APC and Smad7 link TGFβ type I receptors to the microtubule system to promote cell migration

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ABSTRACT **Cell migration occurs by activation of complex regulatory pathways that are spatially and temporally integrated in response to extracellular cues. Binding of adenomatous polyposis coli (APC) to the microtubule plus ends in polarized cells is regulated by glycogen synthase kinase 3β (GSK-3β). This event is crucial for establishment of cell polarity during directional migration. However, the role of APC for cellular extension in response to extracellular signals is less clear. Smad7 is a direct target gene for transforming growth factor-β (TGFβ) and is known to inhibit various TGFβ-induced responses. Here we report a new function for Smad7. We show that Smad7 and p38 mitogen–activated protein kinase together regulate the expression of APC and cell migration in prostate cancer cells in response to TGFβ stimulation. In addition, Smad7 forms a complex with APC and acts as an adaptor protein for p38 and GSK-3β kinases to facilitate local TGFβ/p38–dependent inactivation of GSK-3β, accumulation of β-catenin, and recruitment of APC to the microtubule plus end in the leading edge of migrating prostate cancer cells. Moreover, the Smad7–APC complex links the TGFβ type I receptor to the microtubule system to regulate directed cellular extension and migratory responses evoked by TGFβ.**

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INTRODUCTION

Cell migration in response to growth factors or cytokines is of fundamental importance during physiological processes such as embryogenesis, wound healing, and inflammatory processes, as well as in pathological processes such as tumor progression. Cell migration implicates complex regulatory pathways that are spatially and temporally integrated with changes of cytoskeleton of the cells (Ridley *et al.*, 2003). Directional cell migration is characterized by dynamic changes in the actin cytoskeleton in the leading edge of the cell facing the direction of the movement, which is referred to as membrane ruffling (Etienne-Manneville, 2004, 2008; Raftopoulou and Hall, 2004). During cell migration the organization of the major components in the cytoskeleton—the actin filaments and microtubules—have to be coordinated. The interactions between microtubules and the actin-rich cell cortex are crucial during cell migration for epithelial cell polarization, membrane extension, and retraction. Cell-adhesion molecules and cellular organelles such as the Golgi complex and the microtubule-organizing center (MTOC) are known to be redistributed in front of the nucleus in polarized, migrating cells (Ridley *et al.*, 2003; Etienne-Manneville, 2004).

Transforming growth factor-β (TGFβ) is a key determinant of cell fate during embryonic development, including cell migration. In the adult, TGFβ also controls processes characterized by cell migration, such as wound healing, inflammation, and tumorigenesis.

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Abbreviations used: APC, adenomatous polyposis coli; ca, constitutively active; EGF, epidermal growth factor; GFP, green fluorescent protein; GSK-3β, glycogen synthase kinase 3β; HA, hemagglutinin; HaCaT, human immortalized keratinocyte; HEK, human embryonic kidney cell; JNK, c-Jun N-terminal kinase; kd, kinase dead; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; MTOC, microtubule-organizing center; NGF, nerve growth factor; PC-3U, human prostate cancer cell; PI3K, phosphatidylinositol 3-kinase; siRNA, short interfering RNA; TGFβ, transforming growth factor-β; TβRI, transforming growth factor-β type I receptor; wt, wild type.

TGFβ exerts its cellular effects by binding to type II (TβRII) and type I (TβRI) serine/threonine kinase receptors (Moustakas and Heldin, 2008). Important intracellular mediators of TGFβ signaling are Smad proteins, of which there are three classes: receptor-activated Smads (Smad2 and Smad3 in the TGFβ signaling pathway), common-mediator Smads (Smad4), and inhibitory Smads (Smad6 and Smad7; Derynck and Zhang, 2003; Shi and Massagué, 2003). Smad7 expression is induced by TGFβ as well as by other cytokines and growth factors, such as interferon-γ, tumor necrosis factor-α, and epidermal growth factor (EGF), suggesting that Smad7 is involved in cross-talk between different signaling pathways (Nakao *et al.*, 1997; Massagué, 2000; Heldin *et al.*, 2009; Mu *et al.*, 2012). We previously reported that Smad7 is additionally required for TGFβ-induced activation of p38 mitogen-activated protein kinase (MAPK) and Cdc42 and regulation of cytoskeletal responses in human prostate cancer (PC-3U) cells (Edlund *et al.*, 2003, 2004, 2005). Smad7 has also been found to cause activation of another stress-activated MAP kinase—the c-Jun N-terminal kinase (JNK; Mazars *et al.*, 2001). Thus Smad7 has effects beyond its well-known inhibitory effect of the canonical TβRI–Smad pathway.

Adenomatous polyposis coli (APC) is a multifunctional tumor suppressor protein (Bienz, 2002). Mutations in the APC gene are frequently found in colorectal cancers. Germline mutations cause familial adenomatous polyposis, and a majority of sporadic colorectal tumors also acquire APC mutations. Of interest, these mutations almost always cause loss of the C-terminal functions of the APC protein required for microtubule binding, cell polarity, and chromosome segregation. Moreover, epigenetic transcriptional silencing of APC is frequently found in prostate cancers (Yegnasubramanian *et al.*, 2004). APC also plays a key role in the Wnt-signaling pathway, where the interaction between APC and β-catenin is important for presentation of β-catenin to the Ser/Thr kinase glycogen synthase kinase 3β (GSK-3β). Active GSK-3β phosphorylates β-catenin, marking it for proteasomal degradation, thereby preventing accumulation of β-catenin (Nelson and Nusse, 2004). However, the interaction between β-catenin and APC has recently been suggested to be crucial for migratory responses of cells, as β-catenin might anchor APC to the cell membrane (Sharma *et al.*, 2006). The localization of APC to plasma membrane is important for proper cell migration (Näthke *et al.*, 1996). In neuronal cells APC is known to be important for establishment of cell polarity and for axonal outgrowth in response to neuronal growth factors. However, the role of APC in relation to GSK-3β and regulation of microtubules for growth factor–induced extension of cells is not clearly understood (Barth *et al.* 2008).

The activity of GSK-3β is regulated by Wnt, as well as by other growth factors such as TGFβ and EGF (Cheon *et al.*, 2004; Edlund *et al.*, 2005; Woodgett, 2005). The Ser/Thr kinases Akt, protein kinase A, and protein kinase C phosphorylate GSK-3β on Ser-9, leading to its inactivation (Doble and Woodgett, 2003; Jope and Johnson, 2004). p38 MAPK has also been shown to inactivate GSK-3β directly by a phosphorylation at Ser-389 (Thornton *et al.*, 2008), and a report by Bikkavilli *et al.* (2008) suggests that Wnt-induced inactivation of GSK-3β is a result of p38-dependent phosphorylation at Ser-9.

In this study, we investigated the role of Smad7, p38 MAPK, and APC in TGFβ-induced migratory responses in prostate cancer cells and other cells. We found that TGFβ promoted the formation of a complex between Smad7 and APC in the leading edge of migrating cells in a p38 MAPK– and GSK-3β–dependent manner.

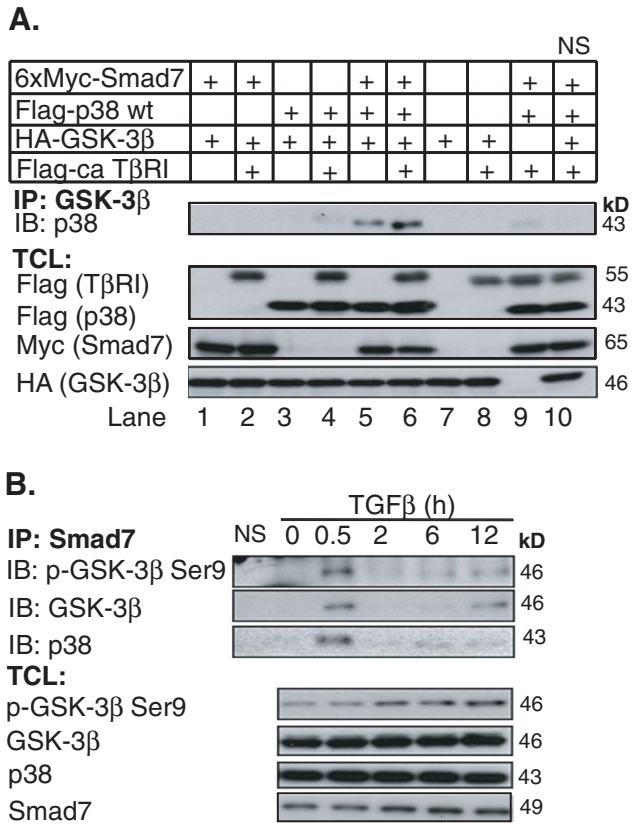


FIGURE 1. TGFβ-dependent interactions between Smad7, GSK-3β, and p38. (A) COS1 cells transiently transfected with HA-GSK-3β, 6xMyc-Smad7, FLAG-p38, or FLAG-ca TβRI, either alone or in combinations, were lysed and subjected to immunoprecipitation with an antiserum against GSK-3β (N-part), followed by immunoblotting for p38. The corresponding total cell lysates were subjected to immunoblotting with antisera against TβRI (FLAG), p38 (FLAG), Smad7 (Myc), and GSK-3β (HA). (B) Human PC-3U cells treated with TGFβ for indicated time periods were lysed and subjected to immunoprecipitation with Smad7 antiserum (N-19), followed by immunoblotting with p-GSK-3β Ser-9, total GSK-3β, or p38 antisera. The corresponding total cell lysates were subjected to immunoblotting with antisera against p-GSK-3β Ser-9, total GSK-3β, total p38, and Smad7 (N-19). NS, precipitation with nonimmune IgG served as negative control.

