Lefty Inhibits Receptor-regulated Smad Phosphorylation Induced by the Activated Transforming Growth Factor-β Receptor*

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Transforming growth factor-β (TGF-β) is a pleiotropic cytokine that regulates growth and differentiation of diverse types of cells. TGF-β actions are directed by ligand-induced activation of TGF-β receptors with intrinsic serine/threonine kinase activity that trigger phosphorylation of receptor-regulated Smad (R-Smad) protein. Phosphorylated R-Smad proteins bind to Smad4, and the complexes formed move into the nucleus, where they act as components of a transcriptional complex. Here, we show that TGF-β signaling is inhibited by lefty, a novel member of the TGF-β superfamily. Lefty perturbed TGF-β signaling by inhibiting the phosphorylation of Smad2 following activation of the TGF-β receptor. Moreover, lefty inhibited the events that lie downstream from R-Smad phosphorylation, including heterodimerization of R-Smad proteins with Smad4 and nuclear translocation of the R-Smad-Smad4 complex. Lefty repressed TGF-β-induced expression of reporter genes for the p21, cdc25, and connective tissue growth factor promoters and of a reporter gene driven by the Smad-binding element. Similarly, lefty inhibited both BMP-mediated Smad5 phosphorylation and gene transcription. The action of lefty does not appear to depend on protein synthesis, including synthesis of inhibitory Smad proteins. Thus, lefty provides a repressed state of TGF-β- or BMP-responsive genes and participates in negative modulation of TGF-β and BMP signaling by inhibition of phosphorylation of R-Smad proteins.

TGF-β1 is a potent and pleiotropic cytokine that regulates cell growth and differentiation, embryonic patterning, deposition of the extracellular matrix, and fibrosis (1–7). To exert these functions, TGF-β brings together two transmembrane serine/threonine kinases, the type I and II receptors. The assembly and oligomerization of TGF-β receptors lead to phosphorylation of receptor-regulated Smad (R-Smad) proteins, heterodimerization of R-Smad proteins with Smad4, and subsequent nuclear accumulation of these complexes (8–11). Activated Smad complexes interact with other transcription factors in the nucleus (12–15), bind to DNA by their N-terminal Mad homology-1 domains (16–21), and activate transcription of TGF-β-responsive genes through the C-terminal Mad homology-2 domains (1, 3, 4).

In view of its diverse and potent activities, the signaling by TGF-β is under tight regulation both by positive and negative feedback mechanisms. The two principal events in TGF-β signaling, TGF-β receptor and Smad phosphorylation, are controlled by a network of regulatory proteins, which modulate the magnitude of signals induced by TGF-β (22). Positive regulators include ligand accessory receptors and substrate-anchoring factors such as membrane-anchored proteoglycan, betaglycan, and SARA (Smad anchor for receptor activation). Betaglycan, also known as the TGF-β type III receptor, binds TGF-β and increases its affinity for the signaling receptors (23, 24). By binding to the Mad homology-2 domains of Smad2 and Smad3, SARA facilitates the interaction of these Smad proteins with TGF-β receptors (25). A second set of factors participating in the negative regulation of TGF-β signaling includes the cytosolic growth factor-sequestering protein FKBP-12, the pseudoreceptor transmembrane protein BAMBI, and a set of inhibitory Smad proteins that include Smad6 and Smad7. Some of these factors inhibit the function of TGF-β by interacting with TGF-β receptors or Smad proteins. By virtue of binding to TGF-β type I receptor, FKBP-12 prevents its transphosphorylation by the TGF-β type II receptor (26). BAMBI, on the other hand, blocks TGF-β-mediated signaling by forming inactive dimers with the type I receptor (27). By binding to receptor-activated Smad1, Smad6 forms a Smad1-Smad6 complex, which appears to be inactive (28), whereas a second inhibitory Smad protein, Smad7, binds to the activated TGF-β receptor, blocking the phosphorylation of receptor-regulated Smad (34, 35). Among the intracellular transcriptional repressors thus far identified that block TGF-β signaling are two closely related members of the Ski/sno family of nuclear oncoproteins, SnoN and Ski (29–35). Ski was identified on the basis of homology to v-Ski, the transforming protein of the Sloan-Kettering virus. SnoN and c-Ski both inhibit TGF-β signaling by interfering with Smad function (36). TGF-β-mediated Smad signaling integrates with and is controlled by other signaling factors such as those in the Ras/Erk and Jak1/Stat pathways (37, 38). Erk, activated by growth factors such as epidermal growth factor and hepatocyte growth factor, induces the phosphorylation of R-Smad proteins in the linker region and without inhibiting the association of R-Smad proteins with Smad4 and prevents the nuclear accumulation of Smad proteins (38). Thus, the magnitude of the TGF-β effect is the net outcome of actions of these positive and negative signals.

