Functional Characterization of Transforming Growth Factor β Signaling in Smad2- and Smad3-deficient Fibroblasts*

A prominent pathway of transforming growth factor (TGF)-β signaling involves receptor-dependent phosphorylation of Smad2 and Smad3, which then translocate to the nucleus to activate transcription of target genes. To investigate the relative importance of these two Smad proteins in TGF-β signal transduction, we have utilized a loss of function approach, based on analysis of the effects of TGF-β on fibroblasts derived from mouse embryos deficient in Smad2 (S2KO) or Smad3 (S3KO). TGF-β caused 50% inhibition of cellular proliferation in wild-type fibroblasts as assessed by [3H]thymidine incorporation, whereas the growth of S2KO or S3KO cells was only weakly inhibited by TGF-β. Lack of Smad2 or Smad3 expression did not affect TGF-β-induced fibronectin synthesis but resulted in markedly suppressed induction of plasminogen activator inhibitor-1 by TGF-β. Moreover, TGF-β-mediated induction of matrix metalloproteinase-2 was selectively dependent on Smad2, whereas induction of c-fos, Smad7, and TGF-β1 autoinduction relied on expression of Smad3. Investigation of transcriptional activation of TGF-β-sensitive reporter genes in the different fibroblasts showed that activation of the (Smad binding element)α-Lux reporter by TGF-β1 was dependent on expression of Smad3, but not Smad2, whereas activation of the activin response element-Lux reporter was strongly suppressed in S2KO fibroblasts but, on the contrary, enhanced in S3KO cells. Our findings indicate specific roles for Smad2 and Smad3 in TGF-β1 signaling.

Transforming growth factor (TGF)-β is the prototypic member of the TGF-β superfamily and mediates a multiplicity of biological effects on different cell types. TGF-β regulates cellular proliferation, induces synthesis of extracellular matrix proteins such as fibronectin and plasminogen activator inhibitor-1 (PAI-1), modulates the immune response, and plays an important role in embryonic development and cellular differentiation (1).

TGF-β evokes its biological effects by signaling through two different receptor serine/threonine kinases, TGF-β receptor type (TβR)-I and TβR-II, that form a tetrameric complex after binding of TGF-β to TβR-II. TβR-II activates TβR-I by phosphorylation of serine residues in the GS box. The anchor protein SARA (Smad anchor for receptor activation) recruits the cytoplasmic signal transducers Smad2 and Smad3, classified as so-called receptor-activated Smads (R-Smads), to the Tβ R-I kinase domain, resulting in their phosphorylation on serine residues in the C-terminal SXSX motif. Activated R-Smads heterologomerize with the common partner (CO)-Smad4, and these complexes are transported into the nucleus, where they regulate gene expression. R-Smads and CO-Smads contain two highly conserved domains, the Mad homology (MH) I domain and the MH2 domain, which are connected by a linker region. Whereas their MH1 domains can interact with the DNA, the MH2 domains are endowed with transcriptional activation properties.

Down-regulation of TGF-β signaling is effected, in part, by a feedback mechanism involving induction of expression of the inhibitory Smads, Smad6 and Smad7, which then prevent R-Smad activation (2, 3). Absence of Smad2 or Smad3 expression resulting from targeted deletion of the respective Smad genes in mice has revealed different developmental roles for Smad2 and Smad3. Homozygous loss of function mutations of the Smad2 gene by targeted deletion of the MH1 or MH2 domain resulted in embryonic lethality due to failure to establish an anterior-posterior axis, gastrulation, and mesoderm formation (4, 5). These events are controlled by Smad2-dependent signals from the visceral endoderm (6, 7). Postgastrulation-rescued Smad2 mutant embryos survived up to embryonic day 10.5 but showed several malformations such as cyclopia, cranial abnormalities, and impaired left-right patterning as observed by abnormal heart loops and embryo turning (7).

