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Genetics

Targeted deletion of *Smad4* shows it is required for transforming growth factor β and activin signaling in colorectal cancer cells

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ABSTRACT ***Smad4* (*DPC4*) is a candidate tumor suppressor gene that has been hypothesized to be critical for transmitting signals from transforming growth factor (TGF) β and related ligands. To directly test this hypothesis, the *Smad4* gene was deleted through homologous recombination in human colorectal cancer cells. This deletion abrogated signaling from TGF-β, as well as from the TGF-β family member activin. These results provide unequivocal evidence that mutational inactivation of *Smad4* causes TGF-β unresponsiveness and provide a basis for understanding the physiologic role of this gene in tumorigenesis.**

The *Smad4* (*DPC4*) gene was discovered by virtue of its mutational inactivation in a large fraction of pancreatic cancers (1), and has since been found to be mutated in a subset of colorectal cancers (2). *Smad4* is homologous to the *Drosophila Mad* gene, known to be required for signaling by the transforming growth factor (TGF) β family member dpp (3). Based on this homology, it was suggested that the driving force for *Smad4* inactivation is the abrogation of TGF-β signaling (1). This hypothesis was attractive because many cancers seem to be unresponsive to TGF-β, the prototype growth-inhibitory polypeptide (4).

This simple model became complicated when numerous other homologues of *Mad* were identified in vertebrate cells (5–19). Some of these homologues appear to stimulate or mimic responses to TGF-β, whereas others inhibit them. The situation is similar in *Caenorhabditis elegans,* where one of the two *Smad4* homologues antagonizes TGF-β-related receptor signaling (20). The *Drosophila* Mad protein binds to DNA in a sequence-specific manner (21), whereas other Smad proteins contain transcriptional activating domains that function when complexed with DNA-binding partners (22–26). Furthermore, and unlike the situation in *Drosophila*, there are dozens of TGF-β-like ligands in vertebrates with diverse functions (27). And even the response to TGF-β is variable: whereas many mammalian cells are growth-inhibited by TGF-β, others, including some epithelial cancer cells, are growth stimulated (28–31).

Perhaps as a result of these complexities, some experimental observations have supported the idea that *Smad4* mediates TGF-β signaling (8, 22, 26, 32–34), whereas others indicated that *Smad4* is dispensable for such signaling (J.L.D. and S.E.K., unpublished results). However, experimental approaches to this question have so far been indirect, employing overexpression of exogenously introduced *Smad4* constructs. As *Smad4* polypeptides have been shown to interact with several other proteins, including other Smads (8, 22, 25, 26), the interpretation of such overexpression experiments is problematical. Moreover, in some studies, overexpression of *Smad4* had little effect on TGF-β signaling unless other *Smad* genes were simultaneously expressed (8, 22, 26, 32). To unambiguously determine whether *Smad4* is required for TGF-β signaling in human colorectal cancer cells, we have deleted the *Smad4* genes through homologous recombination in cells originally containing two normal *Smad4* alleles.

MATERIALS AND METHODS

Gene Targeting. A promoterless strategy was adapted for targeted disruption of the *Smad4* gene (35). A cosmid clone (c417–46) containing human *Smad4* was used as the source for homologous arms (36). A 2.2-kb *Sac*I/*Bgl*II fragment immediately upstream of exon 3 and a 4.7-kb *Xba*I/*Hin*dIII fragment downstream of exon 4 were assembled in pBluescript surrounding promoterless NEO or HYG cassettes containing simian virus 40 polyadenylation signals (Fig. 1*A*). Homologous recombination should result in the expression of NEO and HYG genes from the endogenous *Smad4* promoter as fusion proteins with the amino-terminal 141 amino acids of *Smad4*. For first allele targeting, exponentially growing HCT116 human colorectal cancer cells (American Type Culture Collection) were transfected with a *Not*I-linearized NEO targeting vector. G418 (0.4 mg/ml)-resistant clones were screened by a reverse transcriptase–PCR approach, by using the primers "a" (5′-CAGCTATAACTACAAATGGAGC) and "c" (5′-TTGTTCAATGGCCGATCCCAT) (Fig. 1*A*), yielding a 338-bp PCR product. Reverse transcriptase–PCR positive clones were expanded and genomic DNA was prepared from them and used for Southern blot analysis with a hybridization probe located immediately outside the 5′ homologous arm (Fig. 1*A*). A clone carrying a homologous recombinant and no additional random integrants was then transfected with the HYG targeting vector. The heterozygote clone 5–60 was a subclone derived from the same parent used to derive *Smad4*−/− clones 5–18 and 5–63, and contained a targeted NEO gene plus a randomly integrated (nontargeted) HYG gene. Hygromycin (0.1 mg/ml)-resistant clones were screened by PCR of genomic DNA, by using primers "a" and "d" (5′-GCAGGTCTTGCAACGTG) (Fig. 1*A*). PCR with primers "a" and "b" (5′-CTGCAGTGTTAATCCTGAGAG) (Fig. 1*A*) and genomic Southern blot analysis were performed to confirm the complete deletion of both *Smad4* alleles and the purity of clones (Fig. 1 *B* and *C*).

