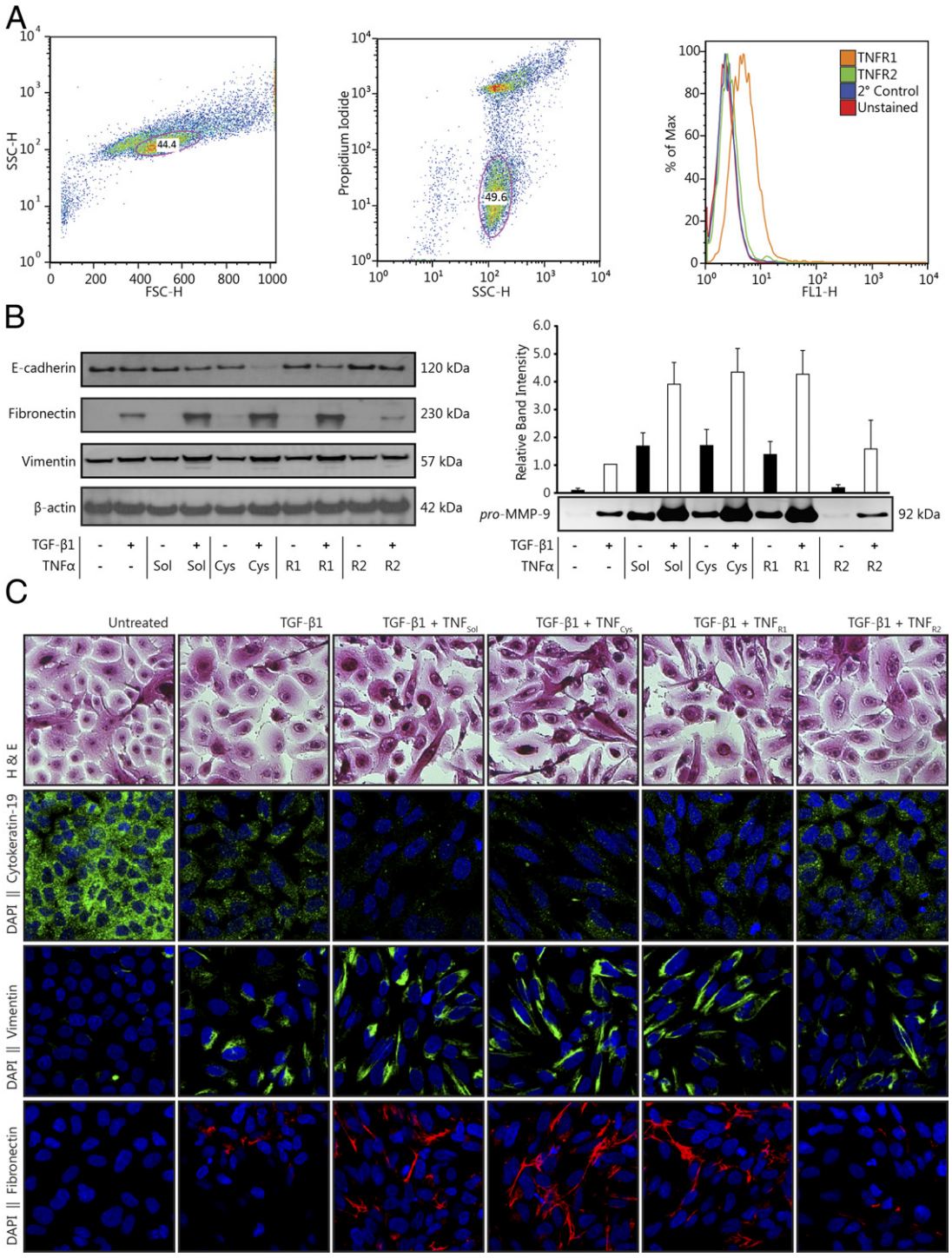


Figure 1. TNF-α accentuates TGF-β1–driven EMT via TNFR1. **A:** Flow cytometry analysis demonstrates that PBECs express TNFR1 on their cell surface, but express little to no TNFR2. **B:** Stimulation of PBECs ($n = 6$) with soluble TNF-α (TNF$_{sol}$), membrane-bound TNF-α (TNF$_{cys}$), TNFR1-specific mutant (Cys-TNF32W/86T) membrane-bound TNF (TNF$_{cys}$R1), or TNFR2-specific mutant (Cys-TNF143N/145R) membrane-bound TNF (TNF$_{cys}$R2) (all 20 ng/mL) for 72 hours alone has no effect on the expression of E-cadherin, vimentin, or fibronectin. Stimulation, however, with TNF$_{sol}$, TNF$_{cys}$, or TNF$_{cys}$R1, but not TNF$_{cys}$R2, increases pro-MMP-9 secretion. TGF-β1 (10 ng/mL) down-regulates the expression of E-cadherin, increases the expression of vimentin and fibronectin, and increases the secretion of pro-MMP-9. Co-stimulation of the cells with TGF-β1 and TNF$_{sol}$, TNF$_{cys}$, or TNF$_{cys}$R1, but not TNF$_{cys}$R2, accentuates the changes in EMT marker expression compared to TGF-β1 alone. **C:** Co-stimulation of PBECs with TGF-β1 and TNF$_{sol}$, TNF$_{cys}$, or TNF$_{cys}$R1, but not TNF$_{cys}$R2, accentuates changes in cell morphology and EMT marker expression compared to TGF-β1 alone. FL1-H, height of fluorescence intensity; FSC-H, height of forward scatter; SSC-H, height of side scatter.

SMAD*i* also reduced EMT in co-stimulated PBECs for all markers compared to untreated control PBECs, however, an accentuated effect was still apparent for all markers with the exception of collagens I-IV when comparing co-stimulated PBECs with TGF-β1 alone stimulated PBECs in the presence of SMAD*i* (Figure 3, B and D; see also Supplemental Figure S2A at *http://ajp.amjpathol.org*). Approximately 90% knockdown of SMAD3 was achieved by

