Targeted Disruption in Murine Cells Reveals Variable Requirement for Smad4 in Transforming Growth Factor β-related Signaling*

(Received for publication, September 24, 1999)

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The tumor suppressor gene Smad4 has been proposed to be a common mediator of transforming growth factor β (TGFβ)-related signaling pathways. We investigated the role of Smad4 in TGFβ-related pathways by targeted disruption of its locus in murine cell lines. TGFβ responses, including growth arrest, induction of the endogenous PAI-1 gene, and other extracellular matrix components, were normal in Smad4-deficient fibroblasts. Assembly of a TGFβ-induced DNA-binding complex on one of two regulatory regions in the human plasminogen activator inhibitor (PAI)-1 promoter did not require Smad4 but was, instead, dependent on a TFE3 binding site. In contrast, Smad4 was required for activation of the Xenopus Mix.2 promoter in response to TGFβ/activin. Smad4 was also involved in the regulation of the Msx homeobox protein family members in response to bone morphogenetic protein (BMP). Interestingly, the expression of the endogenous Msx-2 was reduced, whereas that of Msx-3 was activated in differentiating Smad4−/− ES cells relative to wild-type cells. Moreover, reporter assays of the Msx-2 promoter revealed an absolute requirement for Smad4 in fibroblasts and ES cells for activation. Our results indicate that Smad4 is dispensable for critical TGFβ-induced responses but is required for others in murine fibroblasts. We have identified transcriptional targets for Smad4 in the BMP signaling pathway, which may contribute to the genetic defect observed in the Smad4-deficient embryos.

The TGFβ1-related molecules include TGFβs, activin, and BMP-2/4, and they signal through heteromeric complexes of type II and type I serine/threonine kinase receptors (1). The type II receptor transphosphorylates and activates the type I receptor, which then transmits the signal to a family of intracellular signaling molecules called Smad, which are related to the Drosophila gene Mothers against dpp (Mad) (2). There are three functional categories of Smad proteins: receptor-regulated Smads, common-mediator Smads, and inhibitory Smads. The BMP-regulated Smads consist of Smad1, Smad5, and Smad8, whereas Smad2 and Smad3 are restricted to the TGFβ/activin pathway (3). Smad4 is part of the common-mediator Smads (2, 3), originally cloned as the tumor suppressor gene DPC4, deleted in 50% of pancreatic carcinomas (4).

Heteromeric complexes between the various receptor-regulated Smads and Smad4 have been suggested to be a critical prerequisite for functional signaling. Activation of the TGFβ/activin pathway results in the phosphorylation of Smad2 or Smad3 by the type I receptor followed by their interaction with Smad4. A similar phosphorylation-dependent interaction of Smad1 and Smad5 with Smad4 has been observed in BMP signaling (2, 3). Such heteromeric complexes are translocated to the nucleus where Smad4 appears to function as a transcriptional coactivator (5). Furthermore, activation of the TGFβ/activin pathway leads to induction of the Mix.2 and goosecoid promoters through the formation of an activin responsive factor (ARF). The ARF is composed of Smad2, Smad4, and either FAST-1 or FAST-2, members of the forkhead transcription factor family (5). Smad4 in the ARF promotes transactivation by binding to DNA at a site adjacent to the FAST site (6, 7). Although Smad binding to the goosecoid promoter and several Drosophila dpp-responsive genes is mediated via a GC-rich sequence (5), the optimal Smad binding element appears to be the sequence AGAC (8), which is present on various promoters of TGFβ-responsive genes (5), including at several sites within the human PAI-1 promoter (9–11). However, experiments designed to determine the requirement for Smad4 in the activation of the promoters, such as PAI-1, relied primarily on reporter constructs with artificially modified promoters rather than activation of the endogenous gene.

BMP2/4 signaling is required throughout development for
cell fate determination, structural morphogenesis and apoptosis. These processes are mediated, in part, by the Smad4 homeobox gene family. Although the expression of Msx-3 is restricted to the neural tube and hind brain region during early mouse development (12, 13), that of Msx-1 and -2, as well as BMP-4, is extended to other tissues undergoing epithelial-mesenchymal transition (14, 15). It has been well established that BMP4 can induce apoptosis in the cephalic neural crest (15) and in the chick limb (16). Circumstantial evidence has initially implicated Msx-1 and Msx-2 in this process based on the close association of their temporal-spatial expression patterns with sites undergoing program cell death (17) and on their transcriptional response to BMP4 (15). Moreover, the introduction of a dominant-negative BMP-receptor in the chick limb results in decreased inter-digital death and a specific decrease in the levels of Msx-2 transcripts (14). Overexpression of Msx-1 can mimics BMP-induced responses in Xenopus, including ventral mesoderm formation and epidermal induction (18). Although these studies establish a fundamental role for the Msx genes in BMP signaling pathway, the role of Smad4 in these BMP-induced responses is not known.

