Molecular Biology of the Cell
Vol. 16, 4672–4683, October 2005

The Endogenous Ratio of Smad2 and Smad3 Influences the Cytostatic Function of Smad3[D]

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Submitted January 20, 2005; Revised August 1, 2005; Accepted August 2, 2005
Monitoring Editor: Carl-Henrik Heldin

Although Smad2 and Smad3, critical transcriptional mediators of transforming growth factor-β (TGF-β) signaling, are supposed to play a role in the TGF-β cytostatic program, it remains unclear whether TGF-β delivers cytostatic signals through both Smads equally or through either differentially. Here, we report that TGF-β cytostatic signals rely on a Smad3-, but not a Smad2-, dependent pathway and that the intensity of TGF-β cytostatic signals can be modulated by changing the endogenous ratio of Smad3 to Smad2. Depleting endogenous Smad3 by RNA interference sufficiently interfered with TGF-β cytostatic actions in various TGF-β-sensitive cell lines, whereas raising the relative endogenous ratio of Smad3 to Smad2, by depleting Smad2, markedly enhanced TGF-β cytostatic response. Consistently, Smad3 activation and its transcriptional activity upon TGF-β stimulation were facilitated in Smad2-depleted cells relative to controls. Most significantly, a single event of increasing this ratio by Smad2 depletion was sufficient to restore TGF-β cytostatic action in cells resistant to TGF-β. These findings suggest a new important determinant of sensitivity to TGF-β cytostatic signaling.

INTRODUCTION

Transforming growth factor-β (TGF-β) is a prototypic antimitogenic cytokine that delivers cytostatic signals to most epithelial, neuronal, and immune cells (Massagué *et al*., 2000; Attisano and Wrana, 2002; Moustakas *et al*., 2002). TGF-β exerts its cellular actions by binding to a heteromeric receptor complex consisting of type I (TβRI) and type II (TβRII) serine/threonine kinase receptor subunits (ten Dijke *et al*., 1996). The activated receptor in turn phosphorylates the receptor-regulated Smad (R-Smad), Smad2, and Smad3, which then form heteromeric complexes with a common partner, Smad4, and accumulate in the nucleus where they regulate the expressions of TGF-β target genes (Heldin *et al*., 1997; Derynck *et al*., 1998; Massagué and Wotton, 2000; ten Dijke and Hill, 2004). TGF-β responses can also be modulated by Smad7, an inhibitory Smad that associates with the activated receptors to disrupt the further propagation of TGF-β signaling (Nakao *et al*., 1997; Miyazono, 2002).

The antiproliferative effect of TGF-β generally results from growth arrest at the G_1 phase of the cell cycle. It has been shown that a critical set of TGF-β cytostatic gene responses generally involve the repression of the growth-promoting transcription factor *c-myc* and the transcriptional activation of the cyclin-dependent kinase (Cdk) inhibitors $p15^{INK4b}$ and/or $p21^{Cip1}$ (Pietenpol *et al*., 1990; Hannon and Beach, 1994; Datto *et al*., 1995), which results in the inhibition of G_1 Cdk activities and the subsequent accumulation of hypophosphorylated retinoblastoma protein (pRB) (Laiho *et al*., 1990; Koff *et al*., 1993). These cytostatic gene responses by TGF-β cooperatively mediate cell cycle arrest at the G_1 phase.

The TGF-β cytostatic response is of interest because its loss is often considered to be a major step in tumor progression (Derynck *et al*., 2001; Wakefield and Roberts, 2002; Siegel and Massagué, 2003). Mutational inactivation or deregulation of the TGF-β signaling components have been shown to be responsible for the inability of tumor cells to respond to TGF-β cytostatic signals (Kim *et al*., 2000; Massagué *et al*., 2000; Wakefield and Roberts, 2002). However, most cancer cells more commonly lose TGF-β cytostatic responsiveness despite retaining functional TGF-β receptors and the Smad system. The molecular basis for this loss is thus largely unidentified, although aberrant hyperactivation of the oncogenic Ras/MAPK pathway in cancer cells, a hyperactive PI3K/Akt pathway and high levels of FoxG1 in glioblastoma cells, and the inactivation of Smads by extensive Cdk phosphorylation have been suggested to be responsible for this loss, because all of these prevent cytostasis by the TGF-β/Smad pathway (Kretzschmar *et al*., 1999; Matsuura *et al*., 2004; Seoane *et al*., 2004).

Although Smad2 and/or Smad3 have been implicated in cytostatic gene response by TGF-β (Rich *et al*., 1999; Feng *et al*., 2000; Pardali *et al*., 2000), it remains rather obscure as to whether the activities of both Smad2 and Smad3 are essential for the cytostatic effect of TGF-β or whether either is sufficient to deliver TGF-β cytostatic signals. In this study,

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05–01–0054) on August 10, 2005.

[D] The online version of this article contains supplemental material at *MBC Online* (http://www.molbiolcell.org).

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we addressed precisely this question and found that Smad3, but not Smad2, plays a key role in the cytostatic program of TGF-β. In addition, we present evidence that the cytostatic signal intensity of TGF-β depends on the endogenous ratio of Smad3 to Smad2, which is readily modulated by TGF-β depending on cell type. Most importantly, we suggest that the maintenance of this ratio below a required threshold itself can function as a pressure against TGF-β cytostatic effect without loss or mutation of TGF-β signaling system.

MATERIALS AND METHODS

Cell Culture and TGF-β Treatment

HaCaT, Huh7, Mv1Lu, and Panc-1 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. Other cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS and the same antibiotics. For TGF-β treatment, human recombinant TGF-β1 (PeproTech, Rocky Hill, NJ) was rehydrated in 4 mM HCl, and 1 mg/ml bovine serum albumin solution and used at a final concentration of 5 ng/ml in all experiments.

Small Interfering RNA (siRNA) Transfections

The siRNAs used are "Ready-to-Use" synthetic siRNA duplexes of 21 nucleotides each, guaranteed to silence human and mouse Smad2 and Smad3 (Cellogenetics CLG-1107 for siSmad2 and CLG-1108 for siSmad3). The nonsilencing control siRNA was also synthesized by Cellogenetics (Gaithersburg, MD) and confirmed to be not complementary to any mammalian mRNA sequence by BLAST analysis. They were transfected as described previously (Kim *et al*., 2004). Briefly, cells were transfected with each siRNA for 4 h using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). After adding normal medium, cells were further incubated for 12 h before treating TGF-β.

Adenoviral Infections

Recombinant adenoviruses expressing Smad2, Smad3, or β-galactosidase were kindly provided by Dr. K. Miyazono (The Cancer Institute, Tokyo University, Tokyo, Japan) and used individually at a multiplicity of infection (MOI) of 50, as described previously (Fujii *et al*., 1999).

Cell Cycle Analysis and the Measurement of Cell Proliferation

For cell cycle analysis, cells were harvested after various treatments, washed with phosphate-buffered saline, fixed with 70% ethanol for at least 1 h, and stained with 20 μg/ml propidium iodide (Sigma-Aldrich, St. Louis, MO) containing 10 μg/ml RNase A (Sigma-Aldrich). Cellular DNA contents were determined by FACSCalibur flow cytometry (FL-2) (BD Biosciences, San Jose, CA). Ten thousand events were counted for each analysis. For the cell proliferation assay, depending on cell type, $0.5–1.5 \times 10^5$ cells were seeded onto a 35-mm culture dish in triplicate. After the various transfections or infections, cell numbers were determined 2 or 3 d after TGF-β stimulation using a Coulter counter (Beckman Coulter, Fullerton, CA) or a hemocytometer.

Antibodies and Immunoblotting

Anti-Smad2 (S-20), anti-Smad4 (B-8), anti-Smad7 (H-79), anti-c-Myc (9E10), anti-$p15^{INK4b}$ (C-20), anti-Cdk2 (M2), anti-Lamin B (C-20), and anti-α-tubulin antibody (B-7) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Smad2 and anti-phospho-Smad3 antibody were purchased from Cell Signaling Technology (Beverly, MA) or kindly provided by Dr. E. B. Leof (Mayo Clinic Cancer Center, Rochester, MN), respectively. Anti-$p21^{Cip1}$ antibody (OP64) was obtained from Calbiochem (San Diego, CA), and anti-pRB antibody (14001A) was from BD Biosciences PharMingen (San Diego, CA). Anti-Smad3 and anti-FLAG (M2) antibody were from Zymed Laboratories (South San Francisco, CA) and Sigma-Aldrich, respectively. To determine the expressions of protein of interest in the various cell types, immunoblotting was performed with the above-mentioned antibodies, as described previously (Park *et al*., 2004). Immunoblotting with anti-α-tubulin was used routinely as an internal loading control.

