The epithelial-mesenchymal transition (EMT) is a crucial morphological event that occurs during the progression of epithelial tumors. EMT can be induced by transforming growth factor (TGF)-β in some tumor cells. Here, we demonstrate the molecular mechanism whereby Snail, a key regulator of EMT, is induced by TGF-β in tumor cells. Snail induction by TGF-β was highly dependent on cooperation with active Ras signals, and silencing of Ras abolished Snail induction by TGF-β in pancreatic cancer Panc-1 cells. Transfection of constitutively active Ras into HeLa cells led to induction of Snail by TGF-β, while representative direct targets of TGF-β, including Smad7 and PAI-1, were not affected by Ras signaling. Using mitogen-activated protein kinase inhibitors or Smad3 or Smad2 mutants, we found that phosphorylation at the linker region of Smad2/3 was not required for the induction of Snail by TGF-β. Taken together, these findings indicate that Ras and TGF-β-Smad signaling selectively cooperate in the induction of Snail, which occurs in a Smad-dependent manner, but independently of phosphorylation at the linker region of R-Smads by Ras signaling.

Transforming growth factor (TGF)-β, a prototypical member of the TGF-β family, regulates a broad range of cellular responses, including cell proliferation, differentiation, and apoptosis. TGF-β and related factors exhibit pleiotropic effects through binding to transmembrane serine-threonine kinase receptors type I (TβR-I) and type II (TβR-II). Upon ligand-induced heteromeric complex formation between TβR-I and TβR-II, TβR-I is phosphorylated and activated by TβR-II kinase and mediates specific intracellular signaling through phosphorylation on the C-terminal SSXS motif of receptor-regulated Smads (R-Smads). Phosphorylated R-Smads interact with common-partner Smad (co-Smad, Smad4 in mammals) and translocate into the nucleus, where they regulate transcription of target genes in cooperation with various transcription factors and transcriptional coactivators or corepressors (1–3).

TGF-β elicits potent anti-proliferative effects in a wide variety of cells, including epithelial cells, endothelial cells, and hematopoietic cells, although under certain conditions it promotes the proliferation of mesenchymal cells, including fibroblasts, chondrocytes, and osteoblasts. In early stages of carcinogenesis, TGF-β inhibits the growth of epithelial cells, and insensitivity to this growth-inhibitory effect of TGF-β is associated with the development of tumors (4). In contrast to its anti-oncogenic effects in the early stages of carcinogenesis, TGF-β also acts as a promoter of tumor cell invasion and metastasis in advanced stages of tumorigenesis (5). TGF-β is often overexpressed in various tumor tissues and induces migration and invasion of cancer cells (6). Blockade of TGF-β signaling thus leads to suppression of tumor cell motility, intravasation, and metastasis. One mechanism by which TGF-β induces formation of spindle cell carcinomas and promotes tumor cell motility and invasion is epithelial-mesenchymal transition (EMT) (7, 8).

EMT is the differentiation switch directing polarized epithelial cells to differentiate into mesenchymal cells. During the process of embryonic development and that of wound healing and reorganization in adult tissues, epithelial cells may lose their epithelial polarity and acquire mesenchymal phenotype. The process of invasion of tumor cells involves the loss of cell-cell interaction together with acquisition of migratory properties and is often associated with EMT of cells. EMT is characterized by the loss of epithelial markers, including E-cadherin, down-regulation of cytotkeratins, up-regulation of mesenchymal markers, including fibronectin, N-cadherin, and vimentin, and acquisition of a fibroblast-like motile and invasive phenotype (9).

Recent studies on the molecular mechanisms by which expression of E-cadherin is repressed in epithelial cells have revealed that several transcription factors, including the zinc-finger factors Snail and Slug, the two-handed zinc-finger factors of bZIP family proteins (ZEB1 and SIAH1), and the basic helix-loop-helix factors Twist and E12/E47, are involved in this...
process. These transcription factors repress expression of E-cadherin and elicit EMT when overexpressed in normal epithelial Madin-Darby canine kidney and Eph4 cells. In addition, when overexpressed in cancer cells, these factors induce EMT with the development of metastatic properties, such as cell migration and invasion in vitro and in vivo (10, 11).