Besides the vast network of positive and negative intracellular regulatory factors, there are extracellular proteins that, by directly interacting with TGF-β, prevent its binding to receptors (22). The latency-associated peptide noncovalently
binds TGF-β, keeping it in a latent form incapable of binding to betaglycan or its signaling receptors (23). Some indirect evidence suggests that lefty, a member of the family of morphogens that is thought to encode a signal for “leftness,” is poised to act as an inhibitor of TGF-β family members (39–41). Anti-vin, a lefty-related gene product, acts as a specific competitive inhibitor for activin during embryo development in zebrafish (42, 43), and the lefty-related factor Xatv acts as a feedback inhibitor of nodal signaling in induction of mesoderm and left/right axis development in Xenopus (44). Overexpression of Xatv in the marginal zone in Xenopus suppresses mesoderm formation, causes defects in gastrulation, and inhibits the secondary axis formation induced by Xnr1 and Xatv, suggesting that Xatv acts as a feedback inhibitor of activin signaling (45). Injection of lefty into mouse blastocysts leads to neurogenesis, a function attributable to BMP inhibitors such as chordin, noggin, and follistatin (41, 44–47). These findings suggest (but do not prove) that lefty is an inhibitory member of the TGF-β superfamily. In this report, we carried out experiments to determine whether lefty is capable of blocking TGF-β and BMP signaling and the associated gene transcriptional activity.

**EXPERIMENTAL PROCEDURES**

**Materials—**The full-length 1.96-kilobase lefty A (ebaf) cDNA was derived from a human placental cDNA library (48). The materials used in this study included an enhanced chemiluminescence system (Roche Molecular Biochemicals), polyvinylidene difluoride membrane (Bio-Rad), and Kodak Onat film (Sigma). Unless otherwise indicated, all other chemicals were from either Sigma or Fisher. The P19 cell line was obtained from American Type Culture Collection (Manassas, VA). Recombinant TGF-β1 was obtained from Sigma, and recombinant BMP-4 was from R&D Systems (Minneapolis, MN). Recombinant Escherichia coli lefty A, expressed and refolded from Ser136 to Phe369, was obtained from Regeneron Pharmaceuticals (Tarrytown, NY). Anti-Smad2/3 (R-Smad proteins) and anti-Smad4–7 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The constitutively active TGF-β type I receptor was a gift from the laboratory of Dr. J. Massagué (37).

**Cells, Transfection, and Protein Preparation—**P19 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and 1% antibiotics/antimycotic mixture (Life Technologies, Inc.). For transfection, cells were seeded into 6-well plates (Falcon, Franklin lakes, NJ) at a concentration of 1.5 × 10⁴ cells/ml and maintained in a CO₂ chamber at 37°C for 16 h. When 60% confluent, cells were transfected with cDNA from the TGF-β type I receptor and reporter constructs using Superfect transfection reagent (QIAGEN Inc.) or Fugene (Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturers’ protocol. The amount of protein in the cytosolic, nuclear, and cell lysates was determined with the Bio-Rad protein assay kit.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—**The proteins in the total cell lysates or cell fractions (15 µg of protein/lane) were resolved on a 12% gel together with a prestained protein ladder (Life Technologies, Inc.) and were subsequently blotted onto polyvinylidene difluoride membranes in a Mini-Trans-Blot apparatus (Bio-Rad). The blots were stained using protein-specific antibodies, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology). The specific bands were detected with the enhanced chemiluminescence system as described by the manufacturer. The relative optical densities of the bands were quantitated by laser scanning densitometry.