RESULTS

Smad7 induces a physical interaction between p38 and GSK-3β in a TGFβ-dependent manner

We previously observed that TGFβ treatment of human PC-3U cells and human immortalized keratinocytes (HaCaTs) leads to inactivation of GSK-3β and to an increase of β-catenin levels in a Smad7-dependent manner (Edlund *et al.*, 2005).

Moreover, Smad7 is required for activation of p38 (Edlund *et al.*, 2003), which appears to be important for the regulation of GSK-3β in the TGFβ pathway (Edlund *et al.*, 2005). In the present study, we investigated whether Smad7 could act as an adaptor protein for p38 and GSK-3β. Overexpression of Smad7 induced an interaction between ectopically expressed hemagglutinin (HA)-tagged GSK-3β and FLAG-tagged p38 (Figure 1A; compare lanes 3 and 5). Of note, the Smad7-induced interaction between GSK-3β and p38 was further increased by expression of constitutively active (ca) TβRI, which was used to mimic an active TGFβ signal (Figure 1A, compare lanes 5 and 6). We observed an enhanced interaction between GSK-3β

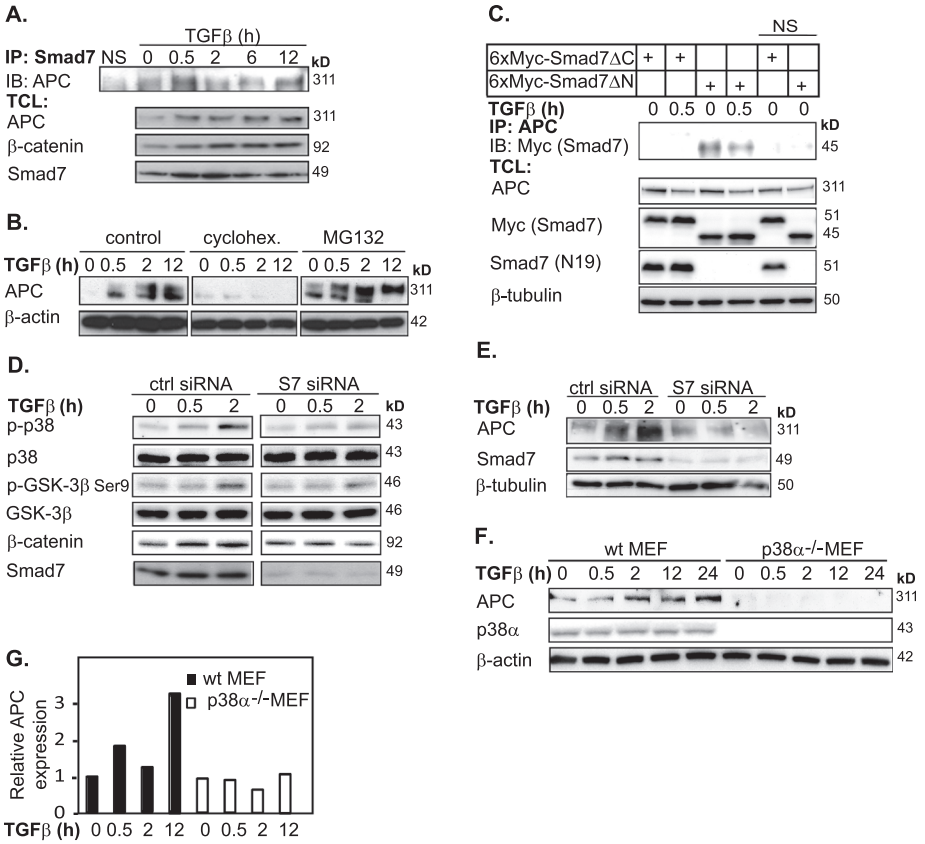


FIGURE 2: TGFβ-dependent regulation of APC and complex formation of Smad7, p38, and APC. (A) Human PC-3U cells treated with TGFβ for indicated time periods were lysed and subjected to immunoprecipitation with Smad7 antiserum (N-19), followed by immunoblotting using APC antiserum (antibody-1). The corresponding total cell lysates were subjected to immunoblotting for APC (antibody-1), β-catenin, and Smad7 (N-19). NS, precipitation with nonspecific IgG served as negative control. (B) PC-3U cells were treated with TGFβ for indicated time periods in the absence or presence of the inhibitor of protein synthesis cyclohexamide (10 μM) or the proteasomal inhibitor MG132 (10 μM). The cells were lysed and subjected to immunoblotting for APC (antibody-1). The corresponding total cell lysates were subjected to immunoblotting with actin to confirm equal loading of protein in each lane. (C) Endogenous APC interacts strongest with the C-terminal part of Smad7. PC-3U cells transiently transfected with 6xMyc-Smad7 ΔC (N-terminal part of Smad7) and 6xMyc-Smad7 ΔN (C-terminal part of Smad7) were lysed and subjected to immunoprecipitation with APC (Ab5) and immunoblotted for Smad7 (Myc). A light chain–specific antibody was used to avoid cross-reaction with IgG heavy chain. The corresponding total cell lysates were immunoblotted for Smad7 (Myc), N-terminal Smad7 (N-19), and APC (Ab1). One filter was immunoblotted for β-tubulin to verify equal loading. NS, precipitation with nonspecific IgG or beads only served as negative control. (D) PC-3U cells transiently transfected with either control siRNA or Smad7 siRNA were treated with TGFβ for various time periods and then subjected to immunoblotting using antisera for p-p38, p38, p-GSK-3β Ser-9, GSK-3β, and β-catenin. The expression of Smad7 was analyzed by immunoblotting. (E) PC-3U cells transiently transfected with either control siRNA or Smad7 siRNA were treated with TGFβ for various time periods and then subjected to immunoblotting using antisera for APC by immunoblotting with the antibody-1 antiserum. β-Tubulin served to confirm equal loading of protein in each lane. (F) The protein expression level of APC was compared between wt and p38α$^{-/-}$ MEFs treated with TGFβ for indicated time periods. The cells were lysed and subjected to immunoblotting using an APC antiserum (antibody-5) and a p38α antiserum to verify the knockdown of p38α. The same amount of cell lysate was subjected to immunoblotting using actin antibodies to confirm equal loading of proteins in each lane. (G) quantitative real-time PCR analysis of expression of *APC* was performed on mRNA extracted from wt and p38α$^{-/-}$ MEFs treated with TGFβ for indicated time periods. N = 3; one representative experiment is shown from three independent experiments in duplicate.

and kinase-dead (kd) p38 compared with wild-type p38 (Supplemental Figure S1A, compare lanes 7 and 8), which suggests that GSK-3β is a substrate for p38, since kinases often dissociate from their substrates after phosphorylation (Johnson and Hunter 2005). Of importance, endogenous GSK-3β was coimmunoprecipitated with Smad7 and p38 in TGFβ-treated PC-3U cells (Figure 1B). The interaction was maximal 30 min after TGFβ treatment. We did not observe any specific interaction between Smad7 and Akt, another potential regulator of GSK-3β (unpublished data). Taken together, these results show that Smad7 promotes physical association between p38 and GSK-3β and that the interaction is enhanced by activated TβRI.

Smad7 promotes TGFβ-induced p38 activation, inactivation of GSK-3β, and accumulation of APC

Inactivation of GSK-3β is a key event for establishment of cell polarity and leads to recruitment of APC to microtubule plus ends in migrating polarized cells (Etienne-Manneville, 2004). To explore whether APC interacts with regulatory components in the TGFβ-signaling pathway, we performed coimmunoprecipitation analyses, using ectopically overexpressed proteins in human embryonic kidney (HEK) 293T cells. Immunoprecipitated APC associated with both Myc-tagged Smad7 (Supplemental Figure S1B, lanes 1 and 2) and FLAG-tagged kd p38 (Supplemental Figure S1B, lane 6). The observation that kd p38 associates with APC more strongly than does wt p38 (Supplemental Figure S1B; compare lanes 3 and 4 with lanes 5 and 6) suggests that p38 phosphorylates APC and then dissociates from its substrate upon phosphorylation. Expression of ca TβRI resulted in stronger interaction between APC and Smad7 (compare lanes 1 and 2) or kd p38 (compare lanes 5 and 6).

Of importance, an interaction between endogenous APC and endogenous Smad7 was also demonstrated; this interaction was enhanced by TGFβ, particularly after 30 min of stimulation (Figure 2A). Of interest, stimulation of PC-3U cells with TGFβ resulted in increased levels of endogenous APC (Figure 2A). Therefore we investigated next the importance of protein synthesis and degradation for TGFβ regulation of APC levels in PC-3U cells. Treatment of cells with the protein synthesis inhibitor cyclohexamide prevented the TGFβ-induced increase of APC (Figure 2B). Moreover, treatment of cells with the proteasomal inhibitor MG132 led to a higher amount of APC, especially after 12 h of TGFβ stimulation. Taken together, our data suggest that the level of APC in PC-3U cells is regulated by TGFβ by increased synthesis and, at later time points, by modulation of proteasomal degradation. Moreover, Dobashi *et al*. (1996) and Votin *et al*. (2005) showed that nerve

growth factor (NGF) also increases the expression levels of APC in neuronal cells, suggesting that the expression of APC might be regulated by various growth factors. The observation that expression of ca TβRI reduced the levels of APC in HEK 293T cells (Supplemental Figure S1B), is consistent with the finding that TGFβ causes a reduction of APC protein levels in the nontransformed epithelial cell line Mv1Lu cells (Satterwhite and Neufeld 2004). Another possible explanation for the difference between the regulation of APC by ectopic expression of ca TβRI and stimulation of cells with TGFβ is that ectopic expression of ca TβRI will not alone fully reconstitute activation of the TGFβ pathway, in line with our recent observations (Sorrentino *et al.*, 2008; Mu *et al.*, 2011).