In contrast, mice harboring homozygous deletions of the Smad3 gene are viable and survive for several months, indicating that Smad3 is dispensable for embryonic development. However, Smad3 knockout mice are smaller than wild-type littermates and show forelimb malformations (8, 9). Mice lacking expression of Smad3 die from chronic inflammation of several organs as a consequence of impaired immune function.
including defects in mucosal immunity, as revealed by abscesses in tissues adjacent to mucosal membranes, and expansion of activated T-cell populations. This can be attributed in part to the lack of responsiveness of Smad3-deficient T cells to the growth-inhibitory effects of TGF-β as well as to a defective chemotactic response of Smad3-deficient neutrophils to TGF-β (8, 9). Smad3-deficient mice show accelerated wound healing compared with wild-type littermates, which is a consequence of enhanced re-epithelialization by proliferating keratinocytes and reduced wound infiltration as well as TGF-β production by monocytes (10). Homozygous Smad3 knockout mice generated by Zha et al. (11) die from colon carcinomas between 4 and 6 months of age, a phenotype that was not observed in Smad3-null mice derived by Datto et al. (8) or Yang et al. (9).

To investigate the relative importance of Smad2 and Smad3 in TGF-β1 signaling, we have established mouse embryo-derived fibroblasts lacking expression of the Smad2 or Smad3 gene (7, 9). In contrast to analysis of the function of Smad2 and Smad3 by overexpression studies, these loss of function cell systems provide a more appropriate model to investigate the physiological roles and relative importance of these R-Smads in TGF-β signaling and provide insight into the consequence of impaired TGF-β R-Smad function in relation to pathophysiology. Our data show that expression of Smad2 or Smad3 in fibroblasts is important for TGF-β1-mediated growth inhibition as well as for synthesis of PAI-1, whereas Smad2 and Smad3 contribute uniquely to TGF-β1-induced activation of several luciferase reporter constructs. We further show that certain genes are selectively dependent on only one of these two TGF-β receptor-activated Smads, such as, for example, the matrix metalloproteinase MMP-2, which is critically dependent on Smad2 but not Smad3 expression. Collectively, our results indicate nonredundant roles for Smad2 and Smad3 in TGF-β1-mediated signaling and provide insight into the targets of these specific signaling pathways in vivo.

**EXPERIMENTAL PROCEDURES**

**Generation of Mouse Embryo-derived Fibroblasts and Primary Derivative Fibroblasts**—With the embryonic purification method (Qiagen, Santa Clara, CA) following the manufacturer's protocol. For Northern blot analysis, RNA (10 µg) was electrophoresed on 1% agarose gels and transferred to Nytran-N nylon membrane (Schleicher & Schuell). Membranes were hybridized with the relevant 32P-labeled cDNA probes in Church buffer (16) or QuickHyb solution (Stratagene) according to the manufacturer's protocol and analyzed by phosphorimaging or by exposure to X-ray film. Equal RNA loading was established by ethidium bromide staining of 28S and 18S rRNA, or, alternatively, membranes were hybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18S RNA cDNA probe. Hybridizations were performed using the following cDNA inserts: 1300-bp Nhel/Xho fragment of murine p15(ink4b) (420-bp EcoRI fragment of murine p121(ink4a)), 650-bp Nhel/Xho fragment of mouse p27(kip1), 2200-bp BamHI/HindIII fragment of rat c-fos, 1300-bp EcoRI/HindIII fragment of murine Smad7, and 1000-bp HindIII/XbaI fragment of TGF-β1.

**Extracellular Matrix Protein Assays—**TGF-β1-induced fibroinectin and PAI-1 synthesis was assessed by as described by Whara et al. (17). MMP-2 was co-purified with fibroinectin using gelatin-Sepharose beads in the fibronectin assay. MMP-2 zymography was basically performed as described by Kleiner and Stetler-Stevenson (18), using gelatin-Sepharose affinity-purified proteins from fibroblast-conditioned medium. Briefly, protein samples were separated under nonreducing conditions on an 8% SDS-polyacrylamide gel containing 1 µg/ml gelatin. The gel was incubated for 1 h at room temperature in 2.5% Triton X-100 on a rotary shaker, followed by incubation for 18 h at 37°C in enzyme buffer containing 50 mM Tris, pH 7.5, 0.5 mM CaCl2, 5 mM NaCl, and 0.5% NP-40. The gel was then washed with H2O, stained with Gelcode Blue Stain Reagent (Pierce, Rockford, IL) according to the manufacturer's protocol, and dried on 3MM paper.

