Cancer-Associated Transforming Growth Factor β Type II Receptor Gene Mutant Causes Activation of Bone Morphogenic Protein-Smads and Invasive Phenotype

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Abstract

Transforming growth factor β (TGFβ) plays a key role in maintaining tissue homeostasis by inducing cell cycle arrest, differentiation and apoptosis, and ensuring genomic integrity. Furthermore, TGFβ orchestrates the response to tissue injury and mediates repair by inducing epithelial to mesenchymal transition and by stimulating cell motility and invasiveness. Although loss of the homeostatic activity of TGFβ occurs early on in tumor development, many advanced cancers have coopted the tissue repair function to enhance their metastatic phenotype. How these two functions of TGFβ become uncoupled during cancer development remains poorly understood. Here, we show that, in human keratinocytes, TGFβ induces phosphorylation of Smad2 and Smad3 as well as Smad1 and Smad5 and that both pathways are dependent on the kinase activities of the type I and II TGFβ receptors (TβR). Moreover, cancer-associated missense mutations of the TβRII gene (*TGFBR2*) are associated with at least two different phenotypes. One type of mutant (*TGFBR2*^{E526Q}) is associated with loss of kinase activity and all signaling functions. In contrast, a second mutant (*TGFBR2*^{R537P}) is associated with high intrinsic kinase activity, loss of Smad2/3 activation, and constitutive activation of Smad1/5. Furthermore, this *TGFBR2* mutant endows the carcinoma cells with a highly motile and invasive fibroblastoid phenotype. This activated phenotype is TβRI (Alk-5) independent and can be reversed by the action of a dual TβRI and TβRII kinase inhibitor. Thus, identification of such activated TβRII receptor mutations in tumors may have direct implications for appropriately targeting these cancers with selective therapeutic agents. [Cancer Res 2008;68(6):1656–66]

Introduction

In normal epithelia, transforming growth factor β (TGFβ) plays a key role in maintaining tissue homeostasis by inducing cell cycle arrest, differentiation, and apoptosis and ensuring genomic integrity. In this manner, TGFβ functions as a tumor suppressor. In addition, TGFβ orchestrates the response to tissue injury and mediates repair by inducing epithelial to mesenchymal transition (EMT) and by increasing cell motility and invasiveness in a time- and space-limited manner. Escape from the tumor-suppressive actions of TGFβ seems to be an early and frequent event in carcinogenesis (1). Although tumor cells are generally refractory to TGFβ-mediated growth arrest, many retain other functions involved in tissue repair, such as EMT and migration (2). In this case, the TGFβ pathway acts in a pro-oncogenic manner to promote the invasive and metastatic tumor phenotype. Although this uncoupling of the two arms of the TGFβ effector pathway frequently occurs in cancer, the underlying molecular mechanisms have remained elusive. Approximately 40 different cancer-associated missense mutations of the TGFβ type II receptor (TβRII) gene (*TGFBR2*) have been reported, and the vast majority of these mutations are clustered within the receptor kinase domain (3, 4). To determine whether some of these mutations are associated with an oncogenic gain of function, we undertook a detailed analysis of two human head and neck squamous cell carcinoma (HNSCC) lines, A253 and SqCC/Y1, which had been previously reported to carry missense mutations in subdomain XI of the receptor kinase (5). A253 cells are homozygous for a *TGFBR2* mutation that results in a substitution of arginine to proline at codon 537 (R537P), whereas SqCC/Y1 cells are homozygous for a *TGFBR2* mutation that encodes a glutamate to glutamine change at position 526 (E526Q). SqCC/Y1 cells were completely unresponsive to TGFβ. In contrast, although A253 cells were also refractory to TGFβ-mediated growth arrest, they were constitutively in an EMT-like state and highly motile and invasive. Moreover, this phenotype could be directly ascribed to the constitutive activation of bone morphogenic protein (BMP)-Smads, Smad1 and Smad5, and could be reversed using a dual TβRI/II kinase inhibitor. Identification of such activated TβRII receptor mutations in tumors has direct implications for appropriately targeting these cancers with selective therapeutic molecules.

Materials and Methods

Reagents. Human recombinant TGFβ1 (Austral Biologicals) and BMP2 (R&D Systems) were dissolved in 4 mmol/L HCl, with 1 mg/mL bovine serum albumin (BSA). Human recombinant activin A (R&D Systems) was dissolved in sterile PBS, with 1 mg/mL BSA. SB-431542 (Tocris), SD-093 and NPC-30345 (provided by Scios, Inc.), and LY2109761 (provided by Eli Lilly and Co.) are selective chemical competitive inhibitors of the TβR kinases. SB-431542 was dissolved in ethanol. SD-093, NPC-30345, and LY2109761 were dissolved in DMSO. *In vitro* IC_{50} against TβRI ranges from 33.2 nmol/L for SD-093 to 140 nmol/L for NPC-30345. IC_{50} against TβRII is 300 nmol/L for LY2109761 and >10 μmol/L for the other three compounds.

Cell culture. HaCaT immortalized human keratinocytes (obtained from Dr. P. Boukamp, German Cancer Research Center, Heidelberg, Germany), HNSCC cell lines A253 and SqCC/Y1, and the somatic cell hybrid line FaDu × A253 were all maintained in MCDB153^{++} medium supplemented

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with 1% fetal bovine serum (FBS) as described by Pirisi et al. (6). T47D human breast carcinoma cells were obtained from the American Type Culture Collection and maintained in RPMI 1640 (Invitrogen Corp.) supplemented with 10% (v/v) FBS and gentamicin (10 μg/mL). WI-38 human lung fibroblasts were maintained in DMEM supplemented with 10% (v/v) FBS.

Detection of Smad proteins by Western blot. Western blot analyses were carried out as previously described (7). Smad proteins were detected using mouse monoclonal anti-Smad2 (1:1,000; Cell Signaling), anti-Smad3 (1:500; Invitrogen/Zymed), and rabbit monoclonal anti-Smad1 (1:500) and anti-Smad5 (1:1,000; Epitomics) antibodies. Phosphorylated Smads were detected using rabbit monoclonal anti-pSmad2 (1:1,000), rabbit polyclonal anti-pSmad3 (1:1,000), and rabbit polyclonal anti-pSmad1/5 (1:1,000) antibodies (Cell Signaling). TβRII protein was detected using a rabbit polyclonal TβRII (1:200; Santa Cruz Biotechnology) antibody.

Smad dephosphorylation assays. Following treatment of cells with TGFβ (100 pmol/L) for 1 h, the medium was replaced with fresh medium followed by treatment with SD-093 (1 μmol/L), NPC-30345 (10 μmol/L), SB-431542 (10 μmol/L), or LY2109761 (2 μmol/L) for various time points (0–5 h). Whole-cell extracts or nuclear and cytoplasmic fractions (Active Motif Nuclear Extraction kit, Active Motif North America) were subjected to Western blot analysis of Smad proteins as described above.

Gene silencing by small interfering RNA transfection. Transfections were performed in 24-well cluster dishes using cultures at 70% to 80% confluence. GAPDH-specific, control pooled–specific, and Smad-specific small interfering RNAs (siRNA) were obtained from Dharmacon, Inc. Transfection conditions were optimized using GAPDH siRNA according to the manufacturer's protocol. Optimal gene silencing was achieved when transfections were performed using the DF1 reagent (1.5 μL/well) under sterile RNase-free conditions. Twenty-four hours following transfection, cells were lysed and protein extracts were subjected to Western blot analysis as described above. For TGFβ-induced Smad phosphorylation analysis, TGFβ (100 pmol/L) was added 24 h following transfection, and the experiment was terminated 24 h later.

Detection of filamentous actin and E-cadherin. Cells were plated in chamber slides and allowed to adhere overnight. Following treatment with TGFβ (100 pmol/L), SD-093 (1 μmol/L), or LY2109761 (2 μmol/L), TGFβ plus inhibitor, or vehicle only for 72 h, filamentous actin (F-actin) was detected using Alexa Fluor 488 phalloidin (Molecular Probes) and E-cadherin was detected using anti–E-cadherin antibody (Transduction Laboratories) as previously described (8).

Cell migration and invasion assays. Cell migration and invasion assays were performed in MCDB153^{++} culture medium supplemented with 1% (v/v) FBS as previously described (8).

Cell proliferation assays. A total of 10^4 cells were plated in duplicate wells of 24-well plates and allowed to adhere overnight. Cells were then treated with dual TβR kinase inhibitor, LY2109761, at concentrations ranging from 0 to 2,000 nmol/L for 96 h. Cell numbers were determined using a Model 0039 Coulter Counter (Beckman Coulter).

Detection of Smad proteins in A253 xenografts *in vivo*. Exponentially growing A253 tumor cells (1×10^6) were injected s.c. into the flank of viral antibody-free 5- to 6-wk-old athymic nude mice (Harlan Laboratory). Once tumors reached a size of ~0.5 cm, mice were sacrificed and tumors were excised, fixed, and embedded into paraffin. For immunostaining, 5-μm sections were prepared and processed as previously described (9). Phosphorylated Smads were detected using rabbit monoclonal anti-pSmad2 (1:200) and rabbit polyclonal anti-pSmad1/5 (1:200) antibodies (Cell Signaling).