To address more precisely the role of Smad4 in TGFβ-related signaling, we have generated cell lines in which Smad4 was disrupted by homologous recombination (19). Signaling pathways were investigated in ES cell lines and fibroblasts derived from chimeric embryos. In Smad4-deficient fibroblasts, the physiological responses induced by TGFβ/activin remained unchanged relative to wild-type as determined by growth inhibition and the induction of extracellular matrix (ECM) gene expression. Interestingly, we identified Smad4-dependent and -independent elements in the PAI-1 promoter, which might explain why Smad4 is dispensable for some TGFβ-induced responses. However, Smad4 was necessary for other TGFβ/activin-responsive genes. In Smad4-deficient ES cells, endogenous expression of the BMP-responsive gene, Msx-2, was reduced during EB differentiation compared with wild-type, and activation of the Msx-2 promoter element was Smad4-dependent in ES cells and fibroblasts. Interestingly, endogenous expression of another Smad family member, Msx-3, was induced in the absence of Smad4. These results suggest that the Msx family member could be relevant biological targets for Smad4 and provide a mechanism by which perturbation of BMP-induced responses may contribute to the developmental defects of Smad4-deficient embryos.

**EXPERIMENTAL PROCEDURES**

**Generation of Smad4−/− Homozygous Fibroblasts**—Primary mouse embryonic fibroblasts (MEF) were generated from pooled E12.5 chimeric embryos obtained by injecting Smad4−/− ES cells (of 129/J origin) into C57BL/6 blastocysts (wild-type). MEFs were grown in the absence of methionine-free medium. The supernatants were collected and brought to a concentration of 0.3% Triton X-100. The fibroblasts present in 250 μl of supernatant was purified with the equivalence of 50 μl of gelatin-Sepharose beads incubated overnight at 4 °C and processed as described elsewhere (21).

**Electrophoretic Mobility Shift Assay (EMSA)**—Fibroblasts were stimulated with TGFβ (5 ng/ml) for 45 min. in 0.2% fetal calf serum, and cell extracts and binding assays were performed as described elsewhere (22). Proteins were observed by end-labelling the oligonucleotide primers with [γ-32P]ATP, and double-stranded fragments were generated by annealing with a 10-fold molar excess of the unlabeled complementary primer. Competing oligonucleotide fragments were added 10 min prior to the radiolabeled probe, and supershifting of the complex was performed by adding 1 μl of polyclonal antibody together with the probe.

**Embryoid Body Differentiation and RT-PCR Analysis**—The conditions for ES cell differentiation into embryoid bodies have been previously described (19). Extracted RNA was reverse-transcribed with the Advantage RT-PCR kit (CLONTECH) and amplified for 30 cycles using the following primers (sense:antisense): Msx-1, 5′-CCGAACCACGAGCACGGACCA-3′/5′-ACTCCGGCTGCTCTGCTCAAA–3′; Msx-2, 5′-CCGGGCTCTCTGCTGAAAG-3′/5′-CCGGCTATATGATGTCTGCTTT-3′; Msx-3, 5′-GCCACACAGAGATGGCAACAACA-3′/5′-GACATCCGCGGCAGCAAC-3′; TSC-22, 5′-CAGAGGTGACGGCAGTGK-3′/5′-TTCTCTTACGTTGTGTTCG-3′.

To control for the amount of RNA in different samples, expression of glyceraldehyde-3-phosphate dehydrogenase was analyzed. The PCR products were Southern-blotted and hybridized with end-labeled primers of sequences internal to the amplified product.

**Western Blot Analysis**—A ratio of 10 3 cells were lysed in 500 μl of CHAPS buffer (10 mM tris-HCl pH 7.5, 1 mM MgCl2, 1 mM EDTA, 0.1 mM benzamidine, 5 mM 2-mercaptoethanol, 0.5% CHAPS, 0.5% Triton X-100 and 50 μCi/ml [35S]methionine). Cells were lysed, and the ECM attached to the surface of the dish was harvested as described elsewhere (21). For fibrocartin and collagen induction, cells were seeded at a density of 2 × 104/35-mm dish. TGFβ-1 (100 ng) was added 16 h later for an additional 24 h. Proteins were labeled as described above for 2–3 h in 1 ml of methionine-free medium. The supernatants were collected and brought to a concentration of 0.3% Triton X-100. The fibroblasts present in 250 μl of supernatant was purified with the equivalence of 50 μl of gelatin-Sepharose beads incubated overnight at 4 °C and processed as described elsewhere (21).

