Smad4/DPC4 silencing and hyperactive Ras jointly disrupt transforming growth factor-β antiproliferative responses in colon cancer cells*

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Smad4/DPC4 is a tumor suppressor gene frequently mutated or deleted in pancreatic and metastatic colon cancers. Smad4 acts as a cofactor that binds transforming growth factor-β (TGF-β) receptor-activated Smad2 and Smad3 and generates transcriptional complexes. Using SW480.7 colon carcinoma cells, defective in Smad4 function, we have investigated whether this loss plays a role in the resistance of colon cancer cells to the antiproliferative effects of TGF-β. SW480.7 cells contain only one Smad4 allele, which we found encodes a wild type protein that is not expressed. We generated SW480.7 cells conditionally expressing Smad4 via an edcsosynase-inducible system. Smad4 expression in these cells failed to rescue TGF-β antiproliferative and gene responses (e.g., down-regulation and induction of p21/Cip1 and plasminogen activator inhibitor-1). SW480.7 cells contain an activated Ki-ras oncogene. Hyperactivation of Ras can inhibit Smad nuclear accumulation by their phosphorylation at mitogen-activated protein kinase sites. Cotransfection into SW480.7 cells of Smad4 together with a Ras phosphorylation-resistant Smad3 (but not with wild type Smad2, Smad3, adenomatous polyposis coli (APC), or TGF-β type II receptor) restored the TGF-β antiproliferative response. These results suggest that loss of Smad4 function by both deletion and silencing and inhibition of Smad2/3 function by a hyperactive Ras pathway jointly prevent TGF-β antiproliferative responses in SW480.7 colon cancer cells.

The molecular events leading to colon cancer involve the sequential mutation of specific genes that transform a normal colonic epithelium to an adenomatous polypl and, ultimately, to an invasive cancer. Inactivating mutations in APC (adenomatous polyposis coli) and activating mutations in Ki-ras occur generally early in colon cancer, whereas p53 inactivation occurs generally late, as the adenoma progresses into a carcinoma (1). Another relatively late event is loss of responsiveness to TGF-β (2, 3). Under normal conditions, TGF-β is a potent antimitogen in epithelial, including intestinal, cells (4, 5). Loss of TGF-β antiproliferative responses in colon carcinoma cells may compromise the turnover of the colonic epithelium, thus favoring tumor formation. This loss of responsiveness can result from inactivating mutations in TGF-β receptor genes. The most prominent example of this is the loss of TGF-β type II receptor function in the vast majority of colon cancers with microsatellite instability, which are a small subset (around 10%) of all colon cancers (6–8). In most other cases, however, loss of TGF-β responsiveness is due to post-receptor defects, the molecular nature of which is only now beginning to emerge.

Inactivating mutations in Smad4/DPC4 are the most important of the known TGF-β post-receptor defects in colon cancer. Following the initial identification of Smad4 mutations in half of all pancreatic carcinomas (9), Smad4 mutations were reported in colon cancer (10–12) and other gastrointestinal cancers (13). In colon cancer, the incidence of Smad4 mutations appears to increase with tumor progression. Although rare as an initiating event (12) and relatively infrequent (approximately 10% of cases) in colon adenomas and nonmetastatic carcinomas (14), Smad4 mutations have been reported in more than 30% of invasive metastatic carcinomas and in colon cancer metastases (15).

Although the importance of Smad4 as a tumor suppressor gene in colon cancer is becoming increasingly apparent, little is known about how the loss of Smad4 function contributes to tumor progression. Smad4 plays a central role in TGF-β signaling by serving as a common partner of other Smad proteins (16). TGF-β binding brings together two types of transmembrane serine kinases, the type I and type II receptors. In this complex, the type II receptor phosphorylates and activates the type I receptor, which in turn phosphorylates Smad2 or the highly related protein Smad3. Other TGF-β family members act in a similar fashion. Activin induces phosphorylation of Smad2 and Smad3, whereas bone morphogenetic proteins induce phosphorylation of the related Smad1, Smad5, and Smad8. Upon phosphorylation, these Smads associate with Smad4 and move into the nucleus where they assemble transcriptional complexes that activate specific sets of genes. Thus, Smad4 is a shared key component of these various signaling pathways.