Chromatin Immunoprecipitation (ChIP) Assay

siRNA-transfected HaCaT cells (1×10^6 cells) were incubated for 12 h in the presence or absence of TGF-β, and in vivo ChIP assay was performed using EZ ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's recommendations. Briefly, cells were cross-linked by addition of 1% formaldehyde for 10 min and glycine was added (0.125 M final) for 5 min to stop the cross-linking reaction. Cells were then lysed with a lysis buffer and sonicated to shear genomic DNA to lengths between 200 and 2000 base pairs. One-tenth of the total chromatin lysate was used for purification of total genomic DNA. The rest of the lysate was used for immunoprecipitation with anti-phospho-Smad3 antibody. After the collection of immunoprecipitates using protein G agarose, protein–DNA complexes were eluted and heated at 65°C to reverse cross-linking. After digesting proteins by proteinase K, DNA fragments were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA). Either total or immunoprecipitated DNA were analyzed by PCR of 30 or 35 cycles, respectively, at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. Specific primer sets were designed to amplify a target sequence within the human $p15^{INK4b}$ gene promoter (5′-ATGCGTCCTAGCATCTTTGG-3′ and 5′-GGCAAAGAATTCCGTTTTCA-3′) and the human $p21^{Cip1}$ gene promoter (5′-CTCACTTCGTGGGGAAATGT-3′ and 5′-GGCTCCACAAGGAACTGACT-3′).

Coimmunoprecipitation and Cdk2 Kinase Assay

siRNA-transfected SNU-368 cells were treated or not treated with TGF-β for 12 h and then lysed with lysis buffer (50 mM Tris-Cl, pH 7.5, 250 mM NaCl, 1% NP-40, 5 mM EDTA, 50 mM NaF, 0.1 mM $NaVO_4$, 100 mM phenylmethylsulfonyl fluoride, 0.1 mM pepstatin A, 0.1 mM antipain, 0.2 mM leupeptin, 10 μg/ml aprotinin, and 1 mM benzamidine). Endogenous Smad4 was immunoprecipitated with anti-Smad4 antibody and coimmunoprecipitated phospho-Smad2 or phospho-Smad3 was analyzed by immunoblotting with anti-phospho-Smad2 or anti-phospho-Smad3 antibody. For the immune complex kinase assay, siRNA-transfected or adenovirus-infected HaCaT cells were treated with or without TGF-β for 12 h before lysis and Cdk2 immunoprecipitation followed by the kinase assay was performed, as described previously (Kim *et al*., 2001).

Nuclear Fractionation

siRNA-transfected HaCaT cells were incubated for 12 h in the presence or absence of TGF-β and collected for lysis. Nuclear fractions were prepared using NE-PER extraction reagents (Pierce Chemical, Rockford, IL), according to the manufacturer's instructions. Briefly, after removal of the cytoplasmic fraction using cytoplasmic extraction reagents, the insoluble pellet obtained was resuspended in nuclear extraction reagent (100 mM KCl, 10 mM HEPES, pH 7.9, 10% glycerol, 1 mM dithiothreitol, 5 mM $MgCl_2$, 0.1% NP-40, and 10 mM NaF) containing protease inhibitors. After vigorously vortexing every 10 min during incubation on ice for 40 min, the nuclear fraction was isolated by centrifugation. Immunoblotting for Lamin B and α-tubulin was performed to confirm the nuclear fraction and to exclude cytoplasmic contamination, respectively.

Luciferase Reporter Assays

To measure the effects of depleting endogenous Smad2 or Smad3 on their self-mediated transcriptions, HaCaT cells were transfected with ARE-Luc (0.25 μg)/FAST-1 (0.25 μg) or $(SBE)_4$-Luc (0.5 μg) (kindly provided by Dr. S. J. Kim, National Cancer Institute, Bethesda, MD) together with different siRNAs (0.25 μg). After adding normal medium, cells were further incubated for 12 h and then stimulated with TGF-β for 18 h. To inhibit autocrine TGF-β activity in Panc-1 cells, neutralizing anti-TGF-βs antibody (R&D Systems, Minneapolis, MN) was added after transfecting with $(SBE)_4$-Luc and different siRNAs. Cells not treated with exogenous TGF-β treatment were incubated for 30 h. pSV-β-Gal (0.25 μg) was also transfected to normalize transfection efficiencies. Luciferase activity was measured using a TR717 microplate luminometer (Applied Biosystems, Foster City, CA).

RESULTS

TGF-β-mediated Cell Cycle Arrest and Growth Inhibition Principally Depend on Smad3 in Epithelial Cell Systems

To identify and compare the relative contributions of individual R-Smads to the TGF-β cytostatic program, we first analyzed the effects of Smad2 or Smad3 depletion on TGF-β-induced growth inhibition using siRNA in a variety of TGF-β-sensitive cell types. For this, we first confirmed that endogenous Smad2 or Smad3 were efficiently and selectively depleted by each siRNA transfection (Figure 1A) and that these depletions did not induce significant change of cell proliferation without TGF-β treatment (our unpublished data). When TGF-β was treated in HaCaT human keratinocytes, the inhibition of cell proliferation by TGF-β was less effective in Smad3-depleted cells than in mock or nonsilencing control siRNA transfected cells, whereas, interestingly, cell proliferation was much more inhibited by TGF-β in Smad2-depleted cells (Figure 1B). These enhancing or inhibitory effects of respective Smad2 or Smad3 depletion were also consistently observed in other TGF-β-sensitive cell types originating from epithelium, such as HepG2, Hep3B,

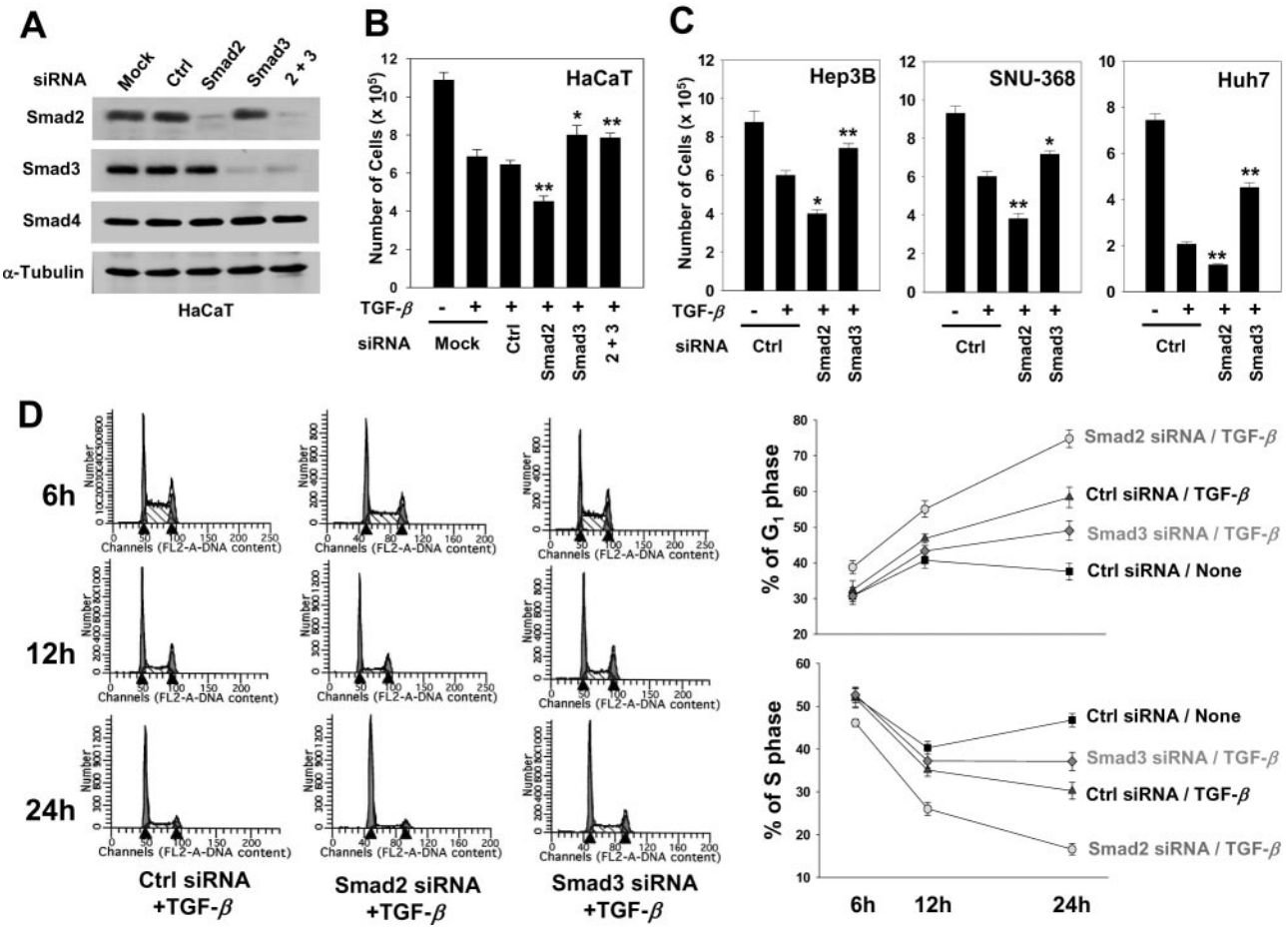

Figure 1. Effects of Smad2 or Smad3 depletion on TGF-β-induced cell cycle arrest and growth inhibition. (A) HaCaT cells were transfected with mock, nonsilencing control (Ctrl), Smad2, Smad3, or Smad2/3 (2 + 3) siRNA and incubated for 24 h. Whole cell lysates were immunoblotted with the indicated antibodies. Anti-α-tubulin was used as an internal loading control. (B) HaCaT cells transfected with the indicated siRNAs were treated with (+) or without (−) TGF-β. Cell proliferation was determined by cell number on day 3 after treating TGF-β. Error bars represent the SDs of three experiments (*$p < 0.05$, **$p < 0.01$). (C) Transfection of the different siRNAs in Hep3B (left), SNU-368 (middle), or Huh7 (right) and cell proliferation measurements were performed as described in B (*$p < 0.05$, **$p < 0.01$). (D) HaCaT cells transfected with the indicated siRNAs were incubated in the presence or absence of TGF-β. At the indicated times after treating TGF-β, cells were fixed, stained with propidium iodide, and subjected to flow cytometric analysis. The percentages of cells in the G_1 and S phases were determined based on the DNA content histograms (left) and graphed (right). The graphs shown in the right represent means ± SD of three independent experiments.