Expression Vector Construction. An expression construct for the TGF-β RII gene was constructed by placing a TGF-β RII cDNA (generously provided by H. Lodish and R. Wein-

Abbreviations: TGF, transforming growth factor; TβRII, TGF-β type II receptor; αGT, α1,3-galactosyltransferase.

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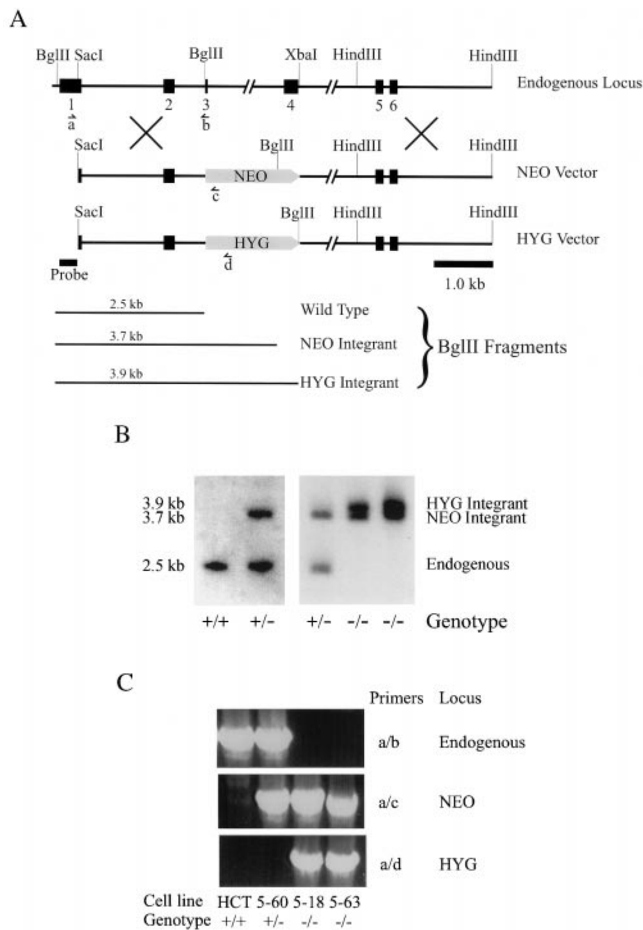

FIG. 1. Targeted disruption of the human *Smad4* gene. (*A*) Alignment of endogenous *Smad4* locus with the two targeting vectors. Solid boxes represent exons 1–6. Drug markers are shown as shaded boxes with pointed ends indicating the simian virus 40 polyadenylation signals. Also shown are the locations of the PCR primers and the probe used for Southern blot analysis. The expected *Bgl*II restriction fragments hybridizing to the probe are diagrammed at the bottom. As a result of recombination between the vector and endogenous locus, exons 3 and 4 are entirely deleted. (*B*) Southern blots of *Bgl*II-digested genomic DNA. Genomic DNA was prepared from parental (+/+), heterozygote (+/−), and *Smad4*-null (−/−) HCT116 clones. After digestion with *Bgl*II and agarose gel electrophoresis, DNA was blotted and hybridized with the probe mapped in *A*, corresponding to sequences outside the 5′ homologous arm. (*C*) Genomic PCR of the clones used in this report. Genomic DNA was prepared from parental HCT116 cells (HCT) (+/+), and clones 5–60 (+/−), 5–18 (−/−), and 5–63 (−/−). Primer pairs (see *A* for positions) were specific for the deleted portion of endogenous *Smad4* gene (*Top*), the NEO-targeted locus (*Middle*), or the HYG-targeted locus (*Bottom*).