The Smad signaling pathway is activated by TGF-β and regulated by other signaling pathways. Among them, mitogen-activated protein kinases (MAPKs) are reported to positively and negatively regulate the Smad pathway in phosphorylation-dependent or -independent fashions. MAPKs are activated by various cellular stimuli, phosphorylate R-Smads at their linker regions, and modulate Smad signaling (12, 13). The MAPK family consists of three subfamilies, i.e., extracellular signal-regulated kinase (Erk) 1 and 2, the c-Jun N-terminal kinase (JNK), also known as the stress-activated protein kinase), and p38 MAPK. The classic MAPKs, Erks, are principally activated in response to growth factors, whereas JNK and p38 are activated by various stresses, including TNF-α treatment, UV light, x-ray irradiation, and H2O2 treatment (14).

TGF-β and bone morphogenetic proteins (BMPs) induce phosphorylation of the C-terminal SSXS motif of Smad2 and -3 and that of Smad1, -5, and -8, respectively. Erk, activated by growth factors, phosphorylates several serine residues in the linker region of Smad1, and inhibits its nuclear localization and transcriptional activity (15). Recently, it has been reported that phosphorylation of Smad1 at the linker region accelerates the interaction of Smad1 with Smad ubiquitin regulatory factor 1 (Smurf1), resulting in Smurf1-mediated ubiquitination of Smad1 and inhibition of BMP signaling (16, 17). Smad2 is also phosphorylated at several serine/threonine-proline sites in its linker region by activated Erk in response to growth factors and induces cytoplasmic retention, allowing attenuation of TGF-β signaling (18). On the other hand, in Ras-transformed cells, Smad2/Smad3 with phosphorylation at the linker regions have been reported to be predominantly located in the nucleus and to transmit certain signals (19). Smad2 and -3 phosphorylated at the linker region were also observed in late-stage invasive and metastatic colorectal cancer (20). Phosphorylation at the linker region thus plays important roles in the integration of TGF-β and other signaling pathways, leading to modulation of cellular responses induced by TGF-β. However, the functional importance of phosphorylation of R-Smads at the linker region in TGF-β signaling, particularly in cancer cells, is not well understood.

In the present study, we investigated the molecular mechanisms by which TGF-β induced Snail transcription in the process of EMT in pancreatic carcinoma Panc-1 cells. TGF-β induced expression of Snail in Panc-1 cells, which have endogenous active K-ras. In addition to Panc-1 cells, we have shown that some cancer cells with active ras mutations exhibited induction of Snail by TGF-β. Knockdown of K-Ras by siRNA attenuated TGF-β-mediated Snail up-regulation, while induction of Smad7, a representative target gene of the TGF-β-Smad pathway, by TGF-β was not affected by knockdown of K-Ras. Interestingly, Snail induction by TGF-β was observed even in the presence of MAPK inhibitors. In addition, Snail was induced by TGF-β in a Smad-dependent manner and in cells expressing the Smad3-4A or Smad2-4A mutant with conversion of the conserved MAPK phosphorylation sites in the linker region. These findings suggest that TGF-β induces Snail in cooperation with Ras signaling, which occurs independently of phosphorylation at the linker region of R-Smads.

EXPERIMENTAL PROCEDURES

Cell Culture—Human cancer cells (HeLa, Panc-1, MCF7, Huh7, A431, PC-3, and A549) and human keratinocyte HaCaT cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 4.5 g/liter glucose, 10% fetal bovine serum, 50 units/ml penicillin, and 100 μg/ml streptomycin. HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium in the presence of 10% fetal bovine serum, 100 μM minimal essential medium non-essential amino acids (Invitrogen), and the same antibiotics. All cells were grown in a 5% CO2 atmosphere at 37 °C.

Plasmid Construction—Human Snail cDNA was obtained from Dr. F. van Roy (Ghent University). Human Snail and human H-RasG12V were subcloned into pcDEF3 and pcDNA3.0 vectors, respectively, to obtain their expression plasmids (21). The Smad3-4A (T179A, S204A, S208A, and S213A) mutants were generated by point mutagenesis.3

Reagents and Antibodies—Recombinant human TGF-β1 was obtained from R&D Systems (Minneapolis, MN). Recombinant human epidermal growth factor (EGF) and hepatocyte growth factor (HGF) were purchased from Sigma-Aldrich and Pepro Tech (London, UK), respectively. Mouse monoclonal anti-FLAG M2 and anti-α-tubulin antibodies were purchased from Sigma-Aldrich, and mouse monoclonal anti-E-cadherin, anti-fibronectin, and anti-Smad2/3 antibodies were from BD Transduction Laboratories (Lexington, KY). Rat monoclonal anti-Snail and rabbit anti-phospho-p44/42 MAPK antibodies were from Cell Signaling (Danvers, MA). Mouse monoclonal anti-K-Ras antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit monoclonal anti-phospho-Smad2 (Ser-465/467) and mouse monoclonal anti-HDAC1 antibodies were from Upstate (Lake Placid, NY). Rat monoclonal anti-HA antibody was from Roche Applied Science.