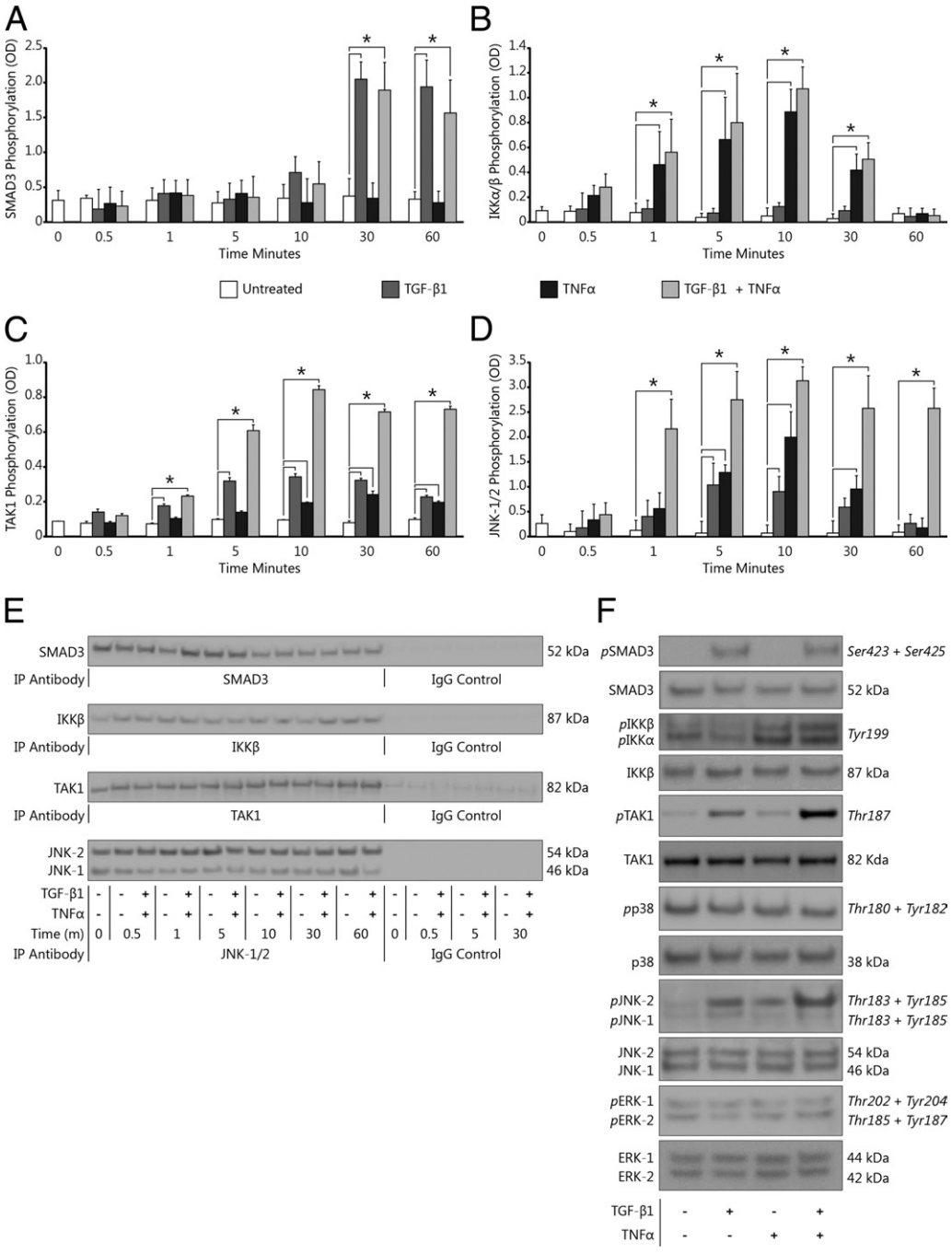


Figure 2. TAK1 and JNK-2 display accentuated and prolonged phosphorylation in response to TGF-β1 and TNF-α stimulation. Indirect ELISA for the phosphorylation of SMAD3, IKKβ, TAK1, and JNK-1/2 were performed on PBEC lysates immunoprecipitated for SMAD3, IKKβ, TAK1, and JNK-1/2, respectively. *$P < 0.05$ ($n = 3$). **A:** Stimulation with TGF-β1 (10 ng/mL) induces significant SMAD3 phosphorylation (Ser423 + Ser425) from 30 minutes onwards. Stimulation with TNF-α (20 ng/mL) does not significantly increase SMAD3 phosphorylation. Co-stimulation with TGF-β1 and TNF-α do not increase the phosphorylation of SMAD3 compared to TGF-β1 alone. **B:** TGF-β1 does not induce significant IKKβ phosphorylation (Tyr199) at any time point. Stimulation with TNF-α induces significant phosphorylation of IKKβ from 1 minute and lasting up to 30 minutes. Co-stimulation with TGF-β1 and TNF-α does not accentuate the phosphorylation of IKKβ compared to TNF-α alone, although an additive increase in IKKβ phosphorylation is observed. **C:** TGF-β1 induces significant TAK1 phosphorylation (Thr187) from 1 minute, peaking at 10 minutes, and lasting to at least 60 minutes. Stimulation with TNF-α induces significant phosphorylation of TAK1 from 10 minutes, peaking at 30 minutes, and lasting beyond 60 minutes, although the amount of phosphorylation was lower than that seen with TGF-β1 alone. Co-stimulation with TGF-β1 and TNF-α induces significant phosphorylation of TAK1 from 1 minute, peaking at 10 minutes, and again lasting beyond 60 minutes. A significant accenting effect is observed from 5 minutes onward compared to cells treated with TGF-β1 or TNF-α alone. **D:** TGF-β1 induces significant JNK-1/2 phosphorylation (Thr183 + Tyr185) at 5 and 10 minutes. Stimulation with TNF-α induces significant phosphorylation of JNK-1/2 from 5 minutes and lasting up to 30 minutes. The degree of phosphorylation induced by treatment with TNF-α is greater than that with TGF-β1. Co-stimulation with TGF-β1 and TNF-α induces significant phosphorylation of JNK-1/2 from 1 minute, peaking at 10 minutes and lasting beyond 60 minutes. A significant accenting effect is observed at 1, 30, and 60 minutes in co-treated cells compared to cells treated with TGF-β1 or TNF-α alone. **E:** Selected immunoprecipitates and matching IgG controls were analyzed by Western blot to demonstrate the efficacy and specificity of the immunoprecipitates. Significant specificity for all of the antibodies used compared to IgG controls is observed. **F:** Total lysate from PBECs stimulated with TGF-β1 or TNF-α for 30 minutes were analyzed by Western blot for total and phosphorylated forms of key signaling proteins. Phosphorylation of SMAD3, IKKβ, TAK1, and JNK-1/2 is similar to that observed by indirect ELISA. In addition, neither p38 nor ERK-1/2 displays any change in phosphorylation in response to treatment.

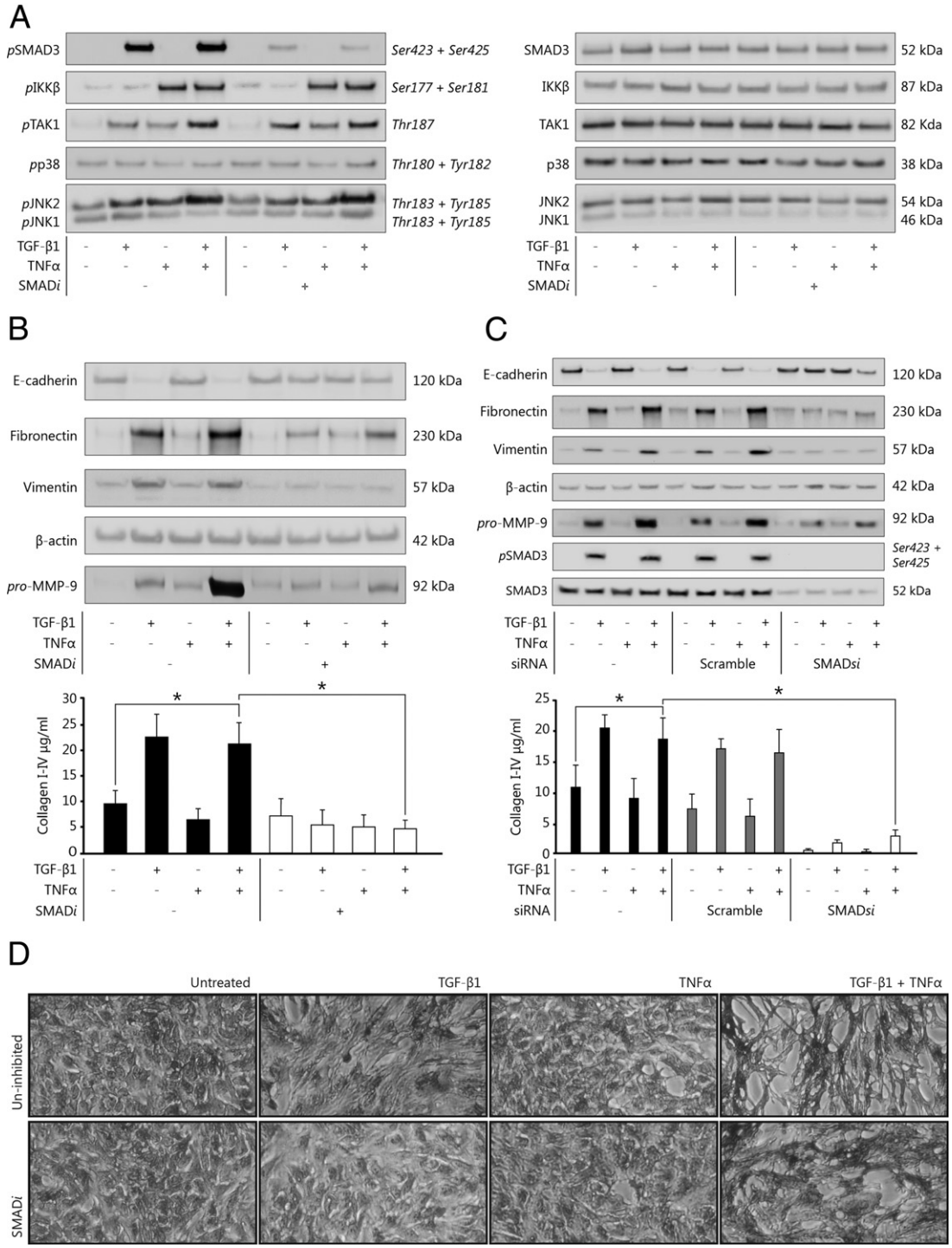


Figure 3. Blocking SMAD3 activity limits TGF-β1–driven EMT. **A:** Stimulation of PBECs (n = 3) with TGF-β1 (10 ng/mL) or TNF-α (20 ng/mL) induces phosphorylation of p38, SMAD3, IKKβ, TAK1, and JNK-1/2, as described in Figure 2. Pretreatment with specific inhibitor of SMAD3 (SMAD*i*) (10 μmol/L) strongly attenuates the phosphorylation of SMAD3, but has no effect on the phosphorylation of p38, IKKβ, TAK1, and JNK-1/2. **B:** Stimulation of PBECs (n = 4) with TGF-β1 down-regulates E-cadherin expression, increases fibronectin and vimentin expression, and increases pro-matrix metalloproteinases (MMP)-9 secretion. This change in EMT marker expression is accentuated by co-stimulation with TGF-β1 and TNF-α. Collagen I-IV secretion is significantly up-regulated by stimulation with TGF-β1, however, no accentuation is observed on co-stimulation. Pretreatment with SMAD*i* attenuates both TGF-β1–driven and TNF-α–accentuated changes in EMT marker expression and collagen I-IV secretion, returning levels to near baseline. **C:** Treatment of PBECs (n = 3) with SMAD*si* (5 nmol/L) induces an approximate 90% knockdown of total SMAD3 after 24 hours, with no phosphorylation of SMAD3 detected after stimulation with TGF-β1 or TNF-α for 30 minutes. Knockdown of SMAD3 inhibits both the TGF-β1–driven and TNF-α–accentuated down-regulation of E-cadherin expression, the increase in fibronectin and vimentin expression, and the increase in collagen I-IV and pro-MMP-9 secretion compared to uninhibited controls. A sequence scrambled control has no effect on EMT marker expression. *$P < 0.05$. **D:** A549 cells treated with TGF-β1 begin to lose their epithelial morphology and acquire a more elongated mesenchymal morphology, and this change is accentuated by co-stimulation with TNF-α. Pretreatment with SMAD*i* attenuates this morphological change in both TGF-β1 and co-treated cells.

treatment with SMAD*si* for 24 hours with a complete loss of detected phospho-SMAD3 30 minutes after stimulation with TGF-β1 or TNF-α. SMAD*si* inhibited TGF-β1–driven and TNF-α–accentuated EMT in a similar fashion to SMAD*i* (Figure 3C; see also Supplemental Figure S2B at *http://ajp.amjpathol.org*).