RNA extraction and reverse transcription-PCR. RNA was extracted from HaCaT, A253, SqCC/Y1, and WI-38 cell lines using the Qiagen RNeasy RNA Extraction kit (Qiagen, Inc.). Reverse transcription-PCR (RT-PCR) for Alk-1, Alk-2, Alk-3, Alk-5, and Alk-6 was performed using 25 ng total RNA using the Qiagen OneStep RT-PCR kit. The following primers were used: *Alk-1*, 5′-GGCTCCCCCAGGAAAGGCCTT (forward) and 5′-GGACTCTCCCAGCTCGCTGTG (reverse); *Alk-2*, 5′-GTAGATGGAGTGATGATTCTT (forward) and 5′-AGGGAAGGATTTTCCTTTAGT (reverse); *Alk-3*, 5′-TTGGGAGCCTATTTGTTCATC (forward) and 5′-TTGGGAGCCTATTTGTTCATC (reverse); *Alk-5*, 5′-CACCTCTGTACAAAAGACAAT (forward) and 5′-GCAGATATAGACCATCAACAT (reverse); and *Alk-6*, 5′-CGTCCAAAGGTCTTGCGTTGT (forward) and 5′-AGCCCTGTGGTGTATAGGTCC (reverse).

Site-directed mutagenesis. A 4.63-kb *Eco*RI fragment containing the full-length cDNA sequence of the human *TGFR2* gene was excised from pH2-3FF (obtained from H. Lodish, Whitehead Institute, Cambridge, MA) and subcloned into pcDNA3 at the *Eco*RI site. The 1.8-kb amino acid coding sequence of TβRII was then amplified by PCR using primers that contained a *Kpn*I site at the 5′ end and an *Eco*RI site at the 3′ end. The PCR product was digested with *Kpn*I and *Eco*RI and then ligated into pcDNA3 at the *Kpn*I/*Eco*RI sites. The resulting construct (TBRII/pcDNA3) was then used to generate specific mutants using the QuikChange II XL Site-Directed Mutagenesis kit (Stratagene). Forward and reverse primers were designed to generate the individual mutants, E526Q and R537P. In addition, a *Hae*II site was introduced in the E526Q-specific primers, whereas a *Xho*I site was introduced into the R537P-specific primers to allow initial screening of mutant clones. Mutations in plasmid DNA were confirmed by restriction digestion and DNA sequencing at the Cancer Institute of New Jersey DNA Synthesis and Sequencing Shared Resource.

Reporter gene assays. Cloned *TGFBR2* mutants and/or wild-type receptors were expressed in the *TGFBR2*-null T47D human breast carcinoma cells using transient transfection with Lipofectamine 2000 (Invitrogen) as previously described (5, 10). The following TGFβ-responsive reporter genes were used: *p3TP-lux* (gift from Dr. Michael Centrella, Yale University, New Haven, CT), *SBE4-luc* (obtained from Dr. Bert Vogelstein, Johns Hopkins University, Baltimore, MD), *Col7A1-luc* (obtained from Dr. Alain Mauviel, Institut National de la Sante et de la Recherche Medicale U697, Paris, France), and *pDel-5-myc-luc* (gift from Dr. Fang Liu, Rutgers University, Piscataway, NJ).

Results

We first examined the effects of TGFβ superfamily members on Smad activation in human keratinocytes. HaCaT cells expressed a basal level of phosphorylated Smad2 and Smad3 even in the absence of exogenous TGFβ (Fig. 1*A, left*). As expected, TGFβ treatment resulted in a robust activation of Smad2 and Smad3. In addition, and quite unexpectedly, TGFβ treatment also induced phosphorylation of the classic BMP pathway Smads, Smad1 and Smad5 (Fig. 1*A, left*). The identity of pSmad1 and pSmad5 was confirmed using a pSmad1/5-directed antibody that does not cross-react with pSmad3. Furthermore, treatment of cells with BMP2 induced phosphorylation of Smad1 and Smad5, coincident with those induced by TGFβ treatment. Conversely, activin A only activated Smad2 and Smad3 but failed to induce pSmad1 and pSmad5. The kinase inhibitor SD-093 inhibits not only the TβRI (Alk-5) kinase but also the related activin type I receptor kinases Alk-4 and Alk-7. Pretreatment of cells with SD-093 inhibited both TGFβ- and activin-induced phosphorylation of Smad2 and Smad3 (Fig. 1*A, left*). Importantly, SD-093 treatment also inhibited TGFβ-mediated induction of pSmad1 and pSmad5, indicating that their phosphorylation was dependent on TβRI (Alk-5) activity. BMP2-induced Smad1 and Smad5 phosphorylation was not affected, consistent with the fact that SD-093 does not target BMP type I receptors (8).

We previously described two human squamous carcinoma cell lines (SqCC/Y1 and A253) that are homozygous for missense *TGFBR2* gene mutations (5). Although endogenous autophosphorylated wild-type TβRII and transphosphorylated TβRI could be detected in HaCaT cells (5), neither receptor species was phosphorylated in SqCC/Y1 *TGFBR2*^{E526Q}-mutant cells. On the other hand, in A253 *TGFBR2*^{R537P}-mutant cells, the levels of both TβRII and TβRI phosphoproteins were significantly higher than in HaCaT cells independently of the presence of exogenous TGFβ (5).

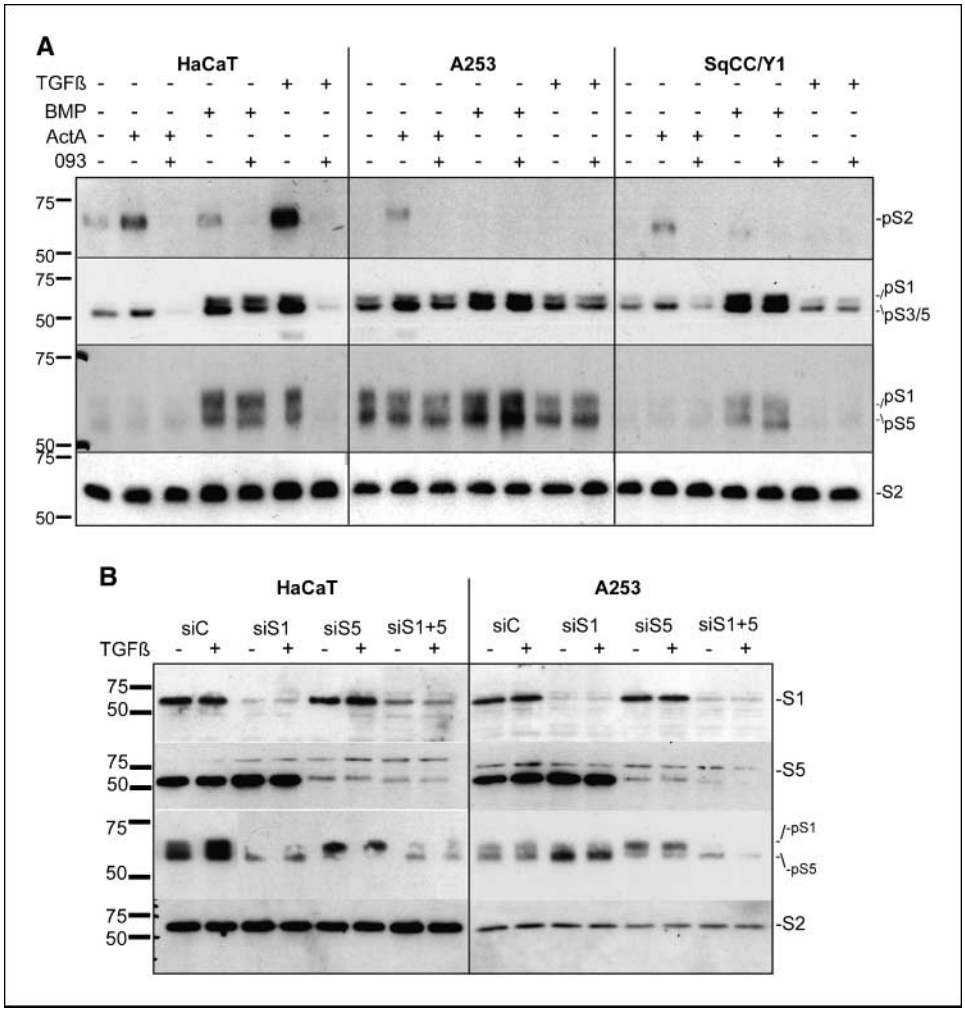

Figure 1. Effects of TGFβ superfamily ligands on Smad activation. *A,* TGFβ (100 pmol/L) treatment of HaCaT cells for 1 h resulted in induction of pSmad2 (*pS2*) and pSmad3 (*pS3*) as well as of pSmad1 (*pS1*) and pSmad5 (*pS5*), all of which were blocked by SD-093 pretreatment. A253 and SqCC/Y1 expressed no detectable pSmad2 or pSmad3 even in response to TGFβ. Note that the pSmad3-directed antibody that was raised against the COOH-terminal phosphorylation site of Smad3 cross-reacts to some extent with the BMP-Smads Smad1 and Smad5. Thus, the upper band recognized by the pSmad3 antibody in HaCaT cells most likely represents pSmad1, whereas the lower band represents pSmad3 and/or pSmad5, which are so close in molecular size that they seem to comigrate. Whereas SqCC/Y1 cells expressed very little pSmad1 and pSmad5, A253 cells constitutively expressed high levels of pSmad1 and pSmad5 (detected by either anti-pSmad1/5 or anti-pSmad3). This BMP-Smad activation was unaffected by SD-093. Control experiments included treatment of HaCaT, A253, and SqCC/Y1 cells with activin A (50 ng/mL) for 1 h, which resulted in activation of Smad2 and Smad3. Pretreatment with SD-093 resulted in blocking this activation in each of the lines. Conversely, BMP2 (100 ng/mL) treatment for 1 h resulted in induction of BMP-Smads Smad1 and Smad5 in each of the cell lines, which was not blocked by SD-093 pretreatment. *B,* transient transfection of Smad-specific siRNA (siSmad) resulted in effectively reducing the level of total Smad protein in a dose- and time-dependent manner for up to 72 h (data not shown). Compared with untransfected cells, as little as 25 nmol/L siSmad was sufficient to knock down the Smad target, whereas 100 nmol/L of control scrambled siRNA had no effect. Furthermore, the effect of any of the siSmads was specific to the Smad target because the levels of closely related Smads were unaffected. In both HaCaT and A253 cells, transient transfection of siRNA specific to Smad1 or to Smad5 resulted in a significant reduction of total Smad1 and Smad5 protein levels, respectively, whereas control pool siRNA had no effect. In addition, cotransfection of siSmad1 (*siS1*) and siSmad5 (*siS5*) effectively silenced both Smads and resulted in loss of both phosphorylated Smad species. Most importantly, these results show that the activated pSmads in A253 represent pSmad1 and pSmad5 but not pSmad3.