Smad accumulation in the nucleus is a highly regulated process. In addition to the positive effect of the receptor-mediated phosphorylations, which occur at C-terminal sites (17, 18), extracellular signal-regulated kinase and mitogen-activated protein kinase-mediated phosphorylation at other sites, in response to activators of the Ras/mitogen-activated protein kinase pathway, inhibit Smad1, Smad2, and Smad3 accumulation in the nucleus (19, 20). In mammalian epithelial cells transformed by a ras oncogene, this inhibitory effect is suffi-
ciently pronounced to significantly limit the activity of the TGF-β/Smad pathway (20).

Because Smad4 is a central component of the TGF-β signaling pathway, it has been assumed that the absence of TGF-β antiproliferative responses in Smad4-defective colon cancer cells is due to the loss of Smad4 function. However, this hypothesis has not been directly tested, and the possibility that the loss of TGF-β signaling in Smad4-defective cancer cells may be caused by other oncogenic alterations remains open. We have investigated these questions using SW480.7 cells, a colon carcinoma cell line defective in Smad4 function (21–23). Here we show that restoration of Smad4 function into SW480.7 cells is necessary but not sufficient to rescue the TGF-β antiproliferative response. We provide evidence that, in addition to Smad4, the rescue of this response requires restoration of Smad3 function, which is suppressed by oncopgenic Ras. The surprising presence of two distinct Smad inhibitory hits in the same cell raises the possibility that Smad4 has other roles in the maintenance of colonic epithelial homeostasis besides mediating TGF-β antiproliferative responses.

EXPERIMENTAL PROCEDURES
Cell Lines—SW480.7, COS-1, HaCaT, and MDA468 cells were maintained in Dulbecco’s modified Eagle medium with 10% fetal bovine serum and antibiotics in an incubator at 37 °C and 5% CO2. SW480.7–Smad4 clones were maintained in selective conditions by adding 0.3 mg/ml Zeocin™ (Invitrogen) and 0.7 mg/ml G-418 (Sigma) to the medium.

Constructs—A cDNA encoding FLAG epitope-tagged human Smad4 was subcloned into the pN2 vector (Ecdysone-inducible system, Invitrogen) at HindIII and BamHI sites. pN2 firefly luciferase was constructed by polymerase chain reaction (PCR) amplifying the firefly luciferase gene and ligating it to pN2 (sites KpnI and BamHI). pC25 vector encoding human Smad2 and pCMV5 vectors encoding human Smad3, Smad3-EP5M, Smad4, and Fast2 have been described (16, 20, 24).

Duplex PCR, Reverse Transcriptase-PCR, and Smad4 Sequencing—For the duplex PCR, total DNA was extracted from MDA468, SW480.7, and HaCaT cells with TRI™ reagent (Sigma). 50 ng of DNA were used for the duplex PCR, Reverse Transcriptase-PCR, and Smad4 Sequencing. The SW480.7–Smad4 clones were maintained in selective conditions by adding 0.3 mg/ml Zeocin™ (Invitrogen) and 0.7 mg/ml G-418 (Sigma) to the medium.

The PCR conditions were 95 °C for 2 min and 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 74 °C for 4 min. The amplification product was gel-purified with Qiaex II (Qiagen), cycle-sequenced with dye terminator chemistry, and analyzed on an Applied Biosystems 377 instrument. Sense and antisense primers were used to obtain a complete overlapping sequence of the Smad4 open reading frame.

Cell Lines with Conditional Smad4 Expression—SW480.7 cells were stably transfected with the pVGKRX vector (Ecdysone-inducible system, Invitrogen), and the resulting clones were assessed by transiently transfecting them with a pLND-luciferase construct and determining fold induction of luciferase after treatment with PA (Invitrogen). The expression level of this reaction was run on a denaturing agarose gel and transferred to a nylon membrane (Nytran® plus, Schleicher & Schuell) in 20 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate). Prehybridization and hybridization were carried out in 50% formamide, 5 × SSC, 5 × Denhardt’s solution, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA. The cDNA probes used contained the full open reading frame of the indicated genes and were labeled with [α-32P]CTP by random priming (Roche Molecular Biochemicals).

