# Functional roles of TGF-$\beta_1$ in intestinal epithelial cells through Smad-dependent and non-Smad pathways.

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## Abstract

***Background & Aims:*** Transforming growth factor-$\beta_1$ (TGF-$\beta_1$) is one of the growth factors expressed in the gut, and has been shown to play an important role in intestinal mucosal healing. We investigated the effects of TGF-$\beta_1$ on the cellular functions of intestinal epithelial cells, and also evaluated its signaling pathways in these cells.

***Methods:*** We used the rat IEC-6 intestinal epithelial cell line for these studies. The expression of TGF-$\beta_1$/Smad signaling molecules was examined. We evaluated the effect of TGF-$\beta_1$ on the proliferation and differentiation by the BrdU incorporation assay and real-time PCR. We manipulated the expression levels of Smad2 and Smad3 using an adenovirus system and small interfering RNA to examine the signaling pathways. The expression of Smad2 and Smad3 along the crypt-villus axis was also examined in the murine intestine.

***Results:*** IEC-6 cells produced TGF-$\beta_1$ and expressed functional TGF-β/Smad signaling molecules. The addition of TGF-$\beta_1$ in the culture medium suppressed the proliferation and increased the expression of a differentiation marker of enterocytes, in a dose-dependent manner. The

adenovirus-mediated and small interfering RNA-mediated studies clearly showed that the growth inhibitory effect and the promotion of differentiation were exerted through a Smad3-dependent and a Smad2-dependent pathway, respectively. IEC-6 cells exhibited upregulated expression of an inhibitory Smad (Smad7) as a form of negative feedback via a non-Smad pathway. Smad2 was predominantly expressed in villi, and Smad3 in crypts.

***Conclusions:*** TGF-$\beta_1$ regulates the cellular functions of intestinal epithelial cells through both Smad-dependent and non-Smad pathways.

**Key words: proliferation, differentiation, intestinal epithelium, Smad2, Smad3, negative feedback.**

## Introduction

In vertebrates, more than 30 genes encode transforming growth factor (TGF) β-related polypeptides, which are processed and secreted as homodimers or heterodimers [1]. TGF-β family members act multifunctionally in numerous tissue types; regulating cell growth, differentiation, adhesion, migration and death, in a developmental context-dependent and cell-type specific manner [2]. The Smad proteins are a group of molecules that function as intracellular signaling mediators and modulators of TGF-β family members. The Smad family of proteins can be classified into three functional groups: the receptor-regulated Smads (R-Smads), common mediator Smads (Co-Smads), and the inhibitory Smads (I-Smads). The TGF-β family can be divided into two groups based on their interaction with Smads: the TGFs, activins, nodal and myostatin, which act through Smad2 and Smad3; and the bone morphogenic proteins (BMPs) and growth differentiation factors (GDFs), which act through Smad1, 5, and 8. A secreted dimeric ligand binds to a heterotetrameric cell surface complex of two type II and two type I receptors. In the complexes, ligand binding induces type II receptors to phosphorylate, and thereby activate, the type I

receptor kinases; which then activates Smads that have been recruited to the receptor complex through the direct phosphorylation on the C-terminal Ser-Ser-X-Ser (SSXS) motif. Two R-Smads form a trimeric complex with a single Co-Smad, and this complex translocates into the nucleus and executes ligand-induced transcriptional activation or repression of responsive target genes, together with other transcription factors [3]. In the TGF-β signaling pathway, Smad2 and Smad3 function as R-Smads and Smad7 functions as an I-Smad. The only mammalian Co-Smad identified thus far is Smad4. In addition to the Smads, non-Smad signaling proteins also participate in the TGF-β signal transductions, such as the small GTPase Ras and the mitogen-activated protein kinases (MAPKs) ERKs, p38 and c-Jun N-terminal kinases (JNKs) [2,4].

The intestinal epithelium is a highly specialized cell population undergoing continuous rapid turnover. The architectural features of the gastrointestinal tract are maintained by a rapid cellular turnover through continuous replication of multipotential stem cells within the crypts, with progeny cells moving in vertical columns toward the villus apex. The intestinal epithelium is functionally divided into a zone of proliferation

confined to crypts and a zone of differentiation situated in the villi. It undergoes continuous renewal throughout the life span, and the terminally differentiated cells of the epithelium are removed by apoptosis[5]. The intestinal epithelium is vulnerable to injury from many different processes, including toxic luminal substances, normal digestion, inflammation, interactions with microbes, oxidative stress, and pharmaceuticals. Once injury occurs, rapid restitution of an epithelial layer is important to maintain the normal function of the digestive tract [6].

Intestinal wound healing is dependent on the precise balance of the migration, proliferation and differentiation of the epithelial cells. Growth factors such as epidermal growth factor, hepatocyte growth factor, and TGF-α play important roles in the regulation of bowel growth, protection, repair and homeostasis [7]. TGF-$\beta_1$ is also involved in modulating proliferation, the commitment to terminal differentiation, induction of extracellular matrix protein formation, and the stimulation of cell migration in intestinal epithelial cells [8,9]. TGF-$\beta_1$ is expressed by various cells in the intestinal mucosa, including the epithelial cells lining the intestinal villi and the lymphocytes in the lamina propria [10].

Smad2/3 and Smad4 are direct mediators of TGF-β signaling, and there is now substantial evidence to suggest that Smad2 and Smad3 have distinct and non-overlapping roles in TGF-β signaling. However, the pathways activated by the TGF-β protein remain to be fully characterized, and even which Smads mediate the signals remains poorly investigated. In the context of proliferation, knockout of Smad2 or Smad3 in mouse embryonic fibroblasts results in only weak growth inhibition by TGF-β in culture, compared to wild-type fibroblasts, the growth of which is inhibited by TGF-β[11,12]. TGF-β induces growth inhibition in Smad3-null mammary gland epithelial cells in culture [11,12]. In contrast, TGF-β treatment of murine hepatocytes with conditional deletion of Smad2 results in TGF-β-mediated G1 arrest, apoptosis, and epithelial mesenchymal transition, but this does not occur in hepatocytes derived from *Smad3*$^{-/-}$ mice [13]. The different cell types may explain the variations in the effect of TGF-β on cell proliferation, because there both context- and cell type-specific differences in TGF-β signaling.

Intestinal epithelial cells express both TGF-β receptors and ligands, so it is possible that the regulation of cellular responsiveness relies on the

production of active TGF-β and its presentation to the signaling receptors [10]. Although TGF-$\beta_1$ was reported to inhibit the proliferation of rat intestinal epithelial cells and to regulate the reconstitution of epithelial integrity after mucosal injury [8,9], the specific TGF-β responses that are mediated by Smad2 and/or Smad3 are not completely understood. The aim of this study was to characterize the functional roles of TGF-$\beta_1$ in intestinal epithelial cells and the Smad-dependency of the signaling pathways. We used the rat IEC-6 small intestinal cell line to conduct experiments using adenovirus-mediated overexpression and small interfering RNA (siRNA)-mediated downregulation of Smad proteins. Although the dominant-negative Smad2/3 mutant was generated by substituting Glu for Asp-407 of Smad3, which is defective in TGF-β receptor-dependent phosphorylation, this mutant possesses a dominant-negative effect on both Smad2 and Smad3 [14,15]. The addition of TGF-$\beta_1$ to the culture medium was found to suppress the proliferation and increase the expression of acyl-CoA synthetase long-chain family member5 (*Acsl5*), a differentiation marker of enterocytes, in IEC-6 cells. Clarifying the molecular mechanisms underlying the signal transduction of TGF-$\beta_1$ in the intestinal epithelium

will enable us to understand the intestinal integrity and to develop a new strategy to treat intestinal mucosal injuries.