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Western Blot Analysis—A ratio of 10 3 cells were lysed in 500 μl of CHAPS buffer (10 mM tris-HCl pH 7.5, 1 mM MgCl2, 1 mM EDTA, 0.1 mM benzamidine, 5 mM 2-mercaptoethanol, 0.5% CHAPS, 10% glycerol, and protease inhibitors) for 30 min on ice. Total protein lysate (50 μl) was fractionated on 8% polyacrylamide gel electrophoresis and immunoblotted with either rabbit polyclonal anti-Smad4 or anti-Smad3 antibodies respectively. The immunoprecipitated antibody was visualized using a horseradish peroxidase conjugated donkey anti-rabbit immunoglobulin and ECL (Amersham Pharmacia Biotech).

**RESULTS**

**Generation of Smad4-deficient ES Cells and Fibroblasts**—To determine the role of Smad4 in TGFβ-related signaling, we generated ES cell lines in which Smad4 was disrupted by homologous recombination (19). Since mice homozygous for the Smad4 mutation die at E7.5 (19, 22), Smad4−/− MEFs were derived from chimeric embryos obtained by injecting mutant ES cells, containing the selectable marker neomycin, into wild-type blastocysts. The mixed population of MEFs was selected in G418 to eliminate wild-type cells, and two immortalized Smad4-deficient 3T3 fibroblast lines (EF6-selC1 and EF7-selE) were established from independentblastocyst injections. In parallel, MEFs were cultured in the absence of selection to generate wild-type 3T3 fibroblasts (EF7). Analysis of G3 isoforms that distinguish between donor and host revealed that the two G418-resistant clones were indeed derived from mutant ES cells (Fig. 1A, lanes 10–12). Homozygosity for the

2 R. Maxson, unpublished data.
disrupted Smad4 allele in these 3T3 fibroblasts was confirmed by Southern blot analysis (data not shown) and immunoblot analysis demonstrated the absence of any detectable Smad4 protein in these cells (Fig. 1B).

**Smad4 Is Dispensable for Physiological Responses Induced by TGFβ—TGFβ signaling exerts a wide range of physiological responses, which include changes in extracellular matrix composition and growth arrest. To determine whether Smad4 is required in this pathway, the activation of extracellular matrix components in response to TGFβ was examined using [35S]methionine labeling in vivo. As expected, PAI-1 expression was induced in wild-type EF7 fibroblasts in a dose-dependent manner by treatment with TGFβ ranging in concentration from 2 to 50 pM (Fig. 2A). However, comparable induction of the endogenous PAI-1 gene was unexpectedly observed in the EF7-selE line of Smad4-deficient fibroblasts (Fig. 2A), implying that Smad4 is not required for this TGFβ-related response. This result prompted us to examine the response of other TGFβ-inducible genes encoding components of the ECM. Fibronectin (240 kDa) as well as several pro-collagens ranging in size from 185 to 205 kDa were induced to similar levels in wild-type and mutant fibroblasts by TGFβ treatment (Fig. 2B). The identity of fibronectin was confirmed using specific binding to gelatin-Sepharose beads (Fig. 2B), whereas the presence of the pro-collagens was revealed by collagenase digestion of these proteins in a dose-dependent fashion (Fig. 2C). A similar induction profile was observed with the second Smad4−/− cell line, EF6-selC1 (data not shown). These results indicate that Smad4 is not required in fibroblasts for the induction by TGFβ of the expression of endogenous extracellular matrix components and are consistent with recent studies demonstrating that Smad4-deficient human cell lines are capable of producing fibronectin in response to TGFβ (23).

Several studies have shown that TGFβ-induced growth arrest is lost during tumorigenesis (2, 3). To investigate whether Smad4 plays a role in this process, DNA replication was examined in fibroblasts by [3H]thymidine incorporation. In both wild-type EF7 and Smad4−/− cells, growth inhibition was first detected at a TGFβ concentration of 2 pg/ml and reached a maximum (2-fold growth inhibition) at 1 ng/ml (Fig. 2D). These values are comparable to those previously reported for TGFβ-responsive epithelial cells (24). These data indicate that Smad4 is not required for TGFβ-induced growth arrest, a finding in apparent disagreement with previous work (25–28). However, these previous studies were conducted using either human Smad4-deficient cell lines derived from carcinomas known to accumulate numerous additional mutations or overexpression of a dominant negative mutation of the Smad4 protein, which could lack specificity.