Pretreatment with IKK*i* did not block the phosphorylation of any of the key signaling proteins investigated, however, phosphorylation of Iκ-Bα, the main substrate of IKK within the NF-κB cascade, was strongly reduced (Figure 4A; see also Supplemental Figure S1B at *http://ajp.amjpathol.org*). Pretreatment with IKK*i* inhibited the TGF-β1 induced down-regulation of E-cadherin expression, the increase in fibronectin and vimentin expression, and, slightly, the increase in collagen I-IV and pro-MMP-9 secretion. IKK*i* strongly reduced the accentuating effect of TNF-α on TGF-β1–driven EMT marker expression, restoring protein levels to those of TGF-β1 stimulated PBECs (Figure 4B; see also Supplemental Figure S2C at *http://ajp.amjpathol.org*). IKK*i* also slightly inhibited the TGF-β1 induced change in cell morphology, but strongly limited the accentuation of this effect by TNF-α (Figure 4D). IKK*si* induced approximately a 90% reduction in detected IKKβ and also strongly reduced the detected levels of phospho-IKKβ. IKK*si* inhibited the TGF-β1 mediated loss of E-cadherin, but displayed only weak inhibitory effects on other EMT markers. However IKK*si* strongly inhibited the TNF-α mediated accentuation of TGF-β1–driven EMT for all markers with the exception of collagens I-IV (Figure 4C; see also Supplemental Figure S2D at *http://ajp.amjpathol.org*).

No difference in the phosphorylation of SMAD3 or p38 following stimulation with TGF-β1 or TNF-α in the presence of TAK*i* was demonstrated, however, TAK1 phosphorylation was completely inhibited and the phosphorylation of IKKβ and JNK-2 was reduced (Figure 5A; see also Supplemental Figure S3A at *http://ajp.amjpathol.org*). Pretreatment of PBECs with TAK*i* prevented the change in cell morphology, the down-regulation of E-cadherin expression, and the increase in fibronectin and vimentin expression in response to TGF-β1, returning levels to near baseline. In addition the increased collagen I-IV secretion seen in PBECs treated with TGF-β1 was returned to near baseline in the presence of TAK*i*, which also reduced the accentuating effect of TNF-α on TGF-β1–driven EMT for the aforementioned EMT markers. TAK*i* decreased pro-MMP-9 secretion from TGF-β1 treated cells, however, in cells treated with TNF-α alone, or TGF-β1 and TNF-α TAK*i* increased the secretion of pro-MMP-9 (Figure 5, B and D; see also Supplemental Figure S4A at *http://ajp.amjpathol.org*). TAK*si* induced a strong reduction in detected levels of total TAK1, along with a strong reduction in detected levels of phospho-TAK1 after 24 hours. TAK*si* inhibited the TGF-β1 induced decrease in E-cadherin expression, increase in fibronectin and vimentin expression, and significantly inhibited the increase in secretion of collagens I-IV seen in control cells or cells treated with the scrambled siRNA, returning expression levels for all proteins to near baseline. TAK1 knockdown also reduced the accentuating effect of TNF-α on TGF-β1–driven EMT in PBECs. In contrast to the described results for TAK*i* in which pro-MMP-9 secretion was enhanced by TAK*i* (Figure 5B), pro-MMP-9 secretion after TAK*si* treatment displayed a return to near baseline across all stimulations (Figure 5C; see also Supplemental Figure S4B at *http://ajp.amjpathol.org*).

JNK*i* inhibited the phosphorylation of JNK-1/2 and c-Jun, with no effect observed on any of the other signaling proteins assayed (Figure 6A; see also Supplemental Figure S3B at *http://ajp.amjpathol.org*). Pretreatment of PBECs with JNK*i* had a similar inhibitory effect to that of TAK*i* on TGF-β1–driven EMT and TNF-α–accentuated TGF-β1–driven EMT (Figure 6, B and D; see also Supplemental Figure S4C at *http://ajp.amjpathol.org*). Knockdown of JNK-2 inhibited the TGF-β1–induced decrease in E-cadherin expression, increase in fibronectin and vimentin expression, and significantly inhibited the increase in secretion of collagens I-IV seen in control cells returning expression levels for all proteins to near baseline. JNK-2 knockdown also reduced the accentuating effect of TNF-α on TGF-β1–driven EMT in PBECs. In contrast, knockdown of JNK-1 did not inhibit the TGF-β1–driven or TNF-α–accentuated decrease in E-cadherin expression or increase in fibronectin and vimentin expression, but did reduce pro-MMP-9 and collagens I-IV secretion compared to untreated controls (Figure 6C; see also Supplemental Figure S4D at *http://ajp.amjpathol.org*).

Disease Relevance of TAK1

Sequential sections from OB lung tissue and unused donor lung as a control were used to investigate the localization and phosphorylation of TAK1 in the airway. Sections were stained for either total TAK1 or phospho-TAK1 and counterstained with the epithelial marker E-cadherin and DAPI as a nuclear marker. Total TAK1 was located throughout all airway cells and there was no detectable difference in the intensity of expression between normal and OB lung tissue. In contrast levels of phospho-TAK1 were higher in epithelial cells in OB tissue compared to normal. It was also noted that phospho-TAK1 displayed evidence of nuclear localization, which was more pronounced in OB tissue (Figure 7A; see also Supplemental Figure S5A at *http://ajp.amjpathol.org*).

To quantify the observations previously described, immunocytochemistry was performed on cultured PBECs treated with TGF-β1 or TNF-α for 30 minutes, and the relative nuclear localization of phospho-TAK1 assessed. PBECs were stained with phospho-TAK1 and β-actin, along with DAPI as a nuclear counterstain. Nuclear localization of phospho-TAK1 was increased following stimulation with both TGF-β1 (74.5 $\pm$ 1.7) and, to a lesser extent, TNF-α (29.8 $\pm$ 1.0) compared to control cells (5.1 $\pm$ 0.1). Co-stimulation with TGF-β1 and TNF-α resulted in an accentuated nuclear localization of phospho-TAK1 (156.2 $\pm$ 5.6) compared to either TGF-β1 or TNF-α stimulation alone (Figure 7, B and C; see also Supplemental Figure S5B at *http://ajp.amjpathol.org*). Alongside this, to confirm the observed nuclear translocation of TAK1, nuclear and non-nuclear fractions were prepared