## Materials and Methods

### *Materials*

Recombinant human TGF-$\beta_1$ was purchased from R&D Systems (Minneapolis, MN, USA). Anti-Smad2, anti-Smad3, anti-Smad2/3 and anti-actin antibodies (Ab) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-phospho-Smad2 and anti-phospho-Smad3 Ab were from Cell Signaling Technology (Heidelberg, Germany). The anti-FLAG Ab was from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG, HRP-conjugated donkey anti-rabbit IgG, HRP-conjugated donkey anti-goat IgG, and Cy3-conjugated donkey anti-rabbit IgG were from Jackson Immuno Research (West Grove, PA). PD98059 and SB203580 were purchased from Merck Millipore (Darmstadt, Germany). SP600125 and LY294002 were from Cell Signaling Technology (Heidelberg, Germany).

### *Cell culture*

A rat intestinal epithelial cell line, IEC-6 (RBRC-RCB0993), was purchased from RIKEN Cell Bank (Tsukuba, Japan). The cells were cultured

in Dulbecco's modified Eagle's medium (DMEM) containing 5 % fetal bovine serum, 4 μg/ml insulin, 25 mM glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified environment of 95% air and 5% $CO_2$. The medium was replaced every 1-3 days, depending on the harvest time and the degree of confluence. All of the experiments were carried out using IEC-6 cells at the 10-25th passage.

### *Adenovirus infection*

Recombinant adenoviruses containing FLAG-tagged Smad cDNAs were kindly provided by Dr. K. Miyazono (University of Tokyo, Tokyo, Japan) and Dr. Hiroshi Yasuda (St. Marianna University School of Medicine, Kawasaki, Japan). For a single adenovirus infection, the cells were infected with a recombinant adenovirus at a dose of 10 plaque-forming units (pfu)/cell in the culture medium described above. In the experiments using double adenovirus infection, the cells were infected with a dominant-negative Smad2/3 adenovirus (AdDNSmad2/3) at a dose of 10 pfu/cell, simultaneously with Smad2 (AdSmad2) or Smad3 (AdSmad3) adenovirus at doses of 5, 10, or 20 pfu/cell. The medium was changed after 12 hours, and TGF-$\beta_1$ was added.

Subsequent experiments were performed at 60 hours after the infection. An adenovirus expressing β-galactosidase (AdLacZ) was used as an infection control.

### *Small interfering RNA (siRNA) transfections*

The siRNAs specific for rat Smad2 and Smad3 (ON-TARGET plus SMARTpool, a mixture of four prevalidated siRNAs) and the matched negative control (non-target control SMARTpool) were purchased from Thermo Scientific Dharmacon (Epsom, UK). The Smad2, Smad3 and negative control siRNAs were transfected twice on two consecutive days using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

### *Measurement of the secretion of TGF-$\beta_1$ in IEC-6 cells.*

IEC-6 cells were seeded at a density of $1x10^5$/ml in plastic 24-well plates and were cultured in DMEM for the indicated lengths of time. The secretion of the TGF-$\beta_1$ peptide was measured by determining the concentration in the supernatant using a commercial ELISA kit (R&D

Systems, Minneapolis, MN) according to the manufacturer's instructions. The protein concentration was measured by the Bradford method using bovine serum albumin as the standard.

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*Immunohistochemical staining*

IEC-6 cells were seeded at a density of 2 x $10^4$/ml on a culture slide, and were cultured in DMEM for 24 hours. Next, the cells were infected with a recombinant adenovirus at a dose of 10 pfu/cell. The medium was changed to DMEM containing 0.1% FBS after 12 hours, and the cells were cultured for a further 48 hours. The cells were fixed in a 2.5% paraformaldehyde with or without stimulation using 10 pM TGF-$\beta_1$ for the last 2 hours of incubation, and were then immunostained sequentially with Blocking Ace (Snow Brand Milk Products, Sapporo, Japan), primary antibodies, and secondary antibodies. Serial sections of formalin-fixed and paraffin-embedded samples of murine intestine were stained in the same manner, using 3,3'-diaminobenzidine tetrahydrochloride as the substrate. The sections were counterstained with hematoxylin. Negative controls were treated the same way, but without the primary antibodies. The samples were examined under

an Olympus BX51 microscope (Olympus Co., Tokyo, Japan). The images were digitized and then processed using the Photoshop software program (Adobe System Inc., Mountain View, CA).

### *Western blotting analysis*

The Western blotting analysis was carried out as described previously [16]. Briefly, for gel electrophoresis, 20-30 μg of protein was loaded onto each lane of 7.5% sodium dodecyl sulfate-polyacrylamide gels and run at 10-20 mA. The proteins were then transferred to nitrocellulose membranes at 60 V for 4 hours. The membranes were incubated sequentially with Blocking Ace (Snow Brand Milk Products, Sapporo, Japan), primary antibodies, secondary antibodies, and then proteins were detected using an enhanced chemiluminescence Western blotting detection reagent (Amersham Biosciences, Piscataway, NJ) to visualize the secondary antibody. The band intensity of actin was used as an internal control.

### *Conventional reverse-transcription polymerase chain reaction (RT-PCR)*

The total RNA was obtained from IEC-6 cells using an RNeasy Mini kit (QIAGEN, Valencia, CA). First-strand complementary DNA was synthesized from total RNA using the Superscript™ First-stranded Synthesis System for Reverse-Transcription Polymerase Chain Reaction (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The PCR primers used in the study are listed in Table 1. The reactions were conducted using denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds, for 25-35 cycles.

### *Quantitative real-time PCR (qRT-PCR)*

The primers used for qRT-PCR are also listed in Table 1. Expression profiling of microdissected enterocytes revealed a set of differentiation markers in the crypt-villus axis, including acyl-CoA synthetase long-chain family member 5 (*Acsl5*), intestinal-type alkaline phosphatase (*Alpi*) and apolipoprotein A-IV (*ApoA4*) [17]. We chose *Acsl5* as a differentiation marker of enterocytes in this study. The reactions were conducted using an ABI PRISM 7900HT instrument (Applied Biosystems, San Francisco, CA) as follows: denaturation at 95°C for 10 seconds, annealing at 60°C for 10

seconds, and extension at 72°C for 10 seconds. The data were analyzed by relative quantification using the comparative threshold cycle method and were normalized to *β-actin*.

### *Measurement of DNA synthesis*

The IEC-6 cells were seeded at a density of 2-4 x $10^4$ cells/ml in plastic 96-well plates and were cultured in DMEM containing 5% FBS. After serum starvation for 24 hours, the IEC-6 cells were treated with TGF-$\beta_1$ at the indicated concentrations for 48 hours in DMEM containing 0.1% FBS. BrdU was added to the culture medium for the last two hours, and the BrdU incorporation was measured using a commercial ELISA kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions.

### *Statistical analysis*

All data are presented as the means ± SD. The statistical significance of the values obtained was evaluated by Student's t-test. A value of $p < 0.05$ was considered to be significant.