Because several studies implicating Smad4 in the TGFβ pathway were based on activation of the 3TP-lux reporter gene (25, 26, 28–30), which contains part of the PAI-1 promoter, we assessed its inducibility in our system. Although transfected 3TP-lux was activated in response to TGFβ in wild-type EF7 fibroblasts, it was not activated in Smad4-deficient fibroblasts (Fig. 2E). TGFβ-dependent activation of 3TP-lux in mutant cells could be restored to wild-type levels by co-transfection with Smad4 (Fig. 2E). Thus, consistent with previous studies, we find that Smad4 is required for activation of the 3TP-lux reporter in our murine fibroblasts. However, because activation of the endogenous PAI-1 gene does not require Smad4 (Fig. 2A), 3TP-lux activation may not be an accurate representation of PAI-1 activation. Rather, it may reflect TGFβ-mediated activation of the 12-O-tetradecanoylphorbol-13-acetate-responsive elements that were added to the construct to enhance TGFβ responsiveness (21). In agreement with this notion, a reporter plasmid comprising only 12-O-tetradecanoylphorbol-13-acetate-responsive elements has been shown to be as responsive to TGFβ as the 3TP-lux reporter (31–33) and Smad3 and -4 can interact with Jun family members and enhance their transcriptional activation (33–35).

**Differential Binding of Smad4 to TGFβ-responsive Regulatory Elements of the PAI-1 Promoter—**To understand the mechanism underlying Smad4-independent activation of TGFβ-inducible genes in fibroblasts, we examined the binding of nuclear protein complexes to the human PAI-1 promoter. Two TGFβ-dependent regulatory regions have been identified in the human PAI-1 promoter at positions −580 and −730 from the transcriptional start site. These regions are flanked by several E boxes (Fig. 3A) capable of binding the basic helix-loop-helix transcription factor, TFE-3, which appears to be important for TGFβ-dependent activation of PAI-1 (36). Two Smad binding sites in the −580 regulatory region have also been implicated in PAI-1 activation (9, 36), one of which (AGNCAGA) is also required for activation in the −730 region (9). Interestingly, EMSA using a probe (PE2.1) specific for the −580 regulatory region (36) revealed that extracts from both wild-type and Smad4-deficient fibroblasts could form a DNA-binding complex in response to TGFβ stimulation (Fig. 3B, lanes 1–5), suggesting that Smad4 is not required for DNA binding within that region. Consistent with this finding, anti-Smad4 as well as anti-Smad2 or -3 antibodies did not result in a supershift of the
FIG. 2. Smad4 is not required for physiological responses induced by TGFβ but is essential for 3TP-lux activation in Smad4-deficient fibroblasts. A, dose-dependent activation of endogenous PAI-1. Wild-type (+/+ ) and mutant (−/− ) cells were treated with increasing concentration of TGFβ (values in pm), and the protein content of the extracellular matrix was assessed by [35S]methionine in vivo labeling and resolved by 10% SDS-polyacrylamide gel electrophoresis. The molecular mass markers are shown at the left of the gel, and the 45-kDa PAI-1 protein is indicated by an arrow. B, fibronectin induction in response to TGFβ treatment. Cells were cultured in the presence (+) or absence (−) of TGFβ. Supernatants of [35S]methionine-labeled cells were either untreated (lanes 1–4) or pre-treated with gelatin-Sepharose beads (lanes 5–8) and resolved using 5% SDS-polyacrylamide gel electrophoresis. The arrow indicates the 240-kDa fibronectin protein, and the bracket indicates pre-collagen bands. C, identification of pro-collagens by collagenase treatment. Supernatants from wild-type TGFβ-treated fibroblasts obtained as described for the fibronectin assay were subjected to increasing concentrations of collagenase (Form III) and resolved using 5% SDS-polyacrylamide gel electrophoresis. Pre-collagen bands are indicated by the bracket and were induced in both wild-type and mutant fibroblasts (see panel B). U, units. D, TGFβ-induced growth inhibition of Smad4-deficient fibroblasts. Thymidine uptake levels of wild-type (squares) and Smad4-deficient (triangles) fibroblasts were examined in response to serial dilutions of TGFβ. The values for thymidine incorporation are expressed relative to cpm obtained in the absence of TGFβ. Error bars represent the standard deviation of the mean. E, Smad4 is required for the ligand-dependent activation of the 3TP-lux reporter construct. Wild-type (open bars) and mutant (hatched bars) fibroblasts were transfected in triplicate with the 3TP-lux reporter in the absence (−) or presence (+) of TGFβ (50 pm). Ligand-dependent luciferase activity was detected only in wild-type cells (+/+ ) but could be restored in mutant cells (−/− ) by co-transfection with Smad4. Error bars represent the standard deviation of the mean. Similar results for all assays described in this figure were reproduced at least twice for both E7-SEIE and EF6-SEL1 mutant clones. Only results from representative experiments using the clone E7-SEIE are shown.