Huh7, SNU-354, -368, -423 human hepatoma cells, SNU-620 human gastric cancer cells, and Mv1Lu mink lung epithelial cells (Figure 1C; our unpublished data). In addition, because the codepletion of endogenous Smad2 and Smad3 efficiently interfered with growth inhibition by TGF-β in a similar manner to Smad3 single depletion (Figure 1, A and B), these data suggest that TGF-β may inhibit cell proliferation through a Smad3-, but not through a Smad2-, dependent pathway.

In general, TGF-β inhibits cell proliferation by inducing growth arrest at the G_1 phase of the cell cycle (Massagué *et al*., 2000; Moustakas *et al*., 2002; Siegel and Massagué, 2003). So, we reasoned that the enhancement or inhibition of TGF-β-mediated growth inhibition by the depletion of individual Smads is driven by alterations in TGF-β-mediated growth arrest. After confirming no significant effect of each Smad depletion on the cell cycle distribution in the absence of TGF-β (our unpublished data), we checked this possibility. Consistent with our notion, Smad2 depletion markedly enhanced both a time-dependent increase in the G_1 phase and a concomitant decrease in the S phase by TGF-β in HaCaT cells (Figure 1D). On the other hand, TGF-β-induced increased G_1, and the reduced S phase disappeared in Smad3-depleted cells (Figure 1D). Similar results were consistently obtained in other TGF-β-sensitive cells (Supplemental Figure S1) and by the double depletion of endogenous Smad2 and Smad3, which also showed an interruption in growth arrest by TGF-β (our unpublished data). These results suggest that a Smad3-dependent pathway plays a major role in the TGF-β-induced cell cycle arrest and consequent growth inhibition.

The TGF-β Cytostatic Program Relies on a Smad3-dependent Pathway

To understand the molecular basis of the effects of depleting individual R-Smad on the G_1 arrest program by TGF-β, we next examined the status of TGF-β cytostatic mediators downstream of Smads. As an initial response to the removal of growth-promoting functions, and facilitating the induction of Cdk inhibitors by TGF-β (Warner *et al*., 1999; Claas-

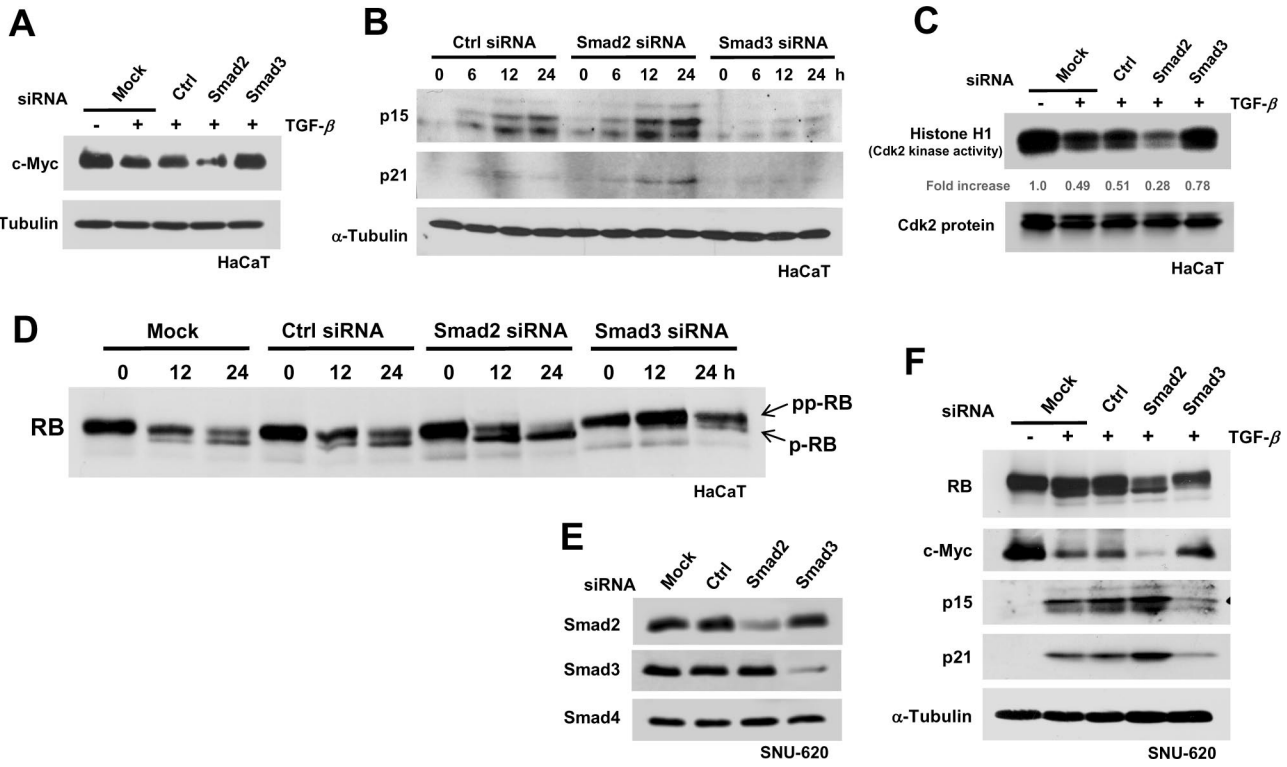

Figure 2. Effects of Smad2 or Smad3 depletion on the TGF-β cytostatic program. (A) HaCaT cells transfected with the indicated siRNAs were incubated with (+) or without (−) TGF-β for 3 h. Total cell lysates were immunoblotted with anti-c-Myc or anti-α-tubulin antibody. (B) Cell lysates were prepared from each siRNA-transfected HaCaT cells treated with TGF-β for the indicated times. Immunoblotting was then performed using the indicated antibodies. (C) The indicated siRNA-transfected HaCaT cells were treated with (+) or without (−) TGF-β for 12 h. Equal amounts of cell extracts (200 μg) were immunoprecipitated with anti-Cdk2 antibody, and its immune complex was used for Cdk2 kinase assay on Histone H1 as a substrate. Fold changes in Cdk2 kinase activity are indicated as numbers. Cdk2 protein levels were determined by immunoblotting. (D) HaCaT cells transfected with the indicated siRNAs were incubated with TGF-β for the indicated times. Whole cell lysates were immunoblotted with anti-pRB antibody. Hyperphosphorylated (pp-RB) and hypophosphorylated (p-RB) pRB are indicated. (E) SNU-620 cells were transfected with the indicated siRNAs and then incubated for 24 h. Whole cell lysates were immunoblotted with the indicated antibodies. (F) SNU-620 cells transfected with the indicated siRNAs were treated with (+) or without (−) TGF-β for 12 h. Cell lysates were then prepared and immunoblotted with the indicated antibodies.

sen and Hann, 2000; Feng *et al*., 2002), TGF-β induced the rapid down-regulation of c-Myc within 3 h in HaCaT cells (Figure 2A, lanes 1–3). Of note, we observed that the individual depletions of Smad2 and Smad3 caused significant facilitation or almost the abrogation of this down-regulation by TGF-β, respectively (Figure 2A, lanes 4 and 5), showing that Smad3 plays a dominant role in the suppression of *c-myc* in response to TGF-β, which is consistent with previous observations (Chen *et al*., 2002; Yagi *et al*., 2002; Frederick *et al*., 2004). Accordingly, Smad2 depletion sequentially promoted the inductions of the Cdk inhibitors $p15^{INK4b}$ and $p21^{Cip1}$ (Figure 2B), the inhibition of Cdk2 kinase activity (Figure 2C), and the progressive accumulation of hypophosphorylated pRB (Figure 2D) by TGF-β. Meanwhile, all these responses by TGF-β efficiently disappeared in Smad3-depleted cells (Figure 2, B–D). We also observed that these effects of depleting individual Smads were consistently valid in the other TGF-β-sensitive cell systems tested (Figure 2, E and F; our unpublished data). Thus, we conclude that Smad3 is the predominant mediator of the TGF-β cytostatic program of G_1 cell cycle arrest and resultant growth inhibition.