**Immunoprecipitation—**Immunoprecipitations of the proteins were performed as described (49). Briefly, for immunoprecipitation, 2–5 µl of specific antibody was added to each milliliter of cell lysate (~10⁶ cells). The immunoprecipitates were subjected to SDS-PAGE and blotting, followed by autoradiography.

**Immunohistochemical Staining of Smad4—**Smad4 was localized in cells grown on coverslips. Cells were fixed for 5 min in 10% buffered Formalin and washed for 5 min with 0.1 M phosphate-buffered saline (pH 7.4). Cells were incubated at room temperature with mouse monoclonal anti-Smad4 antibody for 60 min, followed by a 5-min washing with phosphate-buffered saline. They were then incubated for 60 min at room temperature with a peroxidase-labeled anti-mouse secondary antibody. After washing with phosphate-buffered saline, staining was developed using a diaminobenzidine/H₂O₂ mixture.

**Luciferase Reporter Assay—**Cells were transfected with 0.5 µg of p21-Lux, pCde25-Lux, and pCTGF-Lux using Superfect according to the manufacturer’s instructions. Cytokines (TGF-β and lefty) were added 24 h after transfection, and luciferase activity was measured with a luciferase kit (Promega).

**RESULTS**

**Lefty Represses the Activation of Reporter Genes by TGF-β—**As a first step toward identifying the mode of action of lefty, we examined its effect on TGF-β-mediated gene transcriptional activity. We chose P19 cells since these cells have an intact TGF-β signaling pathway and have been used in elucidating the role of TGF-β proteins in embryogenesis (50). P19 cells were transfected with the artificial construct pSBE-Lux. In this construct, the luciferase gene is under the control of the Smad-binding element, which is in the promoter of TGF-β-responsive genes and is activated by direct binding of the TGF-β-induced transcriptional complex (17). The luciferase activity of cells transfected with the construct was assessed in the presence of TGF-β and varying amounts of lefty. Lefty inhibited the activity of the reporter in a dose-dependent fashion (Fig. 1A). The inhibitory activity of lefty on the reporter could be overridden by increasing the concentration of TGF-β (Fig. 1B).

We next tested the effect of lefty on TGF-β-mediated regulation of the activity of reporters of the cell cycle factors p21 and Cdc25. The G₁ cell cycle events mediated by TGF-β in transformed epithelial cells include up-regulation of p21⁰⁴³ and reduction of Cdc25 (51, 52). P19 cells were transfected with p21-Lux and pCde25-Lux constructs, and the reporter activity was assessed in the presence of TGF-β and lefty. TGF-β increased the p21 reporter activity and decreased the Cdc25 reporter activity (Fig. 1, C and D). Although lefty did not have any discernible effect, it opposed reduction of the Cdc25 reporter activity induced by TGF-β (Fig. 1, C and D). Whereas the reporter activity was increased by lefty close to 50%, the activity of the p21 promoter was reduced 4-fold. These findings imply that lefty inhibits several well known functions of TGF-β that control cell proliferation in epithelial cells. To determine whether lefty actions are primarily confined to TGF-β-mediated control of cell cycle factors or also target other known functions of this cytokine, we further tested the effect of lefty on TGF-β-mediated connective tissue growth factor promoter activity. TGF-β leads to fibrogenesis by activating the transcription of connective tissue growth factor, a cytokine that induces collagen synthesis by fibroblasts (53). Lefty significantly reduced the activity of the reporter induced by 5 µg/ml TGF-β (Fig. 1E and F). Whereas the reporter activity was increased by lefty close to 50%, the activity of the p21 promoter was reduced 4-fold. These findings show that lefty is a broad-range inhibitor of TGF-β actions.

