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Smad4 is Essential for Down-regulation of E-cadherin Induced by TGF-β in Pancreatic Cancer Cell Line PANC-1

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Smad4 is a tumour suppressor gene frequently deleted in pancreatic cancer. To investigate the roles of Smad4 deficiency in invasive and matastatic capabilities of pancreatic cancer, we examined the effects of Smad4 deficiency on regulation of the invasion suppressor E-cadherin in pancreatic cancer cell line PANC-1. TGF-β decreased expression of E-cadherin and β-catenin proteins at the plasma membrane, increased Snail and Slug mRNA expression, and induced fibroblastoid morphology in PANC-1 cells. These effects of TGF-β were abrogated in Smad4-knocked-down PANC-1 cells. We also found that TGF-β-induced down-regulation of E-cadherin expression was partially inhibited in Snail- and Slug-knocked-down PANC-1 cells. Thus, Smad4 mediates down-regulation of E-cadherin induced by TGF-β in PANC-1 cells, at least in part, through Snail and Slug induction. These results suggest that Smad4 deficiency observed in invasive and metastatic pancreatic cancer might not be linked to the loss of E-cadherin.

Key words: pancreatic cancer, E-cadherin, Smad4, TGF-β.

Abbreviations: TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor; siRNA, small-interfering RNA; EMT, epithelial mesenchymal transition.

Smad4 was originally identified as a tumour suppressor gene (DPC4: deleted in pancreatic cancer, locus 4) functionally inactivated in one-half of pancreatic adenocarcinoma (*1*). Subsequent studies have revealed that Smad4 belongs to the Smad gene family, which encodes intracellular signalling mediator of the transforming growth factor-β (TGF-β) superfamily of cytokines (*2*, *3*). TGF-β binds to two different types of serine/threonine kinase receptors, termed type I and type II. The activated heteromeric complex of TGF-β type I and type II receptors induces phosphorylation of Smad2 and Smad3, which form hetero-oligomeric complexes with Smad4. The complexes then translocate to the nucleus and regulate transcriptional responses together with DNA binding co-factors.

Since TGF-β is a potent inhibitor of the growth of epithelial cells (*4*), the tumour-suppressor function of Smad4 in pancreatic cancer has been attributed to its capacity to mediate TGF-β-induced growth inhibition. However, recent studies propose that loss of Smad4 may be associated with invasive and metastatic capabilities of the tumour through various mechanisms (*5–8*). For example, Schwarte-Waldhoff *et al*. (*7*) reported that Smad4-deficient pancreatic cancer cells showed increased vascular endothelial growth factor (VEGF) expression associated with their capabilities of *in vivo* metastasis through enhanced angiogenesis.

E-cadherin is a 120 kDa tramsmembrane glycoportoein that connects cells via homotypic interactions (*9*). Its cytoplasmic domain associates with a family of submembranous cytoplasmic proteins termed the catenins: α-catenin, β-catenin, γ-catenin and p120 (*10*). They link E-cadherin to the cytoskeleton, thus acting as a scaffold for the maintenance of cell architecture. The E-cadherin–catenin complexes represent the main adhesion system responsible for the maintenance of cell–cell contacts in epithelial tissues (*10*).

It is now recognized that E-cadherin may act as a tumour suppressor (*11*). Numerous studies have shown that down-regulation of E-cadherin expression or functional perturbation of the E-cadherin–catenin complexes has been found to occur very frequently during the progression of carcinomas and is associated with an invasive and matastatic phenotype of the tumours. Regarding pancreatic cancer, loss of membranous E-cadherin expression was associated with high grade and advanced stage of the cancer (*12–14*). Furthermore, Perl *et al*. (*15*) elegantly showed that the loss of E-cadherin expression coincided with the transition from well differentiated adenoma to invasive carcinoma in a transgenic mouse model of pancreatic β-cell carcinogenesis, giving further support for a role of E-cadherin as an 'invasive suppressor' of pancreatic cancer.

In this study, we therefore hypothesized that loss of Smad4 might be linked to down-regulation of the

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invasive suppressor E-cadherin in pancreatic cancer cells, resulting in acquisition of highly metastatic capabilities of the tumour. Contrary to our expectations, the current results using Smad4-knockdown pancreatic cancer cell lines suggest that Smad4 is essential for down-regulation of E-cadherin induced by TGF-β in pancreatic cancer cell lines and Smad4 deficiency may not be associated with selective loss of E-cadherin observed in advanced pancreatic cancer.

