Association of β-catenin with P-Smad3 but not LEF-1 dissociates *in vitro* profibrotic from anti-inflammatory effects of TGF-β1

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Summary

Transforming growth factor β1 (TGF-β1) is known to be both anti-inflammatory and profibrotic. Cross-talk between TGF-β/Smad and Wnt/β-catenin pathways in epithelial–mesenchymal transition (EMT) suggests a specific role for β-catenin in profibrotic effects of TGF-β1. However, no such mechanistic role has been demonstrated for β-catenin in the anti-inflammatory effects of TGF-β1. In the present study, we explored the role of β-catenin in the profibrotic and anti-inflammatory effects of TGF-β1 by using a cytosolic, but not membrane, β-catenin knockdown chimera (F-TrCP-Ecad) and the β-catenin/CBP inhibitor ICG-001. TGF-β1 induced nuclear Smad3/β-catenin complex, but not β-catenin/LEF-1 complex or TOP-flash activity, during EMT of C1.1 (renal tubular epithelial) cells. F-TrCP-Ecad selectively degraded TGF-β1-induced cytoplasmic β-catenin and blocked EMT of C1.1 cells. Both F-TrCP-Ecad and ICG-001 blocked TGF-β1-induced Smad3/β-catenin and Smad reporter activity in C1.1 cells, suggesting that TGF-β1-induced EMT depends on β-catenin binding to Smad3, but not LEF-1 downstream of Smad3, through canonical Wnt. In contrast, in J774 macrophages, the β-catenin level was low and was not changed by interferon-γ (IFN-γ) or lipopolysaccharide (LPS) with or without TGF-β1. TGF-β1 inhibition of LPS-induced TNF-α and IFN-γ-stimulated inducible NO synthase (iNOS) expression was not affected by F-TrCP-Ecad, ICG-001 or by overexpression of wild-type β-catenin in J774 cells. Inhibition of β-catenin by either F-TrCP-Ecad or ICG-001 abolished LiCl-induced TOP-flash, but not TGF-β1-induced Smad reporter, activity in J774 cells. These results demonstrate for the first time that β-catenin is required as a co-factor of Smad in TGF-β1-induced EMT of C1.1 epithelial cells, but not in TGF-β1 inhibition of macrophage activation. Targeting β-catenin may dissociate the TGF-β1 profibrotic and anti-inflammatory effects.

Key words: TGF-β1, β-Catenin, P-Smad3, Epithelial–mesenchymal transition, Fibrosis, Inflammation

Introduction

There are two basic but opposing biological functions of transforming growth factor β1 (TGF-β1) in inflammatory diseases: anti-inflammatory and profibrotic (Blobe et al., 2000). The former is beneficial, while the latter is detrimental causing organ fibrosis and loss of function. Similarly in cancer, TGF-β1 inhibits proliferation and induces apoptosis of tumour cells, but promotes metastasis by inducing an invasive phenotype of tumour cells through induction of epithelial–mesenchymal transition (EMT) (Kudo-Saito et al., 2009; Sahai, 2005). The conflicting roles of TGF-β1 make it very difficult to target therapeutically. Why does TGF-β1 exert such diverse functions? Recent research in cytokine signaling suggests that it is the cell, more than the cytokine itself, which determines the cellular response to the cytokine (Fuxe et al., 2010; Massagué, 2000). TGF-β signals predominantly through a Smad-dependent pathway. However, Smads by themselves are unable to bind DNA of target genes (Massagué, 2000). Other largely cell-specific transcription co-factors are required for Smad complex binding and activation of target genes. Thus, the diverse functions of TGF-β1 may well be determined by cell-specific co-factors to Smads.

Promotion of EMT is known to be an important mechanism for TGF-β1-mediated fibrosis (Holian et al., 2008; Lan, 2003), in which β-catenin has been found to play a role (Chilosi et al., 2003; Douglas et al., 2006). We and others have shown that TGF-β1 induces nuclear accumulation of β-catenin in tubular cells, and that β-catenin targeting of certain genes results in EMT (Masszi et al., 2004; Zheng et al., 2009). However, it is unclear whether β-catenin acts as a co-factor for Smad transcriptional activity or as a downstream factor of Smad acting through the canonical Wnt pathway by binding to the LEF-1/TCF family. β-Catenin has both a cell membrane adhesion function involving E-cadherin, and a

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nuclear transcriptional function once translocated to the nucleus. Previous studies using small interfering RNA (siRNA) silencing (Kim et al., 2009b) or gene knockout depleted membrane-bound as well as cytosolic β-catenin. Most importantly, E-cadherin is unstructured without binding to membrane-bound β-catenin (Huber et al., 2001), and loss of E-cadherin itself causes EMT. Thus, proof of the dependence on β-catenin of TGF-β1-induced EMT requires selective targeting of the nuclear function of β-catenin. Despite conflicting evidence about the role of β-catenin in immune cells (Cobas et al., 2004; Mulroy et al., 2003; Xu et al., 2003; Yu et al., 2008), there are no reports of β-catenin involvement in TGF-β signaling in immune cells, particularly in TGF-β's anti-inflammatory actions.

To study the function of β-catenin, a protein knockdown chimera (F-TrCP-Ecad) was developed to selectively block the nuclear activity of β-catenin whilst leaving the membrane activity of β-catenin intact. This model demonstrated that suppression of cytosolic β-catenin inhibited the neoplastic growth of colorectal tumor cells (Cong et al., 2003). In the current study, we examined the role of β-catenin in TGF-β/Smad and Wnt/β-catenin cross-talk, to dissect the profibrotic from anti-inflammatory effects of TGF-β1, using this protein knockdown method to selectively degrade cytosolic β-catenin. This resulted in inhibition of EMT induced by TGF-β1 in renal tubular epithelial cells, but no effect on TGF-β1 inhibition of macrophage activation.

Results

TGF-β1-induced EMT in C1.1 cells is inhibited by cytosolic β-catenin knockdown chimera F-TrCP-Ecad

Subconfluent tubular epithelial cells (C1.1) cultured in the presence of 3 ng/ml TGF-β1 for 24 hours showed a change in morphology from cuboid clustered epithelial cells to spindle-shaped scattered fibroblast-like cells. Cells treated with TGF-β1 lost expression of the epithelial marker E-cadherin and acquired expression of the mesenchymal markers vimentin and fibronectin (Fig. 1A–F). After transient transfection with F-TrCP-Ecad, C1.1 cells with TGF-β1 treatment maintained a cobblestone epithelial morphology and growth pattern, and the transition from epithelial to mesenchymal markers was attenuated significantly, whereas cells transfected with pcDNA3 alone had no effect on TGF-β1-induced EMT (Fig. 1A–F). There were no changes in E-cadherin, vimentin and fibronectin levels in C1.1 cells transfected with F-TrCP-Ecad chimera only (Fig. 1A–F). This confirms the importance of β-catenin in the cellular response to TGF-β1-induced EMT and that targeted degradation of cytosolic β-catenin can suppress the TGF-β1-induced EMT in tubular epithelial C1.1 cells.

We further examined EMT changes downstream of TGF-β1. The repression of E-cadherin promoter activity after TGF-β1 stimulation was abolished by F-TrCP-Ecad chimera. Real-time RT-PCR and gelatin zymography showed that transfection of F-TrCP-Ecad chimera inhibited the expression of known TGF-β1 target genes in EMT, such as snail and MMP-9 (Fig. 2).