Luciferase Assays—Panc-1 cells were seeded in duplicate in 24-well tissue culture plates, followed by transient transfection with human E-cadherin-Luc, Renilla reporter (Promega, Madison, WI), and human Snail expression plasmids using Lipofectamine 2000 reagent (Invitrogen) as recommended by the manufacturer’s instructions. Luciferase activity was determined by a dual luciferase reporter assay system (Promega) using a luminometer (AutoLumat LB953, EG&G Berthold, Natick, MA). Luciferase activity was normalized to the sea pansy luciferase activity of cotransfected pRL-CMV.

Generation and Infection of Lentiviruses—A lentiviral vector encoding full-length human Snail was generated by Gateway technology (Invitrogen). 293FT cells were co-transfected with the expression plasmids, VSV-G and Rev expressing plasmid (pcMV-VSV-G-RSV-Rev), and packaging plasmid (pCAG-

3 A. Nakano, D. Koinuma, K. Miyazawa, T. Uchida, M. Saitoh, M. Kawabata, J.-i. Hanai, H. Akiyama, M. Abe, K. Miyazono, T. Matsumoto, and T. Imamura, manuscript in preparation.
HIVgp) using Lipofectamine 2000. The viral supernatants were collected 72 h after transfection. For lentiviral infection, 3.0 × 10⁶ cells/well in 6-well tissue culture plates were infected with lentiviral vectors. CS-CDF-CG-PRE was used as a green fluorescent protein (GFP)-control.

**Immunostaining—**Panc-1 cells were seeded at a density of 4.0 × 10⁵ cells/well in 8-well culture slides. After 24 h, cells were treated with 1 ng/ml TGF-β1 for 48 h. Cells were fixed in 3.7% formaldehyde in phosphate-buffered saline for 15 min, permeabilized with 0.2% Triton X-100 in phosphate-buffered saline for 5 min, and incubated with primary antibodies diluted with Blocking One solution (Nacalai Tesque, Kyoto, Japan) for 2 h. The cells were then incubated with secondary antibodies for 1 h and TOTO3 (Invitrogen-Molecular Probes) for 5 min. All procedures were performed at room temperature. Fluorescence was examined by confocal laser scanning microscopy (Carl Zeiss, Thornwood, NY).

**SDS-PAGE and Immunoblotting—**Cells were lysed in radiimmunoprecipitation assay buffer solution (20 mM Tris- HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) or in lysis buffer solution (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% phosphatase inhibitor mixture (Nacalai Tesque), 1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride). After clearing with centrifugation, protein concentrations were measured, and equal amounts of total protein per lane were subjected to SDS gel electrophoresis, followed by semidry transfer of the protein to Fluoro Trans W membrane (Pall, Glen Cove, NY). Nonspecific binding of proteins to the membrane was blocked by incubation in TBS-T buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) containing 5% skim milk. Immunodetection was performed with the ECL blotting system (Amersham Bioscience).

**Fractionation of Nuclear and Cytoplasmic Proteins—**HeLa cells transiently transfected with RasG12V or control plasmids were treated with 1 ng/ml TGF-β1 for 1 h, and fractionation of nuclear and cytoplasmic proteins was performed with the NE-PER Nuclear and cytoplasmic extraction reagents following the manufacturer’s instructions (Pierce). Proteins of each fraction were examined by immunoblotting as described above.