MATERIALS AND METHODS

Cell Lines—The Grade III undifferentiated human pancreatic cancer cell line, PANC-1 (*16*), was purchased from the American Type Culture Collection (Rockville, MD, USA). Human pancreatic adenocarcinoma cell lines PK-8 and PK-9 were obtained from Dr M. Kobari (Sendai, Japan) (*17*). Human keratinocyte cell line HaCaT cells were previously obtained from Dr N.E. Fusenig (DKFZ, Heidelberg, Germany) (*18*). These cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco/Invitrogen, Carsbad, CA) containing 10% FCS and 100 μg/ml streptomycin.

Establishment of Smads-knocked-down Cell Lines—We have established Smad2-knocked-down, Smad3-knocked-down, Smad4-knocked-down PANC-1 cells and control Smad4-intact PANC-1 cells, which are designated PANC-1-S2KD, PANC-1-S3KD, PANC-1-S4KD and PANC-1-puro, by using the stable RNA interference (RNAi) method as previously described (*19*, *20*). PK-8 and HaCaT cells stably transfected with the plasmid expressing siRNA that targeted Smad4 (S4KD) or with the empty plasmid (puro) were also established as previously described (*19*, *20*).

Reagents—Human recombinant TGF-β1 was purchased from R&D Inc. (Minneapolis, MN, USA). PD98059, SB203580 and LY294002 were purchased from CALBIOCHEM (San Diego, CA, USA) and SB202190, SP600125 were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). Human Snail and Slug siRNA were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Transfection of Snail and Slug siRNAs into PANC-1 cells was performed according to the manufacturer's instruction.

Western Blot—Western blot analysis was performed by using antibodies for E-cadherin (Beckton Dickinson, San Jose, CA, USA), Smad2/3, Smad4 (Transduction Laboratories Inc.) and β-actin (Cell Signaling Technology Inc., Beverly, MA, USA) as previously described (*19*, *20*).

Immunofluorescence—Cells were grown to confluency on 8-well chamber slides, rinsed with PBS and fixed with ice-cold 100% methanol for 5 min at −20°C. The fixed cells were incubated with primary antibody, anti human E-cadherin (dilution 1:50 in PBS + 1% BSA) (Beckton Dickinson) for 1 h followed by FITC secondary antibodies (dilution 1:50 in PBS + 1% BSA) (Dako Cytomation, Kyoto, Japan). Samples were mounted with mounting medium (Dako Cytomation) and examined under an ultraviolet light microscope.

Quantitative Real-time PCR—Quantitative real-time PCR with specific primers and probes (purchased from Applied Biosystems) was performed using the ABI7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) as previously described (*19*, *20*).

ELISA—Cells were cultured in serum-free DMEM for indicated times and the amounts of TGF-β2 and VEGF in the culture supernatants were determined by using the human TGF-β2 and VEGF ELISA kit (R&D Inc.).

Data Analysis—Values represent the mean ± SD of triplicate samples per group. An unpaired Student's *t*-test was used for the statistical analysis of the results. $P < 0.05$ was considered to be significant.

RESULTS

Smad4 Deficiency Attenuates Down-regulation of E-cadherin Induced by TGF-β in Human Pancreatic Carcinoma Cell Line PANC-1—The human pancreatic carcinoma cell line, PANC-1, expressed functional TGF-β type I and II receptors and Smads (*21–23*). In addition, Nakajima *et al*. (*24*) recently reported that TGF-β decreased E-cadherin expression in PANC-1 cells with a change to a fibroblastoid morphology (*24*). Consistent with these findings, Western blot and immunofluorescence study showed that control PANC-1-puro cells expressed E-cadherin, which was down-regulated by addition of TGF-β (Fig. 1A and B). PANC-1-puro cells also showed a change from epithelial to spindle fibroblastic shaped morphology upon TGF-β stimulation (Fig. 1B). We also found significant β-catenin expression at the plasma membrane in PANC-1-puro cells, which was down-regulated by TGF-β (Fig. 1B). In contrast, treatment with TGF-β did not affect E-cadherin expression, epithelial morphology, and β-catenin expression at the plasma membrane in Smad4-knocked-down PANC-1-S4KD cells (Fig. 1A and B). These results indicated that Smad4 is essential for TGF-β-induced down-regulation of E-cadherin in PANC-1 cells.