Ectopically expressed C-terminal part of Smad7 (ΔN), but not the N-terminal part (ΔC), associated with endogenous APC in vivo (Figure 2C), and we previously reported that the N-terminal part of Smad7 binds to β-catenin (Edlund *et al.*, 2005). Suppression of Smad7 by short interfering RNA (siRNA) resulted in a reduced phosphorylation of p38 and prevented to some extent TGFβ-induced accumulation of β-catenin (Figure 2D). This is in line with our previous observation that p38 and Smad7 play important roles in TGFβ-induced accumulation of β-catenin in PC-3U cells and TGFβ-induced inactivation of GSK-3β (Edlund *et al.*, 2005). Smad7 siRNA did not completely reduce β-catenin levels in this experiment and is probably due to a failure to totally silence endogenous Smad7 levels. However, the TGFβ- and Smad7-induced accumulation of β-catenin is likely to be context dependent, as Smad7 previously was shown to both promote and counteract stabilization of β-catenin in cells. We observed that Smad7 promotes accumulation of β-catenin in PC-3U cells, whereas Smad7 was reported to cause degradation of β-catenin in keratinocytes (Edlund *et al.*, 2005; Han *et al.*, 2006; Tang *et al.*, 2008). We also observed that silencing of Smad7 resulted in reduction of TGFβ-induced levels of APC (Figure 2E). Similar results were observed when knockdown of the expression of Smad7 was achieved by the use of a stable antisense construct (PC-3U/AS-S7; unpublished data). From these data we conclude that Smad7 forms a complex with APC in a TGFβ-dependent manner and that Smad7 is important for TGFβ-induced p38 activation and subsequent GSK-3β inactivation, leading to accumulation of β-catenin in PC-3U cells. Of interest, Smad7 was also found to promote TGFβ-induced synthesis of the APC protein.

Because we observed a TGFβ- and Smad7-induced accumulation of APC protein in PC-3U cells, we investigated the role of the p38 MAPK pathway in this response by performing experiments in wild-type (wt) and p38α$^{-/-}$ mouse embryonic fibroblasts (MEFs). Treatment with TGFβ increased the level of APC in wt MEFs but not in p38α$^{-/-}$ MEFs (Figure 2F). Moreover, treatment of wt MEFs with TGFβ resulted in increased expression of APC mRNA, whereas this response was not observed in p38α$^{-/-}$ MEFs (Figure 2G). From these data we conclude that TGFβ-induced expression of APC is dependent on p38α in both prostate cancer cells and MEFs.

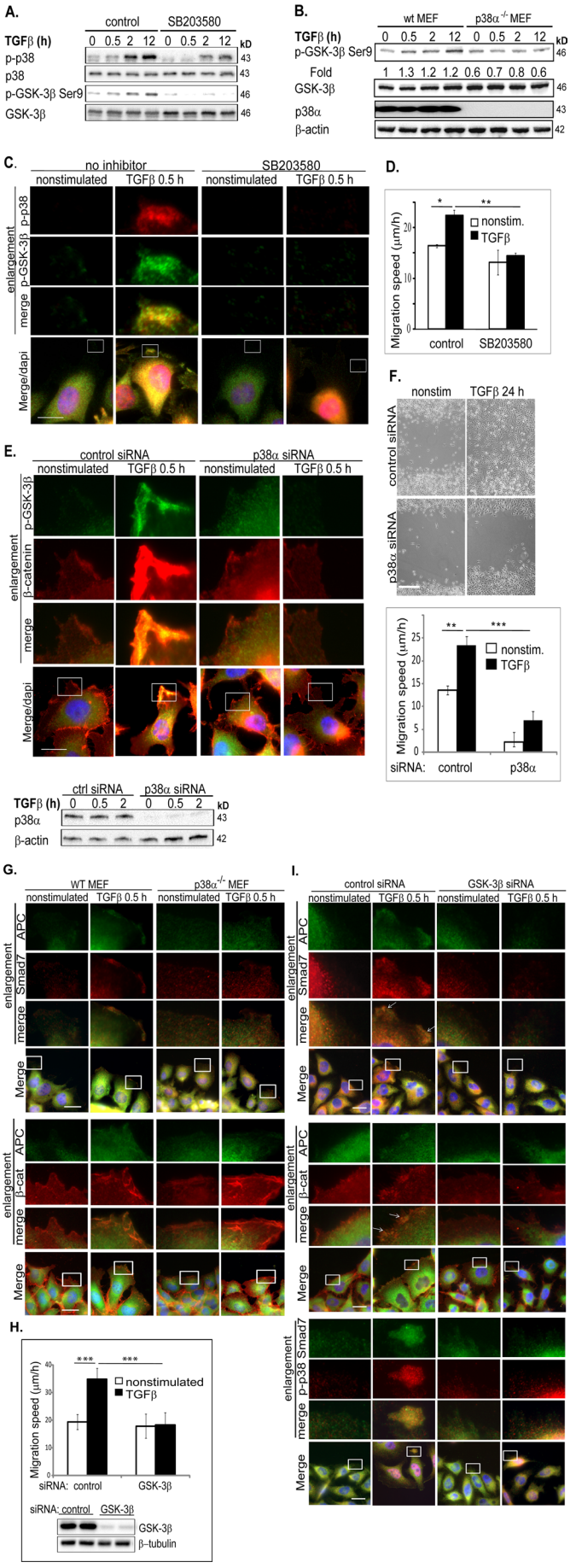
TGFβ-induced inactivation of GSK-3β and accumulation of β-catenin in the leading edge of migrating cells depends on p38α

Inactivation of GSK-3β by its phosphorylation on Ser-9 is regulated by certain kinases, including Akt, protein kinase A, and protein kinase C (Woodgett, 2005; Barth *et al.*, 2008). We found that TGFβ stimulation led to phosphorylation and thereby activation of p38 and inactivation of GSK-3β (Figure 3A). JNK was not activated by TGFβ in PC-3U cells (Supplemental Figure S1C). Of note, treatment of cells with a p38 inhibitor (SB203580 used at 10 μM) or silencing of p38α by siRNA prevented the phosphorylation of GSK-3β (Figure 3A and Supplemental Figure 2A). To further explore the importance of p38α for TGFβ-induced GSK-3β inactivation and its effect on β-catenin accumulation, we investigated wt and p38α$^{-/-}$ MEFs (Porras *et al.*, 2004). Total cell lysates derived from wt and p38α$^{-/-}$ MEFs stimulated with TGFβ for different time periods were subjected to immunoblotting. We observed a reduced inactivating phosphorylation of GSK-3β on Ser-9 in p38α$^{-/-}$ MEFs when compared with wt MEFs (Figure 3B).

Inactivated GSK-3β recruits APC to the microtubule plus end in neuronal cells, and this event is known to be important for microtubule polarization and directed cell migration (Etienne-Manneville, 2004; Raftopoulou and Hall, 2004). To specifically investigate the role of p38α-induced GSK-3β inactivation in human PC-3U cell migration, we used the p38 inhibitor SB203580 as well as siRNA to silence endogenous p38α. In a tissue culture wound-healing assay, immunofluorescence analyses revealed that TGFβ induced a colocalization of p-p38 and p-GSK-3β Ser-9 in membrane ruffles in the leading edge of migrating cells (Figure 3C and Supplemental Figure S2B). In the presence of the p38 inhibitor SB203580, the staining for p-GSK-3β Ser-9 was clearly reduced and was not observed in the leading edge of the cells (Figure 3C and Supplemental Figure S2B), suggesting that GSK-3β is a substrate for p38. As shown in Figure 3D, the p38 inhibitor prevented TGFβ-induced migration. To exclude the possibility that TGFβ-induced proliferation of PC-3U cells contributed to the wound closure, we used the phospho-histone3 Ser-10 antibody to visualize proliferating cells. Because TGFβ did not induce proliferation of the PC-3U cells (Supplemental Figure S2C), we conclude that wound closure was due to increased cell migration. TGFβ treatment of cells induced recruitment of inactivated GSK-3β to the membrane ruffle of control migrating cells, which resulted in a local accumulation of β-catenin, whereas knockdown of p38α led to a reduction in accumulation of p-GSK-3β Ser-9 and β-catenin in the leading edge (Figure 3E). Moreover, we also used siRNA to investigate the functional role of p38α for TGFβ-induced migration in PC-3U-cells. As shown in Figure 3F, knockdown of p38α significantly suppressed TGFβ-induced migration of PC-3U cells, consistent with the report by Zohn *et al.* (2006), which demonstrated that p38 MAPK plays a crucial role for migratory responses via regulation of E-cadherin.

In addition to p38 activation (Figure 2D), Smad7 also contributes to TGFβ-induced activation of Akt (Edlund *et al.*, 2005). We therefore also used inhibitors of phosphatidylinositol-3-kinase (PI3K), acting upstream of AKT, to investigate the importance of PI3K in TGFβ-induced migration of PC-3U cells and HaCaTs. Treatment with p38 or PI3K inhibitor (SB203580 or LY294002, respectively) prevented TGFβ-induced migration in both cell types (Supplemental Figure S2D). Treatment with these inhibitors also reduced the localization of p-p38 in membrane ruffles (Supplemental Figure S2E). Of note, TGFβ treatment of p38α$^{-/-}$ MEFs subjected to cell culture wound-healing assays showed a significant reduction of membrane ruffles when compared with wt MEFs (Supplemental Figure S2F), as well as a clearly reduced migratory response to TGFβ (Supplemental Figure S2G).

We also investigated the subcellular localization of Smad7, APC, and β-catenin in wt and p38α$^{-/-}$ MEFs subjected to cell culture wound-healing assays in the presence of TGFβ. A TGFβ-induced colocalization of Smad7 and APC, as well as of APC and β-catenin, was observed in the leading edge of wt MEFs but not in p38α$^{-/-}$ MEFs (Figure 3G). Next we used GSK-3β siRNA to further validate the role of GSK-3β in TGFβ-induced migration (Figure 3H) and subcellular localization of the APC/Smad7/p38/β-catenin complex in the leading edge of PC-3U cells. As shown in Figure 3I, GSK-3β, is



required for TGFβ-induced recruitment of the polarity complex to the leading edge of migrating cells.