Western Blotting—Cells were lysed in TNE buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 0.5% Triton X-100) containing protease inhibitors. Lysates were separated by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) and transferred to Immobilon-P membranes (Millipore). The monoclonal anti-FLAG antibody (M2) was from Kodak Scientific. Affinity-purified rabbit polyclonal Smad2/3 antibodies have been previously described (20). Smad4 antibodies were raised in rabbits by immunization with a recombinant human Smad4 fragment (amino acids Gly-40 to Val-333) and were affinity-purified with immobilized Smad4 before use. Western immunoblotting was visualized by chemiluminescence (ECL, Amersham Pharmacia Biotech).

RESULTS
Smad4 Allele in SW480.7 Colon Cancer Cells—The SW480.7 human colon carcinoma cell line has been extensively used as a model system for in vitro studies because it contains some of the most characteristic colon cancer genetic alterations. These include activating mutations in Ki-ras, both a complete deletion and a stop codon mutation in APC, and inactivating mutations in both p53 alleles (21). Additionally, these cells have lost one copy of chromosome 18 (21). To address this issue, we analyzed the presence of Smad4 at the level of genomic DNA, mRNA, and protein. Using genomic DNA, we performed a duplex PCR reaction to simultaneously amplify exon 1 of Smad4 and, as an internal reaction control, a phosphoglycerate kinase intron. As additional controls we used genomic DNA from HaCaT, a human keratinocyte cell line that is highly responsive to TGF-β (27, 28), and MDA468, a human breast carcinoma cell line that lacks both copies of p53 (29) and is defective in TGF-β responses (16). A Smad4 amplification product was obtained from SW480.7 cells albeit at a lower yield than from HaCaT, whereas MDA468 failed to amplify Smad4 product (Fig. 1A). Thus, SW480.7 cells appeared to still retain one copy of Smad4 in the remaining chromosome 18. To determine the sequence and expression level of this remaining Smad4 allele, we amplified by reverse transcriptase-PCR from total RNA its entire open reading frame. This reaction yielded a PCR product of the correct molecular weight that
was, however, amplified to a much lower level from SW480.7 than from HaCaT cells (Fig. 1B). Both strands of this SW480.7 product were sequenced and found to correspond to wild type Smad4 (data not shown). This suggested that SW480.7 cells retained one wild type copy of Smad4 that was expressed at a very low level.

We confirmed this point by Northern blot analysis of SW480.7 RNA, which showed barely detectable levels of Smad4 transcript when compared with those found in HaCaT (Fig. 1C). A specific anti-Smad4 polyclonal antibody was raised (Fig. 2) and used in Western blot analysis to determine the level of Smad4 in these cells. Consistent with the mRNA results, these assays detected no Smad4 protein in SW480.7 lysates, whereas Smad4 was readily detected in HaCaT lysates (Fig. 2).

We also determined the expression levels of Smad2 and Smad3 in SW480.7 cells. Like Smad4, Smad2 is located in chromosome 16q21 (30), but unlike Smad4, the remaining Smad2 allele is abundantly expressed, as determined by Northern blot analysis (Fig. 1D). Western immunoblotting of cell lysates using antibodies that specifically recognize Smad2 and Smad3 (20) confirmed that Smad2 was expressed in SW480.7 cells at levels similar to those present in HaCaT cells. Smad3, located in chromosome 15q21–22 (31), was also expressed in SW480.7 cells, both at the mRNA (Fig. 1D) and protein levels (Fig. 2). Collectively these results indicate that SW480.7 cells retain one wild type copy of Smad4 but express it at a very low level owing to a silencing mechanism that does not affect the neighboring Smad2 gene.