berg, Massachusetts Institute of Technology, Cambridge) between a cytomegalovirus (CMV) promoter and an simian virus 40 polyadenylation signal, creating pCEP-Zeo/RII. A cDNA for α1,3-galactosyltransferase (αGT) (37) was cloned into the polylinker of pCEP4 (Invitrogen), generating pαGT. To build pαGT-RII, a *Sal*I fragment containing the TGF-β RII cDNA and surrounding regulatory sequences from pCEP-Zeo/RII was cloned into the *Bst*EII site of pαGT. The resultant vector contained expression cassettes for both αGT and TGF-β type II receptor (TβRII). Details of the construction of expression vectors are available from the authors upon request. The reporters p3TP-lux and pAR3-lux have been previously described (19, 38).

Signaling Assays. For luciferase assays, cells were seeded at 10^5 cells per well in 12-well plates 1 day before transfection with 3 μg of DNA and 9 μl of FuGENE 6 (Boehringer Mannheim). For TGF-β response assays, p3TP-lux (1.5 μg), pαGT-RII or pαGT (1.5 μg), and pCMVβ-Gal (0.2 μg) were cotransfected and cells subsequently incubated for 20 hr with or without 1 ng/ml TGFβ-1 (R & D Systems). For activin response assays, cells were cotransfected with either p3TP-lux (2.0 μg) and pCMVβ-Gal (0.2 μg), or pAR3-lux (1.5 μg), pMyc-FAST-1 (1.5 μg), and pCMVβ-Gal (0.2 μg), and then incubated for 20 hr with or without 10 ng/ml of activin (Research Diagnostics, Flanders, NJ). For experiments involving mutant RI receptors, 1.5 μg of RI constructs were used for transfection. Luciferase activity in cell lysates, normalized for β-galactosidase activity, was determined by using the Promega Luciferase Assay Reagent and the ICN Aurora Gal-XE system. The normalization had an insignificant effect on the relative values. The plasmids p3TP-lux, pAR3-lux, pMyc-FAST-1, TβRI^{T204D}, and ActR1B^{T206D} were generously provided by J. Wrana (The Hospital for Sick Children, Toronto).

For growth response assays, 5×10^5 cells were seeded in T25 flasks 1 day before transfection with 9 μg of either pαGT-RII or pαGT and 18 μl of FuGENE 6, then incubated with TGF-β1 (1 ng/ml) for 36 hr. Three hours before harvesting, BrdUrd was added to the medium at a final concentration of 10 mM. Cells were collected by trypsinization, fixed in 70% ethanol, and stained with fluorescein-conjugated *Griffonia Simplicifolia* lectin I, isolectin B4 (25 μg/ml; Vector Laboratories). For BrdUrd detection, stained cells were fixed in buffered formalin for 10 min, treated with 2 N HCl for 5 min, then incubated for 40 min with an anti-BrdUrd mAb (4 μg/ml; Boehringer Mannheim). The cells were then washed in PBS and incubated for 40 min with a rhodamine-conjugated goat antibody to mouse Ig (Pierce). Cells were washed briefly and then stained with 3 μg/ml 4′6-diamidino-2-phenylindole for 5 min. All steps, except for ethanol fixation at 4°C, were carried out at room temperature.

RESULTS

Targeted Deletion of *Smad4* Gene. The requirements for targeted gene deletion in human cells are significantly more fastidious than those in murine embryonic stem cells, but such targeting can be performed in an analogous fashion. The targeting vectors contained 6.9 kb of *Smad4* sequences interrupted by genes encoding resistance to geneticin (NEO) or hygromycin (HYG) (Fig. 1*A*). Expression of the drug-resistance genes was dependent on the construct's integration downstream of a cellular promoter and predicted to result in the production of a fusion protein with the introduced drug-resistance element at the carboxyl terminus. A PCR-based approach was used to screen for homologous integration of the targeting vectors, and positive results were verified by Southern blotting. Introduction of the NEO vector into HCT116 cells resulted in targeted disruption of the *Smad4* gene in 4 of 300 geneticin-resistant clones. One of these heterozygote clones was then transfected with the HYG vector, resulting in deletion of the second *Smad4* allele in 2 of 254 hygromycin-resistant subclones. Examples of the genomic analyses of these clones are illustrated in Fig. 1 *B* and *C*.