Thus, the *TGFBR2*^{R537P} mutation seems to constitutively activate the TβRII kinase.

To further characterize the two mutant receptors, we examined their effects on Smad activation. In SqCC/Y1 *TGFBR2*^{E526Q}-mutant cells, TGFβ treatment failed to induce phosphorylation of any of the receptor-associated Smad (R-Smad) proteins (Fig. 1*A, right*). In contrast, treatment with activin induced pSmad2 and pSmad3, which was inhibitable by SD-093. Similarly, treatment with BMP2 resulted in activation of pSmad1 and pSmad5, which was not affected by SD-093 treatment. Thus, in SqCC/Y1 cells, TGFβ fails to activate any of the R-Smads, whereas activin and BMP2 retained the ability to induce phosphorylation of their respective target Smads.

As in SqCC/Y1 cells, treatment of A253 *TGFBR2*^{R537P}-mutant cells with TGFβ failed to induce phosphorylation of pSmad2 and pSmad3 (Fig. 1*A, middle*). In contrast, A253 cells constitutively expressed high levels of pSmad1 and pSmad5 (Fig. 1*A, middle*). BMP2 treatment resulted in further increasing the level of these same two phosphoproteins, which was not affected by SD-093 treatment. On the other hand, activin treatment resulted in a modest induction of pSmad2 and pSmad3, inhibitable by SD-093, but not of pSmad1 or pSmad5. Thus, neither the activin nor the BMP pathways seem to be affected by the presence of the *TGFBR2*^{R537P} mutant.

To further substantiate that the bands detected by both the anti-pSmad3 and anti-pSmad1/5 antibodies in HaCaT and A253 cell

extracts did, in fact, represent pSmad1 and pSmad5 and not pSmad3, we silenced endogenous Smad expression using specific siRNAs (Fig. 1*B*). Silencing endogenous Smad1 and/or Smad5 expression using specific siRNAs abrogated the ability of TGFβ to induce pSmad1 and pSmad5 in HaCaT cells and resulted in the loss of the constitutively phosphorylated BMP-Smads in A253 cells (Fig. 1*B*). In contrast, silencing of Smad3 did not affect the ability of TGFβ to induce phosphorylation of Smad1 and Smad5 in HaCaT cells nor did it affect the levels of phosphorylated Smad1 or Smad5 in A253 cells (data not shown). These results further substantiate that the constitutively expressed pSmads in A253 cells indeed represent pSmad1 and pSmad5 and not pSmad3.

Because SD-093 treatment did not affect the level of the constitutively active BMP-Smads in A253 cells, we investigated the possibility that the elevated levels of activated BMP-Smads might be due to a reduced rate of dephosphorylation (Fig. 2*A*). As expected, TGFβ treatment induced a robust activation of Smad2, Smad3, Smad1, and Smad5 in HaCaT cells (time "0," Fig. 2*A*). On

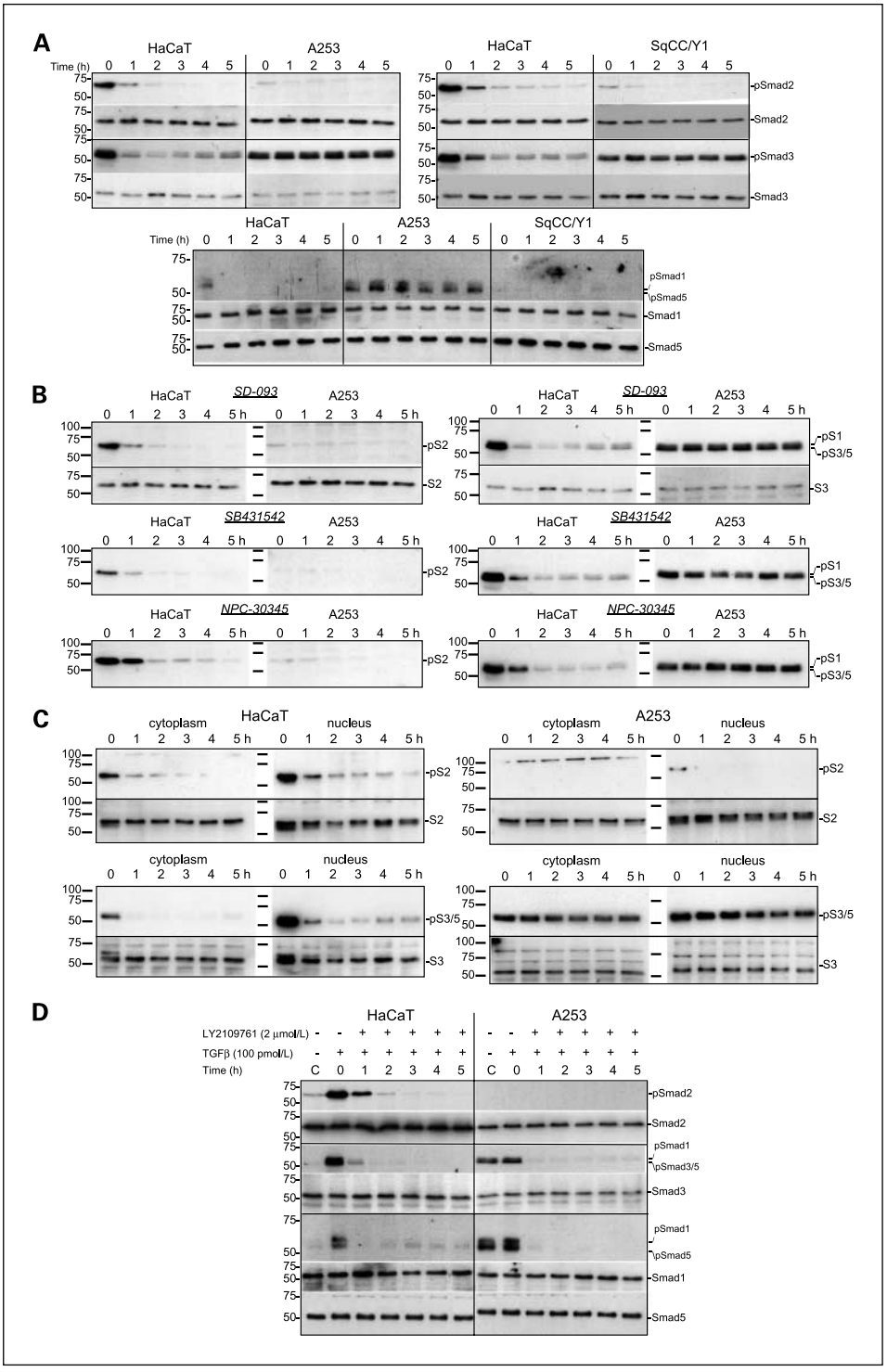

Figure 2. Dephosphorylation of activated Smads. *A,* assays were performed as described in Materials and Methods. In HaCaT cells, inhibition of TβRI (Alk-5) kinase activity using the selective inhibitor SD-093 resulted in dephosphorylation of activated pSmad2 and pSmad3 with a $t_{1/2}$ of 45 min. In addition, TGFβ-induced pSmad1 and pSmad5 were dephosphorylated in response to SD-093 treatment. SqCC/Y1 and A253 cells failed to express pSmad2 and pSmad3. Note that constitutive pSmad activation detected by anti-pSmad3 antibody in SqCC/Y1 cells is a result of longer exposure because these cells lacked pSmad1 and pSmad5. In contrast, treatment of A253 cells with SD-093 for up to 5 h failed to affect the constitutive level of pSmad1 and pSmad5. Total Smad proteins were detected using specific antibodies to Smad1, Smad5, Smad3, and Smad2, indicating that these proteins are expressed in each of the lines. *B,* results obtained using SD-093 were confirmed using other selective TβRI kinase inhibitors SB-431542 (10 μmol/L) and NPC-30345 (10 μmol/L). In HaCaT cells, TGFβ-dependent activated Smad2, Smad3, Smad1, and Smad5 underwent rapid dephosphorylation on treatment with either inhibitor. On the other hand, in A253 cells, pSmad1 and pSmad5 levels were unaffected by either of the two inhibitors. *C,* following dephosphorylation assays performed using SD-093 as described above, extracts were separated into cytoplasmic and nuclear fractions using the Active Motif Nuclear Extraction kit. In HaCaT, TGFβ-activated Smad2 and Smad3 as well as Smad1 and Smad5 were predominantly localized in the nucleus. Activated Smads underwent rapid dephosphorylation in response to SD-093 (1 μmol/L) treatment. In contrast, in A253 cells, nuclear pSmad1 and pSmad5 showed little to no dephosphorylation in response to SD-093. *D,* in HaCaT cells, TGFβ-activated pSmad2 and pSmad3 as well as pSmad1 and pSmad5 were dephosphorylated by treatment with the dual TβRI/II inhibitor LY2109761 with a $t_{1/2}$ of 45 min. In A253 cells, pSmad1 and pSmad5 underwent dephosphorylation in response to LY2109761 treatment with kinetics similar to HaCaT cells.