Modulation of the Endogenous Ratio of Smad3 to Smad2 Affects the Smad3-dependent Cytostatic Signals of TGF-β

In response to TGF-β, Smad2 and Smad3 are activated identically by phosphorylation at the carboxy terminus by TβRI and subsequently form heteromeric complexes with Smad4, which then accumulate in the nucleus (Heldin *et al*., 1997; Derynck *et al*., 1998; Massagué *et al*., 2000; ten Dijke and Hill, 2004). Given that a single event of Smad2 depletion greatly enhances Smad3-dependent TGF-β cytostatic signals, we asked whether a relatively raised endogenous ratio of Smad3 to Smad2 caused by depleting Smad2 facilitates Smad3 activation in response to TGF-β. Indeed, Smad3 phosphorylation and the subsequent formation of heteromeric complex with Smad4 by TGF-β were significantly enhanced in Smad2-depleted cells relative to in control groups (Figure 3, A and B). In addition, Smad2 depletion markedly facilitated the nuclear accumulation of activated Smad3 (Figure 3C). Moreover, interestingly, the reversed intracellular condition caused by Smad3 depletion significantly promoted Smad2 activation and its nuclear accumulation by TGF-β (Figure 3, A–C). So, we then investigated whether modulating this ratio by depleting individual R-Smads influences either Smad-dependent transcriptional activities in response to TGF-β. To approach this issue, we used ARE-luciferase reporter/FAST-1 and $(SBE)_4$-luciferase reporter, which are Smad2- and Smad3-specific reporter genes, respectively (Labbe *et al*., 1998; Zawel *et al*., 1998). In agreement with changes in Smad activation (Figure 3, A–C), Smad2 depletion markedly enhanced Smad3-dependent transcriptional activity in response to TGF-β versus controls

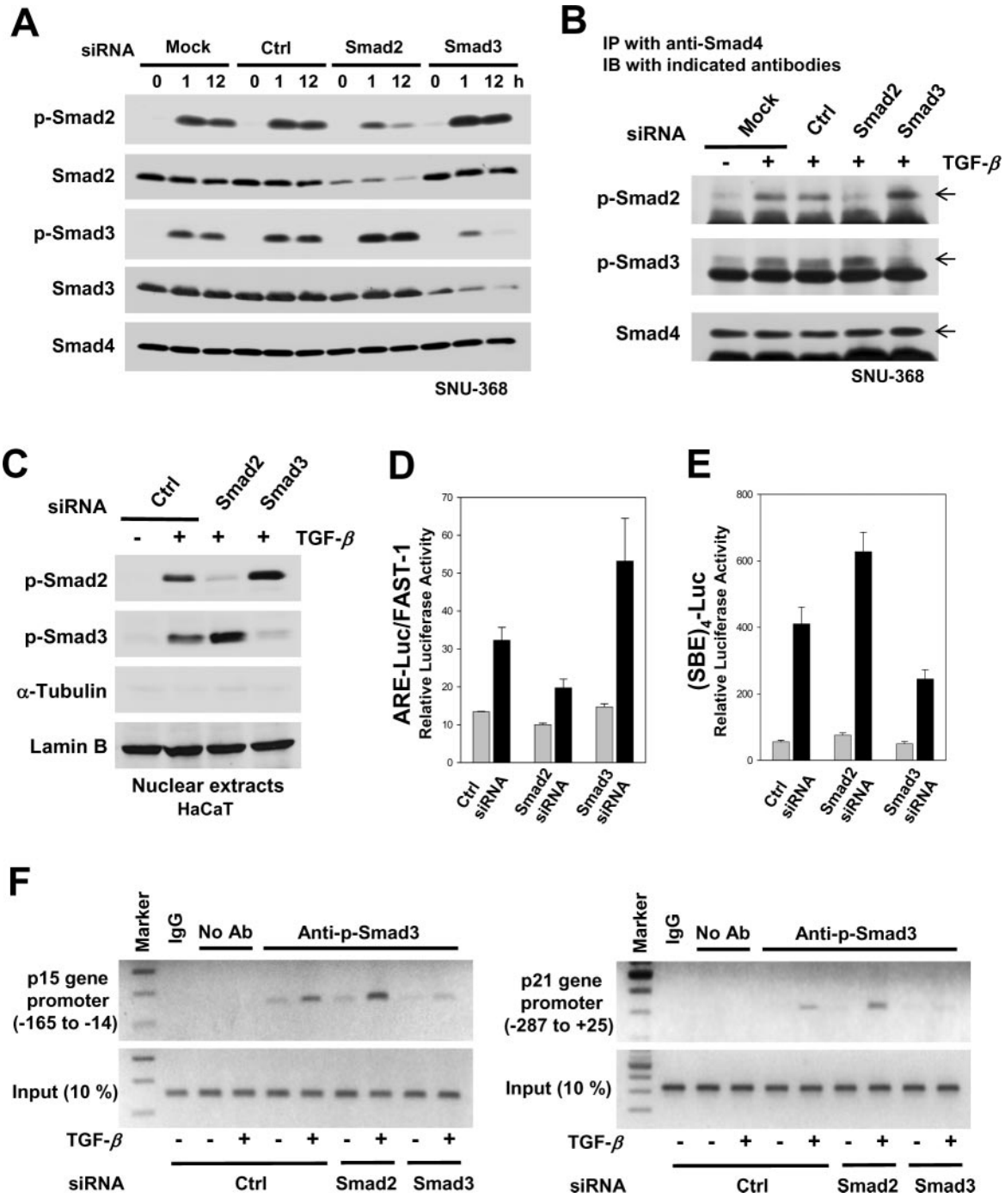

Figure 3. Modulation of the endogenous Smad2/Smad3 ratio by depleting individual R-Smads affects their relative signal intensities in response to TGF-β. (A) SNU-368 cells transfected with the indicated siRNAs were incubated with TGF-β for the indicated times. Whole cell extracts were immunoblotted with the indicated antibodies. (B) Smad4 was immunoprecipitated (IP) from whole cell extracts prepared from SNU-368 cells transfected with each siRNA treated with (+) or without (−) TGF-β for 12 h. Levels of phospho-Smad2 (p-Smad2) or phospho-Smad3 (p-Smad3) associated with Smad4 were evaluated by immunoblotting (IB). The amounts of immunoprecipitated Smad4 were determined by blotting with anti-Smad4 antibody. (C) The indicated siRNA-transfected HaCaT cells were incubated for 12 h in the presence (+) or absence (−) of TGF-β. Nuclear fractions were prepared and immunoblotted with the indicated antibodies. Immunoblotting for Lamin B and α-tubulin were performed to confirm nuclear fraction and exclude cytoplasmic contamination, respectively. (D and E) HaCaT cells were transfected with ARE-Luc/FAST-1 or $(SBE)_4$-Luc together with the indicated siRNAs and pSV-β-Gal and then incubated with (dark bars) or without (gray bars) TGF-β. Luciferase activity was measured 18 h after treating TGF-β. Transfection efficiency was normalized versus β-galactosidase activity, and data are presented as means ± SD of four experiments. (F) The indicated siRNA-transfected HaCaT cells were incubated for 12 h in the presence (+) or absence (−) of TGF-β. ChIP analysis was then performed to detect the presence of phospho-Smad3 on the indicated $p15^{INK4b}$ gene (left) and the $p21^{Cip1}$ gene (right) promoter regions as described in *Materials and Methods*. Normal rabbit IgG and no addition of antibody were used as the negative controls. To confirm the equal chromatin input, 10% of total lysates before immunoprecipitation were used for purification of total genomic DNA, which were used as templates for PCR reactions (bottom).