TGF-β Signaling Requires *Smad4*. The HCT116 colorectal cancer cell line was chosen for targeted deletion because it has a single, well-defined defect in the TGF-β pathway; a truncating mutation in TβRII (39, 55). This allows well-controlled restoration of TGF-β-mediated signaling through introduction of a wild-type TβRII gene. To evaluate TGF-β signaling in these cells, we first used the 3TP reporter (38), containing the promoter from the plasminogen activator inhibitor-1 gene (40). This reporter is a sensitive indicator of TGF-β family member signaling and has been used as the standard for assessing TGF-β-dependent transcriptional responses in mammalian cells. Parental HCT116 cells demonstrated a substantial activation of this reporter upon introduction of the TβRII

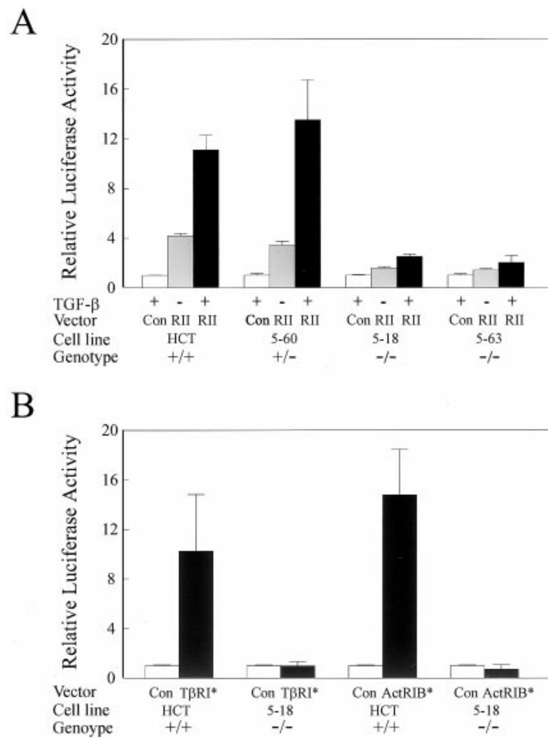

FIG. 2. *Smad4* is required for TGF-β signaling. (*A*) Activation of the 3TP reporter by TβRII plus TGF-β. Cells of the indicated genotypes were cotransfected with p3TP-lux together with a plasmid encoding TβRII (RII) or an identical plasmid devoid of TβRII (Con) as a control. The transfected cells were then cultured in the presence or absence of TGF-β1. Luciferase activity was measured 20 hr after transfection and ligand treatment, and was normalized to the control for each line. Bars and brackets represent the means and standard deviations, respectively, from triplicate transfections. (*B*) Activation of the 3TP reporter by constitutively activated receptors. Cells were transfected as in *A*, except that mutant forms of the RI receptors for TGF-β (TβRI*) or activin (ActR1B*) were used instead of RII. Results were normalized to the luciferase activity achieved with the control vector in each line. Heterozygote *DPC4*+/− cells behaved similarly to the parental HCT116 cells in these assays, and an additional clone of *DPC4*−/− cells gave results identical to those shown for clone 5–18.

gene (Fig. 2*A*). This activation was stimulated by the addition of TGF-β1, though there was significant activity in the absence of added ligand. This activity is likely because of TGF-β present in serum plus that known to be produced endogenously by colorectal cancer cell lines, including HCT116 (41, 42). In the heterozygote clone 5–60, with one wild-type allele of *Smad4* and one deleted allele, the response to TGF-β was similar to that observed in the parental cells (Fig. 2*A*). Two other heterozygote clones were tested, with similar results (data not shown). In clone 5–18, however, in which both alleles of *Smad4* were inactivated by homologous integration, there was little reporter activation upon introduction of the TβRII gene, with or without additional TGF-β (Fig. 2*A*). As a control, we transfected the same clones with a β-galactosidase reporter driven by the CMV promoter instead of the plasminogen activator inhibitor-1 promoter; β-galactosidase activity was no lower in the *Smad4*−/− clone than in parental cells (data not shown). We also assessed activation of 3TP in a second, independent clone (5–63) with both alleles of *Smad4* inactivated through homologous recombination. TGF-β-mediated signaling was equivalently diminished in this clone (Fig. 2*A*).