TβRI (Alk-5) blockade using SD-093, all activated R-Smads were rapidly dephosphorylated with a half-life ($t_{1/2}$) of ~45 min, indicating that their phosphorylation state was also dependent on TβRI (Alk-5) kinase activity (Fig. 2*A*). In contrast, in A253 cells, the constitutively activated BMP-Smads (Smad1 and Smad5) were resistant to dephosphorylation in response to SD-093 treatment. Similar results were obtained using two other TβRI (Alk-5/4/7) kinase inhibitors from different chemical classes, SB-431542 and NPC-30345, indicating that resistance to dephosphorylation was not unique to this particular inhibitor (Fig. 2*B*; refs. 8, 11, 12). Thus, constitutive activation of pSmad1 and pSmad5 in A253 cells seems to have become independent of exogenous TGFβ as well as (TβRI) Alk-5 kinase activity.

Following 1 h of TGFβ treatment, HaCaT cell nuclei were strongly enriched for pSmad2 and pSmad3 as well as pSmad1 and pSmad5. These activated Smads underwent subsequent dephosphorylation in response to SD-093 treatment associated with a rapid reduction of total Smad2 and Smad3 levels in the nucleus, consistent with export of dephosphorylated Smads back into the cytoplasm (Fig. 2*C*). Although little or no pSmad2 or pSmad3 was detected in the nuclei of A253 cells, the nuclear fraction was highly enriched for pSmad1 and pSmad5 (Fig. 2*C*). In addition, SD-093 treatment had minimal effect on dephosphorylation of these activated BMP-Smads (Fig. 2*C*). To determine whether the *TGFBR2*^{R537P} mutant might be responsible for the constitutive activation of BMP-Smads in A253 cells, we made use of LY2109761, a dual inhibitor that targets both the TβRII and TβRI kinases but does not affect BMP-induced cellular responses (13). In HaCaT cells, treatment with LY2109761 caused rapid dephosphorylation of all TGFβ-activated R-Smads (Fig. 2*D*). In sharp contrast to the lack of effect of SD-093 on pSmad1 and pSmad5 levels in A253 cells (Fig. 2*A*), treatment with LY2109761 resulted in rapid dephosphorylation of these BMP-Smads (Fig. 2*D*). Therefore, we tentatively concluded that the effects of LY2109761 on pSmad1 and pSmad5 levels were mediated by its inhibition of the TβRII receptor rather than TβRI (Alk-5), thereby resulting in dephosphorylation of pSmad1 and pSmad5.

TGFβ-induced EMT, which is an important event during wound healing, can be retained in cancer progression (14). F-actin stress fiber formation and loss or redistribution of E-cadherin are hallmarks of EMT (14). As expected, treatment of HaCaT cells with TGFβ resulted in actin stress fiber formation and a redistribution of E-cadherin from the cell membrane to the cytoplasmic compartment (Fig. 3*A*). Furthermore, these TGFβ-induced responses were dependent on TβRI (Alk-5) kinase activity, as the phenotype could be reversed by either SD-093 or LY2109761. In fact, treatment with either of these kinase inhibitors alone resulted in a more cohesive epithelioid morphology with a more pronounced submembranous localization of F-actin and cell surface expression of E-cadherin compared with vehicle (DMSO)-treated controls. In SqCC/Y1 cells, F-actin and E-cadherin were both present at the membrane and the cells had a fairly typical epithelioid morphology (Fig. 3*A*). In addition, there was no demonstrable change in phenotype in response to either TGFβ or kinase inhibitor treatment (Fig. 3*A*). In contrast, in A253 cells, F-actin was diffusely expressed throughout the cytoplasm (Fig. 3*A*) and E-cadherin predominantly in a cytoplasmic and perinuclear pattern independently of exogenous TGFβ (Fig. 3*A*). More importantly, LY2109761 treatment resulted in redistribution of F-actin toward the cell periphery and of E-cadherin to the cell membrane, whereas SD-093 had no effect. Moreover, treatment of A253 cells with LY2109761 alone (in the absence of exogenous TGFβ) induced a more cohesive epithelioid morphology with distinct cell boundaries. Thus, the *TGFBR2*^{R537P} mutant seems to endow A253 with an EMT-like state that is selectively reversible by the dual TβR kinase inhibitor and seems to be dependent on constitutive activation of pSmad1 and pSmad5.

As shown in Fig. 3*B*, TGFβ treatment of HaCaT cells resulted in a 10-fold increase in the rate of cell migration, which could be inhibited by either SD-093 or LY2109761 (Fig. 3*B*). In contrast, SqCC/Y1 cells displayed very little cell migration, which was not affected by TGFβ nor by SD-093 or LY2109761 treatment (Fig. 3*B*). In contrast, A253 cells displayed a very high basal rate of cell migration independently of exogenous TGFβ (Fig. 3*B*). Moreover, whereas SD-093 had no effect on migration of A253 cells, LY2109761 inhibited migration by ~50% ($P < 0.001$). With respect to invasion into Matrigel matrix, SqCC/Y1 cells were completely noninvasive for periods up to 72 h (data not shown). In contrast, A253 cells were highly invasive (Fig. 3*C*). Moreover, A253 invasiveness was reduced by ~50% by LY2109761 treatment but not by SD-093 ($P < 0.01$; Fig. 3*C*). Therefore, it seems that TβRII^{R537P}-mutant cells have acquired novel properties, including constitutive EMT and high rates of migration and invasion that may contribute to the tumorigenicity of A253 *in vivo*. This phenotype is clearly distinct from that of the SqCC/Y1 *TGFBR2*^{E526Q}-mutant cells, which seem to have lost all responses to TGFβ. Moreover, the unique *in vitro* properties of A253 cells were also retained *in vivo*. As shown in Fig. 3*D*, A253 cells gave rise to poorly differentiated invasive squamous cell carcinomas *in vivo*. Consistent with the *in vitro* studies, the levels of pSmad2 expressed by the tumor cells were almost undetectable. On the other hand, the same tumor cells showed strong nuclear staining for pSmad1 and/or pSmad5, on a par with that seen in endothelial cells of the tumor-associated capillaries as well as basal and suprabasal keratinocytes in the epidermis. Interestingly, basal and suprabasal keratinocytes in the epidermis displayed the strongest pSmad1 and/or pSmad5 immunostaining, whereas cells in the stratum granulare and stratum corneum expressed pSmad2, suggesting that the two arms of TGFβ signaling might be activated at different stages of keratinocyte differentiation *in vivo*.

Our results suggest that TGFβ-induced BMP-Smad activation in keratinocytes involves partnering of the TβRII receptor with and activation of one of the BMP pathway type I receptors. As shown in Fig. 4, none of the keratinocyte cell lines (HaCaT, A253, and SqCC/Y1) expressed Alk-1 mRNA, whereas Alk-2, Alk-3, Alk-5, and Alk-6 mRNAs were all expressed in each of the three cell lines (Fig. 4). Although Alk-3 and Alk-6 receptors can activate BMP-Smads, they have not been tied to TGFβ signaling. On the other hand, as Alk-2 has been previously shown to act as a receptor for TGFβ, it is the leading candidate type I receptor to mediate the activation of BMP-Smads in HaCaT and A253 cells.

Finally, we examined the effects of *TGFBR2*^{R537P} on coexpressed wild-type TβRII signaling. First, we compared the phenotype of FaDu × A253 somatic cell hybrid cells derived from the esophageal carcinoma line FaDu and A253 (15) with that of the two parental cell lines. FaDu cells are null for the *MADH4* gene but express wild-type *TGFBR2* (5, 7), whereas A253 is homozygous for the *TGFBR2*^{R537P} mutation. As shown in Fig. 5*A*, treatment of FaDu cells with TGFβ resulted in activation of Smad2 and Smad3, which was inhibitable by SD-093, whereas A253 cells constitutively expressed pSmad1 and pSmad5 but failed to activate Smad2 or Smad3 in response to TGFβ. In contrast, treatment of FaDu × A253 hybrid cells with TGFβ resulted in activation of both