**Transcriptional Reporter Assays—**3TP-Lux reporter construct, ARE-luciferase reporter construct, and forkhead activin signal transducer (FAST)-1 construct and (SBE)2-luciferase reporter construct were provided by Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center),...
Dr. M. Whitman (Harvard Medical School, Boston, MA), and Dr. P. ten Dijke (Ludwig Institute for Cancer Research, Uppsala, Sweden), respectively. For transcriptional reporter assays, fibroblasts were seeded at a density of 10^5 cells/6-well dish. The next day, cells were transfected with the different luciferase reporter constructs using FuGene6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer’s protocol. For Smad reconstitution experiments, pf1Smad2 or pf1Smad3 was co-expressed with the respective luciferase reporter plasmid pSV-b-galactosidase (Roche Molecular Biochemicals) at MOI 40. Smad2, Smad3, and truncated Smad3 were overexpressed in COS-1 cells, and protein extracts were used as controls. The Smad3-specific antibody was raised against the linker region of Smad3. B, effect of truncated Smad3 on Smad2/FAST-1-mediated ARE reporter induction. NMuMG cells were transfected with the ARE-luciferase reporter and FAST-1 in combination with the indicated Smad proteins (control; □, Smad2; ▪, Smad3; △, Smad3ΔN). Representative results are shown as the average of triplicate observations corrected for transfection efficiency as measured by β-galactosidase activity.

**RESULTS**

**Analysis of Smad Expression in S2KO and S3KO Fibroblasts**—Fibroblasts deficient in Smad2 or Smad3 gene expression were derived from mice in which the respective Smad alleles are disrupted by homologous recombination resulting in targeted deletion of exon 2 in Smad2 (7) and exon 8 in Smad3 (9), respectively. Western blot analysis of cellular lysates derived from S2KO fibroblasts and S2KO ES cells using specific antibodies against various peptide sequences in the N-terminal and C-terminal domains of Smad2 demonstrated that the Smad2 deletion in exon 2 resulted in a null allele (7, 17). Western blot analysis of lysates from S3WT and S3KO fibroblasts, primary MEFs, primary DFs, or lung tissue showed that S3WT cells and S3WT lung tissue express full-length Smad3, whereas neither full-length nor truncated Smad3 protein could be detected in S3KO cells or S3KO lung tissue using a specific antibody that was raised against the Smad3 middle linker region (Fig. 1A). The antibody did detect truncated Smad3 that was overexpressed by transient transfection in COS-1 cells.

Although we were unable to detect expression of truncated Smad3 protein in tissues or fibroblasts derived from S3KO mice, we assessed whether expression of a putative truncated Smad3 protein could interfere with Smad2 signaling by using a Smad2-dependent transcriptional reporter assay (20, 21). Using NMuMG murine mammary gland epithelial cells, we observed that TGF-β1 efficiently induced the ARE-luciferase reporter in the presence of co-expressed forkhead activin signal transducer FAST-1 and that this induction was further enhanced after co-expression of Smad2 (Fig. 1B). Whereas overexpression of full-length Smad3 abrogated the TGF-β1-dependent activation of the ARE reporter mediated by Smad2/FAST-1, as reported previously (22, 23), expression of truncated Smad3 enhanced ARE reporter activation, presumably by its ability to act as a dominant negative inhibitor of endogenous Smad3 (9). These data suggest that even if it were expressed, this truncated version of Smad3 would not interfere with Smad2-mediated TGF-β1 signal transduction (Fig. 1B).

Loss of Basal Proliferation and TGF-β1 Growth-inhibitory Response in Smad2- and Smad3-deficient Fibroblasts—Although TGF-β can stimulate proliferation in several fibroblast cell lines, it is known to inhibit the growth of primary MEFs (8, 24). We examined the incorporation of [3H]thymidine to examine the roles of Smad2 and Smad3 in control of basal rates of proliferation and in transducing growth control signals by TGF-β1 in the different fibroblasts.

Basal rates of [3H]thymidine incorporation were 2-fold higher in S2WT compared with S2KO fibroblasts and 8-fold higher in S3WT compared with S3KO cells (Fig. 2). Inspection of cell cultures during the experiments did not reveal significant differences in cell attachment, cell death, or cell densities before and after labeling. These results indicate that lack of either Smad2 or, in particular, Smad3 is associated with decreased cellular proliferation rates in regular growth medium.