SW480.7 Cells Conditionally Expressing Smad4—Next we investigated whether the absence of antiproliferative TGF-β responses in SW480.7 cells was due to the absence of Smad4 function. We reasoned that, if this was the case, transient transfection of Smad4 might cause basal, TGF-β-independent growth inhibition owing to overexpression of the protein. Moreover, stable transfection might select for clones resistant to Smad4 growth inhibitory function due to defects downstream of Smad4. To avoid these potential problems, we decided to generate stable SW480.7 transfectants conditionally expressing Smad4. To this end, we tested in these cells the inducibility of luciferase reporters under the negative control of a tetracycline trans-activator system (32) or the positive control of an ecdysone-inducible system (33). The latter was chosen for our studies because it gave the lowest level of basal reporter expression and the highest induction in the presence of the inducing agent (data not shown). A SW480.7 derivative, clone 15, was generated by stable transfection of the ecdysone system regulatory component (pVgRXR). This clone was then used to generate Smad4 transfectant clones selected in the absence of inducing agent. Upon induction with PA, three of the resulting clones (15.7, 15.8, and 15.13) were found to express Smad4 at levels comparable to those of endogenous Smad4 in HaCaT cells (Fig. 3). In the absence of PA, these clones expressed very low levels of Smad4. These three clones were used in all subsequent studies along with parental SW480.7 cells and clone 15 as negative controls.

Limited Rescue of TGF-β Transcriptional Responses by Smad4—It has been previously shown that transient transfection of Smad4 into SW480.7 cells rescues activation of the 3TP-Lux reporter construct (22). To verify the functionality of Smad4 in the inducible Smad4 clones, we tested their ability to activate this reporter in response to TGF-β. 3TP-Lux is a highly sensitive TGF-β reporter that contains multiple promoter elements from plasminogen activator inhibitor-1 and collagenase (34). TGF-β addition clearly activated 3TP-Lux in clones 15.7, 15.8, and 15.13 in the presence of PA, whereas in its absence only a small response was observed (Fig. 4). This basal response is attributable to leaky expression of exogenous Smad4 (see Fig. 3). Parental SW480.7 and clone 15 showed little, if any, response. These results confirmed that the exogenous, stably transfected Smad4 in these clones was functional.

Next we examined the activation of the A3-Luc reporter
TGF-β Function Restored in Colon Cancer Cells

Fig. 3. SW480.7 clones conditionally expressing Smad4. Equal amounts of total protein from HaCaT, parental SW480.7 cells (P), SW480.7 stably transfected with pVgRXR (15), and three SW480.7 Smad4-inducible clones (15.7, 15.8, 15.13) were subjected to immunoprecipitation with anti-Smad4 antibody, followed by Western blot analysis with the same antibody. The indicated cell cultures were treated as indicated with inducing agent (3 μM PA) for 36 h before lysis.

Fig. 4. Recovery of a Smad4 function in SW480.7 clones. Parental SW480.7 cells (P), clone 15 (15), and three SW480.7 Smad4-inducible clones (15.7, 15.8, 15.13) were transfected with the 3TP-Lux reporter and then incubated with 100 pM TGF-β1 and/or 3 μM PA as indicated. Luciferase activity was measured.

A3-Luciferase response elements from the Xenopus Mix.2 gene. This response is mediated by Smad2 (or Smad3) and Smad4 binding to the activin response elements as a complex with the DNA binding cofactors Fast1 or its mouse homolog, Fast2 (20, 23, 24, 35, 36). We have previously shown that transient transfection of Smad4 along with FAST1 in SW480.7 cells restores the A3-Luc response to TGF-β, and co-transfection of Smad2 enhances this effect (23). The A3-Luc reporter is less sensitive to TGF-β than the 3TP-Lux reporter, possibly because the former is solely based on enhancer elements from an Smad target gene. Using A3-Luc as a reporter together with cotransfected Fast2, we confirmed again the presence of Smad4 function in the inducible Smad4 clones (Fig. 5A). However, A3-Luc activation by TGF-β in these cells was weak (Fig. 5A). Cotransfection of wild type Smad3 enhanced the A3-Luc response but only to a limited extent (Fig. 5B). Collectively, these results demonstrated restoration of Smad4 transcriptional functions in the inducible SW480.7 clones but, at the same time, suggested the presence of other alterations that limited the signaling activity of Smad4 in these cells (see below).

Smad4 Is Not Sufficient to Rescue TGF-β Antiproliferative Responses in SW480.7—We performed 125I-deoxyuridine incorporation assays to determine if restoration of Smad4 expression rescued TGF-β antiproliferative responses in SW480.7 cells. TGF-β had no effect on 125I-deoxyuridine incorporation in any of the Smad4-transfected clones regardless of the presence or absence of Smad4-inducing agent in the media (Fig. 6). To enhance a possible weak response to TGF-β, these experiments were repeated in mitogen-poor media (containing only 0.2% fetal bovine serum). TGF-β also had no effect under these conditions (data not shown). Thus, reintroduction of Smad4 function alone was not sufficient to recover antiproliferative responses in SW480.7 cells.