## Results

### *IEC-6 cells possess functional TGF-β signaling pathways and also produce TGF-$\beta_1$.*

We first examined whether the rat IEC-6 nonmalignant intestinal epithelial cells possess functional molecules in the TGF-$\beta_1$/Smad signaling pathway. As shown in Figure 1A, the IEC6 cells express mRNAs of not only for the receptors (TβRI, TβRII) and *Smad* molecules (R-Smads (*Smad2* and *Smad3*), Co-Smad (*Smad4*), I-Smad (*Smad7*), but also TGF-$\beta_1$ itself. Endogenous R-Smads were detected in naïve IEC-6 cells by a Western blotting analysis and the cellular homogenate of adenovirus infected-cells was used as a positive control (Figure 1B). IEC-6 cells also secreted TGF-$\beta_1$ in a time-dependent manner (Figure 1C). The concentration of TGF-$\beta_1$ was estimated to be about 4nM in the supernatant after 48 hours of incubation.

We next examined whether the Smad-dependent TGF-$\beta_1$ signaling pathways function properly in naïve and adenovirus-infected IEC-6 cells. The cells were infected with the indicated adenovirus at a dose of 10 pfu/cell. The infection efficiency using LacZ-expressing adenovirus (AdLacZ) and *in situ* staining with X-gal was estimated to be more than 98% (data not shown),

which was consistent with our previous study using rat pancreatic stellate cells [15]. IEC-6 cells were incubated with or without 10p M TGF-$\beta_1$ for the last two hours in culture, fixed, and stained with an anti-Smad2/3 or anti-FLAG Ab. In naïve IEC-6 cells without the TGF-$\beta_1$-treatment, the R-Smads (Smad2 and Smad3) exist primarily in the cytosol (Figure 2A, top left panel). When the cells were treated with TGF-$\beta_1$, the R-Smads translocated and accumulated in the nucleus (Figure 2A, top right panel). In AdLacZ-infected IEC-6 cells, R-Smads showed similar localization (Figure 2A, middle panels), whereas they did not accumulate in the nucleus, but remained in the cytosol in AdDNSmad2/3-infected IEC6 cells after the addition of TGF-$\beta_1$ to the culture medium (Figure 2A bottom panels). In order to discriminate Smad2 and Smad3, IEC-6 cells were infected with AdSmad2 or AdSmad3, and were stained with anti-FLAG Ab. Both R-Smads translocated from the cytosol to the nucleus with the addition of TGF-$\beta_1$ (Figure 2B). These results suggested that functional Smad2- and Smad3-dependent TGF-$\beta_1$ signaling pathways are preserved in IEC-6 cells.

***TGF-$\beta_1$ suppressed the proliferation of IEC-6 cells through a***

*Smad3-dependent pathway.*

We next examined the effects of TGF-$\beta_1$ on the proliferation of IEC-6 cells. As reported previously [8,9], the addition of TGF-$\beta_1$ suppressed the proliferation of IEC-6 cells in a dose-dependent manner (Figure 3A). In order to explore the signaling pathway, IEC-6 cells were infected with the indicated adenoviral vectors. After the infection, the cells were incubated with 25 pM TGF-$\beta_1$ for 48 hours. The overexpression of Smad3, but not Smad2, led to growth inhibitory effect (Figure 3B), thus suggesting that TGF-$\beta_1$ inhibits the proliferation of IEC-6 cells through a Smad3-dependent pathway.

However, the possibility remained that the growth inhibitory effect may have resulted from the inhibition of endogenous Smad2 function, and not the involvement of a Smad3-dependent pathway, because Smad2 and Smad3 compete with each other for the receptors and Smad4 during the binding steps. To exclude this possibility, we investigated the Smad2- and Smad3-specific roles in the growth inhibitory effect by co-infection of AdSmad2 or AdSmad3 with AdDNSmad2/3. As shown in Figure 3B, the infection with AdDNSmad2/3 slightly increased the proliferation, and

AdSmad2 co-infection with AdDNSmad2/3 did not alter the cell proliferation. On the other hand, AdSmad3 co-infection with AdDNSmad2/3 led to growth inhibitory effect. To further confirm the Smad3-dependency of this effect, we knocked down the expression of Smad2 or Smad3 using siRNA (Figure 3C) and examined the effects on proliferation. When we knocked down Smad3 expression, the growth inhibitory effect of TGF-$\beta_1$ was abolished (Figure 3D). These data suggest that TGF-$\beta_1$ inhibits the proliferation of IEC-6 cells through a Smad3-dependent pathway.

***TGF-$\beta_1$ upregulated the expression of the Acsl5 gene, a differentiation marker of enterocytes, through a Smad-2 dependent pathway.***

We then examined the effect of TGF-$\beta_1$ on the differentiation of IEC-6 cells. There are two types of enterocytes in the crypt-villus axis, the non-absorptive (located in the upper part of crypts) and the absorptive (located in the middle of villi) cells [17]. In enterocytes lining villi, the expression of a high number of genes related to fatty acid metabolism, such as *Acsl5*, is preferentially found, which was indicative of the absorptive

enterocytes [17]. In the present study, IEC-6 cells were incubated with or without various concentrations of TGF-$\beta_1$ for 48 hours, and a real-time PCR study was performed (Figure 4A). The addition of TGF-$\beta_1$ increased the expression of *Acsl5* mRNA in a dose-dependent manner. To examine the Smad-dependency of this pathway, IEC-6 cells were infected with the adenovirus vector and were incubated with TGF-$\beta_1$. As shown in Figure 4B, the over-expression of Smad2 increased the expression of the *Acsl5* gene, while that of Smad3 showed no alteration, and that of dominant-negative Smad2/3 decreased the expression. Next, we knocked down the expression of Smad2 or Smad3 and examined the effects. When we knocked down the expression of Smad2, the upregulation of Acsl5 was attenuated compared to the control siRNA- and Smad3 siRNA-transfected cells (Figure 4C). These data suggest that the differentiation of IEC-6 cells may be induced through a Smad2-dependent pathway.

### *The expression of Smad2 and Smad3 along the crypt-villus axis of the small intestine.*

To further examine the functional roles of Smad2 and Smad3 *in vivo*,

we immunostained Smad2 and Smad3 in samples of murine intestine. As shown in Figure 5, Smad2 is preferentially expressed in the enterocytes lining villi and Smad3 is predominantly expressed in crypts.

### *The induction of I-Smad in response to TGF-$\beta_1$ occurs independent of the Smad pathways.*

It is well known that I-Smad is induced by TGF-$\beta_1$ and acts as a negative feedback regulator to inhibit the activation of the R-Smads by inducing the degradation of the receptors or by competing with the R-Smads for TβRI binding [18]. As reported in mink lung epithelial cells (Mv1Lu) and human keratinocytes (HaCaT) [19,20], the expression of *Smad7* in IEC-6 cells was upregulated in a concentration-dependent manner by the addition of TGF-$\beta_1$ (Figure 6A). When the cells were infected with AdLacZ or AdDNSmad2/3 and were incubated with 25 pM TGF-$\beta_1$, the expression of *Smad7* in AdDNSmad2/3-infected IEC-6 cells was not decreased compared to that in AdLacZ-infected cells (Figure 6B), thus indicating that the induction of *Smad7* did not occur through a Smad-dependent pathway.

Non-Smad TGF-$\beta_1$ signaling involves MAPKs and PI3K/Akt

pathways [4]. To further delineate whether the induction of *Smad7* expression occurs through ERK, JNK, p38 and/or PI3K signaling, IEC-6 cells were pretreated for 1 hour with an ERK1/2 inhibitor (PD98059), JNK inhibitor (SP600125), p38 MAPK inhibitor (SB203580) or PI3K inhibitor (LY294002), and then incubated in the presence of TGF-$\beta_1$ (25pM). The expression of *Smad7* was not decreased by any of these inhibitors (Figure 6C), suggesting that the induction was not regulated through these pathways. The pre-incubation with LY294002 increased the *Smad7* expression, indicating that PI3K signaling may have an inhibitory effect on its expression, independent of TGF-$\beta_1$.