protein-DNA complex (Fig. 3B, lanes 6–10). Furthermore, binding to the PE2.1 probe could be competed with as little as 3-fold excess of an oligonucleotide mutated in either of the two Smad binding sites; this was similar to the efficiency of wild-type unlabeled oligonucleotide competed with the radiolabeled PE2.1 probe (Fig. 3B, lanes 11–18 and 22–24). However, up to a 30-fold excess of mutant E box oligonucleotides could not compete for binding to the PE2.1 probe (Fig. 3B, lanes 19–21 and 22–24). These results indicate that the TGFβ-inducible DNA-binding complex associated with PAI-1 activation in fibroblasts requires an intact E box but does not depend on Smad4 or any Smad binding elements present in the −580 region.

In contrast to the −580 region, analysis of the −730 PAI-1 region using the TRS probe (10) revealed that a TGFβ-inducible DNA-binding complex could form in extracts from wild-type but not Smad4-deficient fibroblasts (Fig. 3C, lanes 1 and 2 compared with lanes 12 and 13). Further analysis of the complex formed on the TRS site showed that binding was dependent on the Smad element, because unlabeled wild-type oligonucleotide, but not an oligonucleotide mutated in the Smad binding site (TRSm4), was able to compete for binding (Fig. 3C, lanes 3–8). Antibodies against Smad3 and Smad4, but not Smad2, were able to supershift the protein-DNA complex (Fig. 3C, lanes 9–11), indicating that both Smad3 and Smad4 were present in the binding complex. Together, these data indicate that formation of a TGFβ-inducible complex on the TRS is dependent on Smad4 and, in its absence, Smad3 cannot bind to the TRS sequence. Thus, the PAI-1 promoter contains two TGFβ-responsive elements that differ in their requirement for Smad4. Our results suggest that the −580 regulatory region may be the major determinant of endogenous PAI-1 induction in the absence of Smad4.

Smad4 Is Required for Other TGFβ/Activin-dependent Responses—Activation of the TGFβ/activin pathway leads to induction of FAST target genes through the formation of the ARF (5). To investigate whether Smad4 is required for the activation of FAST target genes, we tested the regulation of the A3-lux reporter gene (37) in ES cells that express endogenous FAST-2. In wild-type ES cells, A3-lux was induced by a constitutively activated activin type I receptor (ActRIB) in a dose-dependent manner (Fig. 4A). However, in two independent Smad4−/− ES cell clones, although A3-lux was not activated in response to activin signaling (Fig. 4A), its induction could be restored to wild-type levels by co-transfection of Smad4 (Fig. 4B). Interestingly, A3-lux could also be activated in Smad4−/− ES cells by overexpression of Smad2 and FAST-1 (Fig. 4C). This rescue was Smad2-specific, because overexpression of Smad3 and FAST-1 failed to induce A3-lux responses (Fig. 4C), consistent with the recent demonstration that Smad3 does not activate certain FAST target genes (6). The expression of Smad3 in the transfected cell population was confirmed by Western blotting (Fig. 4C, inset). These results suggest that in the absence of
Smad4, TGFβ/activin-induced target genes can be activated by increased expression of other components of the ARF.