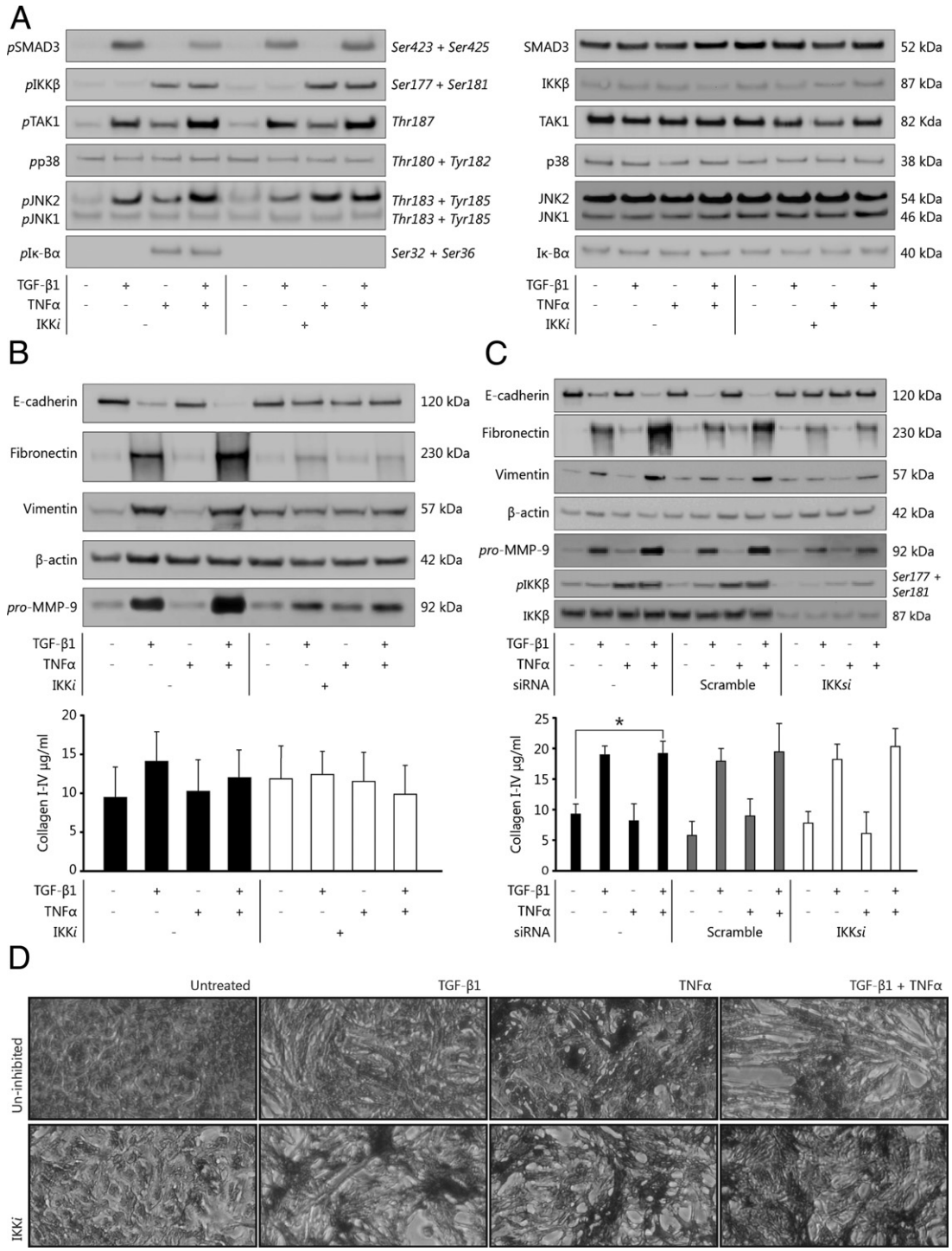


Figure 4. IKK blocking IKKβ activity limits TGF-β1–driven EMT and its accentuation. **A:** Stimulation of PBECs ($n = 3$) with TGF-β1 (10 ng/mL) or TNF-α (20 ng/mL) induce phosphorylation of p38, SMAD3, IKKβ, TAK1, and JNK-1/2 as described in Figure 2. Pretreatment with IKK-2 Inhibitor IV (IKK*i*) (5 μmol/L) for 1 hour strongly reduces the phosphorylation of Iκ-Bα, but has no effect on the phosphorylation of p38, SMAD3, IKKβ, TAK1, and JNK-1/2. **B:** Stimulation of PBECs ($n = 4$) with TGF-β1 down-regulates E-cadherin expression, increases fibronectin and vimentin expression, and increases pro-MMP-9 secretion. This change in EMT marker expression is accentuated by co-stimulation with TGF-β1 and TNF-α. Collagen I-IV secretion is up-regulated by stimulation with TGF-β1, however, no accentuation is observed on co-stimulation with TGF-β1 and TNF-α. Pretreatment with IKKi attenuates both TGF-β1–driven and TNF-α–accentuated changes in EMT marker expression and collagen I-IV secretion, returning levels to near baseline. **C:** Treatment of PBECs with IKK*si* (0.1 nmol/L) induces an approximate 90% knockdown of total IKKβ after 24 hours and strongly reduces detected IKKβ phosphorylation after stimulation with TGF-β1 or TNF-α for 30 minutes. Knockdown of IKKβ has a small inhibitory effect on TGF-β1–driven EMT, but strongly attenuates the TNF-α–accentuated change in EMT marker expression. In contrast, TGF-β1 induced collagen I-IV secretion is not inhibited by knockdown of IKKβ. A sequence scrambled control has no effect on EMT marker expression. $^*P < 0.05$. **D:** A549 cells treated with TGF-β1 begin to lose their epithelial morphology and acquire a more elongated mesenchymal morphology, and this change is accentuated by co-stimulation with TNF-α. Pretreatment with IKK*i* has little to no effect on the TGF-β1 induced change in cell morphology, but strongly attenuates the accentuating effect of TNF-α on cell morphology.

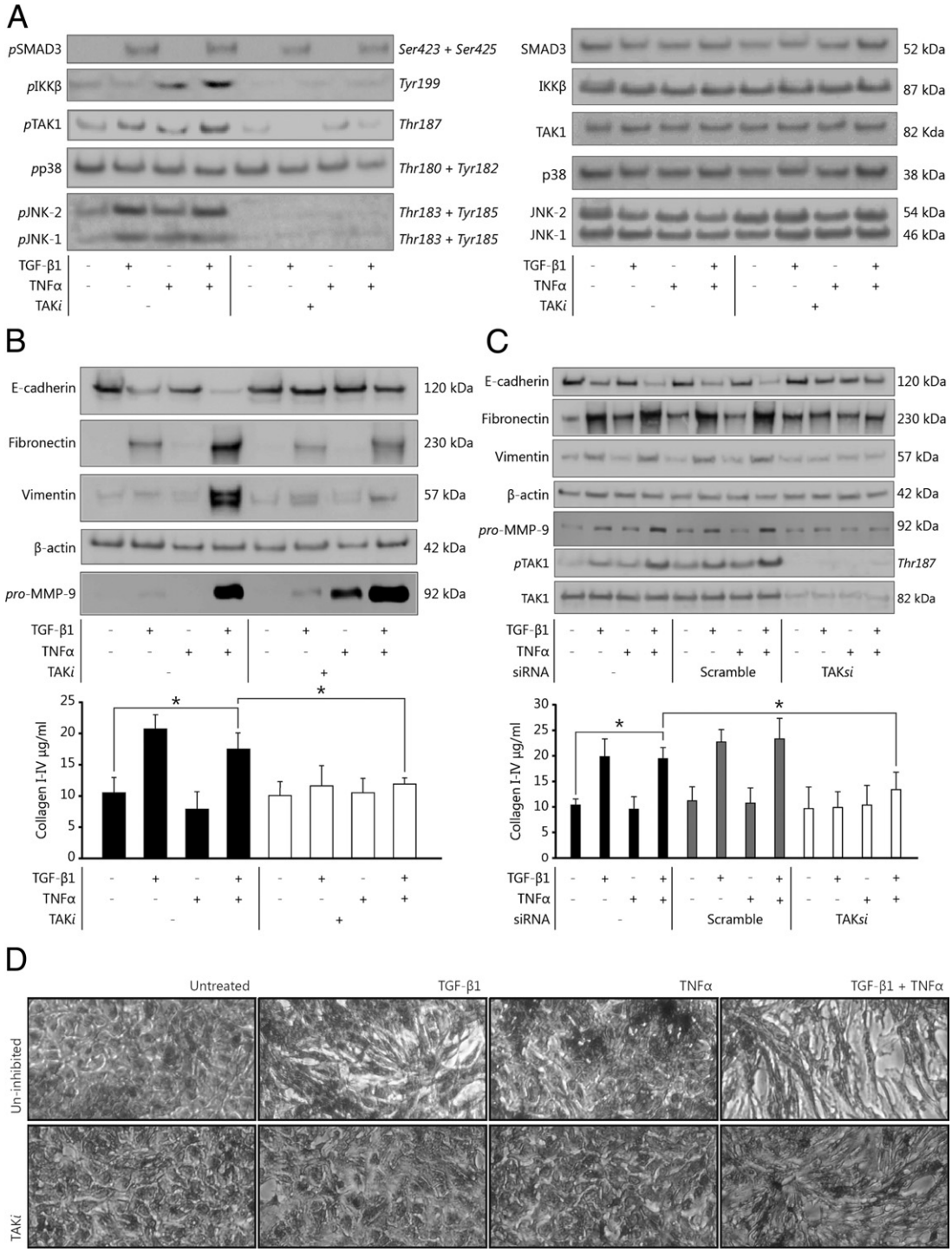


Figure 5. Blocking TGF-β1–activated TAK1 activity limits TGF-β1–driven EMT and its accentuation by TNF-α. **A:** Stimulation of PBECs ($n = 3$) with TGF-β1 (10 ng/mL) or TNF-α (20 ng/mL) induces phosphorylation of p38, SMAD3, IKKβ, TAK1, and JNK-1/2, as described in Figure 2. Pretreatment with (5*Z*)–7-Oxozeaenol, *Curvularia* spp. (TAK*i*) (1 μmol/L) for 1 hour strongly reduces the phosphorylation of TAK1, IKKβ, and JNK-1/2, but has no effect on p38 or SMAD3 phosphorylation. **B:** Stimulation of PBECs ($n = 6$) with TGF-β1 down-regulates E-cadherin expression, increases fibronectin and vimentin expression, and increases pro-MMP-9 secretion. This change in EMT marker expression is accentuated by co-stimulation with TNF-α. Collagen I-IV secretion is significantly up-regulated by stimulation with TGF-β1, however, no accentuation is observed on co-stimulation with TNF-α. Pretreatment with TAK*i* attenuates the TGF-β1–driven changes in EMT marker expression and collagen secretion and the TNF-α–accentuated change in E-cadherin, fibronectin and vimentin expression, and collagen I-IV secretion compared to untreated cells. Conversely, TAK*i* increases secretion of pro-MMP-9 in PBECs treated with TNF-α alone or in combination with TGF-β1. **C:** Treatment of PBECs ($n = 5$) with TAK*si* (5 nmol/L) induces an approximate 80% knockdown of total TAK1 after 24 hours and strongly reduces detected phosphorylation after stimulation with TGF-β1 or TNF-α for 30 minutes. Knockdown of TAK1 also strongly reduces both TGF-β1–driven and TNF-α–accentuated changes in EMT marker expression and secretion of collagen I-IV and pro-MMP-9 compared to uninhibited controls. A sequence scrambled control has no effect on EMT marker expression. $^*P < 0.05$. **D:** A549 cells treated with TGF-β1 begin to lose their epithelial morphology and acquire a more elongated mesenchymal morphology, and this change is accentuated by co-stimulation with TNF-α. Pretreatment with TAK*i* attenuates this morphological change in cells treated with TGF-β1 alone or in combination with TNF-α.