F-TrCP-Ecad chimera preferentially targets soluble nuclear/cytosolic β-catenin for degradation

To confirm F-TrCP-Ecad chimera targeting of cytosolic β-catenin, we separated membrane and cytoplasmic protein from F-TrCP-Ecad transfected and TGF-β1-treated C1.1 cells. As shown in Fig. 3, the level of F-TrCP-Ecad chimera expression in comparison to E-cadherin in C1.1 cells was shown by western blot (Fig. 3A). By design, at the level of F-TrCP-Ecad chimera expression in transiently transfected C1.1 cells, E-cadherin protein level was unchanged. TGF-β1 treatment reduced E-cadherin levels (Fig. 3A) while total β-catenin levels were unchanged (Fig. 3B). This is consistent with EMT of C1.1 cells. However, transfection of the F-TrCP-Ecad chimera in absence of TGF-β1 did not change total or membrane β-catenin protein levels (Fig. 3B,C) whereas it reduced the increase of cytosolic β-catenin in TGF-β1-treated cells (Fig. 3D). These results demonstrated that transient transfection of F-TrCP-Ecad targeted cytosolic β-catenin that had been redistributed to the cytosolic pool by TGF-β1 (Fig. 3C,D).

Knockdown of cytosolic β-catenin inhibits TGF-β1-induced EMT through inhibition of β-catenin/P-Smad3 but not β-catenin/LEF-1

Cross talk between different pathways has been implicated in EMT of both cancerous and non-cancerous epithelial cells. Of relevance, β-catenin/P-Smad3 (Borok, 2009; Kim et al., 2009a) and β-catenin/LEF-1 (Kim et al., 2002; Medici et al., 2006) pathways are activated when EMT occurs. However, it is unclear whether β-catenin acts as a co-factor to the Smad transcriptional complex, or if it acts downstream of Smad signaling through canonical Wnt in the TGF-β1-induced EMT of non-cancerous epithelial cells. As shown in Fig. 4A, we found that formation of β-catenin/P-Smad3 complex (indicated by western blotting of β-catenin and P-Smad3 in Smad3 co-IP of nuclear lysate) significantly increased (3.5 fold, $P<0.01$) 24 hours after TGF-β1 stimulation compared with untreated C1.1 cells, whereas F-TrCP-Ecad transfection markedly attenuated the formation of this complex as indicated by reduction of β-catenin levels in Smad3 co-IP. ICG-001, which selectively modulates β-catenin nuclear activity by reducing its binding to the cAMP-response element-binding protein binding protein (CBP) (Teo et al., 2005), was also found to inhibit the β-catenin/P-Smad3 complex formation in Smad3 co-IP (Fig. 4A). The co-IP result shows that F-TrCP-Ecad blocked β-catenin nuclear translocation by targeting its cytosolic pool (Fig. 4A, input). The effect of ICG-001 was by CBP-dependent inhibition of β-catenin binding to P-Smad3 in the nucleus. F-TrCP-Ecad transfection or ICG-001 treatment in the absence of TGF-β1 did not change the base line level of nuclear β-catenin in Smad3 co-IP. However, both TrCP-Ecad and ICG-001 may also block β-catenin interaction with LEF/TCF of the canonical Wnt pathway, thereby not excluding the dependence on β-catenin as a downstream effect of Smad. To test this, we performed co-IP of β-catenin with LEF-1 and TOP-flash assay to determine canonical Wnt/β-catenin/LEF/TCF signaling in C1.1 cells. Compared with untreated C1.1 cells, TGF-β1 treatment did not increase the level of β-catenin co-precipitated by anti-LEF-1 antibody (Fig. 4B), indicating that no β-catenin/LEF-1 complex formation was stimulated by TGF-β1. Furthermore, the TOP-flash/FOP-flash luciferase activity was increased ~15-fold in C1.1 cells of LiCl-treated a positive control (Fig. 4C), but only minimal TOP-flash/FOP-flash luciferase activity was detected and was almost unchanged ($P>0.05$) when C1.1 cells were treated with TGF-β1 with or without F-TrCP-Ecad transfection or ICG-001 treatment. These results demonstrated an absence of β-catenin/LEF/TCF dependent canonical Wnt signaling in TGF-β1-induced EMT in C1.1 cells, thus excluding a role for β-catenin through canonical Wnt. The presence of non-TGF-β1-stimulated low level β-catenin in LEF-1 co-IP may or may not explain the minimal level of TOP-flash/FOP-flash luciferase activity observed in C1.1 cells.