We conclude from these data that TGFβ treatment, in a p38α-dependent manner, leads to an inactivation of GSK-3β in the leading edge of migrating cells and to subsequent accumulation of β-catenin at the cell membrane, which is required for proper migration.

The TGFβ-induced Smad7–APC complex is required for cell migration and membrane ruffling

APC is a key molecule for establishment of cell polarity in neuronal cells (Barth *et al.*, 2008), and APC is known to localize at the cell membrane in migrating cells (Näthke *et al.*, 1996). Because we found that Smad7 directly associates with APC and is important for TGFβ-induced p38 activation and subsequent inactivation of GSK-3β and accumulation of β-catenin, we next examined the role of the Smad7-APC complex for cell migration in a cell culture wound-healing experiment. Knockdown of Smad7 or APC in PC-3U cells resulted in both cases in a delay of TGFβ-induced closure of the wounds when compared with control cells (Figure 4A). Quantification in three different experiments of the migratory responses of the PC-3U cells stimulated with TGFβ in control cells and cells where endogenous Smad7 or APC was silenced is presented in Figure 4B.

FIGURE 3: TGFβ regulates cellular migration, activity, and subcellular localization of GSK-3β and β-catenin in a p38α-dependent manner. (A) Human PC-3U cells treated with TGFβ in the absence or presence of a p38 inhibitor (SB203580, 10 μM) for indicated time periods were lysed and subjected to immunoblotting using antibodies for p-p38 and p-GSK-3β Ser-9. The filters used for immunoblotting with phosphospecific antibodies were stripped, blocked, and reprobed with corresponding antibodies against total p38 or GSK-3β. (B) The activation status of GSK-3β was analyzed in wt and p38α$^{-/-}$ MEFs treated with TGFβ for indicated time periods. The cells were lysed and subjected to immunoblotting using p-GSK-3β Ser-9 and GSK-3β. p38 antiserum was used to verify the knockdown of p38α and actin antibodies to show equal loading of proteins in each lane. Ratios for signal intensities of p-GSK-3β Ser-9 in relation to total GSK-3β for each sample were normalized to nonstimulated wt MEFs at time point zero and are shown below each sample (N = 4). (C–F) Treatment of PC-3U cells with p38 inhibitor SB203580 or knockdown of p38α prevented TGFβ-induced inactivation of GSK-3β and accumulation of inactivated GSK-3β to leading edge of migrating cells. Both SB203580 and p38α siRNA had significant effects on TGFβ-induced cell migration. PC-3U cells treated with SB203580 (C) or transiently transfected with control, p38α (E), or GSK-3β siRNA (H, I) were treated with TGFβ for 30 min in a cell culture wound-healing assay. Cells were fixed, and coimmunofluorescence stainings for p-p38 and p-GSK-3β (C), or p-GSK-3β and β-catenin (E), or APC/Smad7, APC/β-catenin, and Smad7/p-p38 (I) were performed. The colocalization of the proteins is shown in merge. An enlargement is shown from the part within the white box. Scale bar, 20 μM. (G) The subcellular localization of APC, Smad7, and β-catenin was investigated in wt and p38α$^{-/-}$ MEFs subjected to wound-healing assays and treated with TGFβ for indicated time periods. The colocalization of the proteins is shown in merge. (D, F, G, H) Calculation of the migration speed of PC-3U and MEF cells (see also Supplemental Figure 2, F and G) treated as indicated in wound-healing assays. The wound space at the beginning was ~0.6 mm. After TGFβ stimulation of cells, the cell movement into the gap was imaged with digital camera in a Zeiss microscope. The width of the wound was measured with Zeiss AxioVision 4.6.3 software. The migration rates were calculated by dividing the migrated distance by the time for wound healing. *$p < 0.05$ and **$p < 0.01$ when compared with control siRNA treated with TGFβ. Scale bar, 200 μM.

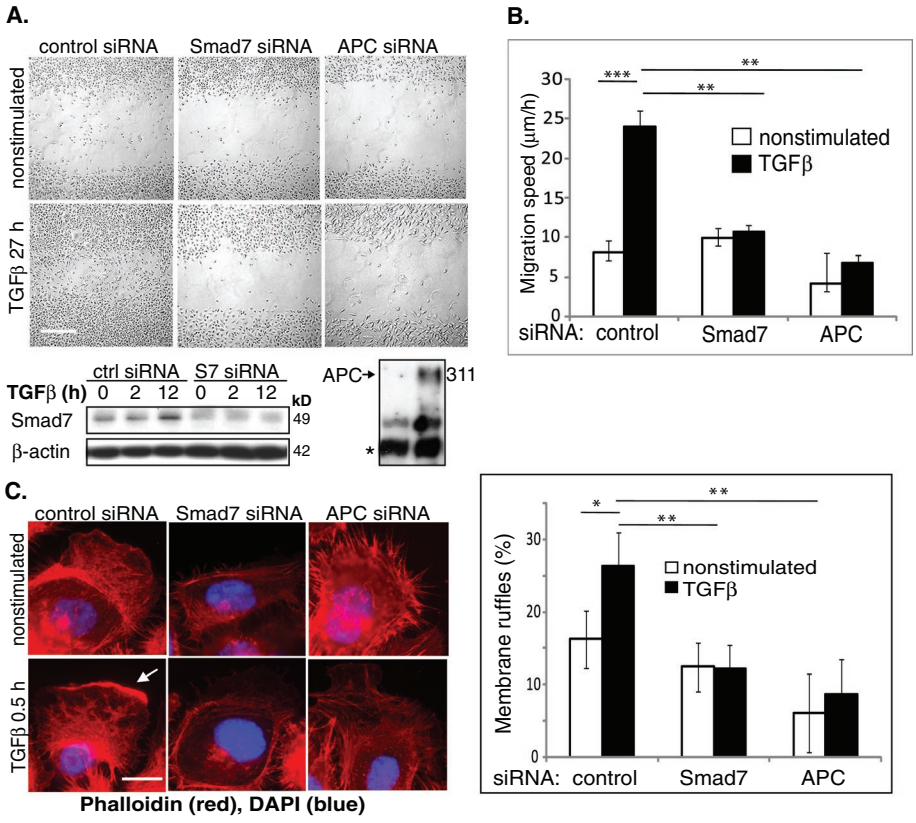


FIGURE 4: TGFβ-induced migration and reorganization of actin filaments is orchestrated by Smad7, p38, and APC. (A) The role of Smad7 and APC in cell migration of PC-3U cells was analyzed by transient transfection of control siRNA, Smad7 siRNA, or APC siRNA. The cells were subjected to cell culture wound-healing assays. The wounded PC-3U cells were treated with TGFβ for 27 h and then fixed in 4% paraformaldehyde. Photographs were taken with a digital camera in a Zeiss microscope. Bar, 200 μm. The expression of Smad7 or APC (specific band is indicated with an arrow) was analyzed by immunoblotting, and the filter used for immunoblotting of Smad7 was reblotted with actin to verify equal loading of proteins in all lanes. Nonspecific bands on the filter used for detection of APC served as internal control for equal loading of proteins and is marked by an asterisk. (B) Calculation of the migration speed of PC-3U cells in wound-healing assay. The migration rates were calculated by dividing the migrated distance by the time for wound healing. The experiment was repeated three times, and from these data the mean value ± SD was calculated for migration rate and then used for statistics. ***$p < 0.001$ and ** $p < 0.01$ when compared with control siRNA treated with TGFβ. (C) A portion of the cells described in A was seeded on coverslips and processed for immunofluorescence analyses of actin reorganization. Filamentous actin was visualized by tetramethylrhodamine isothiocyanate (TRITC)–labeled phalloidin. Arrows indicate membrane ruffling. Nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI). Bar, 20 μm. Effects of control, Smad7, or APC siRNA on the formation of TGFβ-induced membrane ruffles were counted under the microscope and scored (right). Each column represents the mean value from three independent experiments in which 200–300 cells were counted. **$p < 0.01$ and *$p < 0.05$ when compared with control siRNA treated with TGFβ. (D) A portion of the cells described in A was seeded on coverslips and processed for immunofluorescence analyses of the subcellular localization of endogenous Smad7 and APC in PC-3U cells in a cell culture wound-healing assay. The cells were treated as indicated. An overlay of pictures (merge) shows that Smad7 and APC colocalize at the leading edge of migrating PC-3U cells. An enlargement is shown from the part within the white box. Bar, 20 μm.

Moreover, knockdown of the expression of Smad7, by the use of a stable antisense construct (PC-3U/antisense-Smad7; AS-S7 cells) resulted in a diminished capacity to close a wound in response to TGFβ when compared with control PC-3U cells (unpublished data). This was accompanied by a reduced interaction between endogenous APC and β-catenin in TGFβ-stimulated cells (unpublished data).

We also studied the effects of Smad7 and APC on TGFβ-induced membrane ruffling in PC-3U cells subjected to wound-healing assays. A significant reduction in membrane ruffling was noted in cells in which either Smad7 or APC was silenced when compared with control cells (Figure 4C), consistent with their observed importance for TGFβ-induced migration of cells (Figure 4A). Cells treated with APC siRNA acquired spikes all around the cells and loss of TGFβ-induced polarization. Moreover, they did not show accumulation of actin in the membrane ruffles in response to TGFβ, as was the case for control cells. Similar effects were observed in PC-3U cells when APC was silenced, as well as in APC-knockout MEFs (Supplemental Figure S2H). Of interest, a colocalization of endogenous APC and Smad7 in the leading edge of cells was observed (Figure 4D). Of note, knockdown of the Smad7 expression resulted in a loss of APC localization in the leading edge of cells, demonstrating the importance of Smad7 for proper localization of APC during cell migration. Similar results were observed in HEK 293T cells, in which TGFβ stimulated activation of both p38 and JNK (Supplemental Figure S3A; Sorrentino *et al.*, 2008).