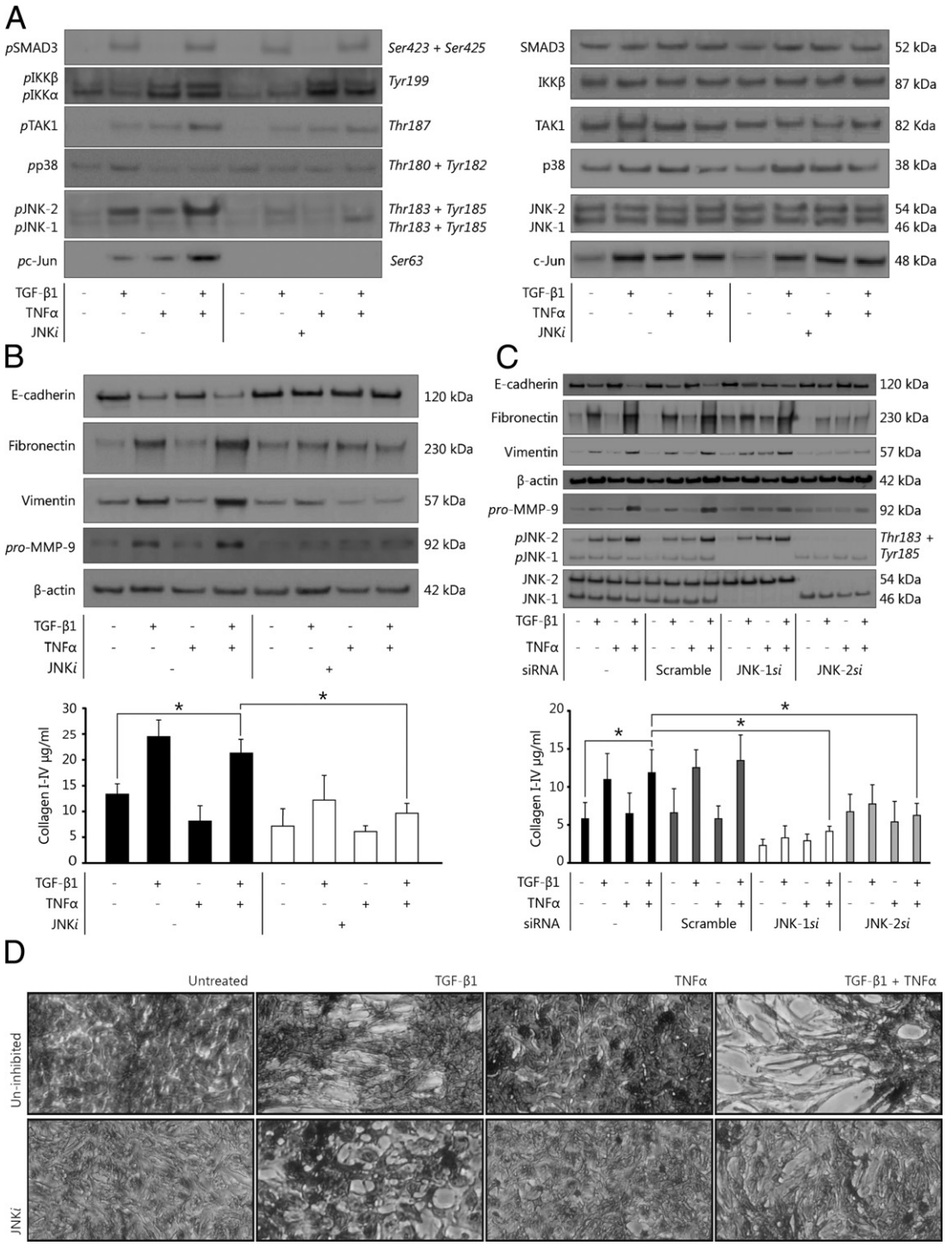
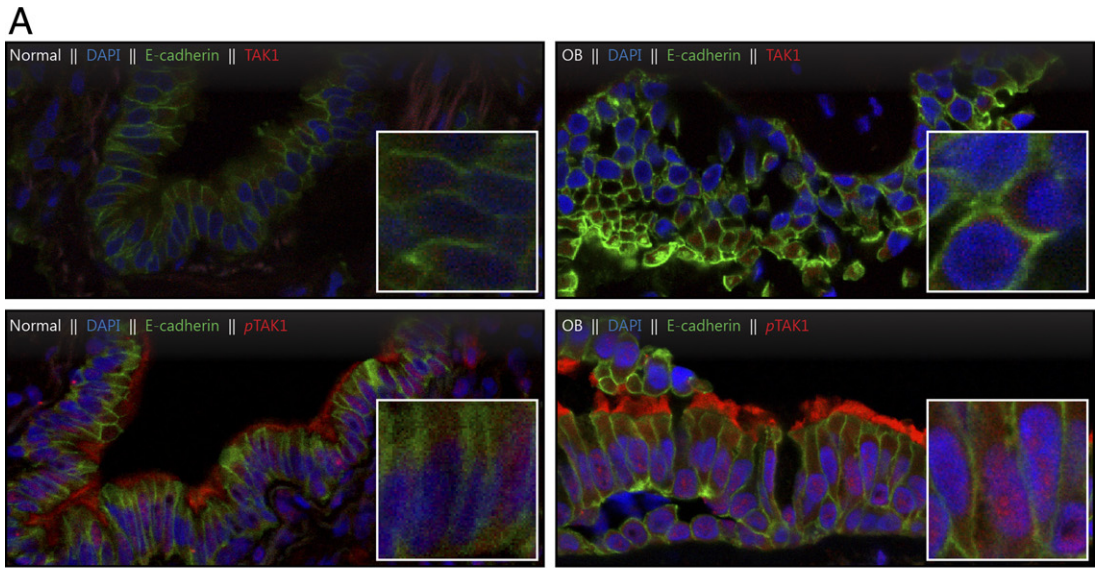
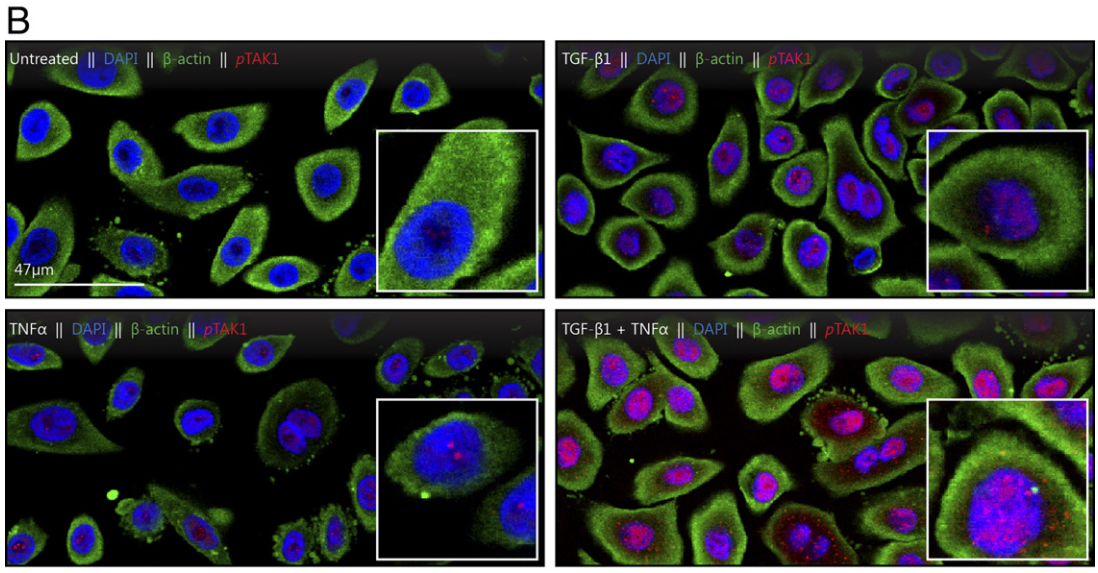