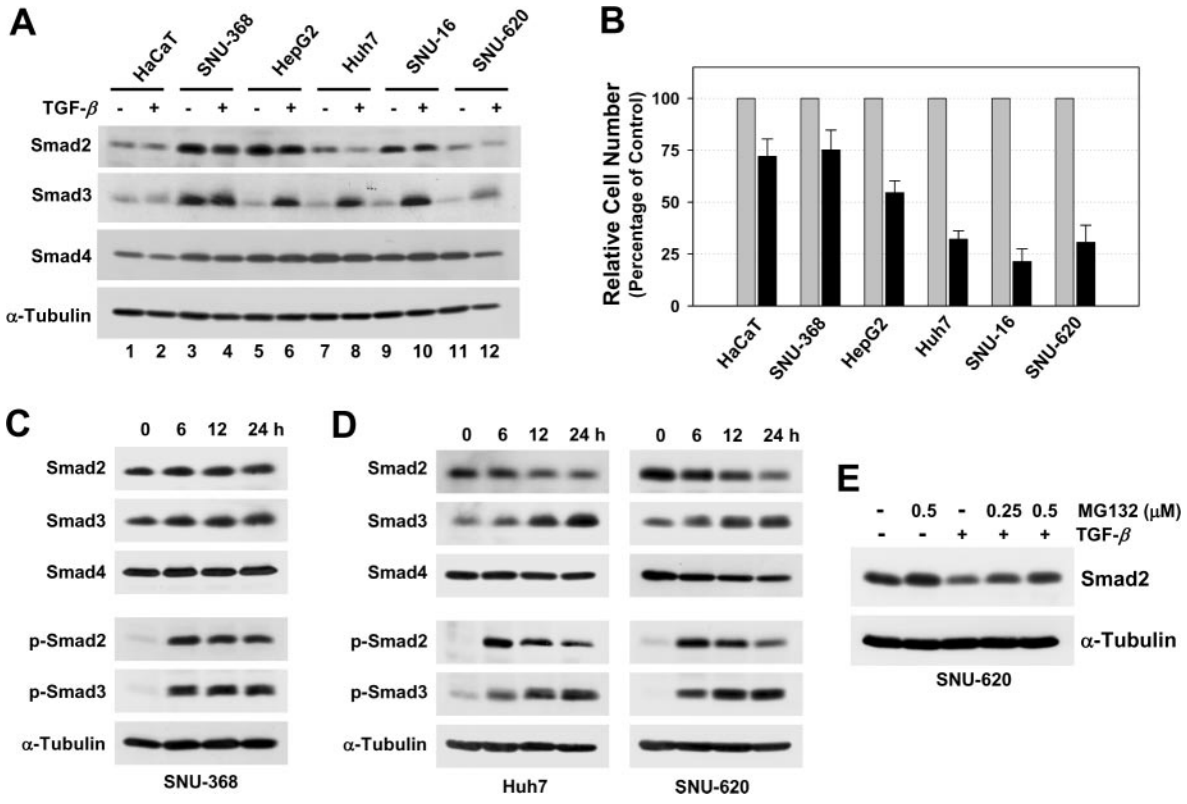

Figure 4. TGF-β can differentially regulate the endogenous ratio of Smad3 to Smad2 depending on cell type. (A) Whole cell lysates were prepared from the indicated cell lines treated with (+) or without (−) TGF-β for 12 h and then immunoblotted with the indicated antibodies. (B) Indicated cell lines were incubated with (dark bars) or without (gray bars) TGF-β and cell proliferation were determined by cell number on day 2 after treating TGF-β. Percentages of TGF-β-treated cell numbers versus untreated controls were calculated and graphed. The error bars represent the SDs of three experiments. (C) SNU-368 cells were incubated with TGF-β for the indicated times. Whole cell extracts were immunoblotted with the indicated antibodies. (D) Whole cell lysates were prepared from Huh7 (left) or SNU-620 cells (right) treated with TGF-β for the indicated times and then immunoblotted as described in C. (E) Cell lysates were extracted from SNU-620 cells treated with (+) or without (−) TGF-β for 12 h in the presence or absence of proteasomal inhibitor MG132 and then immunoblotted as described in C.

(Figure 3E), and Smad3 depletion had the same effect on Smad2-dependent transcriptional activity (Figure 3D). These results provide the possibility that the endogenous ratio of Smad2/Smad3 may be an important regulator of their respective signal intensities in response to TGF-β.

Together, these results suggest that the observed facilitation of TGF-β cytostatic responses by Smad2 depletion (Figures 1 and 2) originally results from a relatively increased endogenous ratio of Smad3 to Smad2, leading to the enhancement of the Smad3-dependent pathway in response to TGF-β. This conclusion is further supported by our finding that, using in vivo ChIP assay with anti-phospho-Smad3 antibody, Smad2 depletion indeed enhances the recruitment of TGF-β-activated Smad3 to the proximal regions in the endogenous promoters of the Cdk inhibitors, $p15^{INK4b}$ (nucleotides −165 to −14) and $p21^{Cip1}$ (nucleotides −287 to +25) (Figure 3F), which directly explains the promoted inductions of these Cdk inhibitors (Figure 2, B and F). Meanwhile, even though Smad2 activation and its-dependent transcriptional activity in response to TGF-β was significantly promoted by Smad3 depletion (Figure 3, A–D), TGF-β cytostatic responses diminished (Figures 1 and 2), strongly suggesting that Smad2 does not act as a direct effector of the TGF-β cytostatic program.

Because alternatively spliced variant of Smad2 lacking exon3 (Smad2Δexon3) has been recently shown to resemble Smad3 in many respects (Yagi *et al*., 1999; Dunn *et al*., 2005), we also estimated the possible involvement of Smad2Δexon3 in the TGF-β-induced cytostasis in our systems. We found that Smad2Δexon3 seems to play a lesser contribution to the TGF-β-induced cytostasis than that of Smad3, because the endogenous level of this variant is generally low in many of the tested cells or undetectable in a certain cell type (Supplemental Figure S2; our unpublished data).

The Endogenous Ratio of Smad3 to Smad2 Is Differentially Regulated by TGF-β, Depending on Cell Type

Because TGF-β cytostatic responses were enhanced by simply elevating the endogenous Smad3 to Smad2 ratio, we investigated whether the basal ratio of Smad3 to Smad2 is indeed related to the sensitivity to TGF-β cytostatic effect in TGF-β-sensitive cell systems. In the unstimulated state, neither the level of Smad3 between cell lines nor the ratio of Smad3 to Smad2 in individual cell lines (Figure 4A, odd lanes) matched cellular sensitivity to the antiproliferative effect of TGF-β (Figure 4B). However, upon TGF-β stimulation, cells relatively highly sensitive to TGF-β, such as HepG2, Huh7, SNU-16, and SNU-620 (Figure 4B), surprisingly showed Smad3 upregulation, which resulted in an increase in endogenous Smad3 to Smad2 ratios (Figure 4A, even lanes), whereas other cells (e.g., HaCaT and SNU-368)

moderately sensitive to TGF-β did not (Figure 4, A and B). These results imply that the endogenous ratio of Smad3 to Smad2 can be differentially modulated by TGF-β, depending on cell type. The up-regulation of Smad3 protein by TGF-β was found to be driven by an increase in Smad3 mRNA expression (Supplemental Figure S3). Thus, in view of Smad3 up-regulation by TGF-β, the endogenous ratio of Smad3 to Smad2 in each cell type correlated well with their sensitivity to the cytostatic effect of TGF-β (Figure 4, A and B).

Next, we further examined the cell type-dependent modulation of the endogenous Smad3 to Smad2 ratio by TGF-β. In cells moderately responsive to the cytostatic effect of TGF-β, the levels of Smad2 and Smad3 were not changed in response to TGF-β, which resulted in the maintenance of the basal Smad3-to-Smad2 ratio (Figure 4C, top). Also, no significant change was observed in the phosphorylation status of R-Smad by TGF-β until 24 h, although Smad2 phosphorylation slightly decreased (Figure 4C, bottom). However, interestingly, cells highly responsive to the cytostatic effect of TGF-β showed time-dependent down-regulation of Smad2 by TGF-β and the concomitant up-regulation of Smad3 (Figure 4D, top), which resulted in a dramatic reversion of the initial Smad3-to-Smad2 ratio. In correlation with this change, Smad3 activation by TGF-β significantly increased in a time-dependent manner, whereas TGF-β-activated Smad2 decreased (Figure 4D, bottom). The down-regulation of Smad2 in this type of cells seems to be caused by the ubiquitin-dependent proteasomal degradation of TGF-β-activated Smad2 (Lo and Massagué, 1999; Seo *et al.*, 2004), because this down-regulation was efficiently blocked in the presence of a potent proteasomal inhibitor *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal (MG132) dose dependently (Figure 4E), and Smad2 mRNA expression was sustained irrespective of TGF-β stimulation (Supplemental Figure S3). Although differential intracellular contexts required for the cell type-dependent fine regulation of Smad expression by TGF-β remain to be identified, these results suggest that TGF-β itself can control the endogenous ratio of Smad3 to Smad2 by increasing Smad3 and/or decreasing Smad2, which favors Smad3-dependent TGF-β cytostasis.

Thus, it seems that the endogenous ratio of Smad3 to Smad2 can be an important regulator of sensitivity to TGF-β cytostatic signals, only if not other TGF-β cytostatic signaling components are deficient or nonfunctional.

Modulation of the Ratio of Smad3 to Smad2 by Overexpressing Each R-Smad Does Not Fully Mimic the Effect of Smads Depletion

Using RNA interference, our evidence shows that the endogenous ratio of Smad3 to Smad2 is involved in the cytostatic response of TGF-β. So, we next evaluated whether modulating this ratio by ectopically overexpressing Smad2 or Smad3 can mimic the effects of individual Smad depletion in response to TGF-β. When Smad3 was overexpressed using adenovirus carrying its cDNA in various TGF-β-sensitive cell types, TGF-β-induced G_1 cell cycle arrest, and growth inhibition were effectively enhanced versus β-galactosidase (LacZ)-infected controls (Figure 5, A and B), which is consistent with the enhancing effect of Smad2 depletion on TGF-β cytostatic action (Figure 1). However, the overexpression of Smad2 showed no inhibitory or enhancing effect on either TGF-β-induced growth inhibition (Figure 5A) or cell cycle arrest (Figure 5B), although, in this case, the overexpressed Smad2 was functional (Figure 5C), and the ratio of Smad3-to-Smad2 was reduced by increasing Smad2 ectopically. Nevertheless, these data still support the notion that TGF-β-induced cytostasis is predominantly under the control of a Smad3-dependent pathway.