To further demonstrate a possible general role of APC for TGFβ-induced polarization and cell migration, as well as the specificity of the siRNA used for APC, we also included studies of the osteosarcoma U2OS

cells. APC has been shown to be crucial for migratory responses in these cells (Kroboth *et al.*, 2007). U2OS cells were transfected with wt or single-point-mutant (SM), green fluorescent protein (GFP)–tagged APC. We observed a TGFβ-induced accumulation of ectopically expressed wt APC labeled with GFP (wt GFP-APC) in membrane ruffles of U2OS cells, whereas this response was prevented in cells in which APC was knocked down by siRNA, which targets both endogenous and ectopically expressed wt APC-GFP (Supplemental Figure S3B). As a control for specificity of the siRNA, we transiently transfected U2OS cells with an SM APC-GFP plasmid and siRNA for APC. As expected, SM APC-GFP was still localized to membrane ruffles in response to TGFβ in cells treated with APC siRNA, as the mutant SM APC-GFP is not recognized by the siRNA for APC (Supplemental Figure S3B). In addition, when the expression of Smad7 was suppressed by siRNA, less APC-GFP was localized in membrane ruffles in TGFβ-stimulated PC-3U cells (Supplemental Figure S3C), which is consistent with data presented in Figure 4D.

From these data, we conclude that knockdown of Smad7 or APC expression reduces TGFβ-induced membrane ruffling and cytoskeletal reorganization in cells. We also conclude that expression of Smad7 is important for proper localization of APC at the leading edge of migrating PC-3U cells in response to TGFβ.

To further validate the generality of the importance of Smad7 and p38 for TGFβ-induced polarization and cell migration, we performed additional studies in the osteosarcoma U2OS cells (Kroboth *et al.*, 2007). Knockdown of Smad7 by siRNA in U2OS cells led to a suppression of p38 activation, whereas TGFβ-induced activation of Smad2 and Erk was not appreciably affected (Supplemental Figure S3D). Of importance, knockdown of endogenous Smad7 resulted in a dramatic loss of TGFβ-induced membrane ruffles in U2OS cells, again demonstrating the importance of Smad7 and p38 for this cellular response (Supplemental Figure S3E). From these data we conclude that loss of Smad7, APC, or p38 by their specific siRNA or by genetic deletion, results in impaired TGFβ-induced membrane ruffling and migration in all investigated cell lines.

Importance of Smad7 and APC for TGFβ-induced establishment of cell polarity and extension of microtubules during directed cell migration

Microtubules are important for the establishment of polarity, directed cell extension, and migration of cells. We used tissue culture wound-healing assays to study the role of Smad7 during cell polarization (Etienne-Manneville, 2004; Barth *et al.*, 2008). A significant dysfunction in microtubule organization and cellular extension was observed in PC-3U cells in which expression of Smad7 was silenced by siRNA (Figure 5A; an asterisk indicates the lack of stretching of microtubules toward the wound). In control cells, endogenous APC was localized at the distal part of the microtubule plus ends after TGFβ treatment for 30 min, whereas the extension and distribution of microtubules, as well as the localization of APC, were significantly perturbed in cells in which Smad7 had been silenced (Figure 5A). Quantification of cells with polarized microtubules stretching toward the wound revealed both a significant TGFβ-induced response and reductions of polarized microtubules in cells in which either Smad7 or APC was silenced (Figure 5B). Treatment of PC-3U cells with the p38 inhibitor SB203580 also prevented TGFβ-induced microtubule polarization (Supplemental Figure S4A). Reorientation of MTOC and Golgi apparatus in front of the nucleus is a hallmark for establishment of cell polarity in migrating astrocytes (Etienne-Manneville, 2004). No clear effect of TGFβ on the localization of Golgi or on MTOC relocalization was seen in rapidly migrating PC-3U cells (unpublished data). We therefore investigated whether TGFβ via p38 or Akt could promote reorientation of Golgi in the human breast cancer cell line MCF10A exposed to TGFβ. As shown in Supplemental Figure 4B, TGFβ induced the localization of Golgi in front of the nucleus within a 120° sector facing the wound. This effect was clearly reduced in cells treated with the p38 inhibitor SB203580 or the PI3K inhibitor LY294002, which inhibits activation of Akt.

To investigate the relationship among the observed Smad7-APC complex, the microtubule system, and β-catenin, we first analyzed a possible interaction between Smad7 and β-tubulin in the presence or absence of APC. A TGFβ- and APC-dependent complex between Smad7 and β-tubulin was observed (Figure 5C). Of interest, TGFβ also induced a Smad7-dependent interaction between APC, β-catenin, and the microtubule system. Smad7 was also important for TGFβ-induced accumulation of β-catenin (Figure 5D), in line with our findings that Smad7 is crucial for p38 activation and subsequent inactivation of GSK-3β.

To further investigate the role of p38 activity for the observed TGFβ-induced association between APC and β-catenin, we investigated the subcellular localization of these proteins in PC-3U cells stimulated with TGFβ. As shown in Figure 5E, TGFβ induced a p38 MAPK–dependent association of APC and β-catenin in membrane ruffles in the leading edge of migrating cells. The TGFβ-induced localization of endogenous APC to the distal part of the microtubule plus ends was also dependent on activation of p38 MAPK (Figure 5F). Because we observed that Smad7 was associated with the microtubule system (Figure 5C), we investigated which part of Smad7 was responsible for this interaction. The C-terminal part of ectopically expressed Smad7 (Myc-tagged ΔN-terminal part) was found to bind to endogenous β-tubulin with higher affinity than the N-terminal part (Myc-tagged ΔC-terminal part). The association was not clearly dependent on TGFβ stimulation of cells but was slightly increased in cells treated with TGFβ (Figure 5G), suggesting that the endogenous Smad7–APC complex could associate with microtubules in a TGFβ-dependent manner, as shown in Figure 5A.

From these observations, we conclude that Smad7 is important for TGFβ-induced polarization and extension of microtubules during directed cell migration. Moreover, Smad7 and p38 MAPK are important for TGFβ-induced synthesis of APC and for the promotion of a complex among accumulated APC, β-catenin, and microtubules during TGFβ-induced cell migration.

TGFβ-dependent APC–TβRI complexes are formed and localized to extended microtubules in the leading edge of migrating cells

The role of microtubules in cell extension has been characterized during polarization of neuronal cells, a process for which APC is known to play an important role. However, less is known about the functional role of microtubules for directed cellular extension and migration of epithelial cells in response to extracellular stimuli such as growth factors (Barth *et al.*, 2008). We therefore investigated whether APC is important for proper localization of the TGFβ receptor complex in migrating cells. Of note, a TGFβ-induced complex of TβRI and microtubules was observed, which was dependent on APC expression (Figure 6A). To further explore the link among TβRI, APC, and the cytoskeleton, we investigated the subcellular localization of TβRI in cells in which APC was silenced by siRNA. Coimmunofluorescence stainings for F-actin and TβRI in PC-3U cells treated with TGFβ revealed a significant accumulation of the receptor in the leading edge of migrating cells in membrane ruffles directed toward the wound. In the absence of APC, the number of cells that formed membrane ruffles was clearly reduced (Figure 4C) and the TβRI was not localized in the leading edge of the cells, suggesting that APC