In low-density cultures (10,000 cells/24-well dish) of S2WT or S3WT fibroblasts, TGF-β1 treatment for 24 h reduced [3H]thymidine incorporation by 46% (p = 0.001) and 54% (p = 0.01) when compared with untreated cells, respectively (Fig. 2). In contrast, TGF-β1 had only a modest effect on [3H]thymidine incorporation in S2KO (12% reduction) and S3KO fibroblasts (16% reduction) compared with untreated cells, respectively. At a higher plating density (15,000 cells/24-well dish), [3H]thymidine incorporation was inhibited in response to TGF-β1 by 36% in S3WT and 9% in S3KO fibroblasts and stimulated by 13% in S2KO fibroblasts compared with untreated cells, respectively (data not shown). Our observations demonstrate that lack of either Smad2 or Smad3 markedly reduces the sensitivity of fibroblasts to growth inhibition by TGF-β1. However, it should be noted that the relative absence of a growth-inhibitory response in these cells is associated with already substantially reduced basal growth rates when compared with the relevant WT fibroblasts.

**Defects in p15 and p21 Regulation in Smad-deficient Fibroblasts**—Regulation of the cyclin-dependent kinase inhibitors p15^INK4b, p21^CIP1/WAF1, and p27^Kip1 has been shown to mediate cell cycle control by TGF-β, depending on cell type and context.
In both S2WT and S3WT fibroblasts, TGF-β1 strongly induced steady-state mRNA expression of p27, peaking at 5.1-fold and 4.9-fold, respectively, at 4 h (2.9-fold, respectively). The importance of Smad3 in induction of p27 mRNA expression by TGF-β1 was further confirmed in primary MEFs and primary DPFs. Thus, adenoviral-mediated reintroduction of Smad3 expression in the S3KO cells (Fig. 1A) restored induction of Smad7 mRNA expression by TGF-β1 (Fig. 4C), whereas infection with recombinant adenovirus expressing β-galactosidase had no effect (data not shown). These observations demonstrate that Smad3 plays an important role in the induction of c-fos and Smad7 expression by TGF-β1.
It is well known that TGF-β1 can induce its own gene expression, in part through the Ras/mitogen-activated protein kinase signaling pathway that impinges on the transcriptional activation complex activator protein 1 (AP-1) (26, 27). Analysis of TGF-β1 autoinduction in the fibroblasts revealed that both basal and autoinduced expression of TGF-β1 was strongly suppressed in S3KO cells, in contrast to S2KO cells (Fig. 4D). This observation was further confirmed in primary S3WT and S3KO MEFs and DFs. Thus, adenoviral reintroduction of Smad3, but not β-galactosidase (data not shown), in the primary S3KO MEFs and DFs restored autoinduction of TGF-β1 to levels observed in S3WT cells (Fig. 4E). Due to early embryonic lethality of the S2KO mice and the technical complications in deriving embryonic fibroblasts, we were unable to perform similar experiments for primary S2WT and S2KO MEFs and DFs. In conclusion, these data show that Smad3 plays an
important role in autoregulation of TGF-\(\beta\)1 expression in both primary and established fibroblast cell cultures.

\textit{TGF-\(\beta\)1-induced Expression of Extracellular Matrix Proteins Is Partially Dependent on Smad2 and Smad3 Expression—} TGF-\(\beta\) is known to induce synthesis of several extracellular matrix proteins, including fibronectin and PAI-1, in many different cell types, and these have recently been shown to be Smad-independent and Smad-dependent, respectively (8, 28).

As shown in Fig. 5A, induction of fibronectin synthesis in WT fibroblasts by TGF-\(\beta\)1 was similar to that in S2KO or S3KO fibroblasts, consistent with the observation that Smad4 is dispensable for TGF-\(\beta\)1-induced fibronectin synthesis and that expression is instead dependent on expression and activation of c-Jun N-terminal kinase (28). However, the possibility that Smad2 and Smad3 can substitute for each other in induction of fibronectin synthesis cannot be ruled out.