**RNA Isolation and Quantitative RT-PCR—**Total RNAs were extracted using the RNeasy Mini Kit (Qiagen) and reverse-transcribed by oligo(dT) priming using Superscript III Reverse Transcriptase following the manufacturer’s instructions (Invitrogen). Quantitative RT-PCR analyses were performed using the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) and SYBR Green (Applied Biosciences). The primer sequences used were as follows; human GAPDH: sense, 5’-GAAGGTGAAGGTCGGTGGCAC-3’; antisense, 5’-GAAGATGGTGATGGGATGTGCTACTCC-3’; human Snail: sense, 5’-TTCTCAGTCCATGGAATTC-3’; antisense, 5’-GACAGGCGACAGAACAGAAA-3’; human E-cadherin: sense, 5’-TGACCCCAACCCCTCATGAGT-3’; antisense, 5’-GTCAGTATCAGCGGTTTC-3’; human Smad3: sense, 5’-TGTGGACGAATGATGCTCC-3’; antisense, 5’-GCCCATCGACTGTA-3’; and human Smad4: sense, 5’-ATCAGAGCGCACCTGTCT-3’; antisense, 5’-GGCAGTTCCAGGATGTCGTA-3’.

**Effect of Ras on TGF-β1-induced Snail Expression**

**FIGURE 1.** TGF-β1 promotes EMT accompanied by E-cadherin repression in Panc-1 cells. A, phase-contrast and immunofluorescence images of cells treated with or without 1 ng/ml TGF-β1 for 48 h. The cells were immunostained with anti-E-cadherin antibodies (green) and stained with propidium iodide to detect nuclei (red). Scale bars indicate 100 μm. B, expression of E-cadherin and fibronectin upon TGF-β1 stimulation. Amounts of E-cadherin and fibronectin proteins were determined by immunoblotting after treatment of Panc-1 cells with or without 1 ng/ml TGF-β1 for 48 h. Levels of α-tubulin were monitored as a loading control for whole cell extracts. C, expression of Snail and phospho-Smad2 upon TGF-β1 stimulation. Panc-1 cells were stimulated with 1 ng/ml TGF-β1 for 2 h and subjected to immunoblot analysis using anti-Snail and anti-phospho-Smad2 (Ser-465/467) antibodies. Levels of α-tubulin were monitored as a loading control for whole cell extracts. D, induction of Snail was examined in cells treated with TGF-β1 for the indicated periods by semi-quantitative RT-PCR analyses. Ratios of mRNAs levels in TGF-β1-treated cells to those in non-treated cells are shown. Values have been normalized to the amounts of housekeeping GAPDH mRNA.

**RNA Interference and Oligonucleotides—**Transfection of short interfering RNAs (siRNAs) was performed according to the protocol recommended for HiPerFect Reagent (Qiagen). Panc-1 or HeLa cells were transiently transfected with siRNAs against K-Ras (5’-TGTTGACGAATGATGCTCC-3’, Stealth RNAi, Invitrogen), Smad2 (5’-AACAGGCTTTACAGCTTCT-3’, Dharmacon Research, Lafayette, CO), Smad3 (5’-CCAGAGCTAGAGACACCAGTGTCT-3’, Stealth RNAi, Invitrogen) and Smad4 (#003902; Dharmacon Research).
**Cell Motility Assay**—Panc-1 cells were seeded at a density of $3.0 \times 10^5$ cells/well in 6-well tissue culture plates, and 12 h later, 1 ng/ml TGF-β1 was added to the media. After 12-h ligand stimulation, wounds were incised by scratching the cell monolayers using 200-μl pipette tips. Photographs were taken under phase-contrast microscopy immediately after incision and 24 h after incision. Areas of migrating cells were estimated by counting numbers of pixels using ImageJ software.

**RESULTS**

**TGF-β Promotes EMT in Pancreatic Cancer Panc-1 Cells**—TGF-β has been reported to induce EMT in some epithelial cell lines and during embryonic development (8, 22). Pancreatic cancer Panc-1 cells were treated with TGF-β, and cell morphology was examined after 48 h of TGF-β stimulation. Upon treatment with TGF-β, cells acquired a spindle cell-type morphology, and the number of cell-cell contacts was reduced due to repressed expression of a representative epithelial marker, E-cadherin, as determined by immunostaining (Fig. 1A). Immunoblotting of whole cell lysates of Panc-1 cells revealed that 48 h TGF-β treatment resulted in down-regulation of E-cadherin with concomitant up-regulation of a mesenchymal marker, fibronectin (Fig. 1B). Short-term treatment (2 h) with TGF-β clearly activated Smad2 phosphorylation on the C-terminal SSXS motif and induced the expression of Snail protein (Fig. 1C). A time-course experiment with semi-quantitative RT-PCR demonstrated that expression of Snail mRNA was rapidly induced to a >5-fold extent by 2 h after TGF-β treatment and remained at high levels until 24 h (Fig. 1D). These findings demonstrate that TGF-β induces EMT in Panc-1 cells.