We next directly activated TGF-β signaling by transfecting a mutant form of the Type I receptor for TGF-β, $T\beta RI^{T204D}$ (TβRI*). Normally, TGF-β binds to TβRII, which then activates the kinase activity of TβRI. $T\beta RI^{T204D}$ turns on TGF-β response pathways by virtue of its constitutively activated kinase, bypassing the need for TGF-β (43). $T\beta RI^{T204D}$ activated 3TP, as expected, in parental HCT116 cells as well as in the *Smad4*+/− clone 5–60 (Fig. 2*B*). In *Smad4*−/− cells, however, there was no reporter activation observed (Fig. 2*B*).

Activin Signaling Requires *Smad4*. Among TGF-β family members, activin most resembles TGF-β in its mode of receptor activation and receptor structure (27). Accordingly, we found that the response to $ActR1B^{T206D}$ (ActR1B*), a constitutively activated activin Type I receptor mutant (44), was dependent on intact *Smad4* (Fig. 2*B*). We also assessed signaling from the endogenous activin receptors present in HCT116 cells, by using either the 3TP or AR3 reporters (19). Addition of activin resulted in a significant increase in the activity of the 3TP reporters in parental and heterozygote *Smad4*+/− cells (Fig. 3*A*). In two independent *Smad4*−/− clones, however, no reporter response was observed (Fig. 3*A*). These experiments, in combination with those depicted in Fig. 2, demonstrated that both endogenous and exogenous receptors for TGF-β ligands were unable to transmit their signals in *Smad4*-deficient cells.

FAST-1-Mediated Signaling Requires *Smad4*. FAST-1 is a DNA-binding component of the multimeric protein complex that transactivates activin responsive elements in *Xenopus laevis* (24, 25). It has since been shown that the response of AR3 promoter to TGF-β is enhanced in the presence of exogenous FAST-1 (19). In HCT116 cells, exogenous FAST-1 was required for response of the AR3 reporter to activin (Fig. 3*B*). This response to activin was totally dependent on endogenous *Smad4*, as demonstrated through analysis of the *Smad4*−/− clones (Fig. 3*B*). To determine whether FAST-1 could affect TGF-β signaling in HCT116 cells, we transfected TβRII with or without FAST-1. In the absence of FAST-1, the AR3 reporter was inactive, whether or not TβRII was introduced (Fig. 3*C*). But TβRII resulted in a dramatic activation of this reporter in the presence of FAST-1, reaching 100–150-fold in parental HCT116 cells and heterozygote *Smad4*+/− derivatives (Fig. 3*C*). In *Smad4*−/− homozygotes, these responses were almost totally abrogated (Fig. 3*C*).

Deletion of *Smad4* Affects TGF-β-Mediated Growth Inhibition. To determine whether the absence of the *Smad4* gene altered the cell's growth response to TGF-β, we employed a new approach for evaluating transfectants. A vector was constructed that contained the gene encoding αGT plus TβRII. HCT116 cells transfected with this vector can be brightly stained with a fluorescently labeled *Griffonia Simplicifolia* lectin. By culturing cells in media containing BrdUrd for 3 hr before analysis, the growth inhibitory effect of expressing TβRII in the presence of TGF-β could be directly assessed by dual-color fluorescence microscopy (Fig. 4, *Inset*). In parental HCT116 cells, TβRII expression resulted in a substantial decrease in BrdUrd incorporation compared with cells transfected with the same vector devoid of TβRII sequences (Fig. 4). The magnitude of the observed responses (≈40% decrease) was consistent with those noted in other human cancer cell lines responsive to TGF-β (45–49). In *Smad4*+/− clone 5–60, a similar degree of growth inhibition was observed. In two *Smad4*−/− clones, however, much less growth inhibition was affected by TβRII (Fig. 4). Though the variability in this assay (see error bars in Fig. 4) precluded us from concluding that there was absolutely *no* growth inhibition from TβRII expression in the *Smad4*−/− cells, there was an obvious and statistically significant difference between the responses in *Smad4*−/− compared with parental cells ($P < 0.05$, Student's *t* test).