Figure 6. Blocking JNK-1/2 activity limits TGF-β1–driven EMT and its accentuation by TNF-α. **A:** Stimulation of primary bronchial epithelial cells (PBECs) ($n = 3$) with TGF-β1 (10 ng/mL) or TNF-α (20 ng/mL) induces phosphorylation of p38, SMAD3, IKKβ, TAK1, and JNK-1/2, as described in Figure 2. Pretreatment with SAPK Inhibitor II (JNK*i*) (5 μmol/L) for 1 hour strongly reduces the phosphorylation of JNK-1/2 and c-Jun, but has no effect on p38, SMAD3, IKKβ, and TAK1 phosphorylation. Total c-Jun is increased in PBECs stimulated with TGF-β1 or TNF-α alone, or in combination, and this was not affected by pretreatment with JNK*i*. **B:** Stimulation of PBECs ($n = 4$) with TGF-β1 down-regulates E-cadherin expression, increases fibronectin and vimentin expression, and increases pro-MMP-9 secretion. This change in EMT marker expression is accentuated by co-stimulation with TNF-α. Collagen I-IV secretion is significantly up-regulated by stimulation with TGF-β1, however, no accentuation is observed on co-stimulation with TNF-α. Pretreatment with JNK*i* attenuates both TGF-β1–driven and TNF-α–accentuated changes in EMT marker expression and collagen I-IV secretion, returning levels to near baseline. **C:** Treatment of PBECs ($n = 3$) with JNK-1*si* (10 nmol/L) and JNK-2*si* (10 nmol/L) induces an approximate 95% knockdown of total JNK-1 or JNK-2, respectively, after 24 hours, and strongly and selectively reduces their phosphorylation after stimulation with TGF-β1 or TNF-α for 30 minutes. JNK-2*si* also strongly reduced TGF-β1–driven and TNF-α–accentuated changes in EMT marker expression and collagen I-IV, and pro-MMP-9 secretion, returning levels to near baseline. In contrast JNK-1*si* has no effect on the down-regulation of E-cadherin expression or the increase in fibronectin and vimentin expression. JNK-1*si*, however, attenuates the increase in pro-MMP-9 and collagen I-IV secretion compared to untreated cells. A sequence scrambled control has no effect on EMT marker expression. $^*P < 0.05$. **D:** A549 cells treated with TGF-β1 begin to lose their epithelial morphology and acquire a more elongated mesenchymal morphology, and this change is accentuated by co-stimulation with TNF-α. Pretreatment with JNK*i* has little to no effect on the TGF-β1–induced change in cell morphology, but strongly attenuates the accentuating effect of TNF-α on cell morphology.





C

Treatment	Red Channel Nuclear Pixel Intensity ± SEM
Untreated	5.1 ± 0.1
TGF-β1	74.5 ± 1.7
TNFα	29.8 ± 1.0
TGF-β1 + TNFα	156.2 ± 5.6

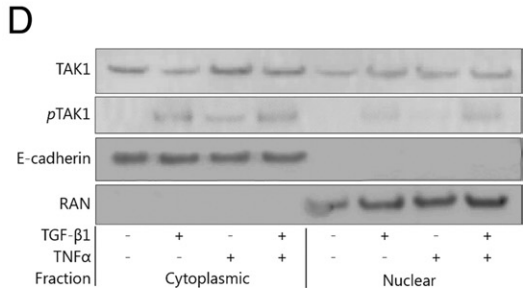


Figure 7. TAK1 nuclear localization in OB tissue and PBECs. **A:** Tissue sections from normal and OB lungs were stained for either total TAK1 or phospho-TAK1, and expression and localization assessed in the epithelium. E-cadherin was used as an epithelial marker. Total TAK1 staining is located throughout the cell with no difference in expression levels between normal and OB tissue. In contrast phospho-TAK1 staining is more intense and shows an increased nuclear localization in OB tissue compared to the normal lung. The phospho-TAK1 antibody displays nonspecific binding of the antibody to the brush border of the airway in both control and OB sections. **B:** Untreated PBECs express little to no phospho-TAK1. Stimulation with either TGF-β1 or TNF-α induces an increased phosphorylation of TAK1 and a pronounced localization of TAK1 to the nucleus. Co-stimulation with TGF-β1 and TNF-α accentuates both the phosphorylation of TAK1, as well as the nuclear localization of the phosphorylated protein. **C:** Mean pixel intensity of phospho-TAK1 staining in the nuclei in response to stimulation in 20 cells quantified from multiple images. Results are expressed as mean ± SEM. **D:** The presence and degree of phosphorylation of TAK1 in both nuclear and non-nuclear fractions isolated from PBECs treated, as indicated was assessed. RAN (a nuclear specific RAS-GTPase) and E-cadherin (a cell membrane marker) ere used to demonstrate purity of nuclear and non-nuclear fractions, respectively. Total TAK1 levels are detected in both nuclear and non-nuclear fractions and remain unchanged in response to stimulation. Phospho-TAK1 is increased in both the nuclear and non-nuclear fractions in response to treatment with TGF-β1 and TNF-α with an accentuation seen on co-stimulation.

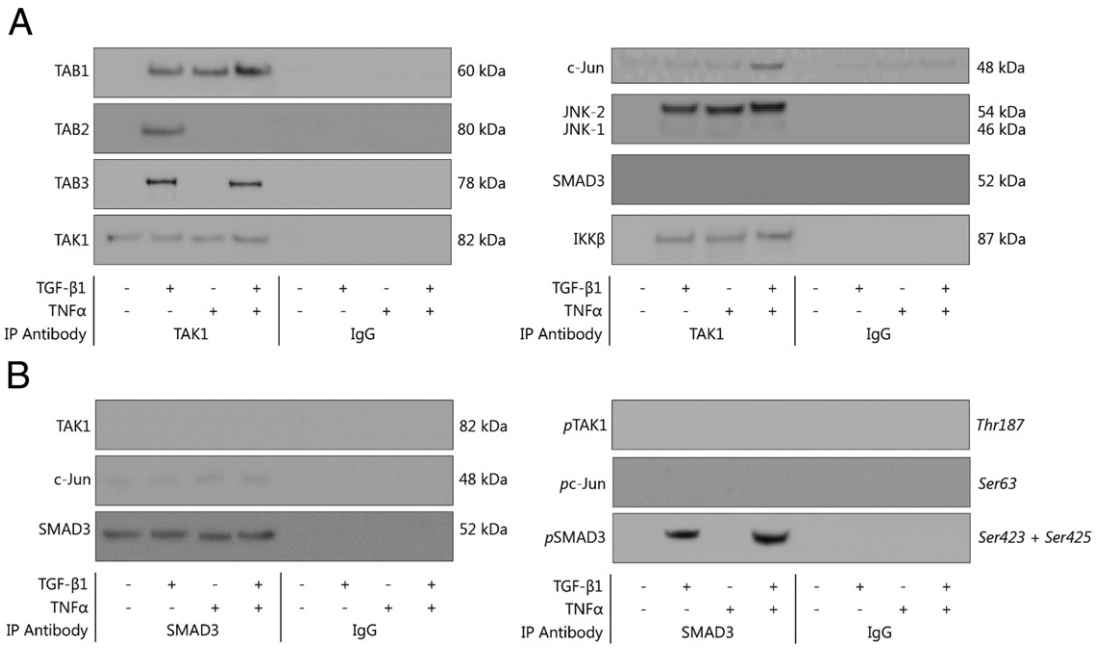


Figure 8. c-Jun as the effector of accentuated and prolonged TAK1, JNK-2 phosphorylation. **A:** PBEC lysates ($n = 3$) from cells treated with TGF-β1 (10 ng/mL) or TNF-α (20 ng/mL) for 30 minutes were immunoprecipitated for total TAK1 or pan-IgG and probed for total TAK1, SMAD3, IKKβ, JNK-1/2, c-Jun, and TAB 1–3. TAB1 is associated with TAK1 on treatment with TGF-β1 or TNF-α, with an accentuated association on co-treatment. Both TAB2 and TAB3 are associated with TAK1 on treatment with TGF-β1, but not TNF-α. On co-treatment TAB3 remains associated with TAK1, but TAB2 is completely lost. IKKβ and JNK-2 are associated with TAK1 after treatment with TGF-β1 or TNF-α alone, or in combination, although no difference in the degree of association between treatments is observed. c-Jun is associated with TAK1 only after co-stimulation with both TGF-β1 and TNF-α. In contrast, SMAD3 and JNK-1 are not associated with TAK1 under any treatment conditions. **B:** PBEC lysates ($n = 2$) treated as previously described were immunoprecipitated for total SMAD3, or pan-IgG and probed for total and phosphorylated forms of TAK1 and c-June. Total c-Jun has a weak association with SMAD3 in response to treatment with TGF-β1 and TNF-α alone or in combination. No association between total or phosphorylated TAK-1 or phosphorylated c-Jun was observed.

from PBECs treated with TGF-β1 or TNF-α for 30 minutes. The cell adhesion protein E-cadherin and rat sarcoma-related nuclear protein (Ran), a nuclear specific rat sarcoma guanosine triphosphate hydrolase enzyme, were used as markers to confirm the purity of the respective fractions. Phospho-TAK1 is expressed in both the nuclear and non-nuclear fractions and displays the same accentuated response to stimulation with TGF-β1 and TNF-α described in both total cell lysate Western blots and immunocytochemistry (Figure 7D).

Potential Role for c-Jun

PBEC lysates from cells stimulated with TGF-β1 or TNF-α for 30 minutes were immunoprecipitated under nondenaturing conditions with TAK1 and SMAD3 antibodies and probed for a variety of markers. The TAK1 associated binding protein (TAB) family facilitate TAK1 autophosphorylation[27] through interaction with receptor scaffold proteins, such as TNF receptor-associated factor 6. TAB1 was associated with TAK1 under all conditions, however, TAB2 and TAB3 associated with TAK1 on stimulation with TGF-β1, but not TNF-α. On co-stimulation, there was no change in TAB3 association compared to TGF-β1 alone, but TAB2 association with TAK1 was completely lost. IKKβ and JNK-2, but not JNK-1 or SMAD3, were associated with TAK1 at 30 minutes after stimulation with TGF-β1 or TNF-α, with no differences in association between stimulations. In contrast, c-Jun was associated with TAK1 only after co-stimulation with both TGF-β1 and TNF-α (Figure 8A). c-Jun was detected in association with SMAD3 under all stimulations, however, the phospho form and the total and phospho forms of TAK1 were not associated with SMAD3 in response to any treatment (Figure 8B).