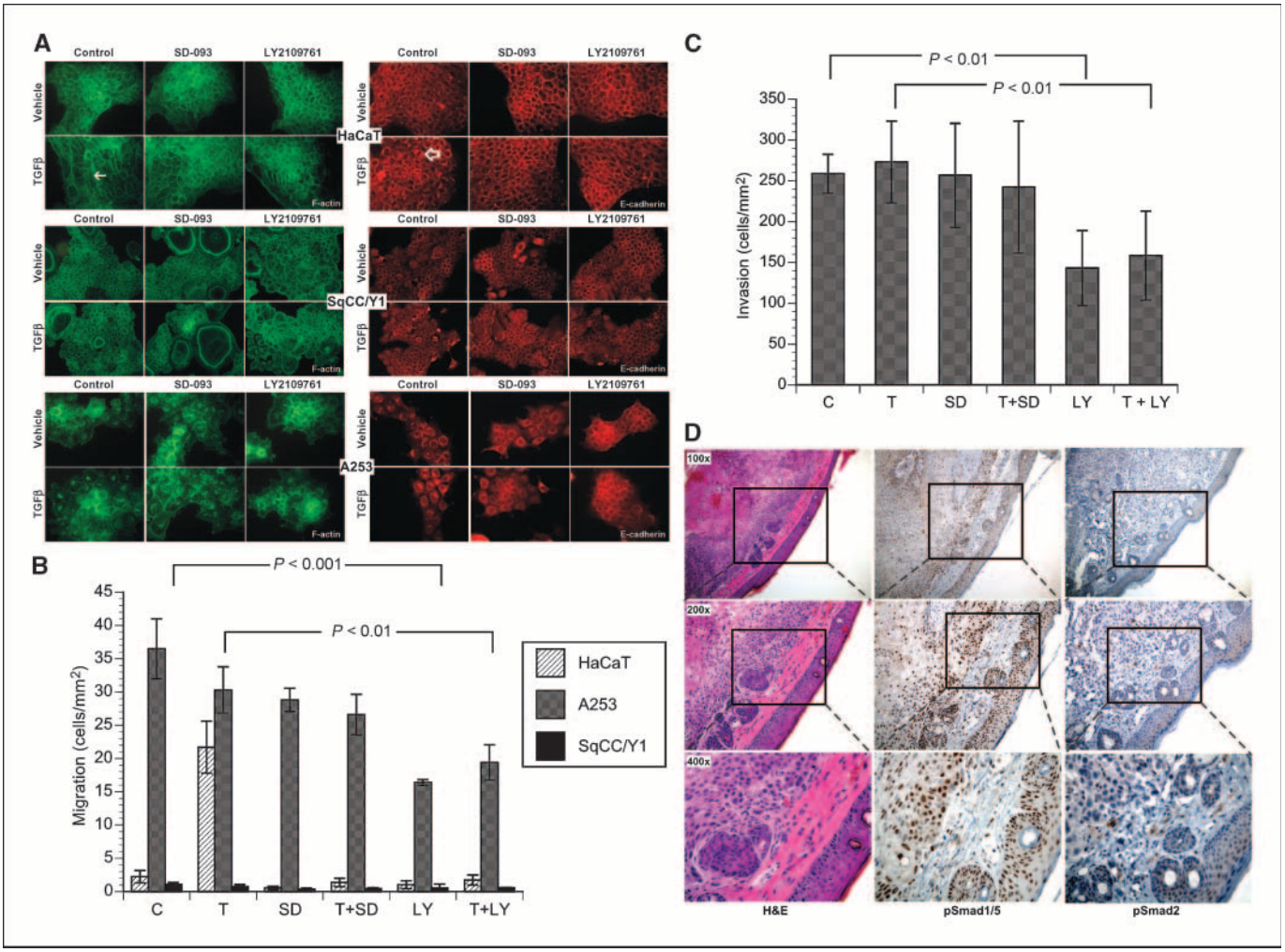

Figure 3. Effects of TβR-kinase inhibitors on cellular responses. *A,* treatment of HaCaT cells with TGFβ (100 pmol/L) for 72 h induced EMT associated with subcellular redistribution of F-actin from a submembranous location to incorporation into stress fibers (→). TGF treatment also resulted in redistribution of E-cadherin from a membranous to cytoplasmic and cytoplasmic and perinuclear localization (⇨). TGFβ-induced actin reorganization as well as E-cadherin redistribution were inhibited by SD-093 (1 μmol/L) or LY2109761 (2 μmol/L) pretreatment. Moreover, drug treatments alone seemed to induce a stronger peripheral F-actin and predominantly membrane-associated E-cadherin staining pattern compared with vehicle-treated (DMSO) control cells. SqCC/Y1 cells showed membrane-associated F-actin and E-cadherin staining. These cells had a strongly epithelial morphology, which was unaffected by treatment with either TGFβ or any of the TβR inhibitors. In contrast, A253 cells displayed diffuse cytoplasmic F-actin staining, whereas E-cadherin was predominantly perinuclear. These cells therefore seemed to be in a state of EMT, which was unaffected by either TGFβ or SD-093 treatment. However, treatment with LY2109761 resulted in redistribution of F-actin to the cell periphery and of E-cadherin to the membrane and restoration of an epithelioid morphology. *B, in vitro* migration assays were performed as described in Materials and Methods. In HaCaT cells, treatment with TGFβ (100 pmol/L) for 24 h induced a 10-fold increase in the rate of cell migration. This increase was completely inhibited by pretreating cells with either SD-093 (1 μmol/L) or LY2109761 (2 μmol/L). SqCC/Y1 cells had a very low basal rate of cell migration, which was unaffected by TGFβ or with either of the inhibitors (SD-093 or LY2109761). In contrast, A253 cells migrated at a rate almost 20 times higher than that of HaCaT cells. Cell migration was not affected by either TGFβ or SD-093 treatment. In contrast, treatment with LY2109761 reduced migration by ~50% independently of the presence of exogenous TGFβ ($P < 0.01$, Student's *t* test). *C,* vehicle control; *T,* TGFβ; *SD,* SD-093; *LY,* LY2109761. *C, in vitro* invasion assays were performed as described in Materials and Methods. A253 cells were constitutively highly invasive. Neither treatment with TGFβ (100 pmol/L) nor treatment with SD-093 (1 μmol/L) had any effect on A253 invasiveness. However, LY2109761 (1 μmol/L) inhibited invasion by ~50% independently of the presence of TGFβ ($P < 0.01$, Student's *t* test). This effect of LY2109761 was not due to growth arrest or cell death due to drug toxicity because treatment with this agent did not affect total A253 cell numbers (data not shown). *D,* 10^6 A253 cells were injected s.c. into 8- to 9-wk-old athymic nude mice and allowed to give rise to palpable tumors. Sections from formaldehyde-fixed, paraffin-embedded, A253-derived tumors were stained by H&E as well as for pSmad2 and pSmad1/5. Magnification, ×200. A253 cells gave rise to poorly differentiated invasive squamous cell carcinomas. pSmad2 expressed by the tumor cells was almost undetectable and certainly significantly lower than the amounts expressed by normal cells as illustrated by differentiated keratinocytes in the overlying epidermis. On the other hand, the same tumor cells showed strong nuclear staining for pSmad1 and/or pSmad5, similar to that seen in endothelial cells of the tumor-associated capillaries. Interestingly, basal and suprabasal keratinocytes in the epidermis displayed the strongest pSmad1 and/or pSmad5 immunostaining, whereas pSmad2 was predominantly expressed in the stratum granulare and stratum corneum, suggesting the possibility that the two arms of TGFβ signaling are activated at different stages of keratinocyte differentiation *in vivo*.

Smad2 and Smad3 (Fig. 5*A*). Most importantly, SD-093 treatment inhibited this induction, suggesting that the presence of wild-type TβRII restored TGFβ-mediated R-Smad phosphorylation in a TβRI (Alk-5)-dependent mechanism. Moreover, the levels of pSmad1 and pSmad5 seemed to be somewhat lower in FaDu × A253 than in parental A253 cells (Fig. 5*A*). As shown previously, the high levels of activated pSmad1 and pSmad5 in A253 cells were not affected by SD-093 treatment (Fig. 5*B*). In contrast, in the FaDu × A253 hybrid cells, TGFβ-activated Smad2 and Smad3 underwent rapid dephosphorylation on SD-093 treatment, with kinetics similar to those observed in HaCaT (Fig. 5*B*). In aggregate, these results indicate that the *TGFBR2*^{R537P} mutant is recessive over the wild-type receptor and that the associated activated cell phenotype only becomes manifest in cells that are either

homozygous for the mutant receptor or have evidence of *TGFBR2* loss of heterozygosity.

To further characterize the function of the *TGFBR2* mutants in the context of wild-type receptor expression, wild-type and mutant TβRII were coexpressed in the *TGFBR2*-null mammary cancer line T47D (10). Cotransfection of wild-type *TGFBR2* and *TGFBR2*^{E526Q} induced 3TP-lux reporter activity in direct proportion to the amount of wild-type *TGFBR2* cDNA (Fig. 5*C, top left*). Thus, the *TGFBR2*^{E526Q} mutant confers a null phenotype and has no dominant-negative properties. Similarly, expression of the *TGFBR2*^{R537P} mutant by itself failed to induce 3TP-lux reporter activity. However, in the presence of both wild-type and *TGFBR2*^{R537P}-mutant receptor, the net reporter gene activity was approximately equal to that obtained with 100% wild-type receptor. Moreover, treatment with either of the two inhibitors, SD-093 or LY2109761, resulted in inhibiting 3TP-lux activity (data not shown). Essentially similar results were obtained with the SBE4-luc reporter gene construct (Fig. 5*C, top right*). On the other hand, when we used the Col7A1-luc reporter, the combination of wild-type and *TGFBR2*^{R537P}-mutant receptor seemed to have an intermediate activity, suggesting that the mutant was not contributing to Col7A1-luc induction (Fig. 5*C, bottom left*). When we examined the effects of the two *TGFBR2* mutants on the TGFβ-repressed Del-5-*myc*-luc reporter gene construct, coexpression of wild-type TβRII and either of the two mutants resulted in a dose-dependent decrease in Del-5-*myc*-luc activity, which was proportional to the amount of wild-type receptor transfected (Fig. 5*C, bottom right*). However, in the sole presence of the *TGFBR2*^{R537P} mutant, the level of Del-5-*myc*-luc activity seemed to be somewhat higher than in the presence of the inactive *TGFBR2*^{E526Q} mutant, suggesting that the *TGFBR2*^{R537P} mutant has modest dominant-negative activity in this case. Total levels of TβRII receptor expression were similar across all conditions (Fig. 5*D*). In aggregate, these results indicate that the TβRII^{E526Q} mutant is a true loss-of-function mutant that has no dominant-negative effect on coexpressed wild-type receptor. The TβRII^{R537P} mutant is equally incapable of activating classic TGFβ target genes when it is the sole receptor gene expressed (as is the case in A253 cells). On the other hand, in the presence of wild-type receptor, the TβRII^{R537P} mutant seems to have retained its capability to activate some target genes (3TP-lux and SBE4-luc), whereas it is inactive with respect to Col7A1-luc and modestly dominant negative with regard to Del-5-*myc*-luc. These results suggests that, in cells that are homozygous for this mutant, the classic TGFβ-regulated gene expression program is eliminated, whereas in the heterozygous situation the TGFβ-regulated gene expression program is likely to be qualitatively altered, giving rise to complex changes in the cellular phenotype.