Smad4 Deficiency Attenuates Down-regulation of E-cadherin Induced by TGF-β in PK-8, PK-9 and HaCaT Cell Lines—To determine whether Smad4 is essential for TGF-β-induced down-regulation of E-cadherin in other cell lines, we examined the effect of Smad4 deficiency on TGF-β-induced down-regulation of E-cadherin in human pancreatic cancer cell line PK-8 and human keratinocyte cell line HaCaT. Both cell lines express Smad4 and respond to TGF-β (*25*, *26*). Similar to the results obtained in PANC-1 cells, immunofluorescence study showed that TGF-β down-regulated E-cadherin expression in control, but not in Smad4-knocked-down, PK-8 and HaCaT cells (Fig. 2A and B). We also found that PK-9 human pancreatic cancer cell line, which lacked Smad4 expression (*25*), expressed E-cadherin, which was not affected by stimulation with TGF-β (Fig. 2C). These results suggest that an essential role of Smad4 in TGF-β-induced down-regulation of E-cadherin is not specific to PANC-1 cells and may be a general phenomenon.

Smad4 Deficiency Attenuates TGF-β-induced Expression of Snail and Slug mRNAs in PANC-1 Cells—Transcriptional repression mechanisms have emerged

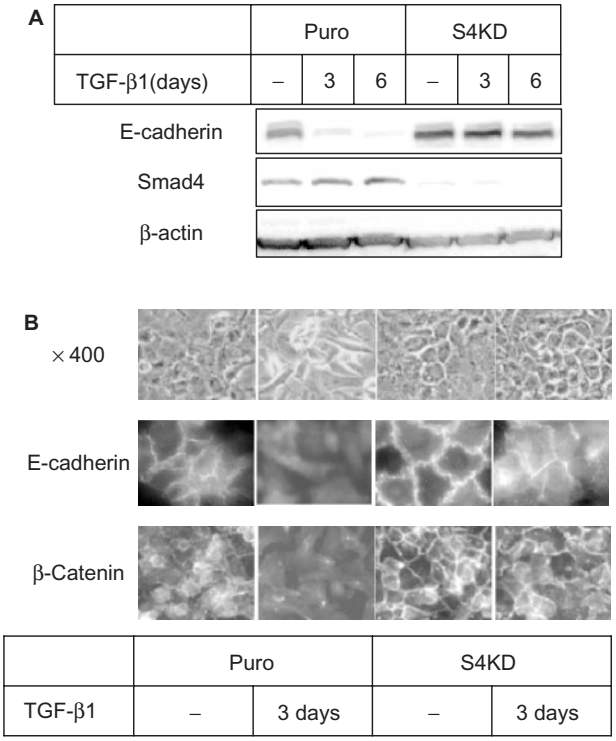

Fig. 1. **Smad4 deficiency attenuates down-regulation of E-cadherin induced by TGF-β in human pancreatic carcinoma cell line PANC-1.** Smad4-knockdown PANC-1 cells (PANC-1-S4KD) and control Smad4-intact PANC-1 cells (PANC-1-puro) were established as described in the section 'Materials and Methods'. (A) Cells were stimulated with 10 ng/ml TGF-β for 3 or 6 days and, then, cell lysates were subjected to Western blot analysis with specific antibodies for E-cadherin, Smad4 and β-actin. (B) (Upper panels) Phase contrast microphotographs showing morphology of a representative PANC-1-puro and PANC-1-S4KD cells in response to 72 h stimulation of 10 ng/ml TGF-β1; (middle and bottom panels) cells were stimulated with 10 ng/ml TGF-β for 72 h and, then, were subjected to immunofluorescence analysis with specific antibodies for E-cadherin and β-catenin. Similar results were obtained at least in three independent experiments.