**Snail Represses E-cadherin Expression during TGF-β-induced EMT**—Because Snail is known to be a key mediator of E-cadherin repression (11), we next investigated the effects of Snail on the E-cadherin promoter in Panc-1 cells during TGF-β-induced EMT. The cells were cotransfected with the E-cadherin promoter-reporter plasmid and various doses of the expression plasmid encoding full-length human Snail. Luciferase assay revealed that E-cadherin promoter activity was repressed in dose-dependent fashion by overexpression of Snail (Fig. 2A). The effect of Snail on E-cadherin expression was determined by immunostaining. TGF-β strongly suppressed the expression of E-cadherin in control cells expressing GFP (Fig. 2B, upper two panels). Snail was located in the nuclei in Panc-1 cells infected with the Snail lentivirus (Fig. 2B, the lowest panels). Interestingly, E-cadherin staining revealed that endogenous expression of E-cadherin was suppressed only in Panc-1 cells ectopically expressing Snail (Fig. 2B). Overexpression of Snail affected E-cadherin expression in Panc-1 cells, whereas expression level of the mesenchymal marker fibronectin between the control cells and Snail-
expressing cells was not changed even in the presence of TGF-β (Fig. 2C).

Because the phenotypic changes induced by TGF-β were also associated with increased cell motility, we determined the effects of Snail on cell motility by an in vitro wounding assay. TGF-β enhanced the motility of Panc-1 cells and accelerated wound closure, whereas ectopically expressed Snail did not significantly affect cell motility in the absence or presence of TGF-β (Fig. 2D). These findings thus suggest that, although Snail induced by TGF-β primarily affected the E-cadherin expression, Snail was not involved in cell motility induced by TGF-β in Panc-1 cells.

Cross-talk between Ras and TGF-β Signaling—Panc-1 cells have the K-RasG12V mutation, which results in constitutive activation of Ras signaling (23). It has been reported that Ras-Raf signaling is required for the EMT induced by TGF-β in epithelial Madin-Darby canine kidney cells (24), and that MAPKs activated by Ras signals can modulate TGF-β signaling through phosphorylation of R-Smads at their linker region (18). We therefore tested whether Ras signals cooperate with TGF-β signaling to induce Snail expression. siRNA against K-Ras was transfected into Panc-1 cells, and down-regulation of endogenous K-Ras protein was confirmed by immunoblotting (Fig. 3A). K-Ras siRNA effectively down-regulated its endogenous protein without off-target effects. In cells transfected with control siRNA, TGF-β led to induction of Snail protein and mRNA, whereas TGF-β failed to induce it in K-ras knockdown cells (Fig. 3A and data not shown). Semi-quantitative RT-PCR and immunostaining revealed that suppression of E-cadherin expression by TGF-β was partially blocked by K-Ras knockdown (Fig. 3, B and C). Because Smad7 and PAI-1 are known to be direct targets of the TGF-β-Smads pathways (25), their mRNAs were also analyzed by semi-quantitative RT-PCR. TGF-β induced up-regulation of Smad7 and PAI-1 mRNAs in K-Ras knockdown cells to extents similar to those in control cells (Fig. 3, D and E). These findings thus indicate that loss of Ras signaling in Panc-1 cells attenuates only Snail induction by TGF-β.

Active RasG12V Mutant Synergistically Affects Snail Induction in Response to TGF-β—To determine the molecular mechanism by which active Ras cooperates with the TGF-β-Smad pathway to induce Snail expression, we performed gain-of-function experiments using HeLa cells transfected with expression plasmid encoding constitutively active Ras (RasG12V). The transfection efficiency of HeLa cells was almost 90% as determined by immunostaining (Fig. 4A). Twenty-four h after transfection into HeLa cells, TGF-β was added to culture media, and expression of endogenous Snail mRNA was analyzed by semi-quantitative RT-PCR. Treatment with TGF-β or transfection of RasG12V alone slightly enhanced expression of Snail, whereas TGF-β treatment of HeLa cells expressing RasG12V synergistically induced Snail expression at both mRNA and protein levels (Fig. 4B and data not shown). Snail promoter activity was also synergistically enhanced by TGF-β in HeLa cells expressing RasG12V (Fig. 4C). On the other hand, no synergism was observed in the induction of Smad7 by TGF-β, and the nuclear translocation of Smad2 induced by TGF-β was slightly reduced in the presence of RasG12V (Fig. 4, D and E), in agreement with the previous reports (16, 18). In addition, we found that total levels of the C-terminal phosphorylation of Smad2 and Smad3 were reduced in RasG12V-expressing cells (supplemental Fig. S1A).