As shown in Fig. 5A, a TGF-\(\beta\)1-induced 72-kDa protein co-purified with fibronectin in these assays. Based on its molecular mass, the fact that it was secreted into the conditioned medium, its affinity for gelatin, and its inducibility by TGF-\(\beta\)1, we hypothesized that this protein could possibly represent collagenase IV/72-kDa gelatinase/MMP-2, which has been reported to be induced by TGF-\(\beta\)1 as assessed by zymography, metabolic labeling, and MMP-2 Western blot analysis, using gelatin-Sepharose-purified conditioned medium from fibroblasts as described above. C, TGF-\(\beta\)1-induced PAI-1 production in S2WT, S2KO, S3WT, and S3KO fibroblasts, as well as in primary S3WT and S3KO MEFs and DFs. Smad3 expression was reintroduced in S3KO cells by adenoviral infection at MOI 40, and adenoviral β-galactosidase (β-gal) infection at MOI 40 was done in parallel as a control. Cells were treated with the indicated concentrations of TGF-\(\beta\)1 for 5 h, and \(^{35}\)S-methionine was added during the last 3 h. Extracellular matrix proteins were extracted and separated on 8% SDS-polyacrylamide gels. Experiments were performed at least three times, and representative results are presented.

\textit{Role of Smad2 and Smad3 in Activation of TGF-\(\beta\)1-induced Transcriptional Reporters—} Receptor-activated Smad proteins including Smad2 and Smad3 function as transcriptional regulators in the nucleus. Whereas overexpression studies have shown that both Smad2 and Smad3 can activate transcription of a variety of TGF-\(\beta\)-dependent luciferase gene reporters, only Smad3 can directly interact with Smad binding elements found in the promoters of many TGF-\(\beta\)-responsive genes (30–33). To investigate the effect of loss of each of these two TGF-\(\beta\)-activated R-Smads on the transcriptional regulation of TGF-\(\beta\)-sensitive reporter genes, we performed transfection studies in the fibroblasts. As shown in Fig. 6A, lack of Smad2 expression only slightly reduced activation of the 3TP-Lux reporter (6.2-fold versus 8.7-fold induction in S2KO versus S2WT fibroblasts, respectively), which is driven by part of the PAI-1 promoter and three tetradecanoyl phorbol acetate-responsive elements (17).

In contrast, absence of Smad3 expression more strongly impaired 3TP-Lux reporter activation by TGF-\(\beta\)1 (2.2-fold versus 5.1-fold induction in S9KO versus S9WT fibroblasts; Fig. 6B). Reconstitution of Smad2 or Smad3 expression in the respective S2KO or S3KO fibroblast cell lines restored TGF-\(\beta\)1-mediated activation of the 3TP-Lux reporter to levels achieved in WT cells (Fig. 6, A and B). TGF-\(\beta\)1-induced activation of the (SBE\(_4\))-luciferase reporter, driven by four repeats of the CAGACA sequence identified as Smad binding element in the \(\text{Jun}B\) promoter (34), was dependent on expression of Smad3, but not on expression of Smad2 (Fig. 6, C and D), consistent with the inability of Smad2 to bind...
DNA. Reconstitution of Smad3 by transient overexpression resulted in efficient activation of the (SBE)4-luciferase reporter in S3KO fibroblasts. Similar results were obtained in primary MEFs and primary DFs, where adenoviral-based reconstitution of Smad3 expression in S3KO fibroblasts also restored the ability of TGF-β1 to induce (SBE)4-luciferase reporter activation (data not shown).

As reported previously (22) and described above (see Fig. 1B), Smad2 and Smad3 differentially affect TGF-β1-induced activation of the ARE-luciferase reporter when overexpressed. To address the effect of loss of each of these Smad proteins, S2KO and S3KO fibroblasts were transfected with the ARE-luciferase reporter and FAST-1. Although the fold induction of ARE-luciferase reporter activity by TGF-β1 was comparable in WT and S2KO fibroblasts, we repeatedly observed that the overall ARE-luciferase reporter levels were suppressed in S2KO fibroblasts (Fig. 4E). On the other hand, absence of Smad3 expression enhanced both absolute levels of activation and the fold induction of the ARE-luciferase reporter by TGF-β1 (Fig. 4F), supporting a suppressive role for endogenous Smad3 in activation of the ARE-luciferase reporter (22). Together, these reporter activation studies clearly indicate differential roles of Smad2 and Smad3 in the induction of specific TGF-β1 target genes.