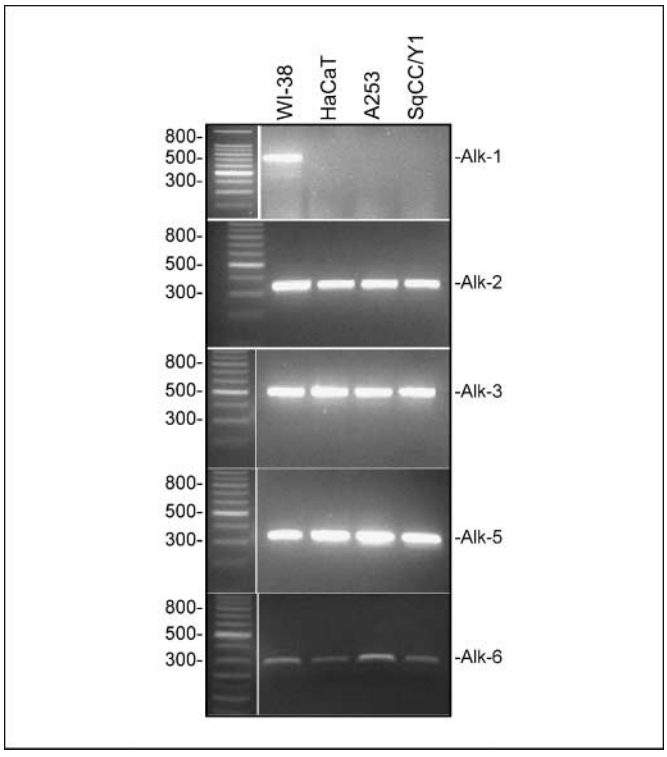

Figure 4. Detection of Alk mRNA expression by RT-PCR. Levels of mRNA expression for each of the Alk receptors were determined by RT-PCR as described in Materials and Methods. *Alk-2, Alk-3, Alk-5*, and *Alk-6* genes were expressed in each of the cell lines to a similar extent. Alk-1 was not detected in any of the keratinocyte cell lines (WI-38 cells are human fibroblasts included as positive control for Alk-1 mRNA expression).

Discussion

Our first important finding is that, besides the canonical TGFβ-Smads Smad2 and Smad3, TGFβ also activates the BMP-Smads Smad1 and Smad5 in HaCaT human keratinocytes (Fig. 6). This ability of TGFβ to cross-activate BMP-Smads has also been observed in several other cell types, including human endothelial cells (16), rat intestinal epithelial cells (17), mouse mammary epithelial cells (18), and human mammary carcinoma cells (19). As in endothelial cells, TGFβ-induced BMP-Smad activation seems to be dependent on Alk-5 kinase activity, as it was inhibitable by several different Alk-5 chemical inhibitors. Although these inhibitors also target the activin receptors Alk-4 and Alk-7, the fact that activin A was not able to activate BMP-Smads practically excludes the involvement of Alk-4 or Alk-7 in BMP-Smad activation. In endothelial cells, TβRII mediates TGFβ-dependent Smad2 and Smad3 activation by partnering with Alk-5 (TβRI) and Smad1 and Smad5 activation by partnering with Alk-1 (20). This is clearly not the case in HaCaT, as these cells do not express Alk-1 mRNA (Fig. 4). Based on their ability to mediate BMP-Smad activation and mRNA expression in keratinocytes, other candidates include Alk-2, Alk-3, and Alk-6. Originally identified as a type I receptor partner for TβRII, Alk-2 (initially named Tsk7L; ref. 21) usually functions as a BMP type I receptor (22). However, Alk-1 and Alk-2 are most similar among the type I receptors. Moreover, Alk-2 is able to weakly bind TGFβ when overexpressed in COS cells, as long as the TβRII receptor is coexpressed, a characteristic of TβRI (21, 23, 24). Although Alk-2 is able to complex with TβRII independently of TGFβ, this requires the kinase activity of TβRII (25). Moreover, TGFβ is able to activate BMP-Smads through Alk-2 in other types of untransformed epithelial cells (16–18). Thus, Alk-2 is the most likely candidate to partner with TβRII and activate Smad1/5 in keratinocytes. Studies to confirm this hypothesis are in progress.

The ability of the TβRII receptor to signal through two distinct type I receptors may be explained by several possible models: TβRII may form heterodimeric complexes with either Alk-5 or Alk-x, with the two types of complexes being in some sort of equilibrium. Alternatively, the three proteins may form heteromeric complexes that include both Alk-5 and Alk-x. In endothelial cells, Goumans et al. (20) reported that Alk-1 and Alk-5 form heteromeric complexes, most strongly in the presence of TGFβ. In addition, efficient heteromeric complex formation between Alk-1 and Alk-5 required