Discussion

Current therapies to limit or reverse fibrosis have failed to demonstrate significant clinical benefits highlighting the need for further research to elucidate the basic mechanisms underlying disease progression, particularly the link between fibrosis and inflammation. Recently, our group and others have shown that TGF-β1 can drive EMT in PBECs, and inflammatory stimuli, such as TNF-α and IL-1β can accentuate this effect,[2,14,15] however, the signaling mechanisms are ill-defined. In this article, we provide novel insight into the signaling events involved in the accentuation of TGF-β1–driven EMT elicited by TNF-α. It is our hope that greater understanding of the interaction between pro-fibrotic and pro-inflammatory mediators will help with the development of new more targeted therapeutics along with the development of potential prognostic biomarkers.

Our data suggest that SMAD3 mediated TGF-β1–driven EMT is indirectly controlled by TAK1/JNK-2 activation possibly through association c-Jun. TNF-α accentuates EMT through IKKβ directly controlled by TAK1, and indirectly by JNK-2, again possibly by the actions of c-Jun. Co-stimulation resulted in accentuated phosphor-

ylation of TAK-1, JNK-2, and c-Jun suggesting that the TAK1>JNK>2-c-Jun cascade plays an important role in the accentuated EMT observed in co-stimulated PBECs.

TAK1 was initially described as a mediator of noncanonical TGF-β signaling,[28] although it is now also known to play a key role in NF-κB signaling. Both TGF-β1 and TNF-α alone promote the phosphorylation, and hence activation,[29] of TAK1, and we demonstrate that this phosphorylation is accentuated and prolonged on co-stimulation, with an associated nuclear translocation. TAK1 does not contain a described nuclear translocation signal and no evidence of direct transcriptional control has been presented in the literature, therefore, the observed localization of TAK1 to the nucleus in response to stimulation was unexpected. We identified two previous studies, however, describing a nuclear localization of TAK1; the first describes localization in response to IL-6 stimulation and the second describes the translocation of TAK1 into the nucleus in association with Ski-related novel protein N (SnoN), an inhibitor of SMAD transcriptional activity. In this instance, TAK1 is required for the degradation of SnoN and the consequent activation of SMAD transcriptional activity.[30] In our system, it is possible that our accentuated activation of TAK1 may result in an even greater inhibition of SnoN activity and an increase in resultant SMAD transcriptional activity, although further investigation is required to test this hypothesis.

The TAB family consists of three members, which are thought to modulate TAK1 function. The most widely investigated member is TAB1, a pseudo-phosphatase that is responsible for the induction of TAK1 activity via promotion of autophosphorylation of residues within the activation loop. It is thought that TAB1 and TAK1 are constitutively associated and that TAB1 is required for TAK1 autophosphorylation,[27] in line with our findings. TAB2 and TAB3 are involved in the recruitment and binding of other proteins to TAK1 to form complexes that can modulate TAK1 activity, and may help explain the observed nuclear translocation and described phosphorylation response. The data presented in this article suggests that the interactions of TAB2 and TAB3 with TAK1 may be of interest in understanding the mechanisms underlying the accentuated phosphorylation of TAK1 and resultant EMT.

JNK-2 binds c-Jun with a higher affinity than JNK-1[31], however, a previous study investigating EMT in primary murine tracheal cells demonstrated an important role for JNK-1, independent of JNK-2, in mediating SMAD3 dependant, TGF-β1–driven EMT.[32] The dose of JNK*i* used in this study does not differentiate between JNK isoforms, and therefore the observed inhibitory effect on EMT could occur due to the inhibition of JNK-1, JNK-2, or both in these experiments. However, our data also demonstrated an increase in JNK-2, but not JNK-1 phosphorylation in response to treatments, and while JNK-2*si* strongly inhibited EMT for all markers, JNK-1*si* only reduced the secretion of pro-MMP-9 and collagens I-IV. Together the data suggest that JNK-1 and JNK-2 may act cooperatively; indeed JNK-1 has been shown to phosphorylate JNK-2 before both phosphorylate p53 at separate activating residues[33], however, we did not observe any change in JNK-1 phosphorylation suggesting that its presence rather than activation may be of importance.

JNK-2 also displayed an accentuated and prolonged phosphorylation on co-stimulation, and its inhibition, unlike other members of the mitogen-activated protein kinase pathway (p38 and ERK1/2), blocked EMT. The lack of response from ERK-1/2 and p38 (see Supplemental Figure S6 at *http://ajp.amjpathol.org*) was surprising, as both have been demonstrated to respond to similar stimuli in other models[34,35] and a role for TAK1 mediated activation of ERK-1/2 and p38 has also been described.[36] However, our results are in agreement with previous reports demonstrating that inhibition of p38 or ERK-1 have no effect on TGF-β1–driven or TGF-β1–driven, TNF-α–accentuated EMT[15,37], suggesting that signaling pathways controlling EMT may be cell or tissue dependent. Further evidence of this varied response in different tissues is demonstrated in two articles specifically investigating the role of TAK1 in fibrosis; the first[38] describes TAK1 as a key mediator of fibrosis and inflammation within the kidney via the activation of p38, JNK, and NF-κB. Whereas the other article demonstrates an opposite effect, with ablation of TAK1 increasing the development of inflammation and fibrosis in hepatic cells.[39]

Throughout the study, we did not observe an additive or accentuated increase in collagen secretion in response to TNF-α stimulation. Previous studies have demonstrated that TNF-α can inhibit collagen production.[40] Taken together with our data on the expression of other markers of EMT, it may be that co-stimulation with TGF-β1 and TNF-α results in a higher proportion of mesenchymal-like cells that produce less collagen on a per cell basis than cells stimulated with TGF-β1 alone. This raises the intriguing possibility that episodic release of TNF-α in the presence of TGF-β1, such as could occur in response to repeated inflammatory insults to the lung, could drive migration and invasion of transformed epithelial cells, but not deposition of collagen until the levels of TNF-α fall again, in which case the transformed cells could then drive fibrosis.

Here, we have identified a role for a TAK1 in orchestrating synergy between TGF-β1 and TNF-α in driving EMT in the development of OB. EMT, however, has also been identified as a potential mechanism contributing to the development of other fibrotic disorders of the lung characterized by elevated TGF-β1, such as idiopathic pulmonary fibrosis[9] and asthma.[41] Furthermore, in the kidney TGF-β1–driven EMT has been suggested to play an important role in the development of fibrosis,[42] whereas a role for inflammatory mediators secreted from leukocytes has also been described.[43] Similarly, a potential role for TGF-β1–driven EMT has been described during the development of liver fibrosis.[44] Considering the ubiquitous expression of TAK1 and the potential role of EMT in tissue fibrosis, we propose that the signaling pathway identified in this article may be important in several fibrotic diseases across multiple organs. Indeed, several mitogen-activated protein kinase inhibitors designed to limit fibrosis in idiopathic pulmonary fibrosis are currently undergoing clinical trials, such as inhibitors of p38 (NCT01203943) and JNK (NCT01366209) and

numerous other trials in other organs are underway. There are currently no clinical trials using inhibitors against TAK-1, and it is unlikely that direct inhibition of TAK-1 itself will be an acceptable therapeutic target, reinforced by the embryo lethal nature of TAK1 knockout mice.[45] Further investigations, therefore, are underway to identify the upstream modulators and downstream effectors of TAK1 and JNK-2 activation that may identify alternative, more suitable therapeutic targets.