**DISCUSSION**

We have investigated TGF-β signaling in established mouse embryo-derived fibroblasts deficient in expression of Smad2 or Smad3 to assess the effect of loss of each of these key signaling intermediates on induction of target gene expression by TGF-β1. We have identified target genes with Smad2- or Smad3-independent patterns of induction, those that are affected by the loss of either R-Smad, and genes that are selectively dependent on one or the other of these two R-Smad proteins. As examples, we have shown that TGF-β1-induced fibronectin synthesis occurs in the absence of Smad2 or Smad3 expression, whereas both Smads have roles in the induction of PAI-1 protein and in the more complex end point of TGF-β1-induced growth inhibition with associated regulation of cyclin/cyclin-dependent kinase inhibitors p15INK4B and p21CIP1/WAF1. We also show for the first time that TGF-β1-mediated induction of c-fos expression requires Smad3 and that induction of MMP-2 is selectively dependent on Smad2. Moreover, similar to that shown for Smad3 null macrophages and keratinocytes (10), we show that autoinduction of TGF-β1 in fibroblasts is strongly suppressed in the absence of Smad3. To test that results shown previously in overexpression systems are truly dependent on Smad2 and Smad3, we have also assessed the activation of several TGF-β-sensitive reporter genes in these Smad-deficient fibroblasts. Together, these experiments demonstrate that Smad2 and Smad3 have both overlapping and distinct roles in TGF-β1 signaling, depending on the target gene and cellular context.

Because of the early embryonic lethal phenotype of the Smad2 knockout mice (4, 5) and the technical difficulties in-
volved in derivation of S2KO embryonic fibroblasts, we were forced to do most of our comparisons between the role of Smad2 and Smad3 in TGF-β signaling using spontaneously immortalized fibroblasts that were cultured over multiple passages. To underscore the validity of our studies, we show that expression of Smad3 is also important for TGF-β1-mediated induction of Smad7, TGF-β1, and PAI-1 in primary MEFs and primary DFs. We also show that induction of expression of these genes by TGF-β1 can be restored after stable reintroduction of Smad3 in these primary Smad3-deficient fibroblasts. In contrast, whereas adenoviral- or retroviral-mediated restoration of Smad2 or Smad3 expression in fibroblasts could restore TGF-β1-responsive reporter gene induction dependent directly on Smads, this strategy was not sufficient to restore induction by TGF-β1 of endogenous gene responses shown to be dependent on Smad2 or Smad3 (data not shown). Similar observations of the inability to rescue responses by stable introduction of Smads or other signaling molecules into established cell systems have been reported (35, 36). Our data suggest that restoration of Smad expression is not sufficient to fully revert the established Smad knockout fibroblasts to their WT counterparts, possibly because loss of Smad expression in combination with multiple genetic alterations, inherently associated with immortalization, irreversibly alters expression of additional genes important in mediating signaling to more complex endogenous targets of TGF-β.

In contrast to previous reports that address the role of different Smads by overexpression in in vitro systems, we show that fibroblasts derived from mouse embryos lacking expression of Smad2 or Smad3 provide a suitable loss of function model system to investigate the different effects of these two R-Smads in TGF-β signaling, as is important for the understanding of their distinct roles in vivo. For example, the different roles of Smad2 and Smad3 are evident in studies of embryogenesis, where targeted deletion of Smad2 or Smad3 results in either early embryonic lethality or viable offspring, respectively (4–9, 11). In wound healing, decreased levels of Smad2 or Smad3 have dramatically different effects (10). Differences are also apparent in carcinogenesis, where Smad2 has been classified as a tumor suppressor based on its mutation frequency in several types of cancer (37, 38), but where evidence for a similar role of Smad3 is lacking (39, 40). Moreover, because auto-induction of TGF-β1, previously shown to involve Ras/mitogen-activated protein kinase/AP-1 signaling (26, 27), is also dependent on Smad3 (Fig. 4, D and E) (10), retention of Smad3 might be selected for in tumor cells because TGF-β1 secreted by tumor cells can promote tumorigenesis by inducing metastasis, invasion, and angiogenesis (reviewed in Ref. 41). It should be noted, however, that R-Smad activity can be blocked by certain oncoproteins including Evi-1, which, in certain cells, could have effects similar to its loss by genetic defects (42).