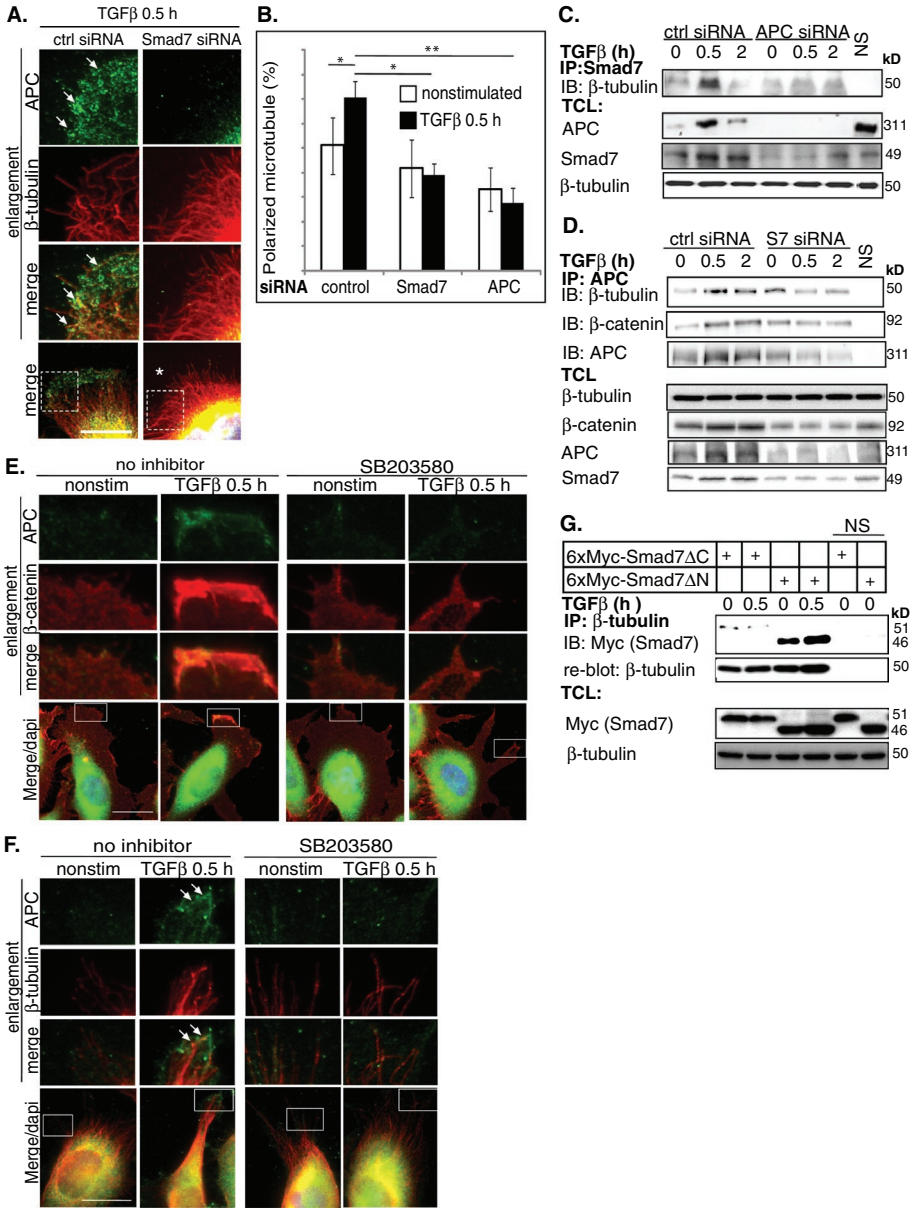


FIGURE 5: TGFβ-induced extension in cellular protrusion and polarization of microtubules is coordinated by Smad7 and APC. (A–D) Smad7 and APC are required for TGFβ-induced cellular protrusion and microtubule polarization. PC-3U cells transiently transfected with control, Smad7, or APC siRNA were treated with TGFβ for 30 min in a cell culture wound-healing assay. Cells were fixed, and coimmunofluorescence stainings for β-tubulin and APC were performed. Arrow indicates APC localized on the distal end tip of a microtubule. Note the lack of extension and orientation toward the wound of microtubules in the absence of Smad7, as indicated with an asterisk. Bar, 20 μm. A fraction of the cells used for experiments shown in A was subjected to immunoblotting to determine the levels of APC and Smad7 (shown in D). (B) The number of cells in which the microtubules were polarized and oriented toward the wound was counted under the microscope. *$p < 0.05$ and **$p < 0.01$. Levels of Smad7 and APC are shown by immunoblotting in C and D. (C) Lysates from PC-3U cells treated as indicated were subjected to immunoprecipitation (IP) with Smad7 antisera (nonspecific IgG [NS] served as negative control), followed by immunoblotting with β-tubulin. The corresponding total cell lysates were subjected to immunoblotting. (D) TGFβ- and Smad7-dependent interaction between APC and microtubules. PC-3U cells transiently transfected with control or Smad7 siRNA and treated with TGFβ for indicated time periods were lysed and subjected to immunoprecipitation with APC antisera (antibody-5) or nonspecific IgG (NS) as negative control, followed by immunoblotting with β-tubulin, β-catenin, and APC. The corresponding total cell lysates were subjected to immunoblotting for β-tubulin, β-catenin, APC, and Smad7. (E, F) PC-3U cells were seeded on coverslips and processed for immunofluorescence analyses of the subcellular localization of endogenous APC and β-catenin (E) or APC and β-tubulin (F), respectively, in PC-3U cells in a cell tissue culture wound-healing assay. The cells were treated as indicated and subjected to coimmunofluorescence stainings to detect endogenous proteins. An overlay of pictures (merge) shows that APC and β-catenin colocalize at the leading edge of migrating PC-3U cells (E). Arrow indicates APC localized on the distal end tip of a microtubule (F). An enlargement is shown from the part within the white box. Bar, 20 μm. (G) Lysates of PC-3U cells transiently transfected as indicated were subjected to immunoprecipitation with β-tubulin or nonspecific IgG or beads only (NS) as negative controls and immunoblotted for Myc. A light chain–specific antibody was used to avoid cross-reaction with IgG heavy chain. The IP filter was reblotted with β-tubulin. The corresponding total cell lysate was subjected to immunoblotting.

is required for a proper localization of the receptor (Figure 6B).

As shown in Figure 6C, inhibitors of p38 (SB203580) also prevented TGFβ-induced accumulation of TβRI in the leading edge of migrating cells. Furthermore, in cells treated with TGFβ in the presence of SB203580, we observed a significant reduction in colocalization of receptors and the microtubule system (Figure 6D), whereas treatment with the PI3K inhibitor LY294002 did not have any effect (unpublished data). Therefore activation of p38 may also be important for recruitment of TβRI to microtubules. In contrast, active PI3K may not be important for TGFβ-induced recruitment of TβRI to the microtubule system, even if it is important for cell migration. Instead, active PI3K is presumably important for the regulation of other molecular events downstream of the observed TβRI/APC/microtubule complex.

Taken together, our data suggest that APC plays a crucial role for the recruitment of the TβRI to the leading edge of migrating cells. Moreover, APC appears to cooperate with Smad7 to build a functional link between the TβRI and the extended microtubules during directed cell migration. Loss of Smad7 expression or p38 activation results in a loss of TGFβ-induced local inactivation of GSK-3β in the leading edge of migrating cells, and as a consequence APC will not be recruited to the distal tip of polarized microtubules in cell extensions.

DISCUSSION

We report here that TGFβ promotes association between Smad7 and APC, which is of crucial importance for promotion of TGFβ-induced cell extension and migration of PC-3U cells. Smad7 acts as an adaptor protein required for TGFβ-induced activation of the p38 MAPK pathway, resulting in local inactivation of GSK-3β in the leading edge of

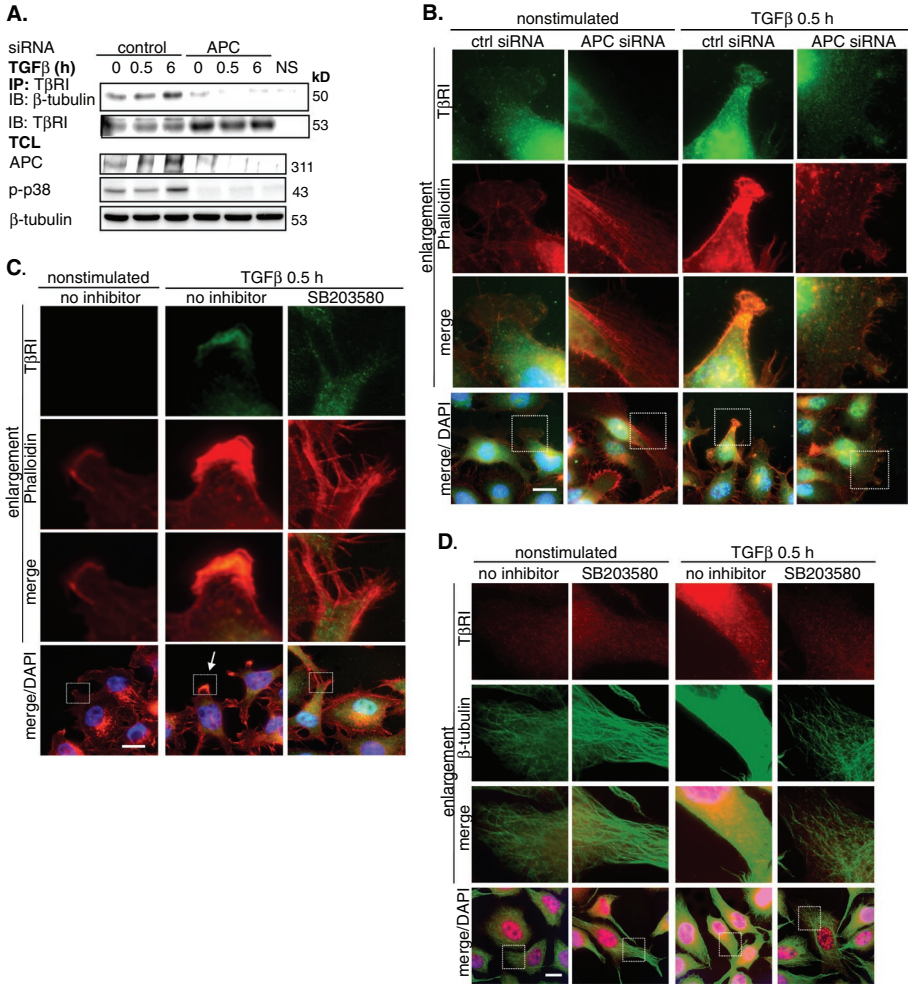


FIGURE 6: TGFβ-dependent complexes of APC, TβRI, and the microtubule system are formed at the leading edge of migrating cells. (A) PC-3U cells transfected with control siRNA or APC siRNA and treated with TGFβ were lysed and subjected to immunoprecipitation with TβRI antisera (V22) (nonspecific IgG [NS] served as negative control), followed by immunoblotting using β-tubulin antisera. The filter was also reblotted with TβRI antisera. The corresponding total cell lysates were subjected to immunoblotting for APC, p-p38, and β-tubulin.
(B) Immunofluorescence stainings to detect the subcellular localization of endogenous TβRI in wounded PC-3U cells treated or not with TGFβ for 30 min in cells subjected to control siRNA or APC siRNA. Filamentous actin was visualized by TRITC-labeled phalloidin. Nucleus was visualized by DAPI. An enlargement is shown from the part within the white box. Bar, 20 μm. Levels of APC are shown by immunoblotting in A. (C) Subcellular localization of endogenous TβRI in wounded PC-3U cells. The cells were treated or not with TGFβ for 30 min in the absence or presence of the p38 inhibitor SB203580 and then subjected to coimmunofluorescence stainings to detect endogenous TβRI. Filamentous actin was visualized by TRITC-labeled phalloidin. Nucleus was visualized by DAPI. An enlargement is shown from the part within the white box. An arrow indicates membrane ruffling. Bar, 20 μm. (D) Coimmunofluorescence stainings of TβRI and β-tubulin to detect the subcellular localization of endogenous TβRI and microtubules in wounded PC-3U cells, treated or not with TGFβ for 30 min in the absence or presence of the p38 inhibitor SB203580. Bar, 20 μM.

migrating cells, which promotes association of APC with plus-end microtubules to control establishment of cell polarity in migrating cells. We also show that TGFβ induces Smad7/p38α–dependent local inactivation of GSK-3β, which leads to an accumulation of β-catenin and increased levels of APC, facilitating cell protrusion in the leading edge of the migrating cell. Our data suggest that inactivated GSK-3β, β-catenin, and APC form a complex with Smad7 in cells treated with TGFβ. Moreover, TGFβ promotes binding of APC to the extended and polarized microtubules in a Smad7- and p38α-dependent manner, consistent with our observation that Smad7 is required for a proper localization of APC to membrane ruffles in the leading edge, as well as to the extended microtubule plus end in migrating cells. We also observed that TβRI is localized in the leading edge of migrating cells in response to TGFβ, consistent with its important role in regulating chemotaxis.