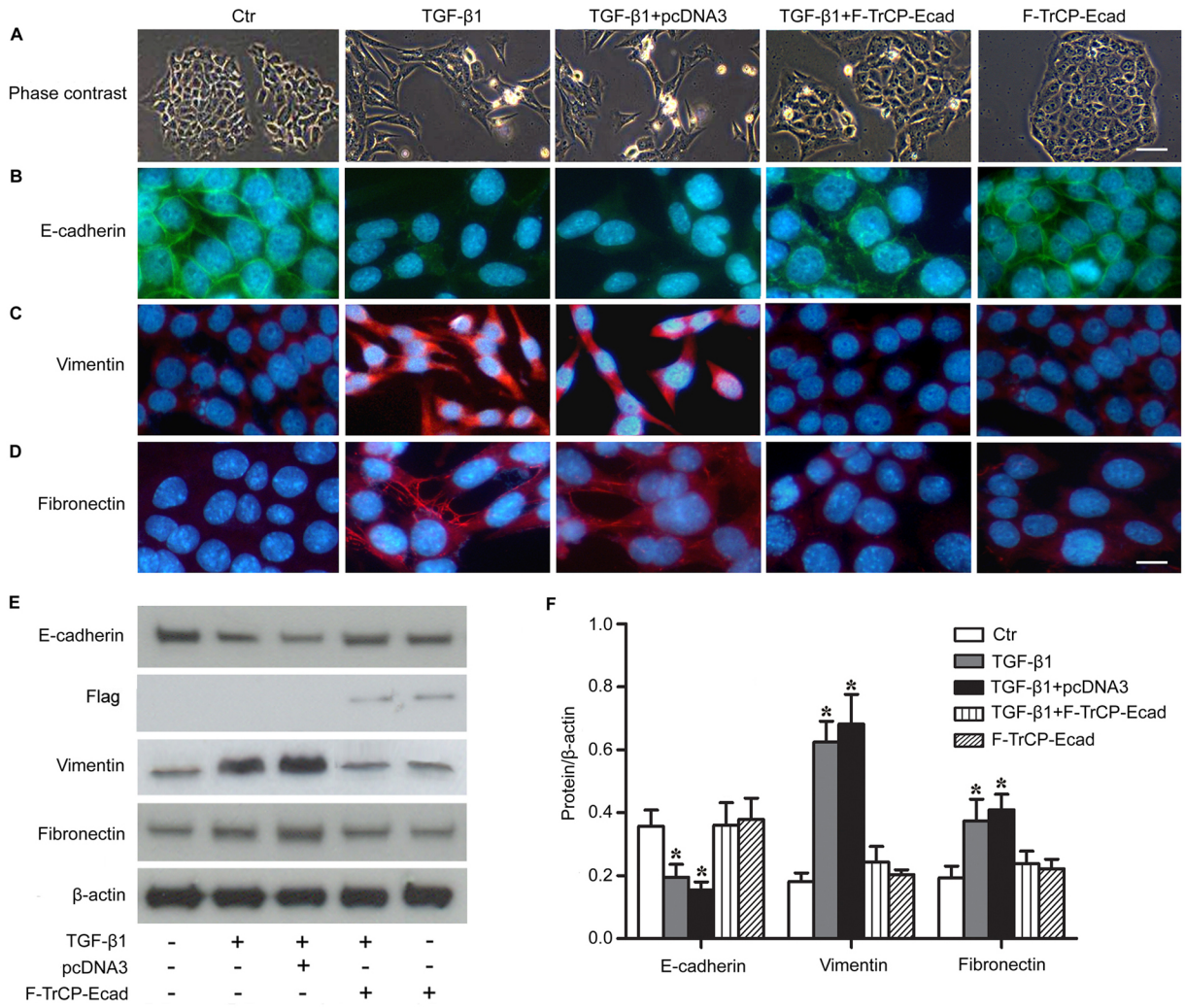

Fig. 1. β-Catenin knockdown chimera F-TrCP-Ecad inhibits TGF-β1-induced EMT in C1.1 cells. (**A**) Phase-contrast images of subconfluent C1.1 cells untreated (Ctr), or treated with TGF-β1 (3 ng/ml) for 24 hours, or transfected with pcDNA3 or F-TrCP-Ecad in the presence of TGF-β1, or transfected with F-TrCP-Ecad alone. Scale bar, 40 μm. (**B**–**D**) Immunofluorescence images of E-cadherin, (B) vimentin (C) and fibronectin (D) staining in C1.1 cells exposed to respective treatments. Scale bar, 10 μm. (**E**) Representative western blots of E-cadherin, Flag, vimentin and fibronectin compared with β-actin control in lysate of untreated or treated C1.1 cells. (**F**) Quantitative analysis of (E) by relative densitometry intensity. Data are presented as means±s.d. *$P<0.05$ vs Ctr, $n=6$.

To confirm β-catenin as a transcriptional co-factor of Smad in TGF-β1-induced EMT, we performed a SMAD reporter assay as an indicator of TGF-β1-induced Smad signaling. As shown in Fig. 4D, TGF-β1 induced a 7-fold increase in Smad reporter activity in C1.1 cells, However, disruption of β-catenin/Smad3 complex formation by F-TrCP-Ecad transfection, or by inhibition of β-catenin/Smad3 using ICG-001 (shown in Fig. 4A) blocked TGF-β1-induced Smad reporter activity to about 1/3, proving a dependence on β-catenin of Smad3 binding to Smad transcriptional responsive elements. The remaining TGF-β1-induced Smad reporter activity after blockade with F-TrCP-Ecad or ICG-001 was presumably non-β-catenin dependent.

Both Smad3 and Smad2 were absent from LEF-1 co-IP (Fig. 4B), whilst no LEF-1 was presented to P-Smad3 Co-IP (Fig. 4A), indicating that there were two distinct pools of nuclear β-catenin, binding to Smads or LEF-1. TGF-β1 did not stimulate an increase of β-catenin expression, nor β-catenin/LEF-1 complex formation and signaling. Together with the results of F-TrCP-Ecad blockade and especially ICG-001 blockade of β-catenin/Smad3 complex formation to inhibit TGF-β1 signaling, our results demonstrated that β-catenin/Smad3 interaction is responsible for TGF-β1-induced EMT in C1.1 cells.

These results suggest that TGF-β1-induced EMT in C1.1 cells involves β-catenin as a transcriptional co-factor for Smad3, but not as a downstream factor of Smad involving β-catenin/LEF-1 interaction. Degradation of cytosolic β-catenin or direct inhibition of β-catenin binding to its co-factors diminished β-catenin/P-Smad3 complex formation and blocked TGF-β1-induced EMT in non-cancer C1.1 cells.

β-Catenin is dispensable for TGF-β1 inhibition of macrophage activation

We used lipopolysaccharide (LPS)-stimulated TNF-α expression and interferon-γ (IFN-γ)-induced inducible NO synthase (iNOS) expression as markers of macrophage activation in J774 cells. TGF-β1 inhibited the expression of LPS-induced inflammatory

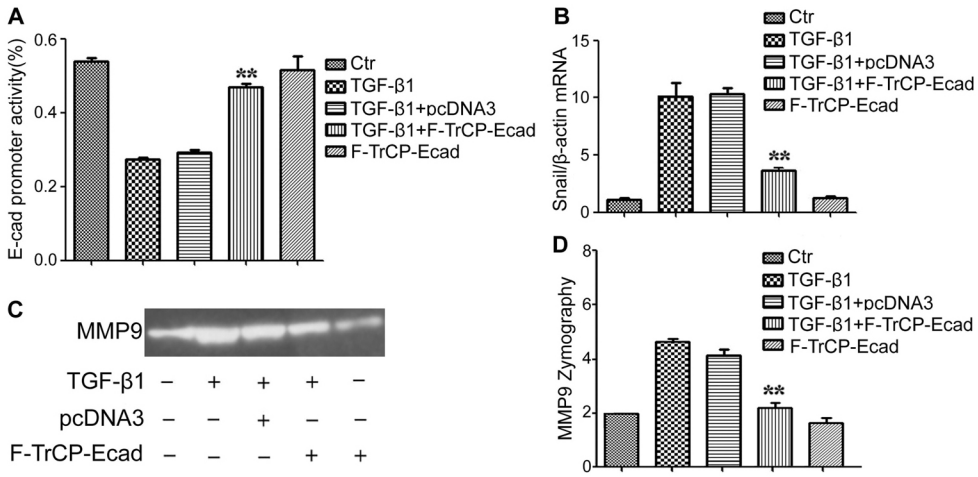

Fig. 2. β-Catenin knockdown chimera F-TrCP-Ecad abolishes TGF-β1-induced E-cadherin promoter repression, Snail transcription and MMP-9 expression in C1.1 cells. (**A**) Relative E-cadherin promoter (−201 to +131) activity in C1.1 cells of control, pcDNA3 or F-TrCP-Ecad transfection treated with TGF-β1 (3 ng/ml) for 24 hours, and F-TrCP-Ecad transfection alone. Values are means±s.d. $^{**}P<0.01$ vs TGF-β1 with or without vector control (pcDNA3), $n=6$. (**B**) The expression of Snail in C1.1 cells with corresponding treatments was analyzed by real-time PCR. Values are means±s.d. $^{**}P<0.01$ vs TGF-β1 with or without vector control (pcDNA3), $n=6$. (**C**) Representative MMP-9 gelatin zymography of supernatants from C1.1 cells with corresponding treatments. (**D**) Quantitative analysis of (C). Relative MMP-9 zymograph intensity was calculated against untreated medium. Values are means±s.d. $^{**}P<0.01$ vs TGF-β1 with or without vector control (pcDNA3), $n=6$.

mediator TNF-α mRNA, and of iNOS mRNA expression stimulated by IFN-γ in J774 cells (Fig. 5E,F). However, TGF-β1-induced downregulation of macrophage activation was not affected in J774 cells transfected with F-TrCP-Ecad or WT β-cat, or treated by ICG-001 (Fig. 5E,F). F-TrCP-Ecad chimera only transfected and ICG-001 or TGF-β1 only treated J774 cells maintained a similar state of activation to controls (Fig. 5E,F).