Next, we used several growth factors, instead of the constitutively active Ras, to stimulate the endogenous Ras protein in HeLa cells. Phosphorylation of Erk was observed in cells treated with either EGF or HGF, and Smad2 was phosphorylated by TGF-β in these cells (Fig. 4F). The intensities of phosphorylation of Erk and that of Smad2 with treatment of EGF/HGF and TGF-β, respectively, were almost the same as that resulting from combined treatments with TGF-β and EGF or HGF (Fig. 4F). Similar to HeLa cells

*K. Horiguchi, K. Miyazono, and M. Saitoh, unpublished data.*
were transiently transfected with RasG12V. At 24 h after transfection, the cells were treated with TGF-
β and then measured. Values were normalized to the amounts of GAPDH mRNA.

FIGURE 4. TGF-β1 and Ras signaling cooperate in promoting Snail expression in HeLa cells. A, HeLa cells transfected with pcDNA3 or FLAG-tagged RasG12V (FLAG-RasG12V) were immunostained with anti-FLAG M2 (green) and stained with propidium iodide to detect nuclei (red). Scale bars indicate 200 μm. B and D, HeLa cells were transiently transfected with RasG12V. At 24 h after transfection, the cells were treated with TGF-β1 for an additional 1 h, and then levels of Snail (B) or Smad7 (D) mRNAs were examined by semi-quantitative RT-PCR. Values were normalized to the amounts of GAPDH mRNA. C, HeLa cells were cotransfected with mouse Snail promoter-reporter construct (Snail-Luc) in combination with RasG12V expression plasmids. At 6 h after transfection, the cells were treated with TGF-β1 for an additional 18 h, and the activities of Snail promoters were then measured. E, HeLa cells transfected with or without RasG12V were treated with TGF-β1 for 1 h. Fractionation of nuclear and cytoplasmic proteins was performed as described under “Experimental Procedures.” F, endogenous levels of Snail were determined by immunoblotting at 2 h after combined treatment with 1 ng/ml of TGF-β1 and 100 or 300 ng/ml of EGF, and with 1 ng/ml of TGF-β1 and 30 or 100 ng/ml of HGF. Levels of α-tubulin were monitored as a loading control for whole cell extracts.

transfected with RasG12V, induction of endogenous Snail was detected only in cells treated with TGF-β in combination with EGF or HGF (Fig. 4F). These findings thus suggest that Ras signaling is required for induction of Snail by TGF-β in both Panc-1 and HeLa cells.

Linker Phosphorylation of R-Smads Is Not Involved in the Synergistic Effects of Ras and TGF-β Signaling—It has been reported that, following the C-terminal phosphorylation by TGF-β receptor kinases, R-Smads are regulated by phosphorylation at the linker region by activated Erk and JNK (18, 20). The linker phosphorylation of both Smad2 and Smad3 in RasG12V-expressing cells was observed at higher levels than that in control cells (supplemental Fig. S1B). To determine whether phosphorylation at the linker region is involved in Snail induction by TGF-β in cooperation with active Ras, MAPK inhibitors, U0126 for MEK1/2 and SP600125 for JNK, were used in Panc-1 cells and in HeLa cells transfected with active Ras. The specificity of effects of U0126 and SP600125 in both types of cells was confirmed by blockade of phosphorylation of their substrates, Erk for MEK1/2 and c-Jun for JNK (Fig. 5, A and B). Under these conditions, Snail induction was examined by semi-quantitative RT-PCR. TGF-β efficiently induced Snail mRNA in both cells, and Snail remained up-regulated even in the presence of these inhibitors (Fig. 5, A and B). TGF-β signaling was not disturbed in the presence of these inhibitors, as evaluated by phosphorylation of Smad2 by TGF-β (Fig. 5, A and B). To further confirm this, luciferase assays using 9xCAGA-Luc and Snail-Luc were performed in HeLa cells transfected with wild-type Smad3 or Smad3-4A mutant. In the Smad3-4A mutant, four serine/threonine residues at the potential MAPK phosphorylation sites in the linker region were mutated to alanine (16). In the presence of wild-type Smad3, the transcriptional activity of 9xCAGA-Luc induced by TGF-β was attenuated in the presence of RasG12V (Fig. 5C). In contrast, these effects of RasG12V were not observed in the Samd3-4A mutant, possibly as a result of efficient nuclear translocation and protection from phosphorylation-dependent degradation of Smad3-4A. These findings suggest that the CAGA reporter activity induced by TGF-β is blocked by Ras signals through phosphorylation of the linker region.