## Discussion

In this study, we have demonstrated that TGF-$\beta_1$ regulates various cellular functions of IEC-6 cells through different intracellular signaling pathways. IEC-6 cells produced and secreted TGF-$\beta_1$ (Figure 1C), and our findings suggest that TGF-$\beta_1$ might exert its effect in an autocrine fashion. However, the concentration of the supernatant after a 48 hour incubation was estimated to be about 4 pM, which did not seem to be sufficient to exert the biological effects. Therefore, we added exogenous TGF-$\beta_1$ to the culture medium and examined the biological responses. Adenovirus-mediated dominant-negative Smad2/3 expression attenuated the growth inhibitory effect of TGF-$\beta_1$ (Figure 3B), and the expression of a differentiation marker, *Acsl5* (Figure 4B). Co-expression of Smad3 with dominant-negative Smad2/3 restored the growth inhibition (Figure 3B). In contrast, overexpression of Smad2 enhanced the expression of *Acsl5* (Figure 4B). The addition of TGF-$\beta_1$ increased the *Smad7* mRNA expression in IEC-6 cells, but the infection of dominant-negative Smad2/3 adenovirus did not inhibit the upregulation (Figure 6). Therefore, our findings demonstrate that TGF-$\beta_1$ inhibits the proliferation, promotes the differentiation, and exerts negative

feedback through a Smad3-dependent, a Smad2-dependent, and a non-Smad pathway, respectively, in IEC-6 cells.

TGF-β signaling is mediated by both Smad-dependent and non-Smad pathways [2,4]. However, it has not been clear if the Smad2 and/or Smad3 proteins are responsible for TGF-β-induced environments in the intestinal epithelium. Evidence in a number of cell types suggests that Smad2 and Smad3 differentially regulate the transcription of TGF-β target genes [1]. For example, in HepG2 human hepatoma cells, the overexpression of the Smad3/4 complex causes higher levels of transcriptional activation of the *p21* promoter than the overexpression of the Smad2/4 complex [21]. Conversely, the Smad2/4 complex associates with the mammalian forkhead domain protein to induce the *goosecoid* (*gsc*) promoter, whereas the Smad3/4 complex represses the *gsc* promoter [22]. We have shown that TGF-$\beta_1$ activated pancreatic stellate cells through a Smad-2 dependent pathway, inhibited the proliferation through a Smad-3 dependent pathway, and upregulated the expression of TGF-$\beta_1$ itself through a non-Smad pathway [15]. It was also shown that TGF-β differentially activates Smad2 and Smad3 in hepatic stellate cells [23].

The cytostatic function of Smad3 over Smad2 in TGF-β signaling was revealed in a study using gene silencing, and Smad3 may have a more important role than Smad2 in TGF-β-mediated cell cycle arrest in epithelial cells [24]. Our study also clearly showed that TGF-$\beta_1$ inhibited the proliferation of IEC-6 cells through a Smad3-dependent pathway.

The crypt-villus axis is a vertical axis with epithelial cells extending bidirectionally upwards and downwards from the stem cells anchored adjacent to the crypt basis. Enterocytes, mucous-producing goblet cells and enteroendocrine cells migrate out of the villus, while Paneth cells migrate towards the crypt-base. We showed that TGF-$\beta_1$ enhanced the expression of a differentiation marker of IEC-6 cells through a Smad2-dependent pathway. Our observations showing that Smad2 is predominantly expressed in villi, and Smad3 in crypts, thus suggest that TGF-$\beta_1$ may play differential roles along the crypt-villous axis. Activin A is a member of the TGF-β superfamily and its signal is also mediated through Smad-dependent and non-Smad pathways [2,3]. Activin A was reported to be involved in the regulation of proliferation and differentiation of intestinal epithelial cells [25]. The activin signaling was increased in intestinal tissues from experimental colitis and

inflammatory bowel disease patients [26,27] and was also shown to be over-expressed in human colorectal cancers [28]. In Smad-dependent pathways, activin A shares common Smad molecules with TGF-$\beta_1$. Further studies are needed to clarify the contribution of TGF-$\beta_1$ and activin A to maintaining the homeostasis of the intestinal epithelium.

I-Smads are induced by TGF-β, activin, or BMP. They act as negative feedback regulators, and inhibit the activation of R-Smads by inducing the degradation of the receptors or by competing with the R-Smads for TβRI binding [18]. TGF-$\beta_1$ induced the expression of *Smad7* mRNA in IEC-6 cells, independent of Smad signaling pathways (Figure 6B). Non-Smad TGF-β signaling involves the small GTPase Ras, MAPKs, PI3K/Akt and protein kinase C [4,29], but MAPKs and PI3K/Akt are apparently not involved in this pathway, at least in IEC-6 cells (Figure 6C).

In conclusion, we showed that TGF-$\beta_1$ regulates the proliferation, differentiation, and *Smad7* mRNA expression in IEC-6 cells through a Smad3-dependent, a Smad2-dependent and a non-Smad pathway, respectively. These observations suggest that TGF-$\beta_1$ is associated with the initiation and termination of proliferation, and/or the maintenance of

differentiation of intestinal epithelial cells.

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## Abbreviations

Ab, antibody; Acsl5, acyl-CoA synthetase long-chain family member5; ALPi, intestinal-type alkaline phosphatase, ApoA4, apolipoprotein A-IV; BMP, bone morphogenetic protein; DMEM, Dulbecco's modified Eagle's medium; FOX, forkhead box; pfu, plaque-forming units; GDF, growth differentiation factor; gsc, goosecoid; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA; TGF, transforming growth factor; TβRI, TGF-β receptor type I; TβRII, TGF-β receptor type II.

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## Table and Figure Legends

Table 1. The primers used in the study.

Figure 1. The expression of TGF-β/Smad signaling molecules in IEC6 cells. A. The expression of the receptors (*TβR1*, *TβR2*), R-Smad (*Smad2, 3*), Co-Smad (*Smad4*), I-Smad (*Smad7*) and *TGF-$\beta_1$* was studied by RT-PCR. B. The expression of the Smad 2 and 3 proteins was examined by a Western blotting analysis. The blots are representative of three independent experiments with similar results. C. The secretion of TGF-β1 was studied by ELISA. The supernatants of IEC6 cells at the indicated times were collected, and the amount of TGF-$\beta_1$ was measured (n=3). The experiments were repeated three times independently with similar results. **, $p < .01$

Figure 2. Nuclear accumulation of Smad2 and Smad3 in IEC6 cells in response to TGF-$\beta_1$ stimulation.

The immunocytochemical analysis of Smad2 and Smad3 was performed using IEC-6 cells that were transfected with AdLacZ, AdDNSmad2/3, AdSmad2, or AdSmad3. The cells were stained with an anti-Smad2/3 Ab (A)

or anti-FLAG Ab (B) with or without the stimulation of 10pM TGF-$\beta_1$ for the last two hours in culture. *Bars*, 100 μm.

Figure 3. The growth inhibitory effect of TGF-$\beta_1$ on IEC-6 cells and the analysis of its Smad-dependency.