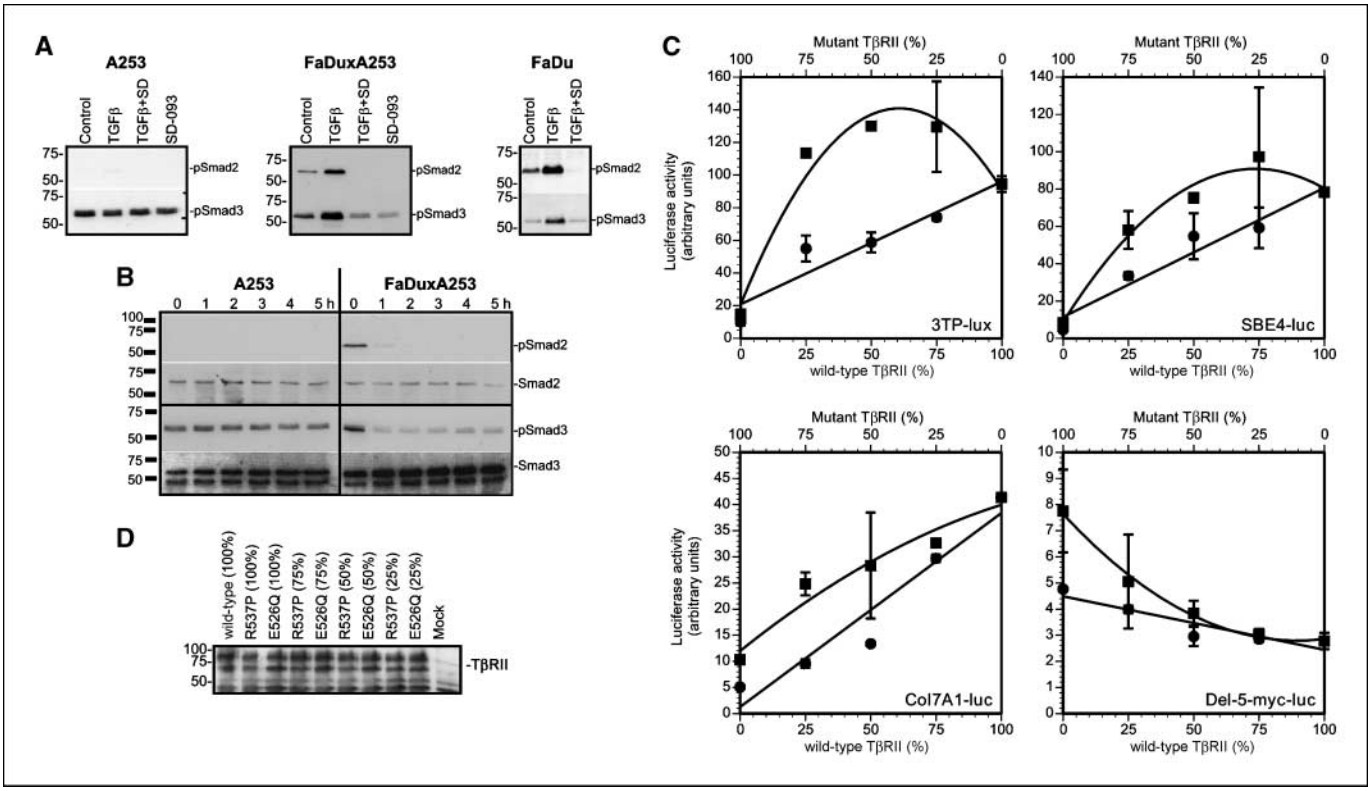

Figure 5. Effects of coexpression of wild-type and mutant TβRII gene. *A,* activation and dephosphorylation of Smads in FaDu × A253 somatic hybrid cells. Treatment of FaDu × A253 with TGFβ (100 pmol/L) for 1 h induced phosphorylation of Smad2 and pSmad3 but not of Smad1 and Smad5. This phenotype was similar to that of parental FaDu cells that express wild-type TβRII. In both cell lines, TGFβ-induced Smad2 and Smad3 phosphorylation was inhibitable by the TβRI kinase inhibitor SD-093. In contrast, TGFβ failed to induce phosphorylation of Smad2 and Smad3 in parental A253 cells that express the *TGFBR2*^{R537P} mutant. *B,* SD-093 dephosphorylation assays were performed as described in Materials and Methods. In FaDu × A253 cells, TGFβ-activated pSmad2 and pSmad3 underwent rapid dephosphorylation in response to SD-093 treatment with a $t_{1/2}$ of 45 min. In contrast, parental A253 cells expressed high levels of pSmad1 and pSmad5, which were unaffected by SD-093. *Top right,* FaDu × A253 cells expressed much lower reduced levels of pSmad1 and pSmad5 than A253 cells. *C,* wild-type and mutant TβRII cDNAs were coexpressed in TβRII-null T47D cells. *Top left,* transfection of E526Q (●) or R537P (■) alone had no effect on 3TP-lux reporter gene activity. When wild-type TβRII was coexpressed with the E526Q mutant, the level of 3TP-lux activity increased in linear proportion with the amount of wild-type TβRII. Thus, the E526Q has no intrinsic activity nor does it act in a dominant-negative fashion on wild-type receptor. In contrast, when wild-type TβRII was coexpressed with the R537P mutant, 3TP-lux activity was similar to that induced by wild-type receptor alone. Thus, although the R537P mutant has no ability to induce 3TP-lux activity on its own, it seems to complement the ability of wild-type receptor to activate this target gene. 3TP-lux activation was inhibited in the presence of either SD-093 or LY2109761. Transfection of E526Q or R537P alone had no effect on SBE4-luc (*top right*) or Col7A1-luc (*bottom left*) activity. When wild-type TβRII was coexpressed with the E526Q mutant, the levels of SBE4-luc (and Col7A1-luc) activity increased in linear proportion with the amount of wild-type TβRII. Thus, the E526Q mutant has no intrinsic activity nor does it act in a dominant-negative fashion on wild-type receptor. In contrast, when wild-type TβRII was coexpressed with the R537P mutant, SBE4-luc (and Col7A1-luc) activity was close to that expected from wild-type receptor alone. Thus, although the R537P mutant has no apparent ability to induce SBE4-luc (and Col7A1-luc) activity by itself, it complements the ability of wild-type receptor to activate this target gene. *Bottom right,* expression of either E526Q or R537P was associated with higher Del-5-*myc*-luc activity than wild-type TβRII. When wild-type TβRII was coexpressed with the E526Q or R537P mutant, the level of Del-5-*myc*-luc activity decreased in linear proportion with the amount of wild-type TβRII. Thus, E526Q and R537P mutants seemed to have lost the ability to induce repression of c-*myc*, which is reversed with the wild-type receptor. *D,* T47D cells cotransfected with wild-type and mutant TβRII cDNAs were subjected to Western blot analysis using antibody specific to TβRII protein. Total TβRII protein was expressed in equivalent amounts in all transfection conditions. Mock-transfected TβRII-null T47D cells were included as negative controls.

the presence of TβRII within the complexes. In endothelial cells, active Alk-5 is required to be present within heteromeric complexes for Alk-1 activation to take place. This is consistent with our finding in HaCaT cells that BMP-Smad activation is dependent on TβRI (Alk-5) kinase activity. In the case of endothelial cells, Goumans et al. (20) found no evidence for direct phosphorylation of Alk-1 by the Alk-5 kinase, but this remains to be shown in HaCaT cells. Besides Alk-5, TβRII is also required for BMP-Smad activation by Alk-1 in endothelial cells, as this response to TGFβ is lost in TβRII-null cells. This is also consistent with our finding that treatment with the dual TβRII/Alk-5 inhibitor LY2109761 abrogated BMP-Smad activation not only in HaCaT cells but also in A253 cells, in which this activation is no longer dependent on Alk-5.

In vitro, TGFβ seems to be able to simultaneously induce pSmad2/3 and pSmad1/5 in keratinocytes (Fig. 1*A*). Of note, immunostaining of normal epidermis (Fig. 3*D*) revealed that pSmad1/5 is predominantly expressed in basal and suprabasal keratinocytes, whereas pSmad2 is expressed in more differentiated cell layers, suggesting the possibility that each of the two TGFβ signaling arms may be activated at different stages of keratinocyte differentiation *in vivo*. In contrast, in the A253 *TGFBR2*^{R537P}-mutant cells, TGFβ no longer induces phosphorylation of Smad2 or Smad3, whereas Smad1 and Smad5 seem to be constitutively activated. These cells are refractory to TGFβ-induced growth arrest, although they seem to be in a permanent EMT-like state, highly motile, and invasive and to express high levels of the TGFβ target proteins, plasminogen activator inhibitor-1 (PAI-1) and fibronectin, independently of treatment with TGFβ (26, 27). Moreover, treatment of A253 cells with the dual TβR kinase inhibitor LY2109761 resulted in dephosphorylation of pSmad1/5, induction of an epithelioid phenotype, and inhibition of cell migration. In aggregate, these findings strongly suggest that, in

keratinocytes, TGFβ/Alk-5–dependent activation of Smad2 and Smad3 results in growth arrest, whereas the TGFβ-induced response to tissue injury, including EMT and cell migration, is mediated by activation of pSmad1 and pSmad5. If this hypothesis is correct, one might speculate that the uncoupling of these two responses commonly seen in epithelial carcinomas might result from inactivation of the Smad2/3 pathway on the one hand with concurrent activation of the Smad1/5 pathway on the other.

Our second major finding is that cancer-associated mutations of the TβRII gene can be associated with at least two distinct phenotypes (Fig. 6). The first is represented by the *TGFBR2*^{E526Q} mutant found in SqCC/Y1 cells. We had previously shown that this mutation abrogates TβRII kinase activity (5, 27). In this case, all downstream responses to TGFβ are eliminated, including phosphorylation of Smads, target gene regulation, fibronectin and PAI-1 production, growth arrest, EMT, and cell migration (26, 27). This phenotype is displayed by SqCC/Y1 cells, which are homozygous for the *TGFBR2*^{E526Q} mutation, as well as by *TGFBR2*-null cells in which the mutant receptor is expressed by itself (5, 26, 27). On the other hand, in cells in which the *TGFBR2*^{E526Q} mutant is coexpressed with wild-type *TGFBR2*, the cellular response to TGFβ seems to be dictated by the wild-type receptor. Thus, transient cotransfection experiments using TβRII-null recipient cells showed that TGFβ target reporter gene activation was proportional to the amount of wild-type receptor expressed (Fig. 5). Thus, the *TGFBR2*^{E526Q} mutant has a true loss-of-function phenotype that does not affect the function of coexpressed wild-type receptor in a dominant-negative manner. As cells that are heterozygous for mutants of this type display attenuated TGFβ signaling, germ-line mutations of this type may be associated with increased cancer susceptibility, similarly to TGFβ1 heterozygous mice (28, 29) and dominant-negative TβRII transgenic mice (30).

The second type of TβRII mutant is exemplified by the *TGFBR2*^{R537P} mutation found in A253 cells (Fig. 6). We had previously shown that this mutation is associated with a constitutively high intrinsic TβRII kinase activity and production of PAI-1 and fibronectin (5, 26, 27). In cells in which this mutant receptor is expressed by itself, TGFβ target gene regulation is largely eliminated (see Fig. 5 and refs. 26, 27). Consistent with these findings, A253 cells that are homozygous for the *TGFBR2*^{R537P} mutation have lost TGFβ-dependent Smad2/3 activation. However, in contrast to *TGFBR2*^{E526Q}-mutant cells, A253 cells display constitutive activation of the BMP-Smad1/5 pathway, which is independent not only of exogenous TGFβ but also of TβRI (Alk-5) kinase activity, as the Alk-5 kinase inhibitor SD-093 failed to induce dephosphorylation of these Smads. On the other hand, BMP-Smad activation was clearly dependent on TβRII receptor kinase activity, as it could be abrogated using the dual TβR kinase inhibitor LY2109761. It is theoretically possible that TGFβ-independent BMP-Smad activation in A253 cells is induced by BMP. However, the fact that