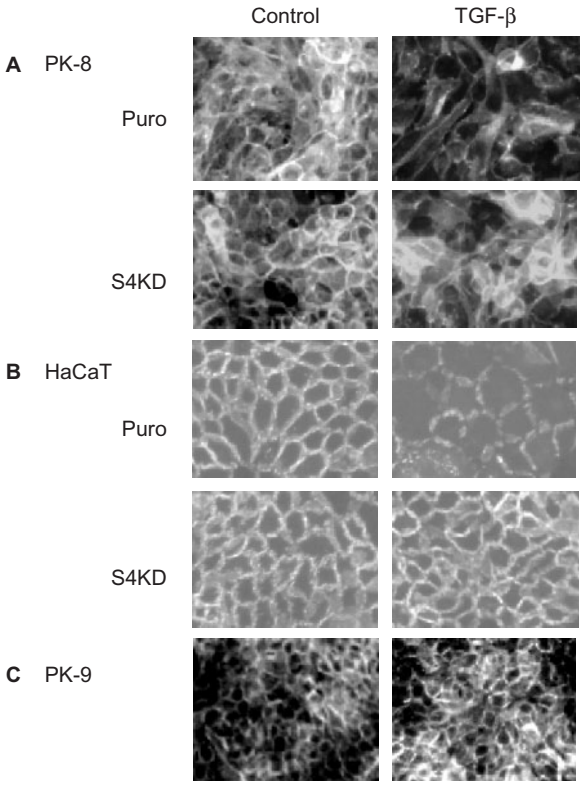

Fig. 2. **Smad4 deficiency attenuates down-regulation of E-cadherin induced by TGF-β in human pancreatic carcinoma cell lines PK-8 and PK-9 and in human keratinocyte cell line HaCaT.** Smad4-knockdown PK-8 cells and HaCaT cells (PK-8-S4KD, HaCaT-S4KD) and control Smad4-intact PK-8 and HaCaT cells (PK-8-puro, HaCaT-puro) were established as described in the section 'Materials and Methods'. PK-8-puro or PK-8-S4KD (A), HaCaT-puro or HaCaT-S4KD (B), or Smad4-deficient PK-9 cells (C) were stimulated with 10 ng/ml TGF-β for 3 days and, then, were subjected to immunofluorescence analysis with specific antibodies for E-cadherin and β-catenin. Similar results were obtained at least in three independent experiments.

as one of the crucial processes for the down-regulation of E-cadherin expression during development and tumour progression (*27*). Several E-cadherin transcriptional repressors have been characterized (Snail, E12/E47, ZEB-1, SIP-1 and Slug) and shown to act through an interaction with proximal E-boxes of the E-cadherin promoter (*27–29*). Recently, TGF-β was reported to be a regulator of Snail and Slug expression and involved in down-regulation of E-cadherin (*30*, 31). We thus examined the effects of Smad4 deficiency on Snail and Slug expression in PANC-1 cells. As shown in Fig. 3A, real-time PCR analysis showed that expression of Snail and Slug mRNA was induced by TGF-β in PANC-1-puro cells. Induction of Snail mRNA by TGF-β was observed in the presence of cyclohexamide (CHX), suggesting that *de novo* protein synthesis is not required for the Snail mRNA induction (Fig. 3A). On the other hand, Slug mRNA expression was increased in the presence of CHX alone and TGF-β did not up-regulate Slug mRNA expression in the presence of CHX. Thus, *de novo* protein synthesis may be required for the Slug mRNA induction (Fig. 3A). In PANC-1-S4KDcells, TGF-β-induced Snail and Slug expression was abrogated (Fig. 3B). To determine whether TGF-β/Smad4-dependent Snail and Slug expression is required for TGF-β-induced down-regulation of E-cadherin, we examined the effects of Snail or Slug deficiency on TGF-β-induced down-regulation of E-cadherin in PANC-1 cells. As shown in Fig. 4A and B, TGF-β-induced down-regulation of E-cadherin was partially suppressed in Snail- and Slug-knocked-down PANC-1 cells. These results suggest that TGF-β/Smad4-dependent Snail and Slug expressions are, at least in part, required for TGF-β-induced down-regulation of E-cadherin in PANC-1 cells.