To further characterize this lack of antiproliferative responses, we analyzed the effects of TGF-β on several endogenous genes. Down-regulation of c-myc mRNA levels is one of the most common antiproliferative gene responses to TGF-β in diverse cell types (37), including human colon cells (38). TGF-β addition rapidly decreased the levels of c-myc transcript in HaCaT cells, used here for comparison, but did not significantly change these levels in any of the SW480.7-derived clones whether or not they were induced to express Smad4 (Fig. 7).

TGF-β has been reported to elevate the expression of the p21/Cip1 cyclin-dependent kinase inhibitor in various cell lines including colon epithelial cells (27, 39, 40). TGF-β induces a small (less than 50%) increase in the levels of Cip1 transcript in the parental as well as the derivative SW480.7 cell lines, but this increase was not significantly enhanced by inducing Smad4 expression (Fig. 7). Furthermore, induction of plasminogen activator inhibitor-1 expression, which is not an antiproliferative gene response (4), was also absent in SW480.7 cells expressing Smad4 (Fig. 7). Together with the limited TGF-β response obtained for the A3-Luc reporter, this general absence of endogenous TGF-β gene responses suggests that SW480.7 cells may have additional oncogenic alterations that limit their ability to activate transcription via the TGF-β/Smad pathway.
Tors encoding for APC or TGF-β, we observed that their accumulation in the nucleus in response to TGF-β was quite blunted when compared with that found in HaCaT cells (Fig. 8A) or in colon cancer and other cell lines not harboring oncogenic Ras mutations (20). As already mentioned, SW480.7 cells contain a Ki-ras-activating mutation. Hyperactivation of the mitogen-activated protein kinase pathway by oncogenic Ras interferes with TGF-β/Smad signaling by inhibiting the accumulation of Smad2 and Smad3 in the nucleus (20). This effect is mediated by phosphorylation of Smad2 and Smad3 at specific mitogen-activated protein kinase sites. Mutant Smad constructs lacking these sites are resistant to the Ras inhibitory effects on their nuclear accumulation (20). The availability of a Ras-resistant Smad3 mutant construct (Smad3-EPSM) allowed us to determine whether this inhibitory effect contributes to the TGF-β-resistant phenotype of SW480.7 cells. Indeed, cotransfection of Smad3-EPSM with Smad4 allowed SW480.7 cells to respond to TGF-β with a 60% inhibition of the E2F-luciferase reporter (Fig. 8A). This effect was specific for the Ras-resistant mutant, as transfection with the wild type Smad3 (or Smad2, data not shown) had only a small effect (Fig. 8B) even though both Smad3 and Smad3-EPSM constructs were expressed at similar levels in these transfections (Fig. 8C).

Transient transfection of Smad3-EPSM also enhanced the A3-Luc response to TGF-β in the SW480.7 derivatives inducibly expressing Smad4 (Fig. 5C). In this experiment, Smad3-EPSM conferred some responsiveness to TGF-β even in the absence of exogenous Smad4, suggesting that the Ras-resistant construct confers high sensitivity to the very limited levels of endogenous Smad4 present in these SW480.7 cells.