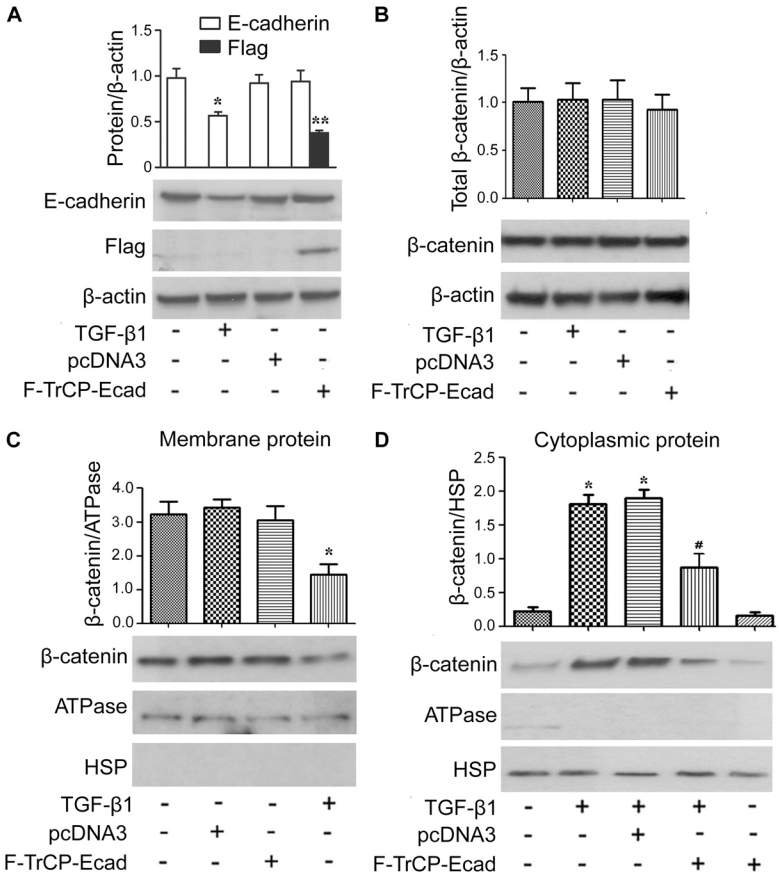

Fig. 3. F-TrCP-Ecad promotes soluble nuclear/cytosolic β-catenin degradation. (**A**) Western blot analysis for E-cadherin and F-TrCP-Ecad (Flag) in C1.1 cells untreated or treated with TGF-β1 (3 ng/ml) for 24 hours, or transfected with pcDNA3 or F-TrCP-Ecad. Values are means±s.d. in relative densitometry intensity. $^{*}P<0.05$ vs untreated group, $^{**}P<0.01$ vs E-cadherin, $n=3$. (**B**) Western blot analysis for total β-catenin in C1.1 cells untreated, treated with TGF-β1 (3 ng/ml) for 24 hours, or transfected with pcDNA3 or F-TrCP-Ecad. Values are means±s.d., $n=3$. (**C**) Western blot analysis of β-catenin levels in membrane protein fraction extracted from C1.1 cells untreated, treated with TGF-β1 (3 ng/ml) for 24 hours, or transfected with vector control (pcDNA3) or F-TrCP-Ecad alone. α1 Na/K-ATPase (ATPase) was used as a membrane protein control. Values are means±s.d. $^{*}P<0.05$ vs the other three groups, $n=6$. (**D**) Western blot analysis of β-catenin levels in cytoplasmic fraction extracted from C1.1 cells untreated, or treated with TGF-β1 (3 ng/ml) for 24 hours and transfected with vector control (pcDNA3) or F-TrCP-Ecad, or transfected with F-TrCP-Ecad alone. Heat shock protein 90 (HSP) was used as the cytoplasmic protein control. Values are means±s.d. $^{*}P<0.01$ vs control, $^{\#}P<0.05$ vs TGF-β1 with or without vector control (pcDNA3), $n=6$.