A. IEC-6 cells were seeded at a density of 2-4 x $10^4$ cells/ml in 96-well plates, and were incubated with the indicated amount of TGF-$\beta_1$ for 48 hours, followed by the determination of DNA synthesis using a BrdU incorporation assay. B. IEC-6 cells were infected with AdLacZ, AdSmad2, AdSmad3 or AdDNSmad2/3. For double adenovirus infection experiments, IEC-6 cells were infected with the combination of AdSmad2 and AdDNSmad2/3 or that of AdSmad3 and AdDNSmad2/3. After 48 hours of incubation, DNA synthesis was measured. C. Smad2, Smad3 or the negative control siRNA were transfected twice on two consecutive days. After 24 hours, the expression of Smad2, Smad3 and β-actin was measured by RT-PCR (25 cycles for Smad2 and Smad3, 15 cycles for β-actin). D. After the transfection of siRNA, IEC-6 cells were incubated in the presence or absence of TGF-$\beta_1$ (25 pM) for 48 hours, and the BrdU incorporation was measured. The values

are expressed as the means ± SD (n=4). The experiments were repeated three times independently with similar results, and a representative figure is shown. *, $p < .05$; **, $p < .01$, by analysis of variance.

Figure 4. The effect of TGF-$\beta_1$ on the differentiation of IEC-6 cells, and the analysis of the Smad-dependency of this effect.

A. IEC-6 cells were incubated with or without the indicated concentrations of TGF-$\beta_1$ for 48 hours, and then were harvested for the isolation of total RNA. The expression of the *Acsl5* gene, a differentiation marker of enterocytes, was measured by quantitative real-time PCR and normalized to the expression of *β-actin*. B. IEC-6 cells were infected with AdLacZ, AdSmad2, AdSmad3, or AdDNSmad2/3. After 48 hours of incubation with 25 pM TGF-$\beta_1$, total RNA was extracted and real-time PCR for *Acsl5* was performed. C. IEC-6 cells were transfected with Smad2, Smad3 or negative control siRNA on two consecutive days. IEC-6 cells were incubated in the presence or absence of TGF-$\beta_1$ (25 pM) for 48 hours, and total RNA was extracted for real-time PCR. Data represent the means ± SD. The experiment (n=3) was repeated at least three times independently, with similar results. *, $p < .05$,

**, $p < .01$.

Figure 5. The expression of Smad2 and Smad3 along the crypt-villus axis. Serial sections of murine intestine were stained with anti-Smad2 and anti-Smad3 antibodies. Sections incubated without the primary antibody did not yield positive immunoreactivity. Bar = 200 μm.

Figure 6. The induction of Smad7 expression in response to TGF-$\beta_1$ and the analysis of its Smad-dependency.

A. IEC-6 cells were incubated with or without the indicated concentrations of TGF-$\beta_1$ for 48 hours, and the expression of *Smad7* was examined by real-time PCR. B. IEC-6 cells were infected with AdLacZ or AdDNSmad2/3. After 48 hours of incubation with 25 pM TGF-$\beta_1$, the expression of *Smad7* was examined. C. IEC-6 cells were pretreated for 1 hour with an ERK1/2 inhibitor (PD98059, 10 μM), JNK inhibitor (SP600125, 10 μM), p38 MAPK inhibitor (SB203580, 10 μM), or PI3K inhibitor (LY294002, 10 μM), and then incubated in the presence of TGF-$\beta_1$ (25 pM) for 48 hours. Data represent the means ± SD. The experiment (n=3) was repeated three times

independently with similar results. *, $p < .05$.

# Table 1

| Gene name | Primer sequence | | GenBank Accession No. | nt | Product size |
|---|---|---|---|---|---|
| Conventional RT-PCR | | | | | |
| TGF-$\beta_1$ receptor I | S | GCT GTG GTT GGT GTC GGA TTA T | NM_012775 | 889-910 | |
| | AS | CCA GAA TAC TAA GCC CAT T | | 1291-1273 | 403 |
| TGF-$\beta_1$ receptor II | S | GCG TGG CCG TGT GGA GGA AGA A | NM_031132 | 502-523 | |
| | AS | GGG CAG CAG TTC CGT ATT | | 979-963 | 481 |
| Smad2 | S | TCA CAG CCA TCA TGA GCT CAA GG | NM_019191 | 548-570 | |
| | AS | TGT GAC GCA TGG AAG GTC TCT C | | 1018-997 | 471 |
| Smad3 | S | CAG GGC TTT GAG GCT GTC TA | NM_013095 | 1274-1293 | |
| | AS | CTG GCA TCT TCT GTG GTT TC | | 1630-1611 | 357 |
| Smad4 | S | GAC CTC CGG CGC TTG TGC AT | NM_019275 | 1536-1555 | |
| | AS | GGG TCC GCA ATG GGC ATG GT | | 1699-1680 | 164 |
| Smad7 | S | CCG CCA CAC TGG ACA ACC CG | NM_030858 | 2452-2471 | |
| | AS | TCG TGG TCA TTG GGC CGC TG | | 2572-2553 | 121 |
| TGF-$\beta_1$ | S | TGG TGG ACC GCA ACA ACG CA | NM_021578 | 483-502 | |
| | AS | TGG GGG TCA GCA GCC GGT TA | | 713-694 | 231 |
| $\beta$-actin | S | TGA GAG GGA AAT CGT GCG TG | NM_031144 | 690-709 | |
| | AS | GAT CCA CAT CTG CTG GAA GGT G | | 1149-1128 | 439 |
| | | | | | |
| Quantitative real-time PCR | | | | | |
| ACSL5 | S | AGC AGC CTG GGT GGG AAG GTT | NM_053607 | 1339-1359 | |
| | AS | AGC CAG CGA CCG ATG TCC CC | | 1709-1690 | 145 |
| Smad7 | S | CCG CCA CAC TGG ACA ACC CG | NM_030858 | 2452-2471 | |
| | AS | TCG TGG TCA TTG GGC CGC TG | | 2572-2553 | 121 |
| $\beta$-actin | S | TGA GAG GGA AAT CGT GCG TG | NM_031144 | 690-709 | |
| | AS | TCA TGG ATG CCA CAG GAT TCC | | 905-885 | 216 |