References

1. Hodge S, Holmes M, Banerjee B, Musk M, Kicic A, Waterer G, Reynolds PN, Hodge G, Chambers DC: Posttransplant bronchiolitis obliterans syndrome is associated with bronchial epithelial to mesenchymal transition. Am J Transplant 2009, 9:727–733
2. Borthwick LA, McIlroy EI, Gorowiec MR, Brodlie M, Johnson GE, Ward C, Lordan JL, Corris PA, Kirby JA, Fisher AJ: Inflammation and epithelial to mesenchymal transition in lung transplant recipients: role in dysregulated epithelial wound repair. Am J Transplant 2010, 10: 498–509
3. Verleden GM, Fisher AJ, Boehler A, Esenne M: Bronchiolitis obliterans syndrome. Lung Transplantation, European Respiratory Society Monograph Vol. 45. Edited by Fisher AJ, Verleden GM, Massard G. Sheffield, UK, European Respiratory Society Journals Ltd, 2009, pp. 197–211
4. Savagner P: The epithelial-mesenchymal transition (EMT) phenomenon. Ann Oncol 2010, 21(Suppl 7):89–92
5. Yamamoto T, Nakamura T, Noble NA, Ruoslahti E, Border WA: Expression of transforming growth factor beta is elevated in human and experimental diabetic nephropathy. Proc Natl Aacd Sci U S A 1993, 90:1814–1818
6. Czaja MJ, Weiner FR, Flanders KC, Giambrone MA, Wind R, Biempica L, Zern MA: In vitro and in vivo association of transforming growth factor-beta 1 with hepatic fibrosis. J Cell Biol 1989, 108:2477–2482
7. Broekelmann TJ, Limper AH, Colby TV, McDonald JA: Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. Proc Natl Acad Sci U S A 1991, 88:6642–6646
8. Massagué J, Wotton D: Transcriptional control by the TGF-beta/Smad signaling system. Embo J 2000, 19:1745–1754
9. Willis BC, Borok Z: TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease. Am J Physiol Lung Cell Mol Physiol 2007, 293:L525–L534
10. Kolosova I, Nethery D, Kern JA: Role of Smad2/3 and p38 MAP kinase in TGF-β1-induced epithelial-mesenchymal transition of pulmonary epithelial cells. J Cell Physiol 2010, 226:1248–1254
11. Matsuzaki K: Smad phosphoisoform signals in acute and chronic liver injury: similarities and differences between epithelial and mesenchymal cells. Cell Tissue Res 2011, 347:225–243
12. Cargnello M, Roux PP: Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol R 2011, 75:50–83
13. Chang L, Karin M: Mammalian MAP kinase signalling cascades. Nature 2001, 410:37–40
14. Borthwick LA, Parker SM, Brougham KA, Johnson GE, Gorowiec MR, Ward C, Lordan JL, Corris PA, Kirby JA, Fisher AJ: Epithelial to mesenchymal transition (EMT) and airway remodelling after human lung transplantation. Thorax 2009, 64:770–777
15. Câmara J, Jarai G: Epithelial-mesenchymal transition in primary human bronchial epithelial cells is Smad-dependent and enhanced by fibronectin and TNF-alpha. Fibrogenesis Tissue Repair 2010, 3:2
16. Locksley RM, Killeen N, Lenardo MJ: The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 2001104:487–501
17. Grell M, Douni E, Wajant H, Löhden M, Clauss M, Maxeiner B, Georgopoulos S, Lesslauer W, Kollias G, Pfizenmaier K, Scheurich P: The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell 1995, 83:793–802
18. Hsu H, Xiong J, Goeddel DV: The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell 1995, 81:495–504
19. Gerondakis S, Grumont R, Gugasyan R, Wong L, Isomura I, Ho W, Banerjee A: Unravelling the complexities of the NF- [kappa] B signalling pathway using mouse knockout and transgenic models. Oncogene 2006, 25:6781–6799
20. Perkins ND: Integrating cell-signalling pathways with NF-kappaB and IKK function. Nat Rev Mol Cell Bio 2007, 8:49–62
21. Thoma B: Identification of a 60-kD tumor necrosis factor (TNF) receptor as the major signal transducing component in TNF responses. J Exp Med 1990, 172:1019–1023
22. Forrest IA, Murphy DM, Ward C, Jones D, Johnson GE, Archer L, Gould FK, Cawston TE, Lordan JL, Corris PA: Primary airway epithelial cell culture from lung transplant recipients. Eur Respir J 2005, 26:1080–1085
23. Bryde S, Grunwald I, Hammer A, Krippner-Heidenreich A, Schiestel T, Brunner H, Tovar GEM, Pfizenmaier K, Scheurich P: Tumor necrosis factor (TNF)-functionalized nanostructured particles for the stimulation of membrane TNF-specific cell responses. Bioconjugate Chem 2005, 16:1459–1467
24. Krippner-Heidenreich A, Tübing F, Bryde S, Willi S, Zimmermann G, Scheurich P: Control of receptor-induced signaling complex formation by the kinetics of ligand/receptor interaction. J Biol Chem 2002, 277:44155–44163
25. Abramoff MD, Magelhaes P, Ram SJ: Image Processing with ImageJ. Biophotonics 2004, 11:36–42
26. Naudé PJW, den Boer JA, Luiten PGM, Eisel ULM: Tumor necrosis factor receptor cross-talk. Febs J 2011, 278:888–898
27. Kim SI, Kwak JH, Na H-J, Kim JK, Ding Y, Choi ME: Transforming growth factor-beta (TGF-beta1) activates TAK1 via TAB1-mediated autophosphorylation, independent of TGF-beta receptor kinase activity in mesangial cells. J Biol Chem 2009, 284:22285–22296
28. Yamaguchi K, Shirakabe K, Shibuya H, Irie K, Oishi I, Ueno N, Taniguchi T, Nishida E, Matsumoto K: Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. Science 1995, 270:2008–2011
29. Singhirunnusorn P, Suzuki S, Kawasaki N, Saiki I, Sakurai H: Critical roles of threonine 187 phosphorylation in cellular stress-induced rapid and transient activation of transforming growth factor-beta-activated kinase 1 (TAK1) in a signaling complex containing TAK1-binding protein TAB1 and TAB2. J Biol Chem 2005, 280:7359–7768
30. Kajino T, Omori E, Ishii S, Matsumoto K, Ninomiya-Tsuji J: TAK1 MAPK kinase kinase mediates transforming growth factor-beta signaling by targeting SnoN oncoprotein for degradation. J Biol Chem 2007, 282:9475–9481
31. Kallunki T, Su B, Tsigelny I, Sluss HK, Derijard B, Moore G, Davis R, Karin M: JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation. Gene Dev 1994, 8:2996–3007
32. Velden JLJ van der, Alcorn JF, Guala AS, Badura ECHL, Janssen-Heininger YMW: c-Jun N-terminal kinase 1 promotes transforming growth factor-β1-induced epithelial-to-mesenchymal transition via control of linker phosphorylation and transcriptional activity of Smad3. Am J Resp Cell Mol 2011, 44:571–581
33. Oleinik NV, Krupenko NI, Krupenko SA: Cooperation between JNK1 and JNK2 in activation of p53 apoptotic pathway. Oncogene 2007, 26:7222–7230
34. Lee MK, Pardoux C, Hall MC, Lee PS, Warburton D, Qing J, Smith SM, Derynck R: TGF-beta activates Erk MAP kinase signalling through direct phosphorylation of ShcA. Embo J 2007, 26:3957–3967
35. Pechkovsky DV, Scaffidi AK, Hackett TL, Ballard J, Shaheen F, Thompson PJ, Thannickal VJ, Knight DA: Transforming growth factor beta1 induces alphavbeta3 integrin expression in human lung fibroblasts via a beta3 integrin-, c-Src-, and p38 MAPK-dependent pathway. J Biol Chem 2008, 283:12898–12908
36. Nishimura M, Shin M-S, Singhirunnusorn P, Suzuki S, Kawanishi M, Koizumi K, Saiki I, Sakurai H: TAK1-mediated serine/threonine phosphorylation of epidermal growth factor receptor via p38/extracellular signal-regulated kinase: nF-{kappa}B-independent survival pathways in tumor necrosis factor alpha signaling. Mol Cell Biol 2009, 29:5529–5539

37. Kasai H, Allen JT, Mason RM, Kamimura T, Zhang Z: TGF-beta1 induces human alveolar epithelial to mesenchymal cell transition (EMT). Respir Res 2005, 6:56
38. Ma FY, Tesch GH, Ozols E, Xie M, Schneider MD, Nikolic-Paterson DJ: TGF-β1-activated kinase-1 regulates inflammation and fibrosis in the obstructed kidney. Am J Physiol-Renal 2011, 300:F1410–F1421
39. Inokuchi S, Aoyama T, Miura K, Osterreicher CH, Kodama Y, Miyai K, Akira S, Brenner DA, Seki E: Disruption of TAK1 in hepatocytes causes hepatic injury, inflammation, fibrosis, and carcinogenesis. P Natl Aacd Sci USA 2010, 107:844–849
40. Regan MC, Kirk SJ, Hurson M, Sodeyama M, Wasserkrug HL, Barbul A: Tumor necrosis factor-alpha inhibits in vivo collagen synthesis. Surgery 1993, 113:173–177
41. Hackett T-L, Warner SM, Stefanowicz D, Shaheen F, Pechkovsky DV, Murray LA, Argentieri R, Kicic A, Stick SM, Bai TR, Knight DA: Induction of epithelial-mesenchymal transition in primary airway epithelial cells from patients with asthma by transforming growth factor-beta1. Am J Respir Crit Care 2009, 180:122–133
42. Liu Y: New insights into epithelial-mesenchymal transition in kidney fibrosis. J Am Soc Nephrol 2010, 21:212–222
43. Lange-Sperandio B, Trautmann A, Eickelberg O, Jayachandran A, Oberle S, Schmidutz F, Rodenbeck B, Homme M, Horuk R, Schaefer F: Leukocytes induce epithelial to mesenchymal transition after unilateral ureteral obstruction in neonatal mice. Am J Path 2007, 171:861–871
44. Zeisberg M, Yang C, Martino M, Duncan MB, Rieder F, Tanjore H, Kalluri R: Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. J Biol Chem 2007, 282:23337–23347
45. Shim J-H, Xiao C, Paschal AE, Bailey ST, Rao P, Hayden MS, Lee K-Y, Bussey C, Steckel M, Tanaka N, Yamada G, Akira S, Matsumoto K, Ghosh S: TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. Gene Dev 2005, 19:2668–2681