DISCUSSION

The results recorded above demonstrate that the deletion of *Smad4* renders cells unresponsive to TGF-β ligands and are

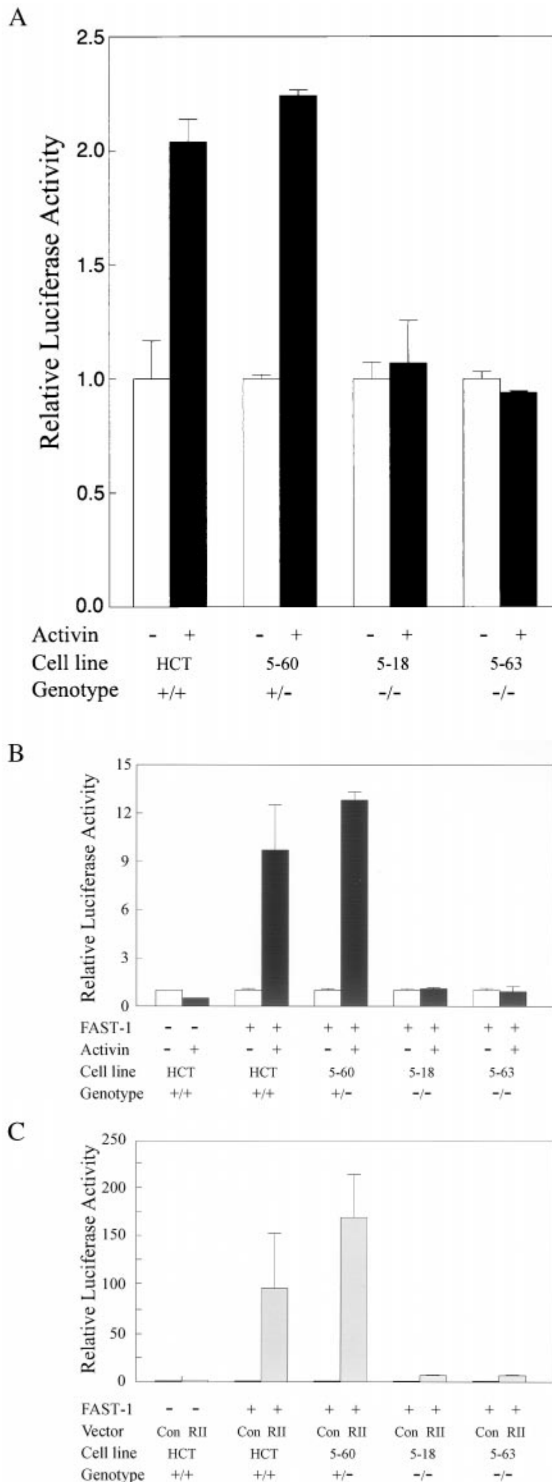

FIG. 3. *Smad4* is required for signaling through activin and FAST-1. (*A*) Activation of the 3TP reporter by activin. Cells of the indicated genotypes were transfected with p3TP-lux and then treated with activin as indicated. (*B*) Activation of the AR3 reporter by activin plus FAST-1. Cells were cotransfected with pAR3-lux and (except as indicated) a plasmid encoding FAST-1 and treated with activin as indicated. For experiments presented in both *A* and *B*, luciferase activity was determined 20 hr after ligand treatment, as in Fig. 2. Results were normalized to the luciferase activity observed in the absence of activin in each case. (*C*) Activation of the AR3 reporter by TβRII plus TGF-β. Cells were transfected with pAR3-lux and TβRII (RII) or a control (Con) vector, and with FAST-1, as indicated, and treated with TGF-β for 20 hr. Results were normalized to luciferase activity of the control transfectants in each case.