TGFβ promotes epithelial–mesenchymal transition (EMT) of epithelial cells (Heldin *et al.*, 2009; Xu *et al.*, 2009). During this process the epithelial cells lose their epithelial hallmarks, such as E-cadherin–regulated cell–cell contacts, and become scattered and mesenchymal-like and achieve migratory capabilities (Heldin *et al.*, 2009; Thiery *et al.*, 2009; Xu *et al.*, 2009). The human PC-3U cells used in these studies have undergone partial EMT, as they express low levels of E-cadherin, have a mesenchymal phenotype, and are scattered. Of interest, they still respond to TGFβ with increased production of fibronectin, which probably promotes their migratory response (Supplementary Figure S6). We therefore conclude that TGFβ-induced formation of the polarity complex we describe in PC-3U cells, MEFs, and other cell lines used in our study occurs independent of the TGFβ-regulated EMT process.

Smad7 is known to bind to the active TβRI (Hayashi *et al.*, 1997; Nakao *et al.*, 1997) and is required for TGFβ-induced activation of p38 (Edlund *et al.*, 2003). Our findings suggest that Smad7 and APC act as scaffold proteins to promote TGFβ-induced local regulation of the polarity complex in migrating cells (Figure 7). Our data demonstrate important roles for APC, Smad7, and active p38α in locally inactivating GSK-3β in the leading edge of migrating cells in response to TGFβ. The local inactivation of GSK-3β also seems to cause accumulation of β-catenin. This is a different function compared with the role in the Wnt-signaling pathway, in which APC presents β-catenin to active GSK-3β to target it for proteasomal degradation (Nelson and Nusse, 2004).

The effects of APC and Smad7 were found to involve activation of p38, consistent with a recent report in which p38 was identified to be a key player for transdifferentiation and migratory responses of cells during embryonic development in mice (Zohn *et al.*, 2006). We demonstrate in this study that p38α is required for TGFβ-induced migration of PC-3U cells, as expected, and this is in line with the report from Zohn *et al.* (2006). Moreover, we observed a reduced TGFβ-induced inactivation of GSK-3β in p38α$^{-/-}$ MEFs and in PC-3U cells in which p38α was silenced with siRNA or treated with the p38 inhibitor SB203580, demonstrating that p38α plays an important role for inactivation of GSK-3β. We also report that p38 interacts with GSK-3β, suggesting that

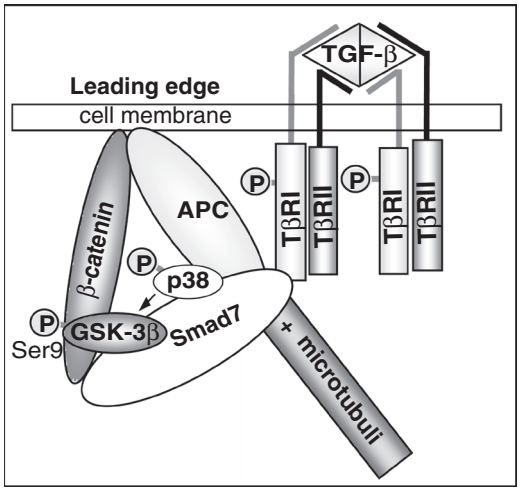


FIGURE 7: Schematic illustration of role of Smad7–APC complex for TGFβ-induced migration of prostate cancer cells. Binding of TGFβ leads to a hetero-oligomerization of the TβR complex, in which the constitutively active TβRII kinase phosphorylates TβRI and activates its kinase. Smad7 is recruited to the activated TβR complex and acts as an adaptor protein to facilitate p38α activation (Figure 2D; Souchelnytskyi *et al.*, 1998; Edlund *et al.*, 2003). We observed a TGFβ-induced complex between TβRI, Smad7, active p38, and APC, which seems to localize to the membrane ruffles in the leading edge of migrating cells. In migrating cells, APC is anchored to the cell membrane (Figures 4D and 5E) in accordance with Näthke *et al.* (1996). TGFβ stimulation of cells leads to both Smad7/p38α-dependent accumulation of APC (Figure 2, E and F) and recruitment of the accumulated APC to the plus-end tips of polarized microtubules (Figure 5A). TGFβ-induced migration of PC-3U cells is dependent on p38α activity (Figure 3, D and F). We propose that Smad7 and active p38 promote inactivation of GSK-3β, which favors association of APC with plus-ended microtubules in the leading edge of migrating cells (Figure 1, Supplemental Figures S1B, S2B, S3, C and E, S4D, and S5, A and F). TGFβ/p38α–induced inactivation of GSK-3β leads to local accumulation of β-catenin (Figure 3, E, G, and I), which binds to APC (Figure 5, D and E) and the N-terminal part of Smad7 (Edlund *et al.*, 2005), whereas the C-terminal part of Smad7 forms a complex with APC (Figure 2C). Of interest, the Smad7 and APC complex seems to form a link between TβRI in the membrane ruffles and the microtubule system (Figure 6). This complex is of importance for directed migration of cells.

GSK-3β might be a substrate for active p38. This is in line with recent reports by Thornton *et al.* (2008) and Bikkavilli *et al.* (2008), who demonstrated that GSK-3β is likely to be a substrate for p38. Thus our data suggest that the observed TGFβ- and Smad7-dependent activation of p38 results in inactivation of GSK-3β, which is an important regulatory event in migrating PC-3U cells.

Javelaud *et al.* (2005) reported that ectopic overexpression of Smad7 results in impaired invasion of cells, which is in apparent contrast to our findings of a positive role for Smad7 in TGFβ-induced migratory response. However, their result may be fully consistent with our finding that siRNA suppression of Smad7 reduces migration, since overexpression of Smad7 may disrupt the functional complexes between p38, APC, and tubulin that we described here.

The role of the actin cytoskeleton in membrane extension and directed cell migration is quite well understood (Pollard and Borisy, 2003; Disanza *et al.*, 2005; Huttenlocher, 2005), whereas the role of the microtubule cytoskeleton in migratory responses to extracellular signals has just recently been put in focus (Dikovskaya *et al.*, 2001; Etienne-Manneville and Hall, 2003; Wen *et al.*, 2004; Zhou *et al.*, 2004). Our present data suggest that Smad7 is important for extension of polarized microtubules in cell migration in response to TGFβ stimulation of cells (Figure 5A). TGFβ induces Smad7 expression (Nakao *et al.*, 1997), which is then used to regulate the activation status of p38 (Edlund *et al.*, 2003; Figure 2D). This induces synthesis of APC (Figure 2, E–G, and Supplemental Figure S5), and Smad7 promotes the association of APC with the distal end of microtubules (Figure 5A) via active p38α MAPK (Figure 5F). This in turn causes local inactivation of GSK-3β in the leading edge of migrating cells, thereby promoting subsequent accumulation of β-catenin (Figure 3E). These observations suggest that Smad7 has an important role in coordinating TGFβ-induced cytoskeletal responses. Moreover, the N-terminal part of Smad7 binds to β-catenin (Edlund *et al.*, 2005), whereas the C-terminal part of Smad7 associates with APC (Figure 2C). Consequently, Smad7 appears to coordinate a complex between TβRI, APC, and β-catenin at the distal end of polarized microtubules. Future studies are required to elucidate whether Smad7 has a role in directed cell migration and polarization induced by other growth factors. Thus it will be interesting to explore whether Smad7 might be used in directed cellular extension and migration in response to extracellular factors other than TGFβ, such as Wnt (Nelson and Nusse 2004), or for integrin-induced polarization of cells (Etienne-Manneville and Hall, 2003). We previously showed that Smad7 is required for TGFβ-induced accumulation of β-catenin and Wnt-induced responses in PC-3U cells, as Smad7, when overexpressed, enhanced transcriptional activity of β-catenin and TCF-4 (Edlund *et al.*, 2005; Supplemental Figure S3). Taken together, the present data and our previous report (Edlund *et al.*, 2005) suggest that Smad7 in a p38α-dependent manner facilitates TGFβ-induced accumulation of β-catenin, thereby promoting migratory responses in prostate cancer cells.

APC has been proposed by several research groups to be a key molecule regulating cell polarity, as well as migratory responses of cells, as it can interact directly and indirectly with both the actin filaments and microtubules (Barth *et al.*, 2008; McCartney and Näthke, 2008). NGF has been shown to induce APC expression at the tips of the neuronal extensions of PC12 cells, which is essential for outgrowth of neurons in response to NGF (Dobashi *et al.*, 1996; Zhou *et al.*, 2004; Votin *et al.*, 2005). Of interest, we observed that TGFβ regulates the levels of APC protein in a Smad7- and p38α-dependent manner (Figure 2, E–G) and that APC and Smad7 colocalized in membrane ruffles in migrating cells (Figures 3, G and I, and 4D). Induction of both APC and Smad7 by TGFβ may be of fundamental importance for the TGFβ-induced migratory response of cells, as increased levels of both proteins could promote their association in the leading edge of the cell and seem to be required for cellular extensions. Whether posttranslational modifications of APC or Smad7 regulate their association or subcellular localization is an interesting topic for future investigations.