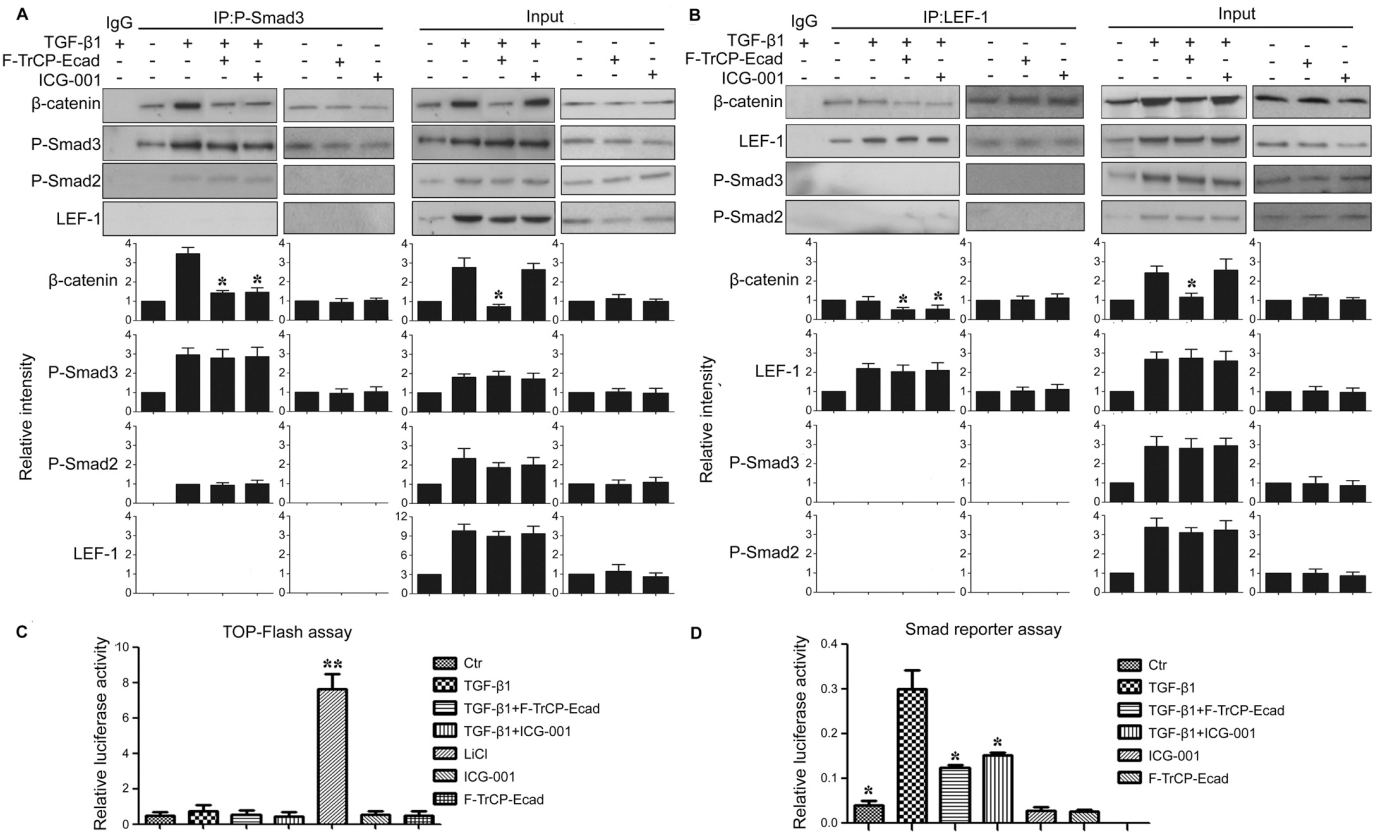

Fig. 4. Knockdown of cytosolic β-catenin inhibits TGF-β1-induced EMT through β-catenin/P-Smad3 but not β-catenin/LEF-1. (**A**) Co-immunoprecipitation (IP) of β-catenin, P-Smad3, P-Smad2 and LEF-1 from C1.1 cell nuclear proteins with rabbit anti-P-Smad3 antibody. Purified rabbit IgG was used as a negative control. Immunoprecipitates were analyzed by western blotting with anti-β-catenin, anti-P-Smad3, anti-P-Smad2 and anti-LEF-1 antibodies. Input refers to analysis of nuclear proteins before immunoprecipitation. Quantification of immunoblots (relative intensity) is shown by fold increase in densitometry intensity (the intensity of the control is arbitrarily defined as 1). Values are means±s.d. *$P<0.05$ vs TGF-β1 treatment group, $n=3$. (**B**) Co-immunoprecipitation of β-catenin, LEF-1, P-Smad3 and P-Smad2 from C1.1 cell nuclear proteins with rabbit anti-LEF-1 antibody. Purified rabbit IgG was used as a negative control. Immunoprecipitates were analyzed by western blot with anti-β-catenin, anti-LEF-1, anti-P-Smad3 and anti-P-Smad2 antibodies. Values are means±s.d. *$P<0.05$ vs TGF-β1 treatment group, $n=3$. (**C**) Interactions between β-catenin and LEF-1 were shown by TOP-flash assay. The relative luciferase activity in C1.1 cells transfected with FOP-flash and TOP-flash vectors are shown in control (Ctr), TGF-β1-treated, TGF-β1-treated and F-TrCP-Ecad-transfected, TGF-β1- and ICG-001-treated, LiCl-treated positive control, and ICG001-alone-treated or F-Trcp-Ecad-alone-transfected C1.1 cells. Values are means±s.d. **$P<0.01$ vs the other six groups, $n=6$. There was no statistical difference in TOP-flash activity among the Ctr, TGF-β1-only and TGF-β1 with F-TrCP-Ecad or ICG-001 groups ($P>0.05$). (**D**) SMAD reporter assay in C1.1 cells. The SMAD reporter relative response luciferase activities in C1.1 cells are shown in control (Ctr), TGF-β1-treated, TGF-β1-treated and F-TrCP-Ecad-transfected or ICG-001-treated, and ICG001-alone-treated or F-Trcp-Ecad-alone-transfected C1.1 cells. Values are means±s.d. *$P<0.05$ vs TGF-β1-only group, $n=6$.

The steady state level of β-catenin was low (Fig. 5A,B) and there was no membrane staining of β-catenin in J774 cells (data not shown). IFN-γ or LPS with or without TGF-β1 had no effect on β-catenin expression (Fig. 5A,B). Similar to C1.1 cells, transfection of F-TrCP-Ecad chimera significantly reduced endogenous β-catenin levels (Fig. 5C,D. $P<0.05$) proving the effectiveness of the chimera in knocking down cytosolic β-catenin in macrophages.

β-Catenin is not involved in Smad3-dependent TGF-β1 inhibition of macrophage activation in J774 cells

We also carried out TOP-flash assay and P-Smad3 co-IP in J774 cells to investigate if β-catenin signaling is involved in Smad3-dependent (Werner et al., 2000) TGF-β1 inhibition of macrophage activation. As shown in Fig. 6A, there was a very low level of nuclear β-catenin present in P-Smad3 co-IP in J774 cells, and it was not changed in response to TGF-β1 treatment. F-TrCP-Ecad chimera or ICG-001 did not change the levels of nuclear β-catenin of either total input or Smad3 co-IP, suggesting no β-catenin nuclear translocation or binding to Smad3, No Smad2 was present in P-Smad3 co-IP. TOP-flash/FOP-flash luciferase activity was low and not changed in J774 cells treated with TGF-β1 with or without F-TrCP-Ecad transfection (Fig. 6B), showing an absence of β-catenin/LEF/TCF dependent canonical Wnt signaling. As a positive control, LiCl stimulated TOP-flash luciferase activity was blocked by F-TrCP-Ecad transfection or ICG-001 treatment (Fig. 6B), proving their effectiveness as inhibitors of β-catenin-dependent canonical Wnt signaling in J774 macrophages.

To further confirm that β-catenin was not involved in TGF-β1 inhibition of macrophage activation, we performed a SMAD reporter assay in J774 cells. TGF-β1 induced Smad dependent luciferase activity in J774 cells, but neither F-TrCP-Ecad transfection nor ICG-001 treatment had any effect on the TGF-β1-induced Smad reporter activity in J774 cells. Interestingly, the TGF-β1-induced Smad reporter activity in J774 cells was at about the same level as in C1.1 cells when β-catenin/Smad

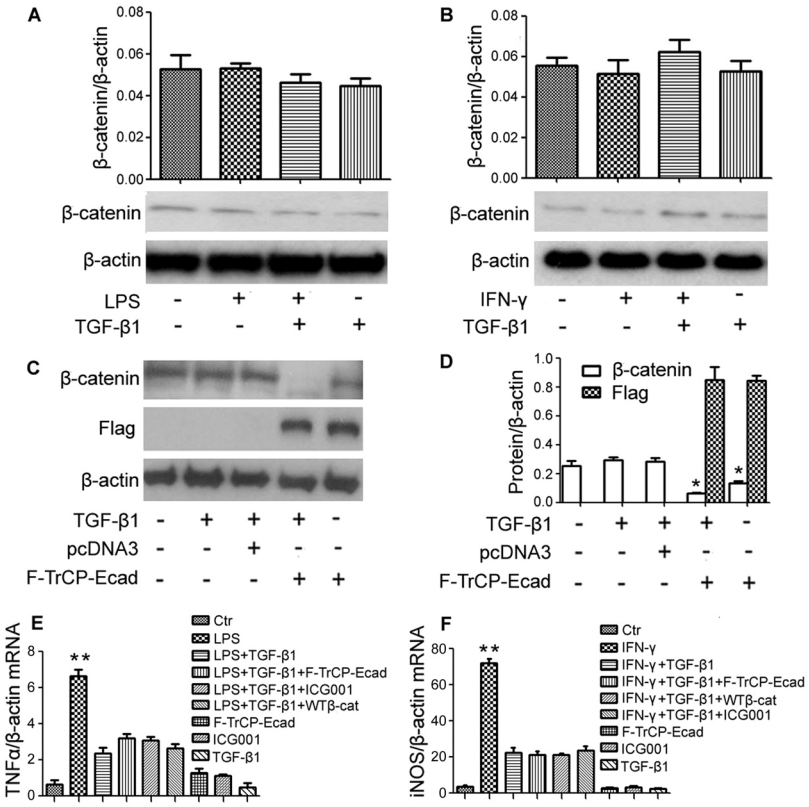

Fig. 5. β-Catenin is dispensable for macrophage activation, and F-TrCP-Ecad or ICG-001 has no effect on anti-inflammatory effects of TGF-β1 in macrophages. (**A**) Total β-catenin protein levels in J774 cells stimulated by LPS (500 ng/ml) in the absence or presence of TGF-β1 for 24 hours, or treated with TGF-β1 alone. Quantification is shown by relative densitometry intensity. Values are means±s.d. (**B**) Total β-catenin protein levels in J774 cells stimulated by IFN-γ (20 ng/ml) in the absence or presence of TGF-β1 (10 ng/ml) for 24 hours, or treated with TGF-β1 alone. (**C**) and (**D**) Western blot analysis for total β-catenin levels and F-TrCP-Ecad expression (Flag) in J774 cells untreated, treated with TGF-β1 (3 ng/ml) for 24 hours with or without transfection of pcDNA3 or F-TrCP-Ecad, or transfected with F-TrCP-Ecad alone. Values are means±s.d. $*P<0.05$ vs other groups, $n=6$. (**E**) Real-time PCR analysis of TNF-α expression in J774 cells stimulated by LPS in the presence of TGF-β1 with F-TrCP-Ecad or WT β-cat transfection or ICG-001 treatment, or treated with TGF-β1 or ICG-001 alone, or transfected with F-TrCP-Ecad alone. Values are means±s.d. $**P<0.01$ vs the other eight groups, $n=6$. (**F**) Real-time PCR analysis of iNOS expression in J774 cells stimulated by IFN-γ in the presence of TGF-β1 with F-TrCP-Ecad or WT β-cat transfection or ICG-001 treatment, or treated with TGF-β1 or ICG-001 alone, or transfected with F-TrCP-Ecad alone. Values are means±s.d. $**P<0.01$ vs the other eight groups, $n=6$.

interaction was inhibited by F-TrCP-Ecad chimera or ICG-001, further confirming an absence of β-catenin from the Smad complexes in macrophages. These results excluded involvement of β-catenin in Smad3-dependent TGF-β1 inhibition of macrophage either by acting as co-factor to Smad or by interacting with LEF-1.