A number of physical differences between Smad2 and Smad3 have been described that might underlie or contribute to their observed functional differences or, in other cases, to their interchageability. Whereas the MH1 domain of Smad3 can interact directly with SBE sequences (CAGAGTCT) in the DNA, Smad2 contains an extra exon that encodes 30 amino acids absent in the MH1 domain of Smad3 and prevents its binding to DNA (31, 32). Consistent with this, we observed that TGF-β1-induced activation of the (SBE)4-luciferase reporter, which consists of four concatemerized SBEs derived from the mouse JunB promoter (34), occurred as efficiently in S2KO as in WT fibroblasts, whereas in S3KO cells, TGF-β1-induced (SBE)4-luciferase reporter activation was impaired. This is in agreement with previous observations that Smad2, in contrast to Smad3, could not be detected in Smad-complexes bound to a JunB probe and that overexpression of Smad2 contributed only weakly to TGF-β1-induced activation of the (SBE)4-luciferase reporter, whereas overexpression of Smad3 potently enhanced reporter induction, even in the absence of TGF-β (34). In a similar manner, Smad3 is involved in activation of the Smad7 gene promoter, whereas Smad2 does not have a functional role in its induction by TGF-β (19, 43). Interestingly, mechanisms exist to alter the DNA binding patterns of Smad2 and Smad3. An alternative splice variant of Smad2 that lacks exon 3 does bind to DNA (32, 44) and might possibly compensate for loss of Smad3 in mediating activation of certain TGF-β-induced responses.

Differential in vivo activities of Smad2 and Smad3 on the same target element are also supported by our studies, suggesting that Smad2 and Smad3 might have distinct affinities for different transcription factors and thereby contribute differently to TGF-β signaling. Thus, overexpression of Smad3 inhibits activation of the goosecoid or Mix2 (ARE) TGF-β target gene promoters that are dependent on FAST, Smad2, and Smad4 (20, 21). This inhibition has been proposed to result from either competition between Smad3 and Smad4 for binding to FAST (23) or competitive affinities of Smad3 and Smad4 for SBE elements in the gene promoters (22). Our loss of function studies further support the dependence of this promoter on Smad2 as well as its negative regulation by endogenous Smad3. Thus, we show compromised activation of the ARE-luciferase reporter in Smad2-deficient fibroblasts, in contrast with enhanced activation of this reporter in Smad3-deficient cells as well as in NMuMG cells in which a truncated dominant-negative form of Smad3 is overexpressed, likely interfering with the function of the endogenous protein (9).

In contrast to direct activation of immediate early genes and TGF-β-sensitive luciferase reporter genes that are likely controlled by low signaling thresholds, regulation of cell growth requires continuous signaling to modulate the tightly balanced cell cycle apparatus that integrates multiple signals at several complex end points. It is therefore more difficult to identify the genes that are directly involved in abrogation of TGF-β-induced inhibition of cellular proliferation. Similar to our findings in Smad2- or Smad3-deficient fibroblasts, it has been reported that the growth-inhibitory effects of TGF-β are lost in Smad3-deficient MEFs and astrocytes (8, 45). Whereas Datto et al. (8) did not observe changes in the induction of p15INK4b or p21cip1/waf1 in Smad3-deficient MEFs, basal expression levels of p15 and p21 were dramatically decreased and increased, respectively, in our S2KO and S3KO fibroblasts compared with WT controls. In agreement with this, suppressed p15 levels and reduced activation of p15 by TGF-β have been observed in Smad3-deficient astrocytes (45). However, the correlation between dysregulated p15 and p21 levels and the observed growth behavior of our KO fibroblasts is unclear at present.

The results presented here demonstrating that activation of genes by TGF-β1 is often dependent on one of the two TGF-β R-Smads, Smad2 or Smad3, suggest that the observed differences in KO phenotypes of Smad2- versus Smad3-deficient mice are not merely a consequence of differential spatially and temporally controlled patterns of gene expression of these two R-Smads during development but rather reflect unique, nonoverlapping roles for Smad2 and Smad3 in control of target gene expression, which allows for more versatility in cross-talk with other signal transduction pathways. Detailed analysis of TGF-β target gene expression in Smad2 versus Smad3 KO cell systems now has the potential to provide insights into their respective roles in regulation of genes that are of critical importance for both normal physiology and development as well as in disease pathogenesis, including carcinogenesis.
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