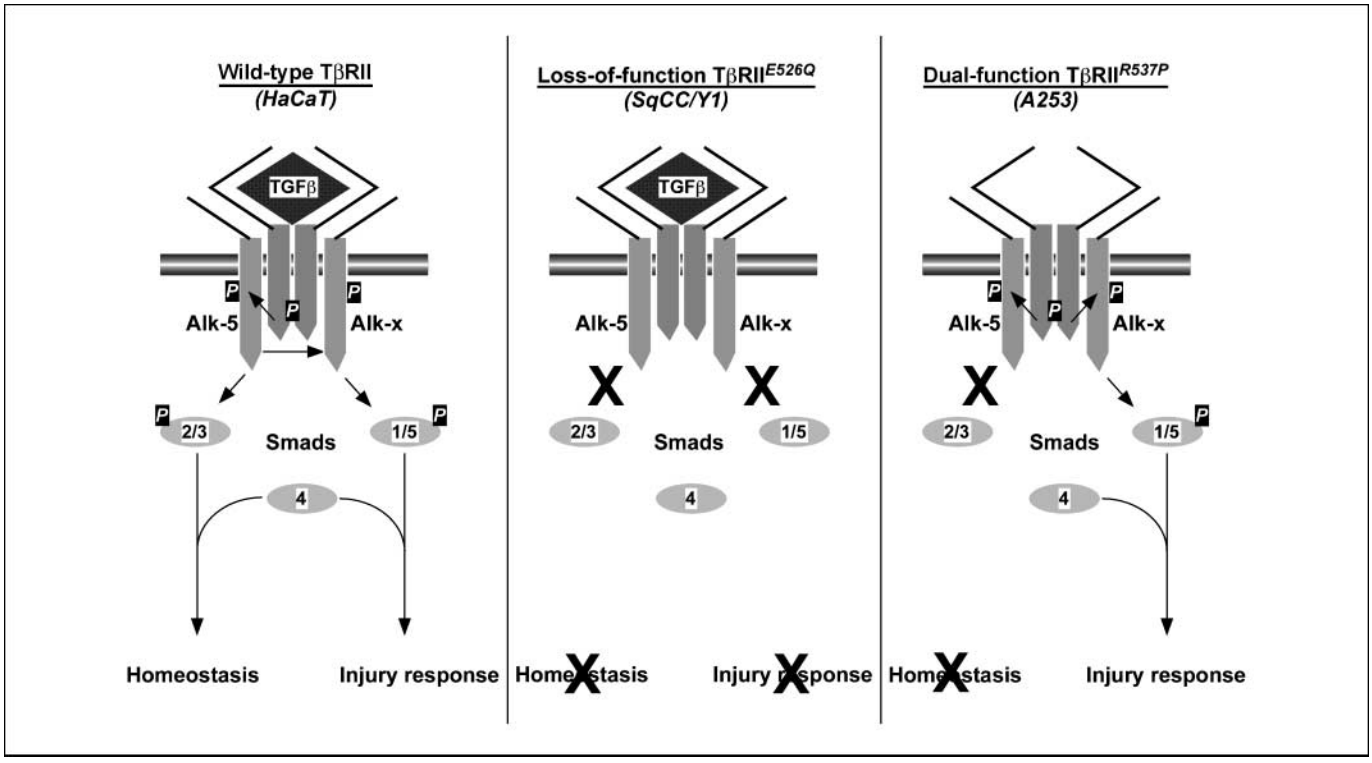

Figure 6. Proposed model of TβR signaling pathway in normal human keratinocytes and squamous carcinoma cells. In normal human keratinocytes (exemplified by HaCaT cells), TGFβ binds to the TβRII and activates the classic Smad2/3 pathway via TβRI (Alk-5). In addition, our studies indicate that TGFβ can activate BMP-Smads (Smad1 and Smad5) in a TβRI (Alk-5)-dependent manner. In analogy with endothelial cells, we assume that one of the BMP type I receptors is involved in this process (Alk-x), with Alk-2 being the leading candidate. Moreover, one might speculate that the Smad2/3 arm of the signaling pathway mediates the homeostatic function of TGFβ, whereas the Smad1/5 arm is involved in the tissue injury response. In the case of some cancer-associated TβRII mutants (exemplified by TβRII^{E526Q} in SqCC/Y1 cells), both arms of the TGFβ/Smad pathway are abrogated. In contrast, in the case of TβRII^{R537P}-mutant A253 cells, the TβRII/Alk-5/Smad2/3 pathway is also abrogated, resulting in loss of TGFβ-mediated growth control. However, the Smad1/5 pathway is constitutively activated in an Alk-5–independent manner, apparently resulting in increased EMT, cell migration, and invasion. Thus, this type of mutant has a dual-function phenotype, associated with loss of one arm of the TGFβ/Smad pathway and activation of the second arm. Moreover, our studies indicate that this gain of function is inhibitable by the dual-receptor kinase inhibitor LY2109761, while it is resistant to selective TβRI kinase inhibitors.

LY2109761 is capable of abrogating the BMP-Smad activation essentially rules this out, as this compound belongs to the series of pyrazole Alk-4, Alk-5, and Alk-7 inhibitors described by Peng et al. (13). These compounds are incapable of inhibiting BMP4-induced xVent2-luciferase activity or reversing BMP4-mediated growth inhibition at concentrations up to 20 μmol/L (13). In fact, these compounds even potentiate the xVent2-lux BMP4 response in mammary epithelial cells at concentrations as low as 0.25 μmol/L.

Although we have not yet elucidated the biochemical mechanisms underlying the *TGFBR2*^{R537P}-mutant cell phenotype, lessons learned from other *TGFBR2* mutants suggest several reasonable possibilities. For example, Chen et al. (31) reported that, in cells overexpressing kinase-inactive truncated TβRII receptors, TGFβ-mediated induction of growth inhibition was abrogated, whereas the induction of fibronectin, PAI-1, and JunB was retained. Recently, Goumans et al. (20) showed that a COOH-terminally truncated TβRII construct dominant negatively inhibited constitutively active Alk-5–dependent activation of the Alk-1/Smad1/5 pathway in primary endothelial cells. In addition, expressing a COOH-terminally truncated TβRII mutant in Alk-5–null cells resulted in TGFβ binding to Alk-1 even in the absence of Alk-5. This shows that a structural mutation of TβRII can dramatically alter its affinity for Alk partners. Thus, perhaps the most likely model is that disease-associated mutations in either the *TGFBR1* or *TGFBR2* receptor genes can affect protein-protein interactions and, specifically, obviate the requirement for Alk-5 in the formation of TβRII/Alk-x complexes and Alk-x activation. Alternatively, mutations might disrupt the interaction of TβRII with Alk-5 while favoring its interaction with alternative Alk-x, somehow resulting in the preferential activation of the Alk-x/Smad1-Smad5 pathway. Whichever mechanism is responsible for the Alk-5–independent and ligand-independent activation of Smad1 and Smad5 in A253 cells is likely to also account for the inability of TGFβ to activate Smad2 and Smad3 in these cells. The fact that activin treatment of A253 cells resulted in the expected phosphorylation of Smad2 and Smad3 shows that both Smads are expressed and able to be appropriately phosphorylated by receptor kinases. Thus, the fact that Smad2 and Smad3 are no longer activated suggests that Alk-5 no longer participates in the TGFβ/TβRII/Alk-5 receptor complex or is no longer activated by the TβRII^{R537P} mutant. We are currently investigating this possibility. Moreover, in endothelial cells, Alk-1 not only induces cellular responses opposite to those of Alk-5 but also directly antagonizes Alk-5–induced transcriptional responses (20, 32). These findings suggest that, in a situation in which the Alk-x/Smad1/5 pathway is selectively hyperactivated (as is the case in A253 cells), this negative cross-talk could further contribute to shutting down signaling through the Alk-5/Smad2/3 pathway.

In contrast to cells that are homozygous for the *TGFBR2*^{R537P} mutation (as is the case in A253), in cells in which the R537P mutant is coexpressed in conjunction with wild-type TβRII, responses to TGFβ seem to be dictated by the wild-type receptor. Thus, in FaDu × A253 hybrid cells (15), the TGFβ-mediated activation of Alk-5/Smad2/3 and dephosphorylation in response to SD-093 seem to have been restored, and the cells no longer express constitutively high levels of pSmad1 and pSmad5. In addition, the cellular responses of FaDu × A253 cells to TGFβ were similar to those of HaCaT keratinocytes (15). Similarly, transient cotransfection experiments of *TGFBR2*^{R537P} mutant and wild-type *TGFBR2* into *TGFBR2*-null recipient cells showed that TGFβ-specific target reporter gene activation was similar to that seen with wild-type receptor alone (Fig. 5). Thus, the *TGFBR2*^{R537P} mutant does not seem to affect the function of coexpressed wild-type receptor in a dominant-negative or dominant-active manner. However, the question whether pSmad1/5-dependent transcripts are upregulated under these conditions is still under investigation.

In cancer, mutations of the *TGFBR2* gene seem to be homozygous; that is, the cancers have undergone loss of the wild-type allele or both *TGFBR2* alleles have undergone missense mutations (3, 33). For the *TGFBR2*^{E526Q} type of mutation, the predominant selective advantage would be loss of TGFβ-mediated growth arrest. Even the putative intermediate heterozygous state would likely be associated with an overall attenuation of TGFβ signal and thus confer a growth advantage. In the homozygous state, the *TGFBR2*^{R537P} type of mutant provides two selective advantages: loss of homeostatic growth control coupled with constitutive EMT, high motility, and high invasion. In this case, our transfection assays suggest that the heterozygous state may not be associated with an altered phenotype. On the other hand, Lu et al. (34, 35) have described a germ-line mutation of the *TGFBR2* gene (T315M) in a family with hereditary nonpolyposis colorectal cancer that is incapable of mediating growth inhibition by TGFβ but has retained the ability to induce PAI-1 in response to TGFβ treatment. Thus, this mutant seems to have a dual phenotype similar to that of the R537P mutant, indicating that heterozygous carriers of this type of mutant may also have increased cancer susceptibility. These studies illustrate that further characterization of individual mutants, in terms of their profiles of Smad activation and a search for alternative signaling pathways, will be key in devising targeted therapeutic strategies for cancer and other diseases that are driven by mutations in TβR genes.

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