Smad4 Deficiency Attenuates TGF-β-induced TGF-β and VEGF Production in PANC-1 Cells—Previous studies have suggested that Smad4 deficiency augments TGF-β and VEGF production of pancreatic cancer cells,

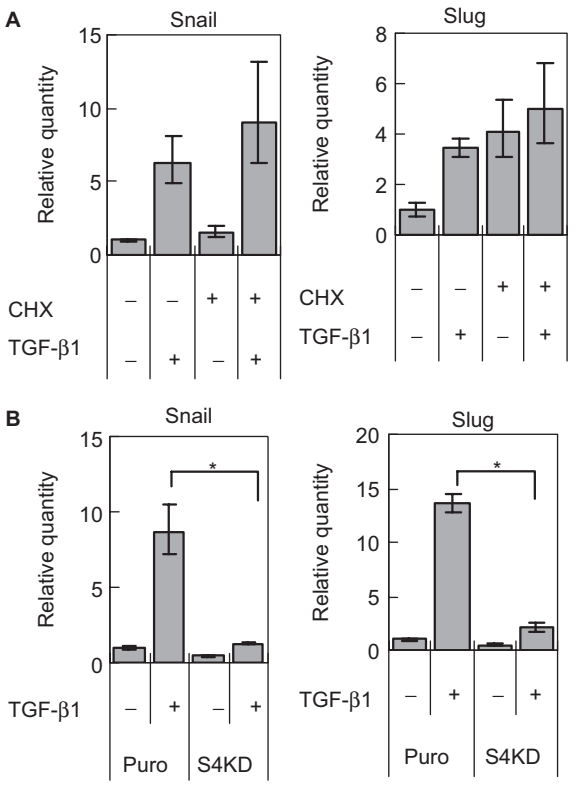

Fig. 3. **Smad4 deficiency attenuates TGF-β-induced expression of Snail and Slug mRNAs in PANC-1 cells.** (A) cDNAs were obtained from PANC-1-puro after 6 h stimulation with 10 ng/ml TGF-β in the presence or absence of 10 μg/ml cyclohexamide (CHX). Then, real-time PCR was performed using specific primers for Snail, Slug, and GAPDH. (B) cDNAs were obtained from PANC-1-puro and PANC-1-S4KD cells after 6 h stimulation with 10 ng/ml TGF-β and, then, real-time PCR was performed using specific primers for Snail, Slug, and GAPDH. The ratio of each gene to that of GAPDH was calculated, and the value of 1.0 was assigned to the PANC-1 wild-type cells that were incubated without TGF-β1. Values represent the mean ± SD of triplicate samples per group. $^{*}P < 0.05$ compared with corresponding control. Similar results were obtained at least in three independent experiments.

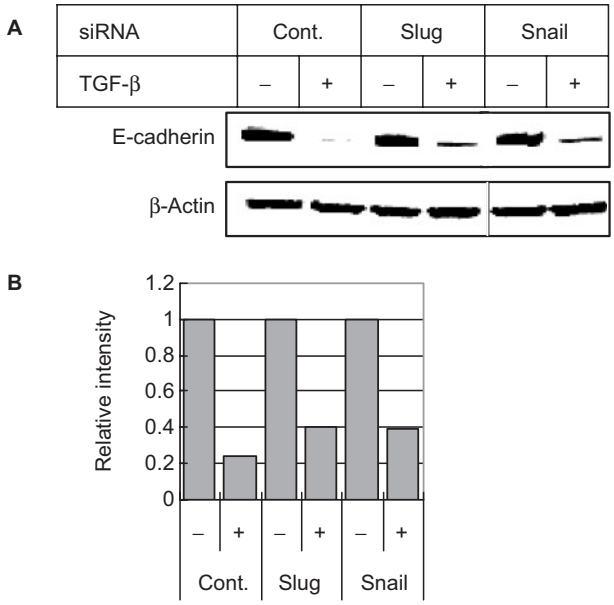

Fig. 4. **Snail and Slug deficiency partially attenuates TGF-β-induced down-regulation of E-cadherin expression in PANC-1 cells.** (A) PANC-1 cells were transfected with Snail, Slug, or control siRNAs according to the manufacturer's instruction. At 72 h after the transfection, cells were stimulated with 10 ng/ml TGF-β for 3 days and, then, cell lysates were subjected to western blot analysis with specific antibodies for E-cadherin and β-actin. (B) The density of the detected bands (E-cadherin and β-actin) was quantified in a Chemi Doc XRS-J, and the ratio (E-cadherin/β-actin) was calculated and presented below the western blot panel.