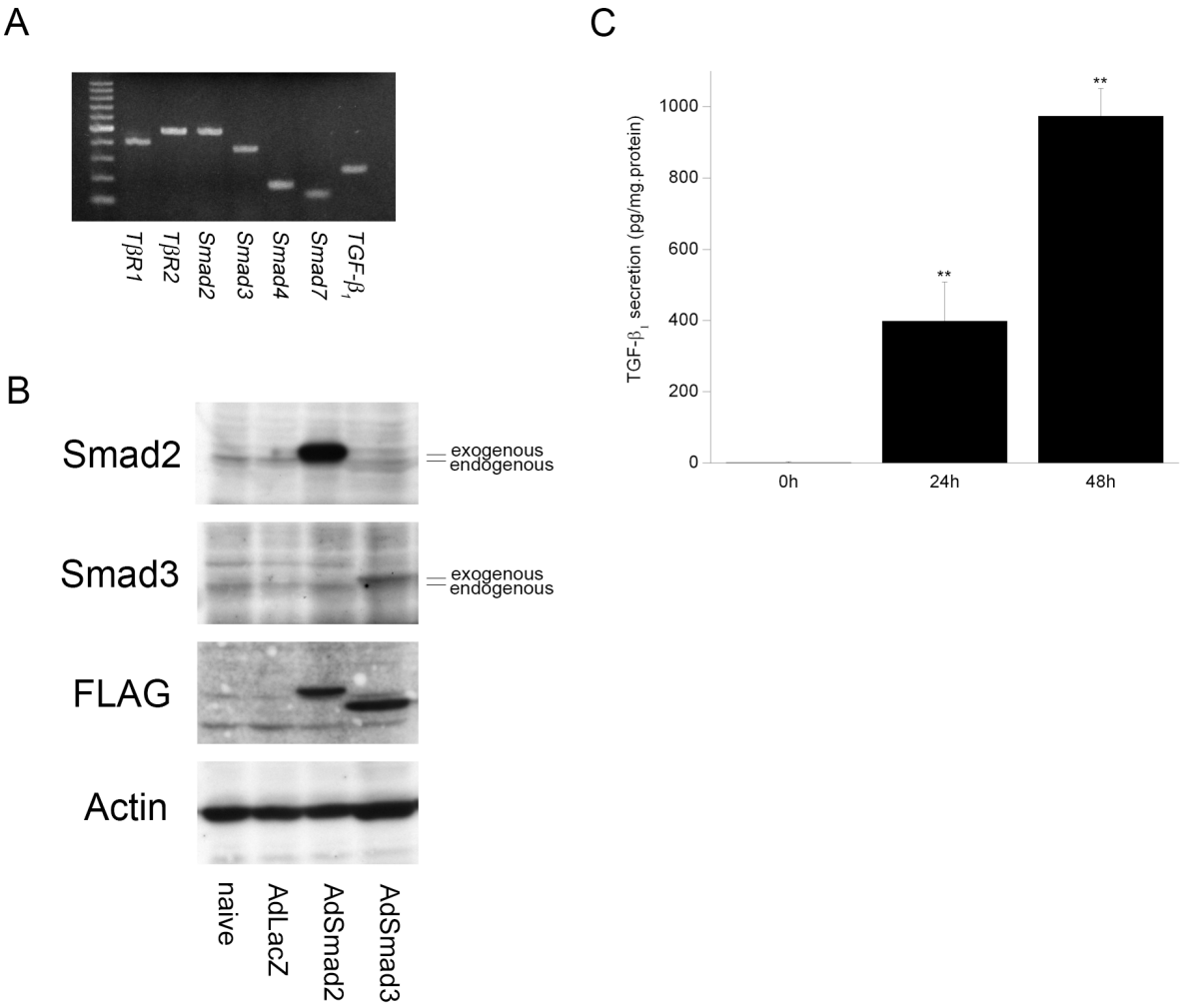
A
TβR1
TβR2
Smad2
Smad3
Smad4
Smad7
TGF-β1
B
Smad2
exogenous
endogenous
Smad3
exogenous
endogenous
FLAG
Actin
naive
AdLacZ
AdSmad2
AdSmad3
C
TGF-β1 secretion (pg/mg.protein)
1000
800
600
400
200
0
**
**
0h
24h
48h

**Figure 1**

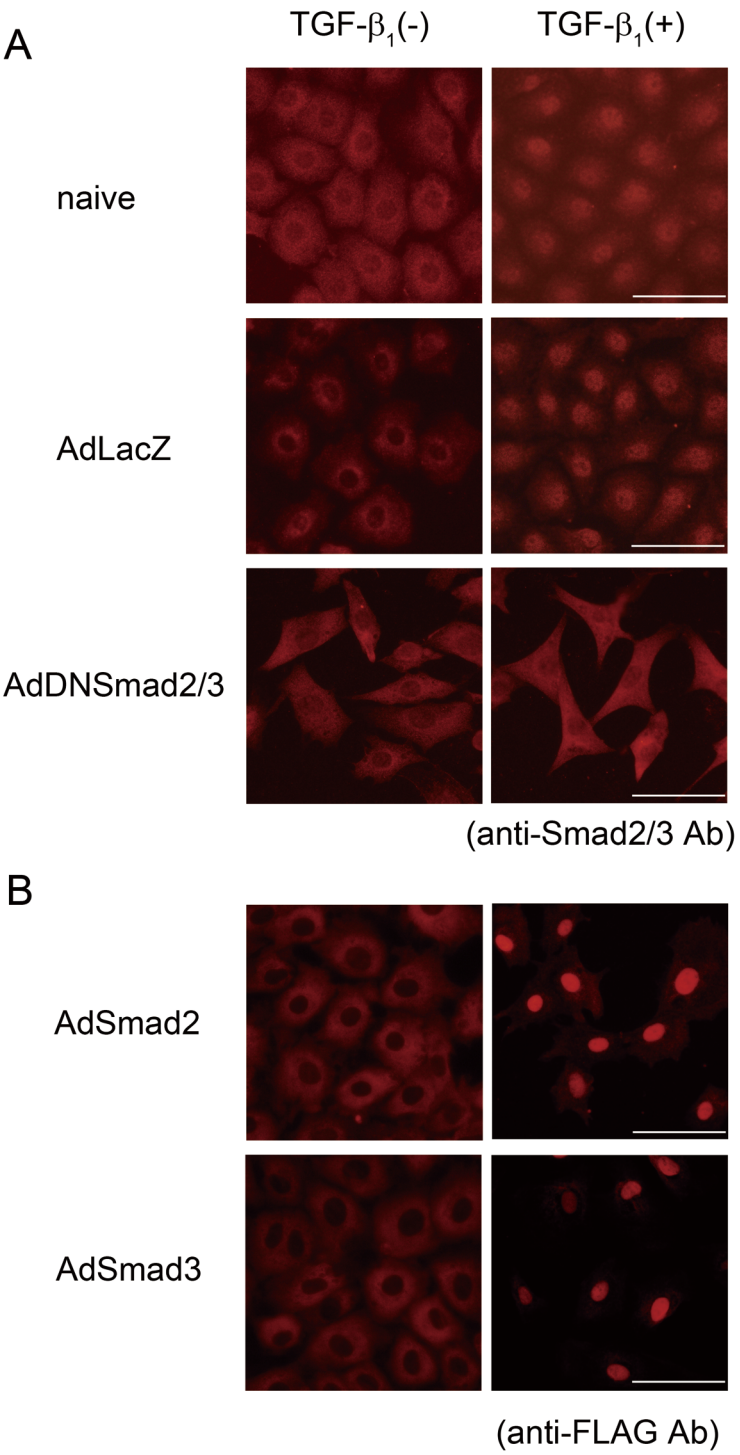
A
TGF-β1(-)
TGF-β1(+)
naive
AdLacZ
AdDNSmad2/3
(anti-Smad2/3 Ab)
B
AdSmad2
AdSmad3
(anti-FLAG Ab)