**Max Family Members Are BMP-responsive Targets for Smad4**—Smad4 has also been implicated in BMP signaling based on its interaction with the receptor-regulated Smad1 and its ability to induce the same mesoderm markers as BMP-2/4 in *Xenopus* (38). BMP2/4 activates the homeobox-containing genes *Msx-1* and -2 in various tissues undergoing epithelial-mesenchyme transition. To determine the role of Smad4 in BMP signaling, activation of the endogenous Max gene family was analyzed during ES cell differentiation into embryoid bodies (EB). RT-PCR analysis of *Msx-1* and -2 expression revealed that these genes were expressed at all time points examined in wild-type and heterozygous ES cells (Fig. 5A). However, in *Smad4*−/− EB, expression of *Msx-2* was markedly reduced, whereas that of *Msx-1* was variable at different stages (Fig. 5A). These results suggest that Smad4 is required for the activation of *Msx-2* and for the suppression of *Msx-3* endogenous expression during EB differentiation. Similar to BMP signaling, TGFβ plays an active role during embryogenesis and ES cell differentiation (39). To address the role of Smad4 in TGFβ signaling in the EB, we examined the expres-

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**FIG. 3.** Differential requirement for Smad4 for two regulatory elements of PAI-1 promoter. A, schematic diagram of the −580 and −730 regulatory regions of the human PAI-1 promoter. The Smad binding elements are shown, including the TRS box (10), the CAGA box (11), the E box (36), the nucleotide changes in the competing oligonucleotide used in EMSA analysis are shown below the probe sequence. B, EMSA analysis of the −580 regulatory region using the PE2.1 radiolabeled probe indicates that Smad4 is dispensable. Two DNA-protein complexes (I and II) are enhanced after TGFβ treatment in both wild-type (wt) and the Smad4-deficient EF7-seI (mut) fibroblast cell lines (lanes 1–3). Supershift of the DNA-protein complex formed with extracts of TGFβ-treated wild-type cells was performed using antibodies (Ab) against Smad2 (S-2, −3 (S-3), and −4 (S-4) (lanes 6–10). EMSA reactions were also competed for binding to the PE2.1 probe with increasing molar excess of wild-type (PE2.1) or mutated oligonucleotides (Sm, CAGm, Em) (lanes 11–13). The c indicates a control probe without extract (lane 5). C, Smad4 is required for the formation of a DNA-binding complex in the −730 region. EMSA analyses were performed as described above, except that the radiolabeled TRS probe was used. TGFβ treatment induced the formation of a DNA-binding complex (arrow) only in wild-type cells (lanes 1–11) and not in Smad4-deficient EF7-seI cells (lanes 12 and 13). Binding of the DNA-protein complex was competed with unlabeled wild-type TRS oligonucleotides but not with oligonucleotides mutated at the Smad binding site (TRSm4). Supershift (bracket) of the protein-DNA complex was obtained with anti-Smad2 and -4 but not anti-Smad3 antibodies. Similar results were obtained for the Smad4-deficient clone, EEF6-seC1, and all results were reproduced at least twice for each clone.

**FIG. 4.** Circumventing the requirement for Smad4 in some TGFβ/activin responses by overexpression of other signaling components. A, Smad4 is essential for the activation of the A3-lux reporter in ES cells. Wild-type ES cells (open bars) and two independent Smad4-deficient ES cell clones C8-24 (hatched bars) and F9-2 (solid bars) were co-transfected with wild-type (WT) or activated (A) ActRIB in addition to the A3-lux reporter. Dose-dependent induction of A3-lux was observed with decreasing amounts of activated ActRIB (values in μg) in wild-type but not in Smad4-deficient cells. B, rescue of A3-lux activation in response to activin signaling by Smad4 over-expression in mutant ES cells. C, rescue of A3-lux activation in response to activin signaling in Smad4-deficient ES cells by over-expression of FAST-1 and Smad2 but not FAST-1 and Smad3. Inset, Western blot analysis of total protein extracted from the various transfected cell population using anti-Smad4 antibody. Although similar results were obtained for both Smad4−/− ES clones, only those obtained for clone C8-24 are shown in B and C.
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However, when Smad4 was co-transfected into mutant fibroblasts, Msx2-lux activity was restored in response to BMP activation. Moreover, when Smad4-deficient cells were transfected in combination with Smad1, Smad4 restored the 10–15-fold increase in the activity of the Msx-2 promoter as seen for the wild-type cells (Fig. 5B). Activation of Msx2-lux was specific to the BMP pathway, because exogenous TGFβ or expression of the activated activin receptor failed to induce Msx2-lux in wild-type fibroblasts even when Smad2 and Smad4 were over-expressed (data not shown). Identical results were obtained in a second Smad4−/− fibroblast clone as well as in two independent Smad4−/− ES clones. These results demonstrate that Smad4 is an obligate signaling component for activation of the Msx-2 promoter.