In contrast, Snail reporter activities in the cells expressing wild-type Smad3 or Smad3-4A mutant were slightly activated by TGF-β alone and further enhanced by active Ras. No difference was observed between the wild-type Smad3 and Smad3-4A mutants in the induction of Snail (Fig. 5C). Similar to Smad3-4A, Smad2-4A mutant showed a similar activity to the wild-type Smad2 to activate the Snail promoter (supplemental Fig. S1C). Thus, in contrast to Smad7, the cooperation of Ras with TGF-β in induction of Snail was not affected by status of phosphorylation at the linker region of Smad2 and Smad3. The
induction of Snail by TGF-β in cooperation with active Ras was thus independent of Erk and JNK activation and of phosphorylation at the linker regions of R-Smads.

Requirement of Smad activation to induce Snail by the cooperation between Ras and TGF-β were evaluated by knockdown experiments using siRNA oligonucleotides against Smad2, Smad3, and Smad4. Specificity and efficiency of these siRNAs were confirmed by silencing of their endogenous gene expression and by inhibiting the direct target genes regulated by TGF-β-Smad signaling (Fig. S2). Following knockdown of both Smad2 and Smad3, or that of Smad4, Snail induction by TGF-β was dramatically reduced in HeLa cells (Fig. 5D). In addition, overexpression of c-Ski, a negative regulator of Smad signaling, partially blocked TGF-β-mediated Snail induction in HeLa cells (data not shown). Thus, these findings suggest that Smad activation is indispensable for Snail induction by the cooperation between Ras and TGF-β.

Snail induction by TGF-β in some cancer cells with active Ras mutations—Ras mutations are frequently observed in various types of cancer cells. To evaluate whether Snail expression following TGF-β is dependent on activating mutation of Ras, we randomly chose several human cell lines, including keratinocyte HaCaT cells, breast cancer MCF7 cells, hepatocellular carcinoma HepG2 and Huh7 cells, epidermoid carcinoma A431 cells, prostate cancer PC-3 cells, and lung cancer A549 cells for examination. Among these cells, PC-3 and A549 each have a K-ras mutation, as previously described (26, 27). All cells were sensitive to TGF-β, as evaluated by up-regulation of Smad7 (Fig. 6A), whereas Snail induction was observed only in the cells with K-ras mutation (Fig. 6B).

**DISCUSSION**

Cross-talk between Ras and TGF-β signaling has been reported to play important roles in various physiological and pathological processes, and Ras signal has been reported to both positively and negatively regulate TGF-β signaling (5). Ras transformation in lung, intestinal, liver, pancreas, and mammary epithelial cells
has been reported to confer resistance to growth inhibition by TGF-β (28–32). Microinjection of oncogenic Ras protein into TGF-β-arrested mink lung epithelial cells overcame growth inhibition by TGF-β and allowed cell-cycle progression into S phase (33). On the other hand, we found that Snail induction by TGF-β was observed only in the presence of active Ras, whereas active Ras did not affect the up-regulation of well known targets of TGF-β, Smad7 and PAI-1, in pancreatic cancer Panc-1 cells. In agreement with these findings, Smad3-4A and Smad2-4A mutants, which were not phosphorylated by MAPKs at the linker region, transmitted signals inducing Snail expression in both the presence and absence of active Ras.