**Lefty Inhibits the Nuclear Localization of R-Smad/Smad4 Complexes Induced by TGF-β—**In the absence of TGF-β, Smad proteins are distributed in the nucleus and cytoplasm (2). Upon stimulation by TGF-β, however, R-Smad proteins (Smad2/3) are phosphorylated by the activated TGF-β type I receptor and heterodimerize with Smad4 (1–5). Therefore, we reasoned that the inhibitory effect of lefty may involve blockage of TGF-β-mediated heterodimerization of Smad proteins and subsequent nuclear translocation of these heteromeric complexes (1–6).
P19 cells were treated with TGF-β in the presence and absence of lefty. After 1 h of treatment, the cytosolic and nuclear lysates of these cells were subjected to Western blotting using antibodies to Smad2/3, Smad4, and Smad5. As compared with the control cells, TGF-β led to the accumulation of Smad2 and Smad4 in the nuclei of treated cells (Fig. 2A, arrows). Endogenous Smad3 was not detected. This could be due to lower levels of Smad3 in P19 cells as well as our electrophoretic conditions. Similar differential endogenous expression of Smad2 and Smad3 proteins has been previously reported (37). TGF-β did not have any effect on nuclear translocation of Smad5, which is an intracellular mediator of the BMP signaling pathway (54). Although lefty did not change the amount of Smad proteins in the cytosol or the nuclei of the treated cells, it inhibited the nuclear translocation of both Smad2 and Smad4 induced by TGF-β (Fig. 2A). Immunolocalization of Smad4 in P19 cells treated with TGF-β and lefty showed nuclear accumulation of Smad4 upon TGF-β treatment. As assessed by the intensity of staining, lefty did not increase the amount of nuclear Smad4 on its own, but inhibited the TGF-β-induced nuclear accumulation of Smad4 (Fig. 2B). These findings show that lefty prevents the TGF-β-mediated nuclear accumulation of the Smad2-Smad4 complex required for gene transcriptional activity.

**Lefty Inhibits the Heterodimerization of R-Smad Proteins with Smad4 Induced by TGF-β**—Biological signaling of TGF-β involves heterodimerization of R-Smad proteins with Smad4 (5–7). In light of the findings, we assessed the extent that lefty can inhibit the TGF-β-mediated heterodimerization of R-Smad proteins with Smad4. R-Smad proteins were immunoprecipitated using anti-Smad2/3 antibody from the cytosol of P19 cells that were treated with TGF-β and/or lefty. The immunoprecipi-
with Smad4 induced by TGF-β in the total amount of R-Smad or Smad4 (Fig. 3). These findings point to Smad2 and Smad4 accumulated in the nuclear fraction and its absence in the cytosolic fraction were prepared from the untreated (−) and treated (+) cells, and equal amounts of proteins (10 µg/lane) were subjected to Western blot analysis for Smad2/3, Smad4, Smad5, and histone 3. The localization of Smad4 in the nuclear fraction and its absence in the cytosolic fraction showed that these preparations were not cross-contaminated. Arrows point to Smad2 and Smad4 accumulated in the nuclear lysates. B. Smad4 was localized by immunoperoxidase staining in the treated cells. Panel a, control cells treated with medium alone; panel b, cells treated with TGF-β; panel c, cells treated with lefty; panel d, cells treated with TGF-β and lefty. Percentages of cells showing nuclear staining were as follows: control 2%; TGF-β 25%; lefty 3%; and TGF-β + lefty 1%. Arrows point to nuclear Smad4.

![Figure 2](image2.jpg)

FIG. 2. Lefty inhibits TGF-β-mediated nuclear translocation of R-Smad proteins and Smad4. P19 cells were treated for 30 min with medium alone (control), TGF-β (5 ng/ml), recombinant lefty (5 ng/ml), and TGF-β (5 ng/ml) plus lefty (5 ng/ml). A, the cytosolic and nuclear fractions were prepared from the untreated (−) and treated (+) cells, and equal amounts of proteins (10 µg/lane) were subjected to Western blot analysis for Smad2/3, Smad4, Smad5, and histone 3. The localization of Smad4 in the nuclear fraction and its absence in the cytosolic fraction showed that these preparations were not cross-contaminated. Arrows point to Smad2 and Smad4 accumulated in the nuclear lysates. B, Smad4 was localized by immunoperoxidase staining in the treated cells. Panel a, control cells treated with medium alone; panel b, cells treated with TGF-β; panel c, cells treated with lefty; panel d, cells treated with TGF-β and lefty. Percentages of cells showing nuclear staining were as follows: control 2%; TGF-β 25%; lefty 3%; and TGF-β + lefty 1%. Arrows point to nuclear Smad4.