Figure 2

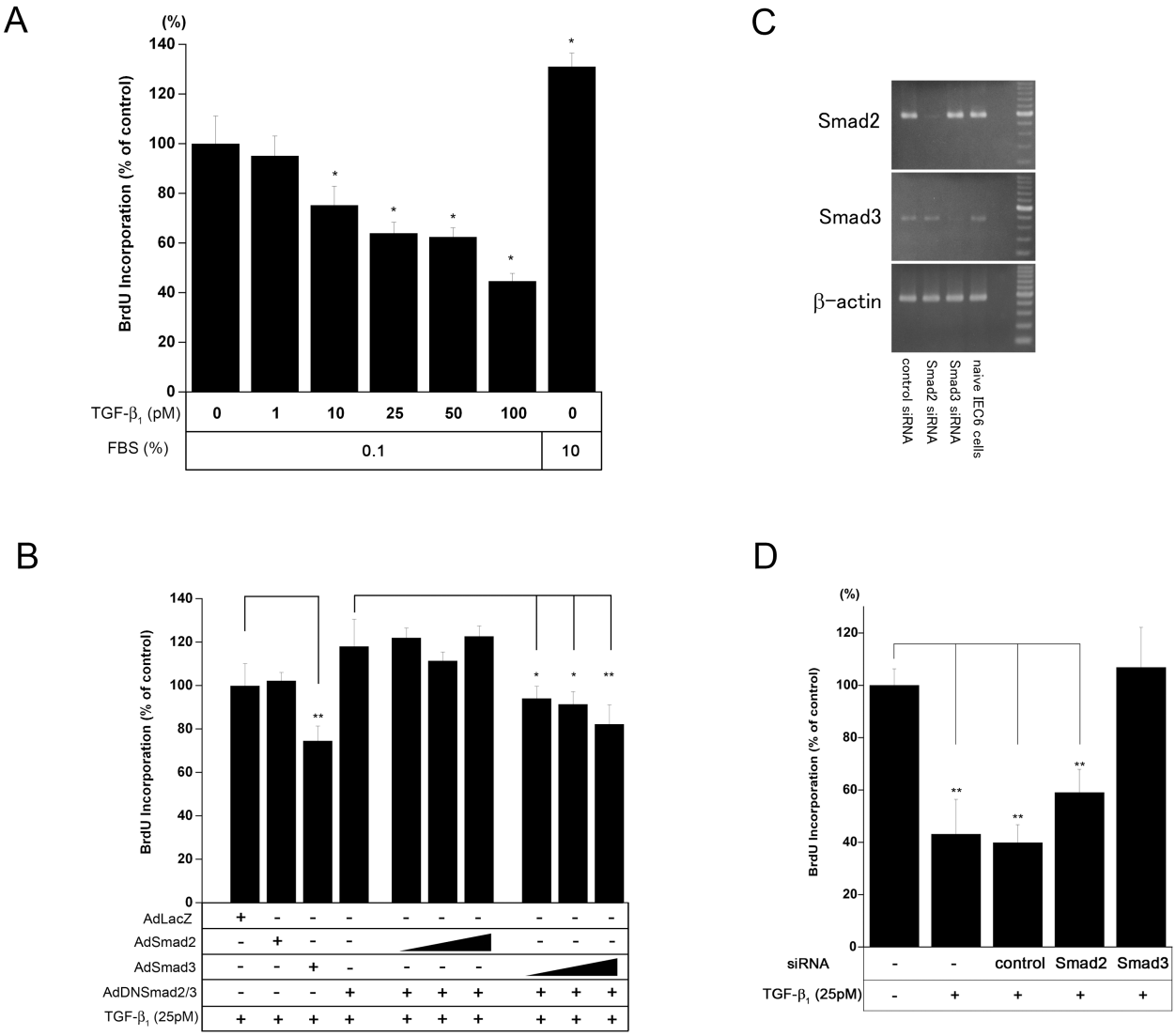
A
(%)
BrdU Incorporation (% of control)
TGF-β1 (pM)
0 1 10 25 50 100 0
FBS (%)
0.1 10
C
Smad2
Smad3
β-actin
control siRNA
Smad2 siRNA
Smad3 siRNA
naive IEC6 cells
B
BrdU Incorporation (% of control)
AdLacZ
AdSmad2
AdSmad3
AdDNSmad2/3
TGF-β1 (25pM)
D
(%)
BrdU Incorporation (% of control)
siRNA
control Smad2 Smad3
TGF-β1 (25pM)

Figure 3

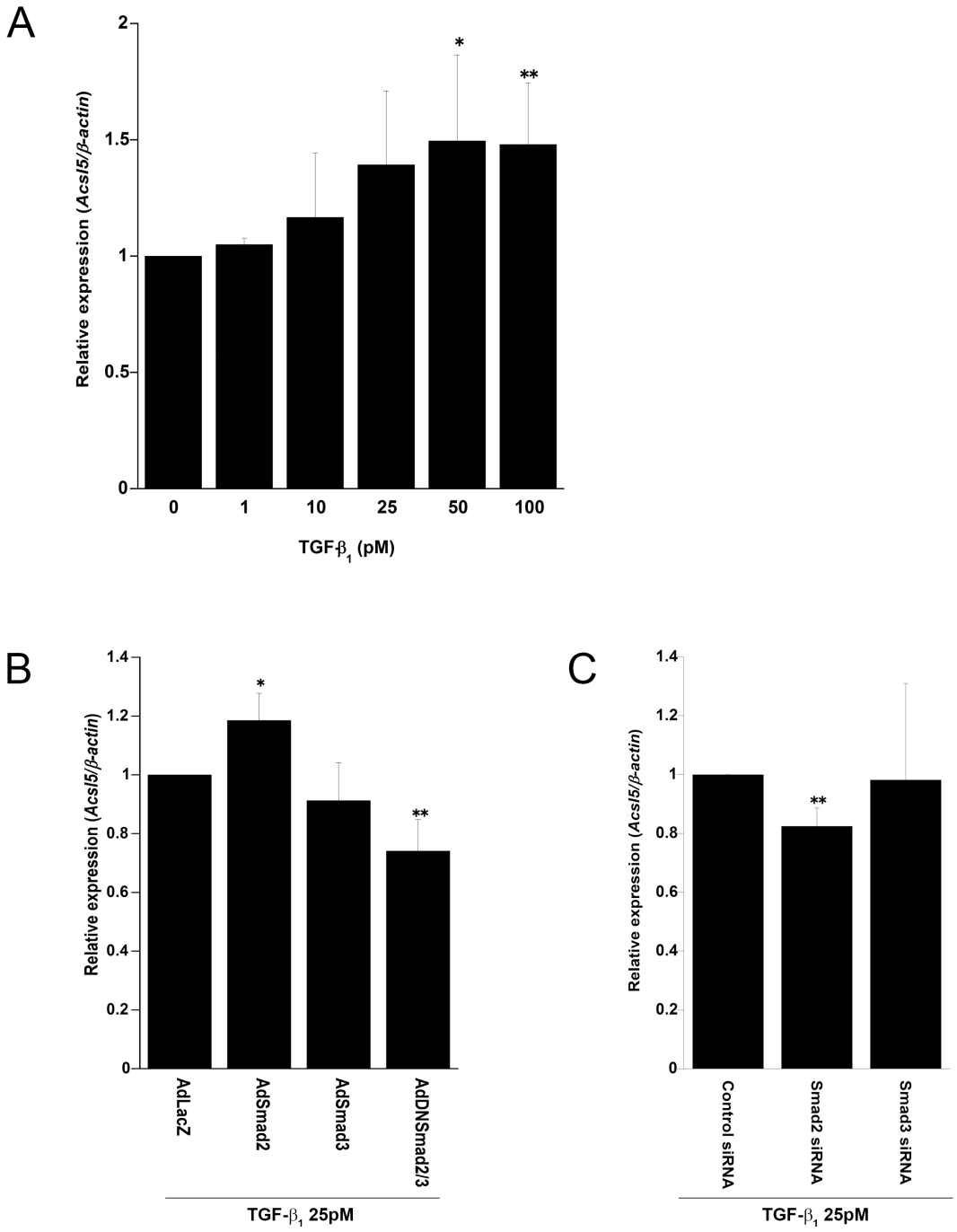
A
Relative expression (Acsl5/β-actin)
2
1.5
1
0.5
0
*
**
0
1
10
25
50
100
TGFβ1 (pM)
B
Relative expression (Acsl5/β-actin)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
AdLacZ
AdSmad2
AdSmad3
AdDNSmad2/3
TGF-β1 25pM
C
Relative expression (Acsl5/β-actin)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
Control siRNA
Smad2 siRNA
Smad3 siRNA
TGF-β1 25pM