which is associated with invasive and metastatic capabilities of pancreatic cancer cells (*7*, *8*). Thus, we further examined the effects of Smad4 deficiency on TGF-β and VEGF production in PANC-1 cells (Fig. 5). Real-time PCR analysis showed that Smad7 mRNA induction, which was dependent of Smad4 (*32*), was abolished in PANC-1-S4KD cells, confirming the specific-knockdown of Smad4 in the cells. TGF-β1 significantly induced TGF-β1 and TGF-β2 mRNA expression in PANC-1-puro cells whereas it was abrogated in PANC-1-S4KD cells. In addition, ELISA assay showed that TGF-β-induced VEGF production was partially, but significantly, suppressed in PANC-1-S4KD cells (Fig. 5B). These results indicated that Smad4 is involved in TGF-β-induced TGF-β and VEGF expression in PANC-1 cells.

Smad3, but not Smad2, Deficiency Attenuates Down-regulation of E-cadherin Induced by TGF-β in PANC-1—Finally, we examined the effects of deficiency of other Smads or non-Smad pathways on down-regulation of E-cadherin induced by TGF-β in PANC-1 cells. As shown in Fig. 6A, Smad2-knockdown and Smad3-knockdown PANC-1 cells showed reduced expression of Smad2 and Smad3. Smad3, but not Smad2, deficiency attenuated down-regulation of E-cadherin induced by TGF-β in PANC-1 cells. In addition, an ERK inhibitor PD98059 and a p38 inhibitor SB203580 marginally suppressed down-regulation of E-cadherin induced by TGF-β in PANC-1 cells (Fig. 6B). In contrast, a JNK inhibitor SP600125 and a PI3K inhibitor LY294002 did not affect down-regulation of E-cadherin induced by TGF-β in PANC-1 cells (Fig. 6B). It was shown that SB203580 (a p38 inhibitor) was not completely specific to p38 and it also inhibited TGF-β signaling (*33*). Therefore, we examined the effects of SB202190, another selective p38 kinase inhibitor, on the down-regulation of E-cadherin induced by TGF-β in PANC-1 cells. SB202190 did not affect TGF-β-induced decrease of E-cadherin expression (Fig. 6B). These results suggested that Smad3 was also necessary for down-regulation of E-cadherin induced by TGF-β in PANC-1 cells and the ERK pathway was only partially involved in the regulation of E-cadherin.

DISCUSSION

Recent studies suggest that loss of Smad4 may enhance tumourgenicity by mechanisms independent of growth