**DISCUSSION**

TGFβ Induces Smad4-independent Physiological Responses—We have shown that targeted disruption of Smad4 in murine fibroblasts does not alter either TGFβ-induced growth arrest or endogenous production of ECM components. Analysis of TGFβ-inducible nuclear complexes binding to the human PAI-1 promoter revealed the existence of both Smad4-dependent (−730) and -independent (−580) regulatory regions. Interestingly, the Smad4-independent region required an intact E box, to which the transcription factor TFE-3 is known to bind and activate PAI-1 expression (36). Moreover, in wild-type cells the DNA-binding complex contains TFE-3 but appears to lack Smad proteins, suggesting that, under physiological conditions in response to TGFβ, Smads might not contribute to TFE3-mediated PAI-1 activation. Our results therefore, define a possible mechanism by which biological responses induced by TGFβ utilize Smad4-independent pathways. Previous studies have suggested that Smad4 act as a stabilizer of the CREB binding protein (CBP/p300 and Smad3 complex in the −730 regulatory region of PAI-1 (11). Our studies support this hypothesis because, in the absence of Smad4, the TGFβ-inducible DNA-binding complex, which normally contains Smad3, cannot bind DNA.

Since the mechanism of growth inhibition by TGFβ is well established in epithelial cells, the dispensability of Smad4 for this response might be a finding specific to fibroblasts. However, we deem this possibility unlikely, because the induction of the T3P-lux and A3-lux reporters, which are both widely employed as TGFβ/activin-responsive vectors, requires Smad4 in both T3P fibroblasts and epithelial cells. These results imply that the fibroblasts are a valid system to use in the study of TGFβ signaling and suggest that Smad4 might not be the only effector of TGFβ-induced growth arrest. Consistent with this notion, evi-1 was shown to antagonize TGFβ-induced growth arrest by interacting specifically with Smad3 (41), and the targeted inactivation of Smad3 renders murine fibroblasts resistant to TGFβ inhibitory effects (42). Alternatively, growth inhibition could be mediated by an as yet unidentified mammalian Smad4 homologue that is able to compensate for Smad4 deficiency. Although such a Smad4 homologue has been identified in Xenopus and shown to possess distinct biological properties (43, 44), its mammalian counterpart has not yet been identified. Several lines of evidence support the notion that some TGFβ-related responses do not require Smad4. For instance, induction of fibronectin in human cells in response to TGFβ requires c-Jun NH2-terminal kinase (JNK) activity independent of Smad4 (23). Similarly, in Drosophila the Smad4 homologue, Medea, was found to mediate only some Dpp responses (45). Thus, our results support the idea that Smad4 may not, in fact, be a central mediator in the transduction of all TGFβ-related signals.

The experiments designed to address the role of Smad4 in
mediating TGFβ responses in the human tumor cell line have been somewhat controversial. Although some studies show that the MDA-MB468 Smad4-deficient cell line is not responsive to TGFβ (26, 28, 29), the growth of COLO 357 Smad4-deficient cell lines can be inhibited by TGFβ (46). Moreover, it has been shown that stable overexpression of Smad4 can restore TGFβ-induced growth arrest in the MDA-MB468 cell line (28) but cannot do so in the SW480 cell line, although it restores other TGFβ-induced responses (47). In another study, in which both loci of Smad4 were interrupted in their M1I domain by an in-frame fusion with selectable markers, it was reported that loss of Smad4 function resulted in an acquired resistance to TGFβ growth inhibition (27). However, because these cell lines are not true null mutants for Smad4, it is unclear whether the remaining amino terminus of Smad4 in the fusion protein could interfere with the signaling of other Smads. Although the mechanism of growth inhibition could differ between fibroblasts and epithelial cells, our results suggest that Smad4 mutations may perturb TGFβ-signaling during tumorigenesis by mechanisms other than a failure in growth inhibition.

Circumventing the Smad4 Requirement by Increasing Other Signaling Components—Smad4 was shown to be essential for the transcriptional activation of the Mix.2 promoter in Smad4-deficient SW480.7 colon carcinoma cell line (48). We find, however, that in ES cells the Smad4 requirement for activation of the Mix.2 gene is dependent on the levels of FAST-1 and Smad2. Thus, in the absence of Smad4, the ability of the target genes to respond to TGFβ-related ligand could depend on the cumulative levels of the other endogenous signaling components of the pathway. When the abundance of signaling components is low, the role of Smad4 in some responses might be to stabilize the transcriptional complex. Such a stabilizing role has been attributed to Smad4 in the ARF (48, 49). It is unclear, however, whether this is its only role and whether an increase in the levels of the other components of the complex consistently bypasses the requirement for Smad4 in other gene targets. This hypothesis remains untested for other known promoters responsive to TGFβ-related ligands, such as Msx-2, because the transcription factor regulating their expression have yet to be identified.