**Figure 4**

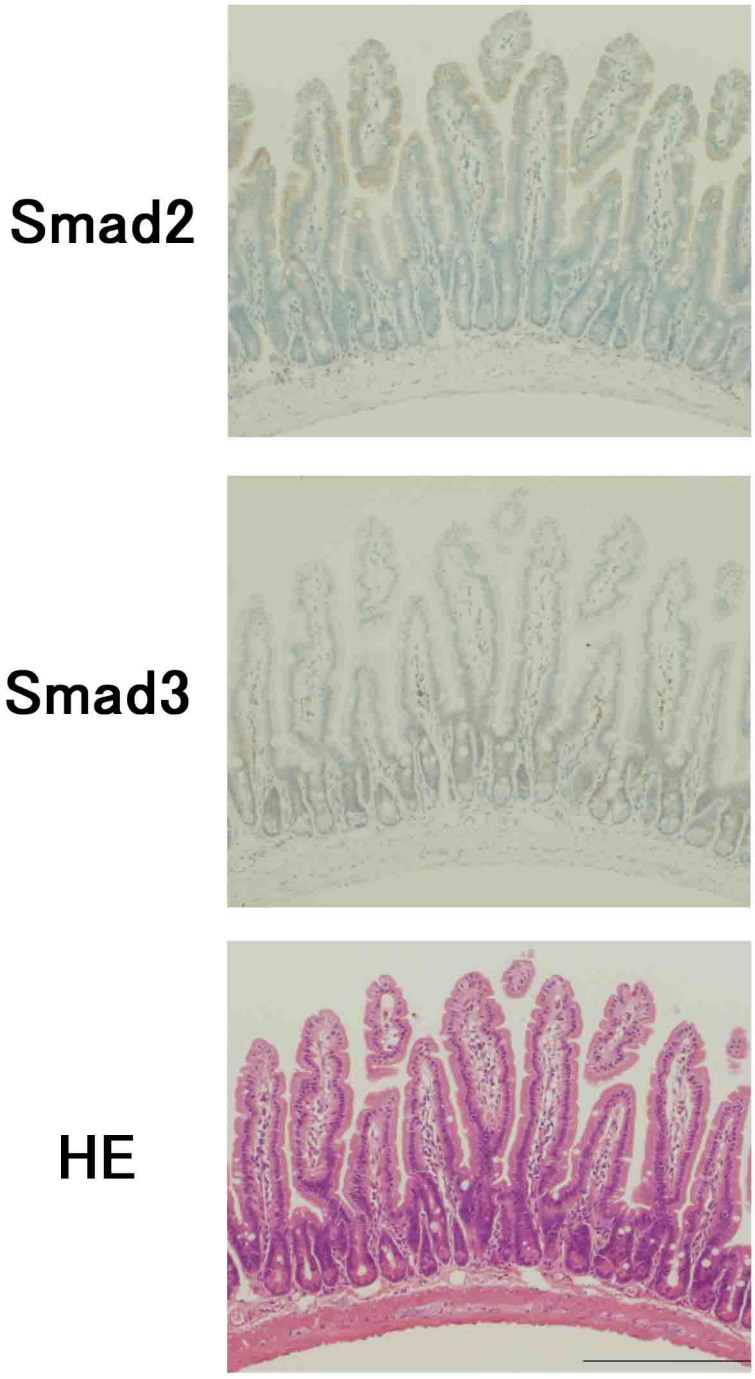


Figure 5

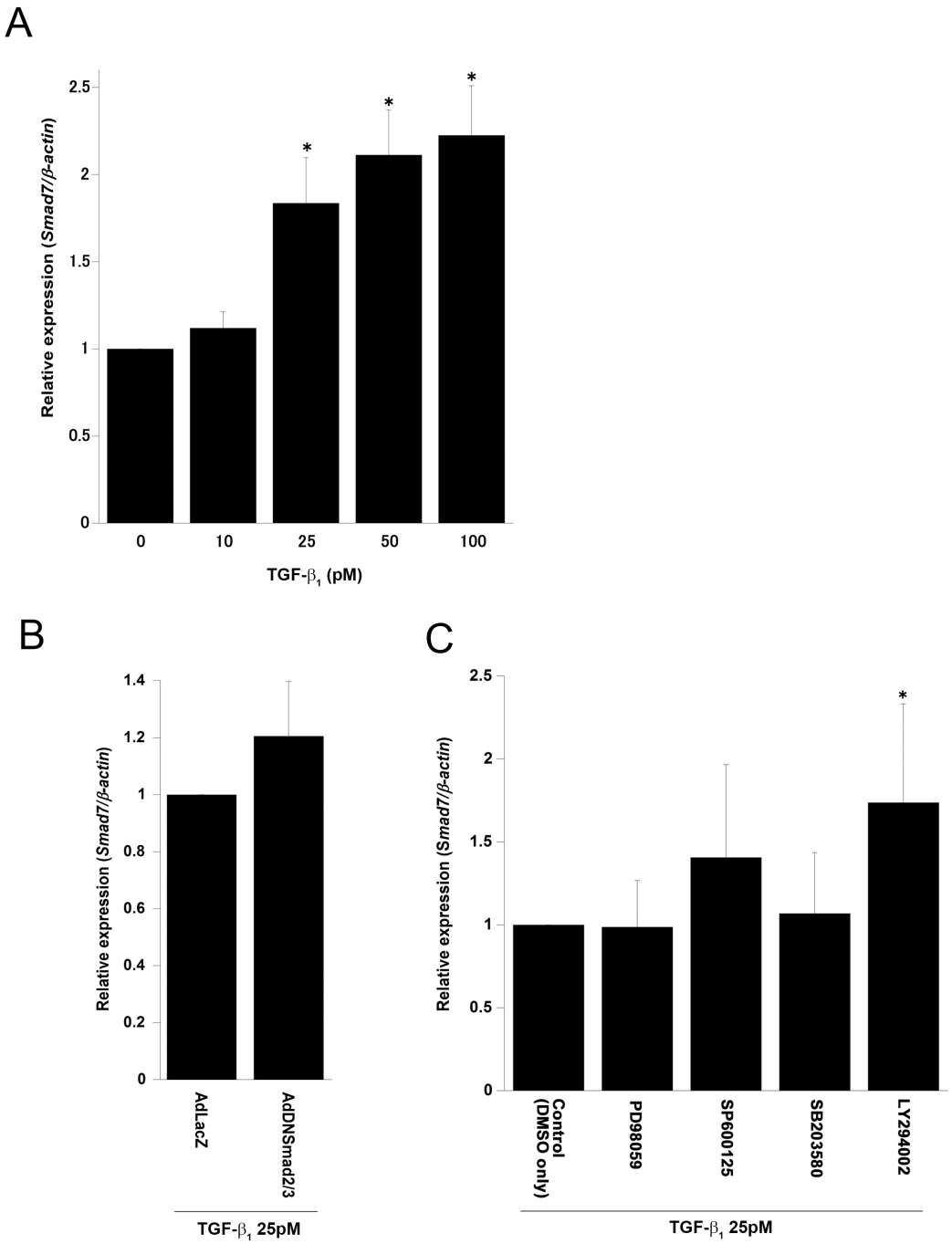
A
Relative expression (Smad7/β-actin)
2.5
2
1.5
1
0.5
0
*
*
*
0
10
25
50
100
TGF-β1 (pM)
B
Relative expression (Smad7/β-actin)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
AdLacZ
AdDNSmad2/3
TGF-β1 25pM
C
Relative expression (Smad7/β-actin)
2.5
2
1.5
1
0.5
0
*
Control (DMSO only)
PD98059
SP600125
SB203580
LY294002
TGF-β1 25pM

Figure 6