The C-terminal part of APC consists of motifs that confer direct and indirect binding of APC to the microtubule system. A novel function for APC in cell migration has emerged during recent years. Epithelial colon cells migrate toward the tip of the villi, where they are shed and undergo apoptosis, a physiological process protecting normal cells from genetic insults and cancer. The loss of the migratory capacity of the colon epithelial cells resulting from a loss of function by genetic inactivation of APC might therefore also be important for the development of colorectal cancer (Näthke, 2006). Our preliminary data show a colocalization of endogenous APC and Smad7 in normal colon and prostate epithelial cells. It is tempting to speculate that genetic inactivation of APC could also result in a loss of some of the responses controlled by TGFβ, such as migration and

apoptosis, in line with the hypothesis proposed by Näthke (2006). Further examination of the role of APC in TGFβ-induced responses is therefore warranted.

Of note, aberrant germline expression of *TβRI*, resulting in a decreased expression of the gene, has been found in 10–20% of familiar colon cancer (Valle *et al.*, 2008), which might result in impaired cell migration. Our observation that APC is induced by TGFβ and required for TGFβ-induced directed cell migration in several investigated cell lines, including fibroblasts, suggests a general role of APC in TGFβ-induced migratory responses, which, of interest, appears to occur independent of EMT and places APC for the first time in the TGFβ pathway.

MATERIALS AND METHODS

Cell culture

The human prostate carcinoma cell line PC-3U, originating from PC-3 cells (Franzen *et al.*, 1993), and PC-3U cells stably transfected with pRKV5 antisense Smad7 (PC-3U/AS-S7 cells) or with pMEP4-Smad7 (PC-3U/pMEP4-Smad7 cells) were routinely grown in RPMI 1640 with 10% fetal bovine serum (FBS) and L-glutamine in the presence of their respective antibiotics to maintain selection pressure, as previously described (Landström *et al.*, 2000). PC-3U/pMEP4-Smad7 cells were stimulated with 1 μM $CdCl_2$ for 12 h to induce expression of FLAG-Smad7; cell lysates were used as positive control for detection of endogenous Smad7 in immunoblotting (Landström *et al.*, 2000, and our unpublished data). COS-1, 293T, and HaCaT cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in DMEM with 10% FBS. In all assays, stimulations with TGFβ1 (referred to as TGFβ in the present article) were performed at 10 ng/ml in medium containing 1% FBS.

Antibodies and reagents

The following antibodies were used: mouse monoclonal anti-APC, antibody-1, and antibody-5 (Calbiochem, La Jolla, CA), anti-FLAG M5 and M2, anti-Myc 9E10, and anti-fibronectin (Sigma-Aldrich, St. Louis, MO), anti–β-catenin and anti–E-cadherin (BD Transduction Laboratories, Lexington, KY), and polyclonal rabbit anti–phospho-Akt Ser-473, anti–GSK-3β, and anti–phospho-GSK-3β Ser-9, anti–phospho-p38, anti-p38, and anti–phospho histone3 Ser-10 (Cell Signaling Technology, Beverly, MA). Anti-p38 (C20) and anti-Akt (akt1, 2) and rabbit anti-TβRI (V22; Santa Cruz Biotechnology, Santa Cruz, CA), the specificity of which was reported previously (Castañares *et al.*, 2007), were purchased from Santa Cruz Biotechnology. Anti-Smad7 (Brodin *et al.*, 1999) and goat anti-Smad7 (N-19; Santa Cruz Biotechnology) were used to detect endogenous Smad7, cell lysates from PC-3U/pMEP4-Smad7 cells were used as positive control, and siRNA for Smad7 was used as a negative control (Edlund *et al.*, 2005, and the present article). We did not observe any detectable expression of the structurally related Smad6, which would migrate as a 62-kDa protein on a SDS–PAGE gel. Rat M-APC antiserum was produced, and its specificity was tested (data not shown). Secondary immunoglobulin G (IgG) horseradish peroxidase–linked whole anti-rabbit, anti-goat, or anti-mouse antibodies were from Sigma-Aldrich. In some experiments, goat anti-mouse IgG, light chain–specific antibodies from Jackson ImmunoResearch Laboratories (West Grove, PA), were used. TGFβ1 was from R&D Systems (Abingdon, United Kingdom). Complete and Pefabloc were from Roche (Indianapolis, IN). The p38α and β inhibitor SB203580 and the PI3K inhibitor LY294002 were purchased from Calbiochem (La Jolla, CA). The JNK inhibitor (L-JNKI1) was purchased from Alexis Biochemicals (San Diego, CA). The general protein kinase C inhibitor GF109203X was purchased from Sigma-Aldrich. All inhibitors were used at a concentration of 10 μM and added to cells 1 h before TGFβ treatment, except for MG132, which was added at the same time as TGFβ.

Western blotting, immunofluorescence, and in vitro and in vivo protein interaction assays

Cells grown on 10-cm dishes were starved at least 12 h and then treated with TGFβ1 for indicated time periods, washed once with ice-cold phosphate-buffered saline, and lysed in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 0.5% [vol/vol] sodium deoxycholate [DOC], 1% [vol/vol] NP40, 10% [vol/vol] glycerol, 1 mM aprotinin, 1 mM Pefabloc, 2 mM sodium vanadate). After centrifugation, supernatants were collected, and protein concentrations were measured using the Protein Assay Kit from Bio-Rad (Hercules, CA). Equal amounts of proteins were subjected to immunoprecipitation. Immunoprecipitates were resolved by SDS-gel electrophoresis in 6, 10, or 12% polyacrylamide gels, blotted onto polyvinylidene difluoride membranes, and subjected to immunoblotting (Edlund *et al.*, 2005). Immunofluorescence and transient transfections were performed as previously described (Edlund *et al.*, 2004, 2005). Wound-healing assays were performed on serum-starved confluent cells growing on sterile coverslides in six-well plates without coating by using a 1000-μl pipette tip for scratching. The wounded cells where then photographed, the wounds were measured, and the cells were treated as indicated in figures. The wound space at the beginning was ~0.6 mm. Expression of Smad7 or APC was silenced by siRNA, and cells were thereafter subjected to wound-healing assays on non-coated glass slides in tissue culture six-well dishes. Control cells and migrating cells facing the wound were subjected to immunofluorescence analyses of cytoskeletal rearrangements by actin stainings or for analysis of subcellular localization of proteins, as indicated in the figure legends. The wound-healing experiments was repeated three to five times, and from these data the mean value ± SD was calculated for migration rate and then used for statistics. Efficient knockdown of Smad7 or APC in each individual experiment was confirmed by immunoblotting. The analyses of F-actin reorganization in membrane ruffles were performed three times, and ~200–300 cells facing the wound were counted in each experiment. Photomicrographs were obtained by a microscope (Axioplan 2; Carl Zeiss MicroImaging, Jena, Germany) with a digital camera (C4742-95; Hamamatsu, Hamamatsu, Japan), using a Plan-Neofluar 40×/0.75 objective lens (Carl Zeiss MicroImaging). Photography was performed at room temperature. Primary images were acquired with the camera's QED software. Image memory content was reduced, and brightness contrast was adjusted using Photoshop 6.0 (Adobe, San Jose, CA).

Plasmids and DNA transfections

The expression vectors for HA-tagged ca TβRI (also called activin-like kinase receptor 5), 6xMyc-tagged Smad7 proteins (wt, ΔN, and ΔC), and FLAG-tagged Smad7 in the mammalian expression vector pcDNA3 (Invitrogen) were gifts from P. ten Dijke (Department of Molecular Cell Biology and Centre for Biomedical Genetics, Leiden University Medical Center, Leiden, The Netherlands). FLAG-tagged TβRI was from K. Miyazono (Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan) and T. Imamura (Division of Biochemistry, Cancer Institute of the Japanese Foundation for Cancer Research, Tokyo, Japan). Myc-APC was from M. Faux and A. W. Burgess (Parkville Branch, Ludwig Institute for Cancer Research, Melbourne, Australia). FLAG-tagged GSK-3β was from E. J. Choi (Laboratories of Cell Death and Human Diseases, School of Life Sciences and Biotechnology, Korea University, Seoul, and School of Pharmacy, Korea University, Chungnam,

South Korea). FLAG(-p38), wt and kd, were from J. Han (School of Life Sciences, Xiamen University, Fujian, China).

RNA preparation

Twenty-one–base pair siRNA duplexes for Smad7 and APC were synthesized by Dharmacon Research (Lafayette, CO). siRNAs for Smad7 siRNA duplex (5′ AA GCU CAA UUC GGA CAA CAA G 3′), APC (5′ AA CUG GAA ACU GAG GCA UCU A 3′) or a nonspecific duplex oligo as a negative control (5′ AAC AGU CGC GUU UGC GAC UGG 3′; 12.8 μg/100 mm plate) were transfected using Oligofectamine (Invitrogen, Carlsbad, CA) at a ratio of 1 μg RNA to 3 μl Oligofectamine. We also used Smart Pool OFF Target for APC and GSK-3β (Dharmacon), and we purchased Signal Silence p38α MAPK siRNA II from Cell Signaling Technology (#6277) and transiently transfected it in PC-3U cells to knock down endogenous p38α.

Expression analyses

Total RNA was isolated from cells using TRIzol (Invitrogen) and treated with DNase before preparation of double-stranded cDNA using superscript II (Invitrogen). The following primers were used for quantitative real-time PCR in the Applied Biosystems (Foster City, CA) 7500 system. APC: forward primer (FP), GCAGCACTCCACAACATCAT, and reverse primer (RP); ATTTTTGTCCTGGTCCATGC. Smad7: FP, TCCTGCTGTGCAAAGTGTTC, and RP, TCTGGACAGTCTGCAGTTGG.

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