**DISCUSSION**

Inhibition of cell cycle progression, facilitation of differentiation, induction of apoptosis, and regulation of cell adhesion and extracellular matrix production are effects of TGF-β through which this factor is thought to contribute to the maintenance of epithelial tissue homeostasis (4, 5). Loss of these TGF-β responses is often observed in carcinoma cells, including colon cancer cells (2, 3). Different lines of evidence point to Smad4 as a tumor suppressor gene in colon cancer; however, the reasons why its loss contributes to tumorigenesis are not yet understood. Specifically, it has not been clear whether loss of TGF-β antiproliferative responses in Smad4-defective colon cancer cells is due to the loss of Smad4 function or to other oncogenic events. There has not been, to our knowledge, reports that restoration of a wild-type Smad4 allele in Smad4-defective tumor-derived cell lines rescues TGF-β antiproliferative responses. We have investigated this question using the SW480.7 colon carcinoma cell line, which is defective in Smad4 function (22, 23). One of the oncogenic defects of this cell line is the loss of one copy of chromosome 18 (21). According to the two-hit model of tumor suppressor gene inactivation (43), the loss of Smad4 function in these cells could have arisen from an inactivating mutation in the remaining Smad4 allele. Surprisingly, we found that SW480.7 contain a Smad4 allele that encodes for a wild type protein. However, expression of this allele is strongly suppressed, as its mRNA and protein products are present at very low, nearly undetectable, levels. The mechanism responsible for this defective expression is not known at present, but this finding raises the possibility that Smad4 silencing is another mechanism leading to the loss of Smad4 function in colon cancer, a possibility worthy of future investigation.

The lack of detectable Smad4 function allowed us to use SW480.7 cells to study the consequences of restoring expression of this gene. It was recently reported that SW480.7 cells stably transfected with Smad4 are not growth-inhibited by TGF-β (44). However, if Smad4 is a mediator of antiproliferative responses in HaCaT, SW480.7 cell lines. Northern blot analysis of 15 μg of total RNA from HaCaT, SW480.7 parental (P), clone 15 (15), or Smad4-inducible clones (15.7, 15.8, 15.13) that were incubated with 3 μM PA for 48 h and then with 150 μM TGF-β1 for 0, 2, or 4 h as indicated are shown. Blots were probed with human c-myc, p21, plasminogen activator inhibitor-1 (PAI-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (used as a loading control) cDNA probes.

**Fig. 6. Absence of TGF-β antiproliferative responses after restoration of Smad4 function in SW480.7 cells.** Clone 15 (15) and three SW480.7 Smad4-inducible clones (15.7, 15.8, 15.13) were incubated as indicated with 3 μM PA for 3 days and with 100 μM TGF-β1 for the last 24 h. 13H-deoxyuridine was added for the last 4 h, and its incorporation into DNA was then assayed. Data are plotted as the mean ± S.D. of triplicate counts.

**Antiproliferative Responses in SW480.7 Cells Are Rescued by Ras-resistant Smad3 Jointly with Smad4—**We investigated whether other known oncogenic alterations might interfere with Smad4-mediated, TGF-β-induced signaling of antiproliferative responses in SW480.7 cells. To efficiently screen various candidate genes we transiently transfected parental SW480.7 cells with Smad4, either alone or in combination with several relevant test constructs. To monitor the effects of TGF-β on cell cycle progression in these assays, we used a E2F-luciferase reporter construct, whose activity is a measure of G1 progression (41). This approach has been previously used to monitor TGF-β antiproliferative responses in other cell types (20). In SW480.7 cells, transient transfection of Smad4 did not mediate any significant decrease in E2F-luciferase reporter activity in response to TGF-β (Fig. 8A). Cotransfection of vectors encoding for APC or TGF-β type II receptor, together with Smad4, again did not restore E2F-luciferase responsiveness to TGF-β (data not shown), suggesting that neither APC inactivation nor low expression levels of TGF-β type II receptor (42) are responsible for the lack of antiproliferative TGF-β responses in these cells.

When we examined endogenous Smad2 and 3 in SW480.7 cells by immunofluorescence, we observed that their accumulation in the nucleus in response to TGF-β was quite blunted...
tive responses, its constitutive overexpression in transfected SW480.7 cells could lead to the selection of Smad4-resistant clones due to secondary mutations. To avoid this potential problem, we generated SW480.7 cell lines conditionally expressing a human wild-type Smad4 allele under the control of the ecdysone system. The levels of Smad4 expressed under inducing conditions by the three SW480.7 derivatives used in this study were similar to those of HaCaT keratinocytes, which is a well characterized, TGF-β-responsive cell line (27, 28, 38, 39). Assays using the highly responsive TGF-β reporter construct 3TP-Lux demonstrated that Smad4 function had been restored in the Smad4-inducible SW480.7 clones. Surprisingly, however, expression of Smad4 in these cells supported only a weak A3-Luc response to TGF-β. The A3-Luc reporter is based on a TGF-β target gene enhancer element (35) and is less sensitive to TGF-β than the 3TP-Lux reporter, which is a composite reporter construct empirically assembled to provide high responsiveness to this factor (34). The limited signaling ability of Smad4 in these cells was further demonstrated by their failure to show endogenous antiproliferative gene responses, including down-regulation of c-myc or up-regulation of p21/Cip1, which are typical of TGF-β action in colonic and other epithelial cells (37, 39). The absence of these responses in Smad4-expressing SW480.7 cells was accompanied by a lack of growth inhibition.