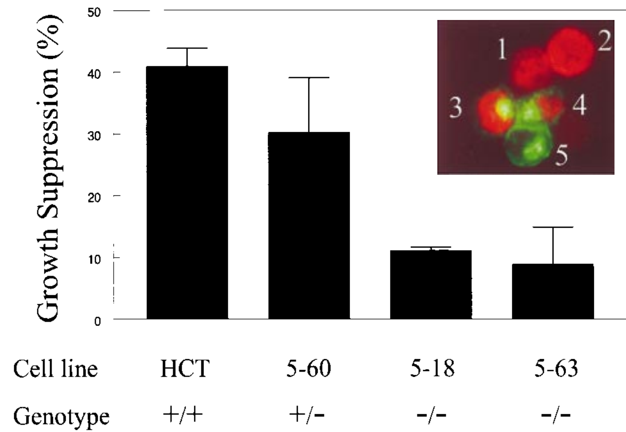

FIG. 4. Effect of *Smad4* on growth suppression by TGF-β. Cells were transfected with a vector encoding αGT plus TβRII (pαGT-RII) or an identical vector without TβRII (pαGT). The cells were then cultured in medium containing TGF-β1 (1 ng/ml), and after 36 hr, BrdUrd was added for an additional 3 hr. The cells were harvested, bound to fluorescein-labeled lectin, and stained with anti-BrdUrd antibodies. (*Inset*) Examples of *Smad4*$-/-$ cells transfected with the TβRII vector pαGT-RII. The green fluorescence at the cell periphery and in the Golgi apparatus indicates expression of αGT. The red nuclear fluorescence indicates DNA synthesis. Cells 1 and 2 were not transfected but were synthesizing DNA. Cells 3 and 4 were transfected and were synthesizing DNA, whereas cell 5 was transfected but was not synthesizing DNA. In the graph, the fraction of BrdUrd-positive cells among pαGT-RII transfectants was normalized to the fraction of BrdUrd-positive cells in the control pαGT transfectants. Bars and brackets represent the means and standard deviations, respectively, determined from at least two independent assessments of 400 transfected (green) cells from a single experiment; similar results were obtained in two other independent transfections. All determinations were performed in a blinded manner. BrdUrd incorporation in pαGT-RII-transfected *Smad4*$-/-$ cells was not statistically different from pαGT transfected cells, but the differences between the *Smad4*$-/-$ homozygotes and parental cells was statistically significant ($P < 0.05$, Student's t test).

consistent with previous studies indicating that exogenous *Smad4* could mediate TGF-β signaling (8, 22, 26, 32, 33). The results are not consistent, however, with observations indicating that *Smad4* is dispensable for TGF-β signaling (J.L.D. and S.E.K., unpublished results). Though these differences may reflect cell-type specific factors, we believe that comparisons between TGF-β responses can only be made unambiguously with lines that are isogenic with respect to all genes other than the one under study. Evaluation of the *Smad4*-deficient lines studied here revealed striking differences in the responses to both TGF-β and activin. Similar effects were observed in independent clones, excluding simple clonal variations as the explanation. The results also demonstrated that the FAST-1 coactivator, which can modulate both TGF-β and activin responses, functions efficiently only when *Smad4* is intact.

Two further implications can be made from our study. First, it is likely that the driving force for *Smad4* mutation in human cancer cell lines is the loss of responsiveness to TGF-β. This conclusion is consistent with the many studies demonstrating that colorectal epithelial cells synthesize TGF-β and that this ligand functions in an autocrine loop to inhibit the growth of cells that are not fully transformed (41, 42, 50–54). Second, our results provide compelling evidence that genetic inactivation of the components of TGF-β signaling occurs in human cancer cells. Conversely, they highlight our lack of knowledge about the basis for TGF-β responsiveness in most human cancers. Like the HCT116 cells used in our study, most other colorectal cancer cells do not respond to TGF-β (4). Yet in only a minority of cases ($\approx$10% with TβRII mutations and $\approx$20% with *Smad4* or *Smad2* mutations) can a genetic defect in the

pathway be identified (2, 7, 12, 39, 55, 56). In cancers other than those of the colon or pancreas, very few mutations in TGF-β response pathway genes have been discovered, despite the fact that TGF-β unresponsiveness is common in most tumor types (56). A search for such genetic defects should illuminate other downstream effectors of TGF-β that play a role both in cancer and in normal development.

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