tates were subjected to Western blotting for Smad4 (Fig. 3). As expected, in the TGF-β-treated cells, Smad4 was present in the R-Smad immunoprecipitates, showing that it had heterodimerized with these Smad proteins (Fig. 3). Lefty alone had no effect on this event, but prevented the TGF-β-mediated heterodimerization of Smad4 with R-Smad proteins (Fig. 3). Treatment of cells with TGF-β or lefty did not lead to any detectable change in the total amount of R-Smad or Smad4 (Fig. 3). These findings point to lefty inhibiting the heterodimerization of R-Smad with Smad4 induced by TGF-β.

Lefty Inhibits the Phosphorylation of R-Smad Proteins Induced by TGF-β—The findings suggested that the effect of lefty may be directly exerted on R-Smad activity. To test this, the phosphorylation of R-Smad was assessed in P19 cells in the presence of both lefty and TGF-β. P19 cells were treated with TGF-β, lefty, or both in the presence of [32P]orthophosphate to label phosphorylated proteins. R-Smad proteins were immunoprecipitated, and the immunocomplexes were subjected to SDS-PAGE, blotting, and autoradiography. The analysis showed that although TGF-β led to the phosphorylation of R-Smad proteins, lefty had no discernible effect. However, lefty reduced the TGF-β-mediated R-Smad phosphorylation (Fig. 4A). Densitometric analysis of the bands showed that lefty inhibited Smad phosphorylation by 80%.

Binding of TGF-β to its receptors leads to several events, including oligomerization of the type I and type II receptors and transphosphorylation of the type I receptor by the constitutively active serine/threonine kinase type II receptors (20). To assess whether the effect of lefty is dependent on the initial events during TGF-β signaling, we transfected P19 cells with a constitutively active TGF-β type I receptor and examined the effect of lefty on phosphorylation of R-Smad proteins. Lefty inhibited the phosphorylation of Smad2 by this receptor (Fig. 4B). Densitometric analysis of the bands showed that lefty inhibited Smad2 phosphorylation by 75%. These findings show that inhibition of R-Smad phosphorylation by lefty is independent of binding of TGF-β to its receptor, does not appear to require complex formation between type I and II receptors, and is not dependent on the phosphorylation of the type I receptor.

To further validate these findings, we assessed the effect of lefty on gene transcription induced by the constitutively active TGF-β type I receptor. P19 cells were cotransfected with the receptor and the pSBE-Lux construct, and the activity of the reporter was assayed in the presence of an increasing amount of lefty. Lefty inhibited the activity of the reporter induced by the receptor in a dose-dependent fashion (Fig. 4C). Taken together, these data show that the effect of lefty is initiated downstream of TGF-β receptor activation.

Lefty Inhibition of the Phosphorylation of R-Smad Proteins Induced by TGF-β Is Not Mediated by Inhibitory Smad Proteins and Does Not Require Protein Synthesis—One possibility for the inhibitory effect of lefty on TGF-β-mediated activities is induction of expression of inhibitory Smad (anti-Smad) proteins. To determine whether the inhibitory activity exhibited by lefty involves synthesis of Smad7, P19 cells were treated with lefty for various periods of time (0–60 min), and the cell lysates were subjected to Western blot analysis for Smad4 (Fig. 5A). Lefty did not induce any change in the total amount of Smad7. Likewise, treatment of cells with lefty failed to induce any change in the amount of Smad6 (data not shown). These data show that inhibition of the activity of TGF-β by lefty does not require synthesis of Smad6 or Smad7.