To identify the reason why Smad2 overexpression has no inhibitory effect on TGF-β-induced cytostasis, we investigated whether ectopically overexpressed Smad2 actually affects the activation status of endogenous Smad3 by TGF-β. Even though the Smad3-to-Smad2 ratio was reduced by expressing Smad2 ectopically, the total level of endogenous Smad3, and most importantly, its phosphorylation by TGF-β, did not change in Smad2-overexpressed cells relative to the LacZ-infected control (Figure 5D, third from top, lanes 2 and 3), which resulted in the maintenance of Smad3-dependent cytostatic responses (Figure 5, E and F, lane 3) even at higher Smad2 overexpression condition (Figure 5E, lane 5). Similarly, Smad3 overexpression also had no effect on the activation status of endogenous Smad2 (Figure 5D, top, lanes 2 and 4). This means that ectopic Smad2 or Smad3 may not be competitive with endogenous Smad3 or Smad2, respectively, for their activation, which is in agreement with the previous observation (Tian *et al.*, 2003). Although an additional question remains to be answered, namely, why modulation of the Smad3-to-Smad2 ratio by overexpression and depletion has different effects on the activation status of endogenous Smads, the above-mentioned results at least explain how Smad2 overexpression does not inhibit Smad3-dependent TGF-β cytostasis. Meanwhile, because ectopically overexpressed Smad3 was sufficiently activated in response to TGF-β (Figure 5D) and subsequently enhanced TGF-β-induced cytostatic responses (Figure 5, E and F, lane 4), these results also explain how Smad3 overexpression facilitates TGF-β-induced cytostasis.

Therefore, in the case of modulating the Smad3-to-Smad2 ratio above physiological levels by adding surplus ectopic Smads, the dependency of the TGF-β cytostatic effect on this ratio seems to be lost. However, even in this case, it remains valid that the input of TGF-β cytostatic signals is determined by the intensity of a Smad3-, but not a Smad2-, dependent pathway.

A Single Event of Smad2 Depletion or Smad3 Overexpression Sufficiently Restores TGF-β Cytostatic Responsiveness in Cells Resistant to TGF-β

Although the loss or mutational inactivation of TGF-β signaling components has been proposed as a mechanism for cells acquiring resistance to the cytostatic effect of TGF-β and hence developing tumors (Kim *et al.*, 2000; Massagué *et al.*, 2000; Derynck *et al.*, 2001; Wakefield and Roberts, 2002), these defects are relatively rare when all human tumors are considered. Rather, many cancer cells have been shown to be resistant to the antiproliferative effect of TGF-β, even when most components of the TGF-β signaling pathway are intact and functional (Jong *et al.*, 2002; Nicolas and Hill, 2003). So, given the dependency of TGF-β cytostasis on the endogenous ratio of Smad3 to Smad2, we presumed that some of these cancer cells may lose TGF-β cytostatic responsiveness by maintaining this ratio below a certain required threshold, thereby attenuating Smad3 activation and its dependent TGF-β cytostatic signals.

To test this possibility, we first selected cell types, such as Panc-1 human pancreatic cancer cells and SNU-398 and SNU-739 human hepatoma cells, which are known to be resistant to the antiproliferative effect of TGF-β despite retaining an intact TGF-β/Smad signaling system (Jong *et al.*, 2002; Nicolas and Hill, 2003), and then investigated the effect of Smad2 depletion on the cytostatic effect of TGF-β in these cells. Surprisingly, consistent with our assumption, the elevation of the endogenous Smad3-to-Smad2 ratio by deplet-

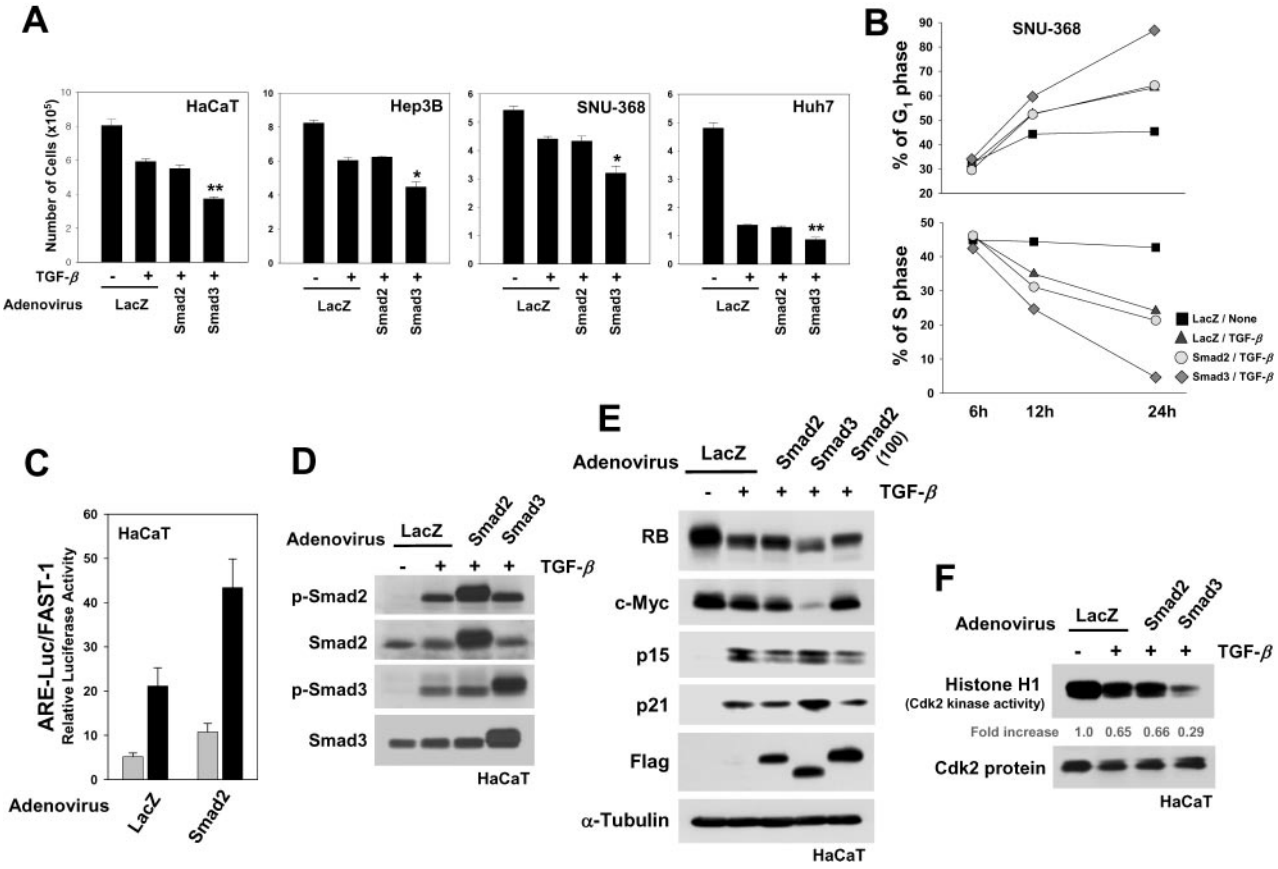

Figure 5. Effects of Smad2 or Smad3 overexpression on the TGF-β cytostatic program. (A) The indicated cell lines infected with adenoviruses carrying β-galactosidase (LacZ), FLAG-tagged Smad2, or Smad3 cDNA were incubated with (+) or without (−) TGF-β. Cell proliferation was determined by counting cell numbers on day 2 after treating TGF-β. Data are presented as means ± SD of three experiments (*$p < 0.05$, **$p < 0.01$). (B) SNU-368 cells infected as described in A were incubated in the presence or absence of TGF-β and subjected to flow cytometric analysis as described in Figure 1D. The percentages of cells in the G_1 and S phases were determined based on the DNA content histograms and graphed. The data shown are representative of four independent experiments. (C) HaCaT cells infected with adenoviruses carrying LacZ or Smad2 were transfected with ARE-Luc/FAST-1 and pSV-β-Gal and then incubated with (dark bars) or without (gray bars) TGF-β. Luciferase activity was measured as described in Figure 3D, and data are presented as means ± SD of four experiments. (D) HaCaT cells infected as described in A were treated with (+) or without (−) TGF-β for 1 h. Total cell lysates were immunoblotted with the indicated antibodies. (E) HaCaT cells were infected with the indicated adenoviruses at 50 MOI (lanes 1–4) or 100 MOI (lane 5) and then incubated for 12 h in the presence (+) or absence (−) of TGF-β. Immunoblotting was performed with the indicated antibodies. (F) HaCaT cells infected as described in E were incubated for 12 h in the presence (+) or absence (−) of TGF-β. A Cdk2 kinase assay was carried out as described in Figure 2C.

ing Smad2 sufficiently restored TGF-β-induced G_1 cell cycle arrest and consequent growth inhibition in these cancer cells (Figure 6, A and B). This implies that the level of endogenous Smad3 in these cells would be enough to trigger TGF-β-induced cytostasis. In addition, elevating the level of Smad3 by overexpressing Smad3 ectopically also efficiently recovered TGF-β-induced cytostasis (Figure 6C), suggesting that the levels of endogenous TGF-β receptors in these cancer cells would be sufficient to activate overexpressed Smad3. Thus, these results indicate that the endogenous ratio of Smad3 to Smad2 below a certain threshold may provide a mechanism for the acquisition of resistance to the cytostatic effect of TGF-β, even in the absence of mutation or loss of the TGF-β/Smad signaling system.