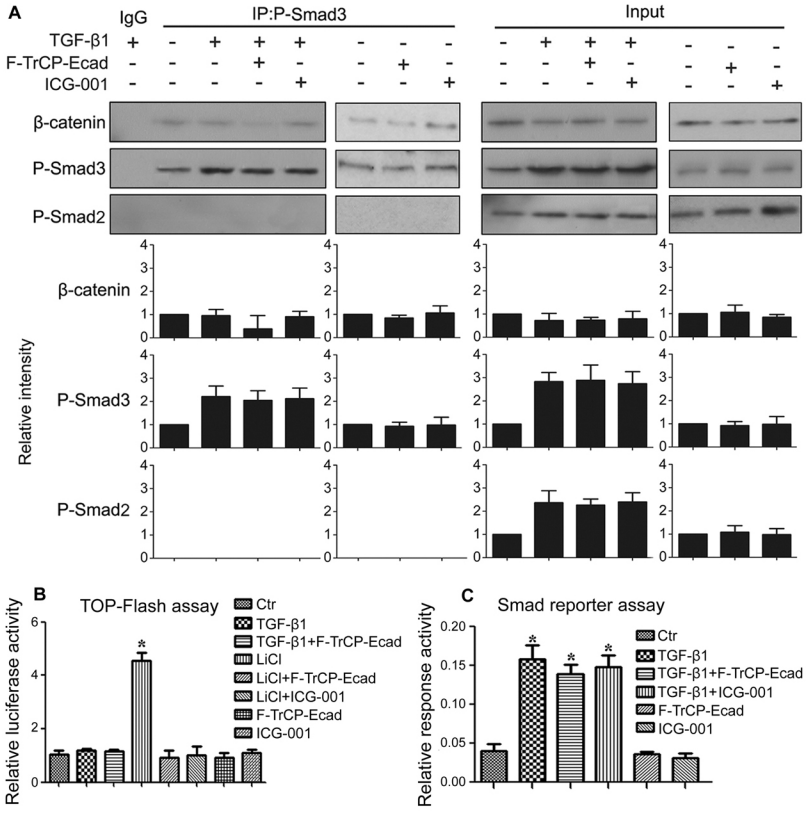

Fig. 6. β-Catenin is not involved in Smad3-dependent TGF-β1 inhibition of macrophage activation in J774 cells. (**A**) Co-immunoprecipitation of β-catenin, P-Smad3 and P-Smad2 from J774 cell nuclear proteins with rabbit P-Smad3 antibody. Purified rabbit IgG was used as a negative control. Immunoprecipitates were analyzed by western blotting with anti-β-catenin, anti-P-Smad3 and anti-P-Smad2 antibodies. Quantification of immunoblots (relative intensity) is shown by fold increase in densitometry intensity (the intensity of the control is arbitrarily defined as 1). Values are means±s.d. (**B**) TOP-flash relative luciferase activities in J774 cells are shown in control (Ctr), TGF-β1-treated cells with or without F-TrCP-Ecad transfection, LiCl-treated cells with or without F-TrCP-Ecad transfection or ICG-001 treatment, F-TrCP-Ecad-alone-transfected or ICG-001-alone-treated groups. Values are means±s.d. $*P<0.01$ vs the other seven groups, $n=6$. (**C**) SMAD reporter relative response luciferase activities in J774 macrophages are shown in control (Ctr), TGF-β1-treated, TGF-β1-treated and F-TrCP-Ecad-transfected or ICG-001-treated, and F-TrCP-Ecad-alone-transfected or ICG-001-alone-treated groups. Values are means±s.d. $*P<0.05$ vs Ctr, $n=6$.

Taken together, our results demonstrated that β-catenin is not involved in the anti-inflammatory effects of TGF-β1 in J774 macrophage cells.

Discussion

In this study, we showed for the first time that β-catenin can be targeted to dissect the profibrotic effect of TGF-β1 in renal tubular epithelial cells from its anti-inflammatory effects through macrophages. We demonstrated that targeted degradation of cytosolic β-catenin or inhibition of β-catenin binding to Smad3 blocked TGF-β1-induced EMT, and that this depends on β-catenin/Smad3 interaction as transcriptional co-activators but does not depend on canonical Wnt signaling in murine renal tubular epithelial C1.1 cells. However, TGF-β1-mediated inhibition of macrophage LPS-induced TNF-α and IFN-γ-stimulated iNOS expression does not involve β-catenin/Smad3 interaction or canonical β-catenin/LEF-1 signaling. Thereby, TGF-β1 inhibition of macrophage activation was not compromised by the targeted inhibition of β-catenin/Smad3 interaction.

EMT plays a substantial role in the pathogenesis of fibrotic diseases (Hewitson, 2009). Although the contribution of EMT to renal fibrosis has been challenged recently (Humphreys et al., 2010), it is still generally accepted that EMT does contribute to the population of myofibroblasts, the main effector cells in kidney fibrosis. Iwano et al. found that 36% of myofibroblasts originate from renal tubular epithelial cells via EMT (Iwano et al., 2002). The process of EMT involves loss of epithelial characteristics with downregulation of epithelial markers such as E-cadherin and gain of a mesenchymal phenotype with expression of mesenchymal markers vimentin and fibronectin together with MMPs (Rastaldi, 2006; Savagner, 2001). Amongst the numerous growth factors, cytokines, hormones and extracellular matrix (ECM) components that regulate EMT, TGF-β1 has been found to be the most important. However, inhibition of TGF-β to prevent fibrosis would also abrogate its protective anti-inflammatory activity.

TGF-β signaling is mediated predominantly through a Smad-dependent pathway. Smads have been shown to cross-talk with components of other pathways such as RhoA, Ras, MAPK, Notch and Wnt/β-catenin. Most importantly, TGF-β/Smad cross-talks with MAPK, ILK and Wnt (Guo and Wang, 2009), which are three major EMT pathways (Chun et al., 2008; Nieto, 2011) that converge at direct or indirect inactivation of GSK-3β (Hu et al., 2008; Thornton et al., 2008). Consequently, activating β-catenin whose target genes (e.g. Snail, Twist, LEF1, Jagged 1) are key transcription factors inducing EMT. Nevertheless, Smad-dependent transcription is further dependent on other transcription co-factors that are both constitutive and cell type specific (Kim et al., 2009a). The transcription co-factors, especially those that are cell type specific, not only stabilize Smad association with DNA, but may also determine which specific gene(s) that the Smad complex targets. β-Catenin of the Wnt pathway has been found to form β-catenin/Smad complexes in TGF-β-induced EMT in lung alveolar epithelial cells (Kim et al., 2009a; Zhou et al., 2012) and to facilitate β-catenin transcriptional activity in chondrocytes (Zhang et al., 2010). However, the detailed molecular mechanisms remain poorly understood. It is still unclear whether the cross-talk between TGF-β/Smad and Wnt depends on β-catenin acting as a transcriptional co-factor to a Smad, or whether it depends on canonical Wnt signaling in which β-catenin is downstream of Smad while its transcriptional activity depends on β-catenin/LEF-1/TCF signaling. In the current study, we provide evidence for pivotal dependence on β-catenin as a co-factor to Smad3 but not LEF-1 in mediating TGF-β1-induced EMT. We found that the β-catenin/p-Smad3 interaction as transcriptional co-factors determines TGF-β1-induced EMT in mouse proximal tubular epithelial C1.1 cells. This contrasts to the finding in chondrocytes that Smad binding of β-catenin facilitates canonical Wnt signaling (Zhang et al., 2010).