Loss of Smad4 Results in Oppositely Deregulated Expressions of Msx-2 and Msx-3—Because important biological responses induced by TGFβ/activin signaling appeared unaffected in Smad4-deficient SW480.7 colon carcinoma cell line (48). We found an obligate requirement of Smad4 for activation of the Mix.2 promoter in response to BMP in both murine ES cells and fibroblasts. Moreover, a consistent decrease of Msx-2 and an increase of Msx-3 endogenous expression was observed throughout in vitro differentiation of Smad4-deficient ES cells relative to wild type. In contrast, expression of Msx-1 appeared more sustained in mutant embryoid bodies. Because Msx-1 and Msx-2 are jointly expressed in the same tissues, these results show that Smad4-deficient ES cells were capable of proper differentiation into tissues where these genes are usually expressed. The unexpected finding that Msx-3 is inversely regulated by Smad4 relative to Msx-2, suggest that Smad4 can regulate gene expression, even within the same family member, in an opposite manner. Thus, it is possible that in response to BMP-4 signaling, Smad4 could interact with positive and negative transcriptional regulators to inversely regulate the expression of Msx-2 and Msx-3, respectively. These results are consistent with the expression pattern of Msx family members in rhombomeres 3 and 5 of the hindbrain. Although the expression of Msx-2 is increased in response to BMP-4 production by neighboring rhombomeres (15), that of Msx-3 is repressed in rhombomeres 3 and 5 (13).

Several studies identified various homeodomain proteins that can bind different Smads (50, 51) and in some cases act as corepressors in the transcriptional complex (52). Moreover, Smad1 and Smad4 can interact with Hox homeodomain proteins and abrogate the Hox-dependent transcriptional repression of a BMP-responsive gene (51). It is possible that a similar mechanism regulates Msx-2 expression, because specific Hox genes determine the rostrocaudal patterning of the rhombomeres (53). It is unclear at this stage whether expression of Msx-3 is regulated by BMP-4 or other TGFβ-related family members such as BMP-2, BMP-7, and DaI-1, which all have dorsaling properties on neural tube development (53). Moreover, the regulation of Msx-3 expression could be induced by other factors, such as fibroblast growth factor, which has been shown to be involved in dorsal/ventral patterning of the neural tube (53) and to activate Msx gene expression (54), or by retinoic acid, known to regulate homeobox genes including those from the Msx family (55). Regardless, the Msx family member appears to be a relevant biological target for Smad4 whereby perturbation of Smad4 signaling could have consequences in neural development.

We have previously shown that Smad4 is important for proper development of the visceral endoderm (VE) in the murine embryo and embryoid bodies and that the latter also failed to cavitate (19). The process of cavitation is mediated by apoptotic signals emanating from the VE surrounding the solid embryonic ectoderm, which transform it into columnar epithelium (56). Recent studies using the dominant-negative BMP receptor have indicated that BMP signaling is involved in proper VE differentiation (57). Thus, the lack of cavitation in Smad4−/− EB possibly results from defective BMP signaling and Msx-2 expression in the VE. This hypothesis is consistent with the lack of cavitation observed in P19 cells, which have reduced levels of Msx-2 compared with ES cells and, when overexpressed, Msx-2 enhances programmed cell death (58). However, to clearly address whether Msx family members are physiological targets of Smad4 in this process, we are currently investigating whether overexpression of Msx-2 in Smad4−/− EB could restore proper differentiation of the VE and induce cavitation. Inversely, overexpression of Msx-3 in wild-type EB could result in perturbed development of the VE.

Thus, we have provided evidence that the loss of Smad4 function in murine fibroblasts does not affect many of the biological responses induced by TGFβ, suggesting that the physiological targets of Smad4 in tumorigenesis could differ from those predicted. Moreover, Smad4 appears to play an intricate role in regulating the expression of some Msx family members in response to BMP, and the deregulated expression of these genes could be responsible for the various defects of Smad4−/− embryos. The identification of transcriptional targets of Smad4 will allow us to understand the mechanisms by which Smad4 contributes to the development of the embryo and tumor suppression.

Acknowledgments—We thank M. Whitman for the A3-lux reporter construct, R. Derynck for Smad3, A. Nakao and P. ten Dijke for the purified collagenase, Alex Grossman, Vuk Stambolic, and José-Luis de la Pompa for critical reading of the manuscript, and Mary Saunders for scientific editing.

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