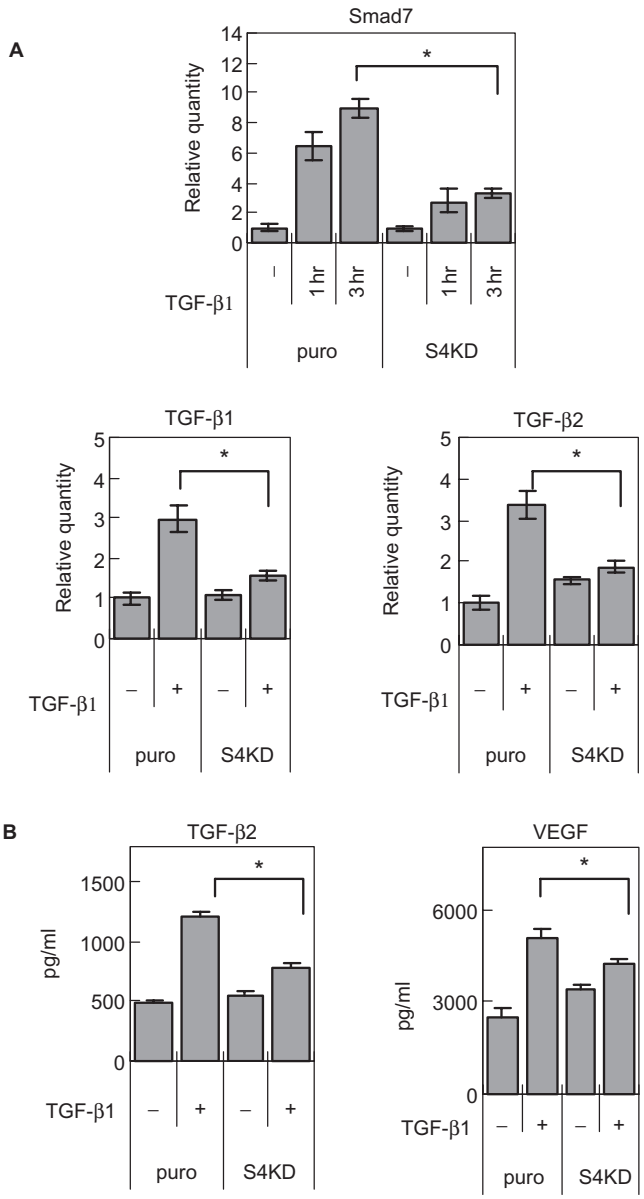

Fig. 5. **Smad4 deficiency attenuates TGF-β-induced TGF-β and VEGF production in PANC-1 cells.** (A) cDNAs were obtained from PANC-1-puro and PANC-1-S4KD cells after indicated hours or 12 h stimulation with 10 ng/ml TGF-β1 and, then, real-time PCR was performed using specific primers for Smad7, TGF-β1, TGF-β2 and GAPDH. The ratio of each gene to that of GAPDH was calculated, and the value of 1.0 was assigned to the PANC-1 wild-type cells that were incubated without TGF-β1. (B) Culture supernatants were obtained from PANC-1-puro and PANC-1-S4KD cells after 48 h stimulation with 10 ng/ml TGF-β1 and, then, concentrations of TGF-β2 and VEGF were measured by ELISA. Values represent the mean ± SD of triplicate samples per group. $^*P < 0.05$ compared with corresponding control.

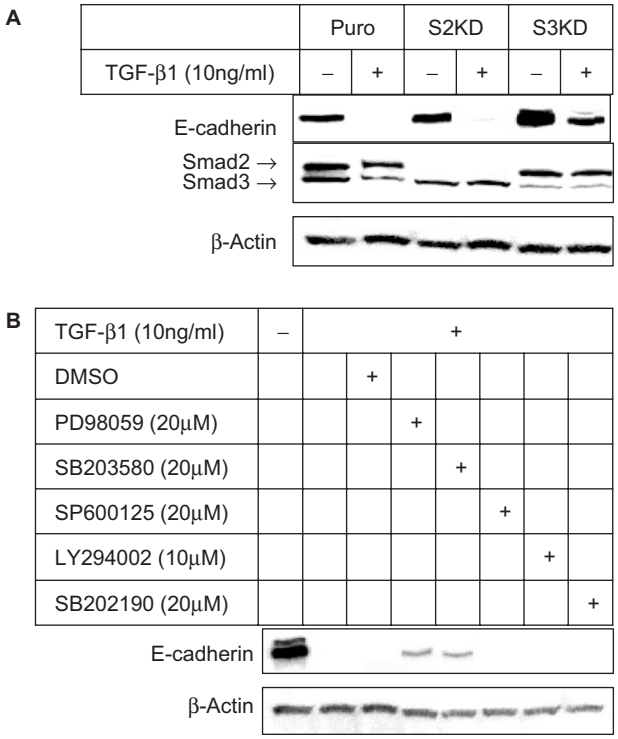

Fig. 6. **Smad3, but not Smad2, deficiency attenuates down-regulation of E-cadherin induced by TGF-β in PANC-1 cells.** (A) Smad2- and Smad3-knockdown PANC-1 cells (PANC-1-S2KD, PANC-1-S3KD) and control Smad4-intact PANC-1 cells (PANC-1-puro) were established as described in the section 'Materials and Methods'. Cells were stimulated with 10 ng/ml TGF-β for 6 days and, then, cell lysates were subjected to Western blot analysis with specific antibodies for E-cadherin, Smad2/3, and β-actin. (B) PANC-1-S4KD or PANC-1-puro cells were stimulated with 10 ng/ml TGF-β in the presence or absence of indicated concentrations of several inhibitors (PD98059, SB203580, SP600125, LY294002, SB212190) for 6 days and, then, cell lysates were subjected to western blot analysis with specific antibodies for E-cadherin and β-actin. Similar results were obtained at least three independent experiments.

inhibition by TGF-β. Indeed, loss of Smad4 expression occurs late in the development of pancreatic cancer (*34*) and may be associated with invasive and metastatic capabilities of the tumour through various mechanisms (*5–8*). Our current results, however, suggest that Smad4 is essential for TGF-β-induced down-regulation of E-cadherin in PANC-1 cells, at least in part, through up-regulation of E-cadherin repressors Snail and Slug (Figs 1, 3 and 4). In addition, Smad4 also appears to be required for TGF-β-induced TGF-β and, although partially, for TGF-β-induced VEGF production by PANC-1 cells (Fig. 5). Thus, loss of Smad4 might not be associated with loss of E-cadherin expression and also up-regulation of TGF-β and VEGF production frequently observed in advanced pancreatic cancer (*12–14*, *35*).