Previous studies using dominant negative β-catenin or β-catenin small hairpin RNA (shRNA) knockdown (Kim et al., 2009b) not only inhibited cytosolic β-catenin, but also disrupted membrane-bound β-catenin that may result in direct disruption of E-cadherin. More importantly, the mechanism underlying dependence on β-catenin of TGF-β-induced EMT was unknown. In support of such a role for cytosolic β-catenin in EMT and resultant fibrosis, a recent publication reported attenuation of bleomycin-induced lung fibrosis in mice by direct inhibition of β-catenin/CBP nuclear function (Henderson et al., 2010).

Canonical Wnt signaling is classically demonstrated by TOP-flash reporter activity when β-catenin binds to LEF-1/TCF and transcriptionally activates the reporter. Consistent with prior reports by others (Kim et al., 2009a), we also found that TOP-flash was not activated in C1.1 cells in response to TGF-β1. We found that LEF-1 is not involved in TGF-β1-induced β-catenin/Smad3 complex formation and does not form complexes with β-catenin in response to TGF-β1 stimulation. This result not only explains the absence of TGF-β1-stimulated TOP-flash activity in epithelial cells, but also demonstrated a difference of TGF-β1-induced EMT in adult epithelial cells (Zeisberg and Duffield, 2010), in contrast to EMT in cancer and embryonic epithelial cells, where canonical Wnt signaling is involved (Bienz, 2005; MacDonald et al., 2009). More importantly, our study suggests that specific targeting of β-catenin/p-Smad but not β-catenin/LEF-1/TCF may prevent TGF-β1-induced profibrotic effects without abolishing canonical Wnt signaling involved in tissue regeneration by stem cells.

In addition to its profibrotic effect, TGF-β1 is anti-inflammatory (Li et al., 2006). TGF-β downregulates inflammatory processes by inhibiting activated immune cells and by inducing suppressive immune cells (Schmidt-Weber and Blaser, 2004). The concomitant profibrotic effect of TGF-β1 has limited its clinical use as an anti-inflammatory agent. We hypothesized that dependence on transcriptional co-factors to Smads in immune cells may differ from that of non-immune cells thereby generating different effects of TGF-β1. Among components of the various Smad and non-Smad signal transduction pathways activated by TGF-β1 (Wang et al., 2010), β-catenin is unique in that it has not, according to the published literature, been linked to anti-inflammatory processes. This suggests that by targeting β-catenin, it may be possible to separate the anti-inflammatory and profibrotic effects of TGF-β1.

Stimulation of macrophages with IFN-γ and/or microbial products such as LPS induces their activation (Bogdan et al., 1992; Takaki et al., 2006). Smad3 is reported to be a critical transcription factor responsible for TGF-β1-mediated inhibition of macrophage activation (Werner et al., 2000). To examine the role of β-catenin in macrophage activation, we chose iNOS and TNF-α as markers of macrophage activation and found that IFN-γ, LPS and TGF-β1 were not able to trigger β-catenin expression in macrophages, and that the addition of WT β-catenin or

inhibition of β-catenin/Smad3 interactions by the β-catenin knockdown chimera or ICG-001 had no effect on TGF-β1-mediated inhibition of macrophage activation. Thus we demonstrated for the first time, that β-catenin is dispensable in TGF-β1-mediated inhibition of macrophage activation.

In this study, we present novel evidence that inhibition of β-catenin/Smad3 interactions can separate the profibrotic from anti-inflammatory actions of TGF-β1, thereby preventing the pro-fibrotic effect of TGF-β without interfering with its anti-inflammatory actions. Further investigations are required in other immune and non-immune cells that TGF-β targets for its pro-fibrotic and anti-inflammatory effects, with the ultimate aim of defining a key therapeutic target in fibrotic inflammatory diseases.

Materials and Methods

Plasmids

F-TrCP-Ecad constructs encoding a chimeric protein with the β-catenin binding domain of E-cadherin fused to βTrCP ubiquitin-protein ligase were kindly provided by Dr Feng Cong. Wild-type (Buchert et al., 2010) β-catenin (WT β-cat) construct: Flag-tagged WT β-cat fused into pcDNA3. E-cadherin promoter luciferase reporter constructs: pmoEcad (−201 to −131)/GL3, with mouse E-cadherin promoter −201 to −131 driving firefly luciferase reporter gene. pRL-RSV (RSV promoter driving *Renilla* luciferase) was used as a transfection control. Plasmids were prepared using EndoFree plasmid kits (Qiagen).

Cell culture and treatment

C1.1 renal tubular epithelial cells (a kind gift from the laboratory of Dr Rudolf Wüthrich, Switzerland) were selected and maintained in DMEM:HAM's F12 (1:1 v/v) medium (Invitrogen) supplemented with 25 μg/ml of EGF (Sigma-Aldrich, St. Louis, MO), 25 μM of HEPES (Invitrogen), 5 μg/ml insulin (Sigma), 1.25 ng/ml prostaglandin E1 (Cayman Chemicals, Ann Arbor, MI), 0.0338 ng/ml 3,3,5-triiodothyonine (Sigma) 5 μg/ml hydrocortisone (Sigma), 1.73 ng/ml transferrin (Sigma), 18 ng/ml sodium selenite (Sigma) and 5% fetal calf serum (FCS) (Invitrogen) at 37℃, in 5% CO2. J774 macrophages were cultured in DMEM (Invitrogen) containing 10% FCS. For treatment of either C1.1 or J774 cells, the corresponding serum-supplemented media were replaced with respective serum free media.

In experiments of TGF-β1 treatments, subconfluent cultures of C1.1 cells were rinsed with PBS (Invitrogen) and then treated with 3 ng/ml TGF-β1 (Biosource). J774 cells were stimulated with either 20 ng/ml IFN-γ (R&D Systems) with or without 10 ng/ml TGF-β1 (Takaki et al., 2006) or 500 ng/ml LPS (Sigma) with or without pre-treatment (30 min) with 15 ng/ml TGF-β1 (Bogdan et al., 1992). After treatment for 24 h, cells were harvested for analysis over three independent experiments. For transfection experiments, 2×10^5 cells were seeded into 12-well plates. After the cells reached about 70–80% confluence, Lipofectamine 2000 (Invitrogen) or PromoFectin-Macrophage (Banksia Scientific) was used to transiently transfect C1.1 or J774 cells according to the manufacturers' protocols.

Subcellular protein extraction

Cultured C1.1 and J774 cell lines were harvested, washed and pelleted in phosphate-buffered saline (PBS) at 850 ***g*** for 2 minutes. Each cell pellet, containing 5×10^6 cells, was subjected to membrane protein extraction using ProteoJET™ Membrane Protein Extraction Kit (Thermo Scientific) and cytoplasmic and nuclear protein extraction using ProteoJET™ Cytoplasmic and Nuclear Protein Extraction Kit (Thermo Scientific) as described by the manufacturer. The isolated membrane and cytoplasmic protein fractions were used directly in SDS-PAGE and western blotting, and nuclear protein was used in co-immunoprecipitation.