We then tested whether the effect of lefty on phosphorylation of R-Smad depends on induction of another protein that blocks the phosphorylation of R-Smad proteins. To do this, the phosphorylation of R-Smad was analyzed in P19 cells treated with cycloheximide and transfected with the constitutively active

![Figure 3](image3.jpg)

FIG. 3. Lefty inhibits TGF-β-mediated heterodimerization of R-Smad proteins with Smad4. P19 cells were treated for 30 min with medium alone (control), TGF-β (5 ng/ml), lefty (5 ng/ml), and TGF-β (5 ng/ml) plus lefty (5 ng/ml). The proteins in the nuclear preparations of untreated (−) and treated (+) cells were immunoprecipitated with an antibody to R-Smad (Sma23), and the immunocomplexes were subjected to Western blotting for Smad4 (upper panel). The arrow points to Smad4. Other bands are the heavy chain of the immunoglobulin in the immunocomplexes. The cell lysates were analyzed by Western blotting for R-Smad and Smad4 to assess the overall amount of these proteins (middle and lower panels).
**FIG. 4.** Lefty inhibits R-Smad phosphorylation. A, P19 cells were incubated for 30 min in culture medium alone (control) and in culture media supplemented with TGF-β (5 ng/ml), lefty (5 ng/ml), and TGF-β (5 ng/ml) and lefty (5 ng/ml) in the presence of [32P]orthophosphate (10 µCi/ml). R-Smad proteins were immunoprecipitated with anti-Smad2/3 antibody, and the immunoprecipitates were subjected to SDS-PAGE, followed by autoradiography (upper panel). The overall amount of R-Smad was assessed by Western blotting of the cell lysates for actin. B, P19 cells were transfected without (−) and with (+) the constitutively active form of the TGF-β type I receptor (TGF-beta RI). Twenty-four hours after transfection, cells were treated with cycloheximide (20 µg/ml) for 1 h. Cells were then treated for 30 min in the presence of [32P]orthophosphate (10 µCi/ml) in culture medium alone (control; −) or supplemented with recombinant lefty (5 ng/ml; +). R-Smad proteins (Smad2/3) were immunoprecipitated from the cell lysates, and the immunocomplexes were subjected to SDS-PAGE and autoradiography (upper panel). The overall amount of Smad2 was assessed by Western blotting (lower panel).

**DISCUSSION**

TGF-β is a potent cytokine capable of modifying and regulating many different cell functions. In view of these diverse activities, there is a great interest in understanding how the signaling by TGF-β is regulated. In view of the potent and diverse activities of TGF-β, the action of this cytokine is tightly controlled by a number of negative feedback mechanisms (29–
oncoprotein interacts with Smad2 and Smad4 and represses their abilities to activate transcription through recruitment of the transcriptional corepressor N-CoR. Ski directly interacts with Smad2, Smad3, and Smad4 on a TGF-β-responsive promoter element and represses their abilities to activate transcription through recruitment of the nuclear transcriptional corepressor N-CoR and possibly its associated histone deacetylase complex (55). Thus, the actions of lefty complement the actions of these intracellular inhibitors of TGF-β responsiveness. The inhibitory effect of lefty is not due to synthesis of a protein, including induction of Smad6 or Smad7, suggesting that lefty acts through an as yet unidentified protein that interacts with either the intracytoplasmic domain of the TGF-β receptor or more likely the R-Smad proteins.