We showed that Smad4 deficiency abrogated down-regulation of E-cadherin induced by TGF-β in PANC-1 cells (Fig. 1). However, Muller *et al*. (*6*) previously reported that Smad4 induced E-cadherin in colon cancer cells, which was in contrast to our results. They showed that re-expression of Smad4 at physiological levels in Smad4-deficient SW480 colon cancer cell line transcriptionally induced E-cadherin and re-established epithelial morphology of the cells although they did not examine expression of Snail family of E-cadherin repressors in the cells. Thus, it is possible that Smad4

deficiency might give different impacts on colon and pancreatic cancer cells.

We showed that Smad4 deficiency abrogated TGF-β-induced TGF-β and VEGF production in PANC-1 cells (Fig. 5). These results were also in contrast to the findings by Subramanian *et al*. (*8*) and Schwarte-Waldhoff *et al*. (*7*). They reported that Smad4 deficiency activated endogenous TGF-β signalling and expression, and VEGF production, respectively, in pancreatic cancer cell lines. Different pancreatic cancer cell lines used in their (BxPC-3 cells, Hs766T cells) and our (PANC-1 cells) studies might explain the different outcomes. This issue, however, remains to be determined in future analysis.

We showed that Smad3, but not Smad2, deficiency attenuated down-regulation of E-cadherin induced by TGF-β in PANC-1 cells (Fig. 6A). It is reported that Smad3 null mutant mice are viable and fertile in contrast to Smad2 null mice that show early embryonic lethality, suggesting distinct roles of Smad2 and Smad3 *in vivo* (*36*). In addition, *in vitro* studies suggest that Smad2 and Smad3 have unique, non-overlapping roles in control of target gene expression by TGF-β (*37*, *38*). Our results also suggest the specific role for Smad2 and Smad3 for TGF-β signalling.

Ellenrieder *et al*. (*39*) reported TGF-β induced epithelial mesenchymal transition (EMT) through MAPK/ERK pathway in PANC-1 cells. They showed that TGF-β induced a fibroblastoid morphology, up-regulated mesenchymal markers, and down-regulated epithelial markers in PANC-1 cells although they did not examine E-cadherin expression as an epithelial marker. Our results showed that PD98059, an ERK inhibitor used in their study, only marginally affected TGF-β-induced decrease of E-cadherin in PANC-1 cells (Fig. 6B). Therefore, E-cadherin expression might undergo different regulation (i.e. Smad-dependent regulation) by TGF-β from other EMT-associated molecular events.

Recently, Han *et al*. (*40*) suggest that down-regulation of E-cadherin/catenin complex by TGF-β may not be associated with metastatic capabilities of skin cancers. They showed that chemically induced skin papilloma in double transgenic mouse expressing TGF-β and dominant negative TGF-β type II receptor (DNRII) progressed to metastasis without losing expression of the membrane-associated E-cadherin/catenin complex and at a rate higher than those observed in non-transgenic, TGF-β1-transgenic, or DNRII-transgenic mice. Thus, mechanisms independent of E-cadherin loss might be also involved in invasive and metastatic capabilities of pancreatic cancer.

In summary, we demonstrated that Smad4 was essential for down-regulation of E-cadherin induced by TGF-β in pancreatic cancer cell line PANC-1 and also in other TGF-β-responsive cell lines. Our results suggest that mechanisms independent of loss of E-cadherin might be involved in invasive or metastatic capabilities of advanced pancreatic cancer cells that lack Smad4. However, the *in vitro* system may not be relevant to the *in vivo* situation of pancreatic cancer. Thus, the roles of Smad4 deletion in the invasive/metastatic phenotype of pancreatic cancer should be investigated using the *in vivo* experimental models in future studies.

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