Luciferase reporter assay

C1.1 cells were split into 12-well plates and each well received 0.5 μg E-cadherin promoter luciferase reporter construct, 0.02 μg *Renilla* luciferase construct pRL-RSV, with or without 1.0 μg of listed plasmids. After treatment for 24 h, cells were lysed and Firefly luciferase and *Renilla* luciferase activities were measured using Dual-Glo luciferase assay system (Promega) by a luminometer (Wallac 1420 Victor Plate Reader, Perkin Elmer Life Sciences). The Firefly luciferase activity was normalized against *Renilla* luciferase activity.

TOP-flash assay

The β-catenin reporter plasmid, TOP-flash, and its mutant control, FOP-flash, were purchased from Millipore Corporation. Cells were plated onto 12-well plates as described above. DNA quantities used in transfections were as follows: Top-flash or FOP-flash at 1 μg/well; F-TrCP-Ecad at 0.5 μg/well; pRL-RSV *Renilla* luciferase plasmid (0.1 μg/well) was used as an internal control for transfection efficiency. After 24 hours, cells were treated as described above. Cells were lysed and Luciferase activities were measured 24 hours after treatment using a Dual-Glo Luciferase Assay System (Promega) in Wallac 1420 Victor Plate Reader. Experiments were performed in triplicate and repeated at least 3 times. Relative luciferase activities were expressed as the ratio of TOP-flash/FOP-flash luciferase activity, each normalized against *Renilla* luciferase activity. The mean values of the normalized ratios were compared.

SMAD reporter assay

The SMAD reporter assay kit (SABiosciences) was used for detecting TGF-β1-induced Smad signaling. The TGFβ-responsive luciferase construct encodes the firefly luciferase reporter gene under the control of a minimal CMV promoter and tandem repeats of the SMAD transcriptional response element (AGCCAGACA). Cells were plated onto 24-well plates. DNA quantities used in transfections were as follows: SMAD reporter, negative control or positive control at 1 μg/well; F-TrCP-Ecad at 0.5 μg/well. Twenty-four hours after transfection, medium was changed and cells were treated with TGF-β1 in the presence or absence of F-TrCP-Ecad or ICG-001 (5 μM) for 24 hours. Dual-Glo Luciferase Assay System (Promega) was used for measurement of Smad reporter activity. All experiments were performed in triplicate and repeated at least 3 times. Relative luciferase activity (RLA) is presented as the ratio between the firefly luciferase activity and the *Renilla* luciferase activity. SMAD reporter relative response activity (RRA)=(RLA Experimental sample-RLA Negative control)/(RLA Positive control-RLA Negative control).

Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) was performed using the Protein-G Immunoprecipitation Kit (Sigma). In brief, cell nuclear proteins were extracted and then quantified by Bradford assay (Bio-Rad). Co-IP was carried out with 2 μg of affinity-purified antibody per 800 μg nuclear proteins supplemented with 30 μl of Protein-G agarose beads overnight at 4℃. The agarose beads were collected by pulse centrifugation, the supernatant was discarded and beads were washed 5 times with 1 ml IP buffer. Finally, immunoprecipitated proteins were subjected to SDS-PAGE protein separation followed by western blot with specific primary antibodies. Western blot analyses of nuclear proteins before immunoprecipitation served as 'input'.

Western blot analysis

The whole cell lysates or nuclear proteins were homogenized in Tris glycine SDS sample buffer (Gradipore). The proteins or precipitates of Co-IP were separated by NuPAGE 4–12% Bis-Tris gel (Invitrogen) and were blotted onto a nitrocellulose membrane. The membrane was blocked by 5% Skim milk in PBS at 37℃ for 1 h, and subsequently exposed to specific primary antibodies [anti-β-catenin, anti-E-cadherin (1:500; BD Bioscience), anti-vimentin (1:200; Abcam), anti-fibronectin (1:100; Santa Cruz Biotechnology), anti-flag (1:500; Sigma), anti-P-Smad2, anti-P-Smad3 and anti-LEF-1(1:200; Cell Signaling)] overnight at 4℃. The membranes were washed and incubated with their respective horseradish peroxidase-conjugated secondary antibodies for 40 min at room temperature. Finally, enzyme activity was detected with the chemiluminescence detection kit. Protein expression was measured with image software (KODAK) by quantifying the relative expression of target protein versus-β-actin.

Morphological and immunofluorescence microscopy

Subconfluent cells, grown on glass coverslips, were treated with listed agents for 24 hours. Cells were washed twice with PBS, fixed with 100% cold methanol for 20 min at −20℃, and nonspecific binding sites were blocked by cellular incubation for 1 h with 2% BSA (Sigma) and 0.2% Triton X-100 in PBS at room temperature. Cells were then incubated in primary antibodies diluted in 2% BSA in PBS for 1 hour at room temperature, followed by four washes with PBS. Cells were incubated with appropriate secondary antibodies: goat anti-mouse IgG2a/2b FITC conjugated antibodies (1:200; BD Biosciences Pharmingen), goat anti-rabbit Texas red conjugated antibodies (1:100; Calbiochem). Cells were washed four times with PBS and nuclei were counterstained by incubation with 4′,6-diamidino-2 phenylindole (DAPI) (Invitrogen) for 5 minutes, then washed again with PBS before mounting with fluorescence mounting medium (Dako). Images were captured using Spot Advanced version 3.4 (Diagnostic Instruments Inc.) with a fluorescence microscope (Olympus BX51) and Spot RT Slider camera.

Real-time RT-PCR analysis

Total RNA was isolated and purified from cells using RNeasy Mini Kit (Qiagen) and RNA samples were quantified by ultraviolet absorbance at 260 nm. cDNA was synthesized using SuperScript III first-strand synthesis system (Invitrogen). The primers were used as follows: Snail, forward: 5′-CTTGTGTCTGCACGACCTGT-3′; reverse: 5′-CTTCACATCCGAGTGGGTTT-3′ (product size 181 bp); (iNOS), forward: 5′-CACCTTGGAGTTCACCCAGT-3′; reverse: 5′-ACCACTCGTACT

TGGGATGC-3′ (170 bp); TNF-α, forward: 5′-GCTGAGCTCAAACCCTGGTA-3′; reverse: 5′-CGGACTCCGCAAAGTCTAAG-3′ (118 bp); β-actin, forward: 5′-GATTACTGCTCT GGCTCCTAGCA-3′; reverse: 5′-GCCACCGATCCACACA-GAGT-3′ (161 bp). Real-time PCR was performed using the Rotogene-6000 Real-Time Thermo cycler and was cycled for 2 min at 50℃, 10 min at 95℃ followed by 40 cycles at 95℃ for 15 sec and 1 min at 60℃.

Zymography and quantification

MMP-9 activity in the supernatants of C1.1 cells was determined with gelatin zymography as described previously (Tan et al., 2010). Briefly, supernatants were mixed with Tris-Glycine SDS native sample buffer (1:1) (Invitrogen) and electrophoresed through 10% Novex® zymogram gelatin gels (Invitrogen). After incubation with zymogram renaturing buffer (Invitrogen) for 30 min, the gel was washed and further incubated with developing buffer (Invitrogen) for 24 hours at 37℃. A band was visualized by staining with 0.5% (w/v) Coomassie Blue R-250 (Bio-Rad) in 50% (v/v) methanol, 10% (v/v) acetic acid for 30 min and destaining with 50% (v/v) methanol, 10% (v/v) acetic acid for 30 min at room temperature. MMP-9 activity was quantified by densitometry using Adobe Photoshop 8 software.

Statistical analysis

Results from at least three independent experiments were expressed as mean ± standard deviation (SD). Student's *t*-test was used to determine the significance of differences between two groups, whereas the one way analysis of variance (ANOVA) was used for comparison of multiple groups. $*P<0.05$ was used to indicate statistical significance.

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