A Certain Threshold of a Smad3-dependent Pathway Is Required for Executing the Cytostatic Effect of TGF-β

Given the restoration of the cytostatic effect of TGF-β by depleting Smad2 in cells resistant to TGF-β, we asked whether this restoration indeed results from the facilitation of a Smad3-dependent pathway. On TGF-β stimulation, both Smad2 and Smad3 were phosphorylated in Panc-1, SNU-398, and SNU-739 cells (Figure 7A). In addition, a set of TGF-β cytostatic gene responses, e.g., c-Myc down-regulation and $p21^{Cip1}$ induction, were readily triggered by TGF-β in these cells (Figure 7C; our unpublished data), showing that TGF-β cytostatic signaling systems are functional in these cells, although they are resistant to the cytostatic effect of TGF-β (Figure 6). Of note, we observed that Smad3 phosphorylation by TGF-β was markedly enhanced when Smad2 was depleted (Figure 7A). Similar enhancement of Smad2 phosphorylation by TGF-β was also observed in Smad3-depleted cells (Figure 7A). Because the expression of the inhibitory Smad7, which functions in a negative feedback manner to prevent TGF-β/Smad signaling, was marginally influenced by depleting each Smad (Figure 7B), we reconfirmed that such facilitated Smad phosphorylation is directly driven by an altered endogenous Smad2-to-Smad3

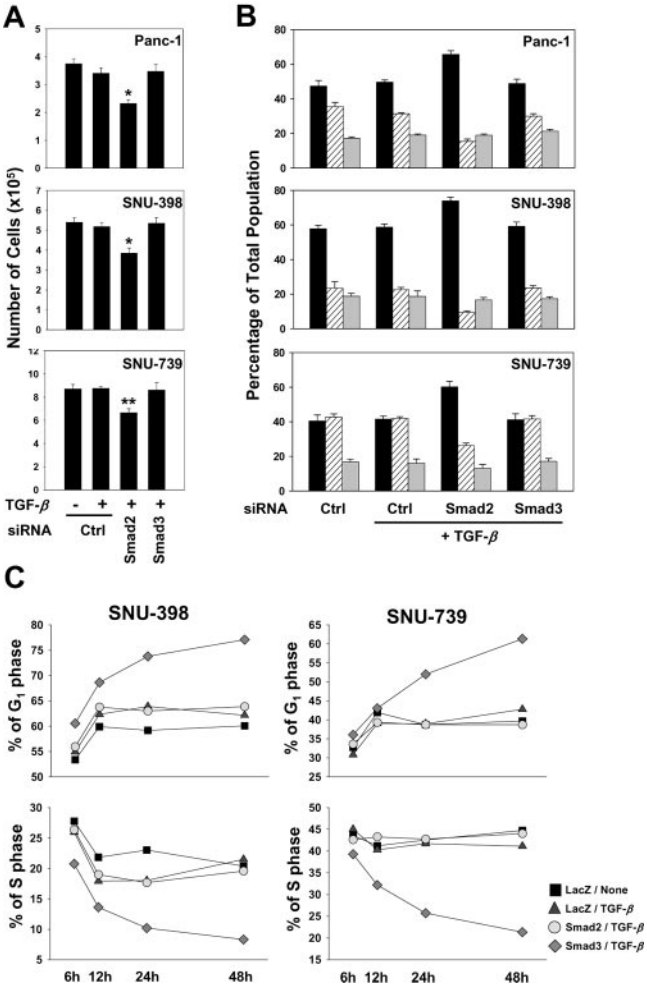

Figure 6. Restoration of TGF-β-induced cytostasis by Smad2 depletion or Smad3 overexpression in cells resistant to TGF-β. (A) The indicated cell lines were transfected with each siRNA and then incubated with (+) or without (−) TGF-β. Cell proliferation was determined by counting cell numbers on day 2 after treating TGF-β. Data are presented as means ± SD of three experiments (*$p < 0.05$, **$p < 0.01$). (B) Indicated cell lines transfected as described in A were treated with or without TGF-β for 24 h and then subjected to flow cytometric analysis. The percentages of cells in the G_1 (black bars), S (hatched bars), and G_2/M (gray bars) phases were determined based on the DNA content histograms and graphed. (C) SNU-398 (left) or SNU-739 (right) cells infected with adenoviruses carrying β-galactosidase (LacZ), FLAG-tagged Smad2, or Smad3 cDNA were incubated in the presence or absence of TGF-β. At the indicated times after treating TGF-β, cells were analyzed as described in B. The percentages of cells in the G_1 (top) and S (bottom) phases were determined based on their DNA content histograms and graphed. The data shown are representative of four independent experiments.

ratio. Subsequently, Smad3-dependent TGF-β cytostatic gene responses were found to be markedly facilitated only when Smad2 was depleted, which resulted in a significant accumulation of hypophosphorylated pRB (Figure 7C, lane 7–9). These results suggest that the recovery of TGF-β cytostasis in Smad2-depleted cells (Figure 6) is caused by the enhancement of a Smad3-dependent pathway.

Interestingly, a small but not significant increase in TGF-β cytostatic gene responses was observed in Smad2-depleted cells even in the absence of exogenous TGF-β (Figure 7C, lane 7). These slight responses disappeared in the presence of neutralizing anti-TGF-βs antibody (Figure 7D), suggesting that raising the endogenous Smad3-to-Smad2 ratio by depleting Smad2 sensitizes cells to TGF-β cytostatic signals to the extent of inducing a response to autocrine TGF-β.

Together, these results indicate that an endogenous Smad3-to-Smad2 ratio below a threshold can function as a pressure against the cytostatic effect of TGF-β and that a certain quantitative threshold of Smad3-dependent pathway is also necessary to efficiently execute TGF-β cytostasis; furthermore, this threshold can be reached below or above the required level depending on the endogenous ratio of Smad3 to Smad2.

DISCUSSION

A growing body of evidence suggests that Smad3 plays important roles in the cytostatic action of TGF-β (Siegel and Massagué, 2003). For example, loss of Smad3 expression increases susceptibility to tumorigenicity in human gastric cancer cells (Han *et al*., 2004). Loss of Smad3 also impairs the inhibitory effect of TGF-β on the proliferation of normal T-cells, which contributes to the promotion of T-cell leukemogenesis in mice (Wolfraim *et al*., 2004). Hyperproliferation is a major constituent of the carcinogenic process and leads to the development of metastatic colon cancer in Smad3 null mice (Zhu *et al*., 1998). In addition, various primary cells from Smad3 null mice were found to be resistant to the growth inhibitory effects of TGF-β (Datto *et al*., 1999; Rich *et al*., 1999; Yang *et al*., 1999), indicating that Smad3 has a key function in cytostatic responsiveness to TGF-β.

Based upon the observed effects of individual R-Smad depletions and overexpressions in a variety of epithelial cell systems in the present study, our results also suggest that Smad3 is the primary key mediator of TGF-β cytostatic signaling, and further that Smad2 is not a direct effector of the TGF-β cytostatic program. The conclusion that Smad2 is not an effector in this cytostatic program is strongly supported by several lines of evidence. First, depleting Smad2 by RNA interference does not prevent TGF-β-induced cytostatic gene responses, G_1 cell cycle arrest, or growth inhibition in various epithelial cells. Rather, all these responses to TGF-β are consistently enhanced. Second, even though Smad2 activation and its dependent transcriptional activity in response to TGF-β are enhanced by depleting Smad3, TGF-β-induced cytostasis is almost abolished. Finally, increasing Smad2 activity by overexpression had no effect on cytostatic responses to TGF-β. Nevertheless, this conclusion does not rule out the possibility that Smad2 plays a role in growth control by TGF-β in nonepithelial systems. Thus, further studies are required to identify the specific biological activities and functions of Smad2 in epithelial as well as nonepithelial systems.

The simultaneous activation of the closely related Smad2 and Smad3 by receptor-mediated phosphorylation and both Smads-derived transcriptional responses are central events in the TGF-β signaling pathway (Derynck *et al*., 1998; Moustakas *et al*., 2001; ten Dijke and Hill, 2004) and occur in most epithelial cells that are responsive to TGF-β. However, a growing body of evidence suggests that the related Smad2 and Smad3 play unique roles downstream of TGF-β and have distinct transcriptional target genes. For example, studies of *JunB* and *Smad7* gene promoters have shown that Smad3, but not Smad2, play a direct role in their inducibility by TGF-β (Jonk *et al*., 1998; von Gersdorff *et al*., 2000). Using Smad2- and Smad3-deficient mouse embryonic fibroblasts,