In the BMP signaling pathway, Smad1 is sequentially phosphorylated at the conserved MAPK and glycogen synthase kinase-3β motifs of the linker region, leading to inhibition of nuclear accumulation, enhanced degradation by Smurf1, and suppression of the transcriptional activity of Smad1 (16, 17). In contrast to BMP signaling, the function of phosphorylation at the linker region of Smad2/Smad3 in TGF-β signaling remains obscure and controversial. MAPKs activated by growth factors or other stimuli in epithelial cells phosphorylate Smad2/Smad3 at the linker regions and induce their cytoplasmic retention, thus inhibiting the TGF-β-Smad signaling pathways (18). In contrast to the suppression of TGF-β-Smad signaling by MAPKs, Karamaju et al. (34) reported that phosphorylation of Smad3 by p38 MAPK at the linker region is required for full transcriptional activation and growth inhibition by TGF-β. In tumor cells with ectopically expressed H-Ras, elevated H-Ras levels are required for nuclear accumulation of Smad2, which is required for the induction of EMT (19). Moreover, Sekimoto et al. (20) reported that phosphorylation at the linker region of Smad2/Smad3 by MAPKs correlates with progression of advanced cancer. In the present study, in pancreatic cancer Panc-1 cells and HeLa cells, induction of Snail by TGF-β was observed only in the presence of active Ras, and silencing of K-Ras resulted in suppression of the induction of Snail by TGF-β. The Smad3-4A mutant was insensitive to the inhibitory effect of Ras and was able to hyperactivate the CAGA-reporter activity induced by TGF-β (Fig. 5C). However, similar to the Smad3-4A mutant, wild-type Smad3 still had the capacity to enhance Snail up-regulation in cooperation with active Ras. These findings suggest that phosphorylation at the linker region of R-Smads is not required for Snail induction by TGF-β. Moreover, although inhibitors of JNK and MEK1/2 specifically and effectively blocked their target kinases, induction of Snail by TGF-β was still observed. We could not evaluate the effects of p38 MAPK inhibitors, SB203580 and SB202190, in the present study due to the lack of specificity of their effects on the TGF-β type I receptor kinase.4 Taken together, these findings suggest that neither phosphorylation at the linker region of R-Smad nor the function of Erk and JNK as kinases downstream of Ras is involved in the induction of Snail resulting from the cooperation between Ras and TGF-β-induced EMT in Panc-1 cells and HeLa cells. In addition, inhibitors for phosphatidylinositol 3-kinase and protein kinase C could not inhibit Snail induction by the cooperation between TGF-β and Ras, suggesting that phosphatidylinositol 3-kinase and protein kinase C are not involved in Snail induction mediated by this cooperation. Therefore, it will be necessary to further evaluate other signaling mechanisms, including small GTPases in the future.

Although we found that Snail was strongly induced in cell lines with active Ras, including Panc-1, PC-3, and A549, Snail may be induced by TGF-β in cooperation with some other signals. Mouse mammary gland epithelial NMuMG cells have been well characterized in TGF-β-induced EMT, and Snail induction by TGF-β is observed in them. Although the results of profiling of Snail mRNA induction by TGF-β in NMuMG cells were very similar to those in Panc-1 cells (22), Ras mutation was not observed in NMuMG cells.4 Moreover, TGF-β as well as BMP can induce EMT through induction of Snail in certain types of cells during embryonic development, although this may occur independently of mutated Ras signaling. Induction of Snail by the TGF-β family in NMuMG cells and in cells during development may thus be regulated by mechanisms different from that examined in the present study.

In human pancreatic adenocarcinoma, Smad4 and TβRII are frequently mutated or deleted, and active Ras gene is also frequently detected (35). A previous study demonstrated that pancreatic-specific TβRII and Smad4 knockout mice with active K-RasG12D mutation developed more aggressive adenocarcinoma than that in control mice, suggesting that loss of TGF-β signaling strongly contributes to early development of pancreatic cancer in collaboration with active Ras (36, 37, 38).
However, because in that study the mice died before metastasis occurred, the invasive and metastatic properties of pancreatic cancer could not be fully evaluated. Recent studies revealed that, although inoculation of breast cancer cells into mice resulted in development of massive primary tumors, metastasis was dramatically blocked by treatment with TGF-β inhibitors or inhibitory Smads (39, 40). Thus, TGF-β may act in cooperation with Ras in the advanced stage of cancer to further promote invasion or metastasis through Snail-mediated EMT.

In conclusion, we have shown that Snail transcription factor repressed E-cadherin expression in the process of EMT induced by TGF-β in Panc-1 cells. Cooperation of Ras signaling is essential for the induction of Snail by TGF-β, whereas phosphorylation at the linker region of R-Smads by Ras is not involved in the induction of Snail. The molecular mechanisms by which Ras cooperates with TGF-β signaling in the process of EMT and in the regulation of other key transcriptional factors, including Slug, ßEF-1, and SIP1, will be examined in further detail in the future.

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