The absence of robust antiproliferative effects and endogenous gene responses to TGF-β in SW480.7 cells induced to express normal levels of Smad4 raises the possibility that other oncogenic alterations present in these cells are limiting Smad4 signaling function. Our evidence indicates that neither the presence of APC mutations nor limitations in TGF-β type II receptor levels contribute to the lack of TGF-β responsiveness in SW480.7. p53, which is inactivated in SW480.7 cells, is not a candidate because it is also inactivated in HaCaT cells, which are highly responsive to TGF-β (27, 28, 38, 39). The TGF-β receptor-regulated Smads, Smad2, and Smad3 are expressed in SW480.7 cells, and their levels do not appear to be rate-limiting because cotransfection of exogenous Smad2 or Smad3 did not very much improve the antiproliferative response in Smad4-transfected SW480.7 cells.

Our results identify the hyperactive Ras pathway present in SW480.7 cells as a rate-limiting event for the signaling function of Smad4. SW480.7 cells harbor a Ki-ras-activating mutation (21), which is a frequent event in colon cancer (45, 46). Ras transformation of intestinal and other epithelial cells inhibits TGF-β antiproliferative effects (2, 47–50). Hyperactivation of the Ras pathway can interfere with Smad signal transduction by inhibiting Smad accumulation in the nucleus (20). Nuclear accumulation of Smads in response to

![Blunted nuclear accumulation of Smads2/3 in TGF-β-treated SW480.7 cells and restoration of TGF-β inhibitory responsiveness by Ras-resistant Smad3 mutant. A, Smad2/3 immunofluorescence pattern in HaCaT and SW480.7 cells that were treated or not for 30 min with 100 pM TGF-β1 and then subjected to immunostaining using affinity-purified anti-Smad2/3 polyclonal antibody. Numbers indicate the percentage of cells with predominant nuclear staining. B, SW480.7 cells were transiently cotransfected with a E2F-luciferase reporter (0.2 μg) and the indicated combinations of constructs (40 ng of Smad4 and/or 8 ng of either FLAG-Smad3 or FLAG-Smad3-EPSM). The amount of DNA used in the transfections was kept equal by using a pCMV5-farnesyl transferase construct. Transfected cells were treated with 100 pM TGF-β1 for 20 h. Results are the average ± S.D. of three determinations and are plotted as either raw values (left panel) or as percentages relative to values without TGF-β treatment (right panel). C, Western blot analysis using anti-FLAG antibody of lysates from SW480.7 cells transfected with equal amounts of FLAG-tagged Smad3 or Smad3-EPSM constructs.](image)
TGF-β is less efficient in colon cancer cell lines harboring Ki-ras mutations than in colon cancer cell lines containing wild type Ki-ras (20). As we show here, SW480.7 cells also have blunted Smad2/3 nuclear accumulation response. Following this lead, we found that a Ras-resistant mutant form of Smad3 lacking inhibitory mitogen-activated protein kinase phosphorylation sites can strongly enhance the ability of Smad3 to rescue the antiproliferative response in SW480.7 cells.

In sum, restoration of Smad4 expression and removal of Smad3 and presumably also Smad2 inhibition by Ras are required for restoration of TGF-β antiproliferative responses in SW480.7 cells. These results provide the first evidence that loss of Smad4 function indeed contributes to deprive colon cancer cells of antiproliferative responsiveness to TGF-β. At the same time, our evidence suggests an important contribution of a hyperactive Ras pathway. The coexistence of Smad4 and Ki-ras mutations is not unique to SW480.7 cells; the high frequency of Smad4 and Ki-ras mutations in metastatic colon cancer (15, 45, 46) and pancreatic cancer (52) predicts the coexistence of both responses, contributing to the emergence of a more malignant phenotype.