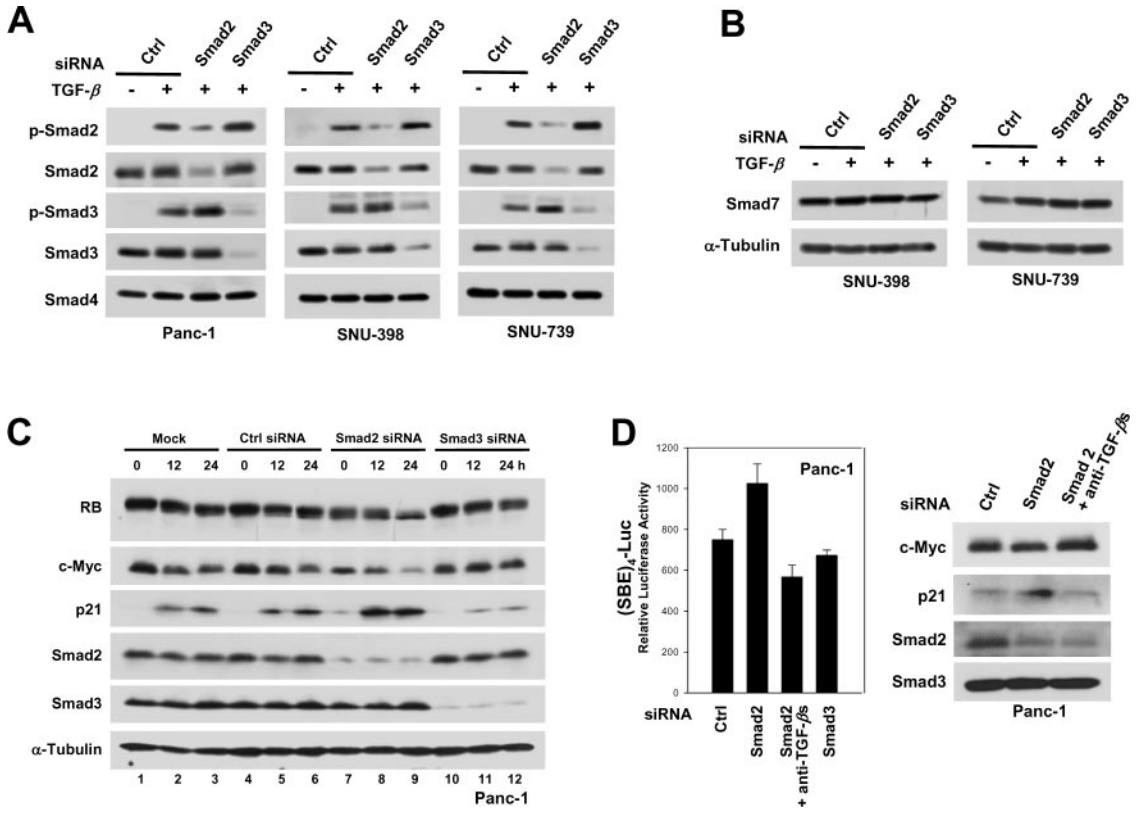

Figure 7. Restoration of TGF-β-induced cytostasis by Smad2 depletion is caused by the facilitation of a Smad3-dependent pathway. (A and B) The indicated cell lines transfected with each siRNA were incubated for 1 h with (+) or without (−) TGF-β. Whole cell lysates were immunoblotted with the indicated antibodies. (C) Lysates were prepared from Panc-1 cells transfected with each siRNA in the presence of TGF-β for the indicated times and immunoblotted with the indicated antibodies. (D) Panc-1 cells transfected with $(SBE)_4$-Luc together with the indicated siRNAs and pSV-β-gal were incubated in the presence or absence of neutralizing anti-TGF-β antibody. Luciferase activity was measured 30 h after transfection. Transfection efficiency was normalized versus β-galactosidase activity, and data are presented as means ± SD of four experiments (left). Whole cell lysates prepared from these cells were immunoblotted with the indicated antibodies (right).

it has been also shown that TGF-β-mediated induction of *matrix metalloproteinase-2* is selectively dependent on Smad2, whereas induction of c-*fos* and *Smad7* relies on Smad3 (Piek *et al*., 2001). Moreover, it has been suggested that Smad2 and Smad3 counteract with each other in the regulation of a subset of TGF-β target genes. Namely, Smad2 acts in combination with FAST and Smad4 to activate the *goosecoid* promoter, whereas Smad3-containing complexes suppress activation of this promoter (Labbe *et al*., 1998). In addition, in response to TGF-β, Smad3 activates a set of immediate-early genes that encode signal transducers and transcriptional regulators, whereas Smad2 seems to negatively modulate a number of these same genes (Yang *et al*., 2003). Thus, these considerable observations suggest that the closely related Smad2 and Smad3 are not redundant but, rather, have distinct activities and transcriptional targets.

Given these distinct activities of Smad2 and Smad3 and the predominant dependency of TGF-β cytostatic signals on a Smad3-dependent pathway in the present study, here, we suggest a crucial and more basic, but not the only, determinant of sensitivity to TGF-β cytostatic signals, namely, the endogenous ratio of Smad3 to Smad2. Our results demonstrate that the intensity of essential cytostatic gene responses to TGF-β, such as, c-Myc repression and the induction of $p15^{INK4b}$ and $p21^{Cip1}$, can be sufficiently modulated by simply changing this ratio, i.e., by reducing either Smad2 or Smad3. Accordingly, the intensity of TGF-β-mediated cell cycle arrest and growth inhibition are also regulated by this ratio. This finding is further supported by the observation that TGF-β stimuli itself can control this ratio by inducing Smad3 and/or reducing Smad2 depending on cell type and that this change in ratio correlates well with sensitivity to the cytostatic effect of TGF-β.

Although the TGF-β-induced up-regulation of Smad3 and down-regulation of Smad2 are not uniformly observed in all cells that are growth inhibited by TGF-β, these responses are of interest because such modulation of Smad expression may make cells more responsive to TGF-β cytostatic signals by increasing the endogenous Smad3-to-Smad2 ratio. Endogenous Smad2 has been known to be targeted for ubiquitination and proteasomal degradation in response to TGF-β (Lo and Massagué, 1999; Izzi and Attisano, 2004; Seo *et al*., 2004). In the present study, we also observed that, in response to TGF-β, Smad2 continuously decreased by proteasomal degradation in several cell types. However, as opposed to the down-regulation of Smad2, significant reduction in Smad3 expression by TGF-β was barely observed in tested epithelial cell types. Rather, Smad3 induction by TGF-β, through as yet unknown mechanisms, is frequently observed in cells highly sensitive to TGF-β cytostasis. This is a novel and more interesting response because it probably works in a positive feed back manner to further propagate Smad3-dependent TGF-β cytostatic signals, like the induction of inhibitory Smad7 by TGF-β acts in a negative feed

back manner (Miyazono, 2002). Thus, the identification of mechanisms responsible for this induction would allow us to approach the question as to why Smad3 response to TGF-β is present in some but not all epithelial cell systems, which ultimately would help us understand the cellular context of the fine regulation of Smads expression by TGF-β and the exact roles of this regulation in TGF-β-induced cytostasis.

One significant physiological function of TGF-β is the inhibition of the proliferation of epithelial, neuronal, and hematopoietic cells, thus TGF-β contributes to the maintenance of homeostasis in these tissues (Attisano and Wrana, 2002; Wakefield and Roberts, 2002; Siegel and Massagué, 2003). This cytostatic function is often lost in cancer cells as a result of mutations or loss that directly inactivates components of the TGF-β/Smad signaling pathway, e.g., TGF-β receptors and Smad4 (Kim *et al*., 2000; Massagué *et al*., 2000; Derynck *et al*., 2001; Wakefield and Roberts, 2002). However, many tumor cells without known mutations in these components are refractory to the cytostatic effect of TGF-β. Thus, the molecular basis for this loss should be understood. Recent studies have addressed this issue and suggested several candidate mechanisms for the loss of TGF-β-induced cytostasis. Ras hyperactivation by oncogenic mutation in several human cancer cells has been shown to induce the additional phosphorylation of MAP kinase sites in Smads and to inhibit the nuclear accumulation of Smads and their ability to mediate TGF-β antiproliferative responses (Kretzschmar *et al*., 1999). It has also been shown that a hyperactive PI3K/Akt pathway and high levels of FoxG1 in glioblastoma cells cooperate to prevent $p21^{Cip1}$ induction and cytostasis by the TGF-β/Smad pathway (Seoane *et al*., 2004). More recently, extensive Smad3 phosphorylation by Cdk2 and Cdk4 has been suggested to inhibit its transcriptional activity and antiproliferative function in response to TGF-β (Matsuura *et al*., 2004).

In addition to these proposed mechanisms, we suggest a new mechanism, namely, that below a certain threshold, the endogenous Smad3-to-Smad2 ratio itself attenuates a Smad3-dependent pathway without mutation or loss in the TGF-β/Smad system and thereby inhibits TGF-β-induced cytostasis. Based on the dependency of a Smad3-dependent pathway on this ratio, we also suggest that reaching a certain quantitative threshold of a Smad3-dependent pathway is essentially required to trigger TGF-β-induced cytostasis. Our results show that several human cancer cells are resistant to the antiproliferative action of TGF-β, even though they have an intact TGF-β receptor/Smad system and thus show Smad3 activation and its dependent cytostatic gene responses by TGF-β. In these cancer cells, simply raising the endogenous Smad3-to-Smad2 ratio by reducing endogenous Smad2 greatly enhances a Smad3-dependent cytostatic pathway, resulting in an efficient restoration of TGF-β-induced cytostasis. Thus, the attenuation of this Smad3-dependent pathway by regulating the endogenous Smad3-to-Smad2 ratio may provide a simple but important mechanism for resistance to TGF-β-induced cytostasis in cancer, which introduces the possibility of its therapeutic use in cancer cells, and also in the many pathological conditions caused by TGF-β signaling deregulation.

ACKNOWLEDGMENTS

We gratefully thank Drs. K. Miyazono for adenoviruses expressing Smads, E. B. Leof for anti-phospho-Smad3, and S. J. Kim for ARE-luciferase reporter/FAST-1 and $(SBE)_4$-luciferase reporter constructs. We also thank Dr. J. W. Lee for manuscript reading and helpful discussion. This work was supported by a grant from the Korean Ministry of Health and Welfare (C99-01-041) and in part by 2004 BK21 Project for Medicine, Dentistry, and Pharmacy.

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