The interaction of lefty proteins with TGF-β and/or BMP and the Smad signaling pathway is likely to play a major role during implantation and embryogenesis as well as oncogenesis. Lefty is a novel subfamily of the TGF-β protein superfamily and is comprised of lefty-1 and lefty-2 in mouse (39, 41) and their homologs, lefty A (ebaf) and lefty B, in human (48, 56). Both lefty-1 and lefty-2 are key embryonic signals that drive development of an asymmetric body plan. Both of these genes are asymmetrically expressed on the left side of gastrulating mouse embryos. However, the major expression domains of the two genes are different. lefty-1 expression is predominantly confined to the left side of the ventral neural tube, whereas lefty-2 is strongly expressed in the left lateral mesodermal plate (39, 41). In zebrafish embryos, lefty-1 is expressed in the central nervous system in the left diencephalon, whereas lefty-2 is expressed in the left heart field (57). Antivin/lefty-1 is also expressed asymmetrically on the left side of the prospective floor plate, notochord, and lateral plate mesoderm of the chick embryo (58). Asymmetric expression of lefty and nodal is perturbed in mouse mutants with laterality defects, and mutation of lefty A is seen in humans who exhibit left/right axis malformations (58–60). Furthermore, the knockout mutation of lefty-1 induces a variety of L/R positional defects in visceral organs. The most notable feature of lefty-1-deficient mice is bilateral expression of nodal and lefty-2, which leads to a left lung isomerism (40). These and other observations support the concept that during embryogenesis, lefty proteins encode a signal for leftness and appear to be involved in neurogenesis. Like lefty, the signaling molecules of TGF-β, including its receptors and Smad proteins, have been implicated in embryo development. TGF-β is intrinsically involved in patterning during developmental processes and neurogenesis. In the chick embryo, TGF-β is involved in the death of ciliary, dorsal root, and spinal motor neurons as well as in neuronal losses that follow limb bud ablation (62). Both overexpression and deletion of TGF-β genes cause significant developmental abnormalities and death in embryos (63–68). Mice genetically deficient in the TGF-beta receptor type II gene die around embryonic day 10.5 from abnormalities that are reminiscent of those described in TGF-β1−/− embryos, including defects in hematopoiesis and visceral yolk sac vasculogenesis (69). In mouse embryos, Smad2 has been found as a key mediator that directs epiblast derivatives toward an endodermal as opposed to a mesodermal fate (70). Recently, some evidence has emerged that directly ties the function of lefty to the signaling molecules of the TGF-β and BMP proteins. Some, if not all, of the developmental processes driven by TGF-β family members may be subject to modulation by lefty. We showed here that lefty inhibits BMP signaling, suggesting that the tissue patterning during embryogenesis might be regulated by the joint effect of these cytokines. Consistent with this thesis, it has recently been shown that regulation of left/right asymmetry in the gut and heart is
not solely induced by lefty; rather, it is driven by context-dependent interactions between lefty and BMP-4 (61). Injection of lefty into two-cell stage Xenopus blastocysts promotes neuralization, an effect that is also inducible by factors such as noggin, follistatin, and chordin that inhibit BMP signaling (41, 44–47). These findings show that the development of asymmetry requires the joint cooperation of various members of the TGF-β superfamily. Similar to lefty, levels of phosphorylated Smad2 have been found to be asymmetrically distributed across both the animal-vegetal and dorsoventral axes in Xenopus embryos, suggesting that Smad2 is a candidate morphogen that regulates primary germ layer formation and dorsoventral patterning (7). Mice trans-heterozygous for both Smad2 and nodal mutations have defects in left/right patterning, indicating that Smad2 is involved in the development of left/right asymmetry and that Smad2 may mediate nodal signaling in these developmental processes (72). Moreover, the gene dosage of Smad2 has been found to be important in tissue patterning, suggesting that lefty might act by regulating the function of Smad2. The left/right asymmetric expression of lefty-2 is controlled by a left side-specific enhancer. The transcription factor FAST2, which mediates signaling by TGF-β and activin, binds to a conserved sequence in this asymmetric enhancer, which is both essential and sufficient for left/right asymmetric gene expression (73, 74). The forkhead transcription factor FoxH1, also known as FAST1, which associates with Smad proteins in response to an activin/TGF-β signal, plays a key role in the development of dorsal axial structures in zebrafish and in specification of mesoderm by TGF-β superfamily signals during early Xenopus embryogenesis (75–77). These findings directly tie the lefty functions to the action of TGF-β and its signaling molecules during embryogenesis.

It is now well established that tumorigenesis is associated with development of resistance to TGF-β signaling, and for this reason, it is thought that TGF-β and Smad signaling molecules act as potent tumor suppressors (78). Since the normal function of the TGF-β signaling pathway is suppression of cellular proliferation and transformation, our results provide a model for the action of lefty as a potential oncoprotein by showing that it counteracts the TGF-β-mediated signaling. According to this model, dominant repression of TGF-β-inducible genes that are negative regulators of cell cycle progression by lefty might confer upon lefty a transforming activity. Lefty is overexpressed in certain forms of human cancer, including those derived from colon, pancreas, ovary, and testis (79). High levels of expression of lefty in human cancers may produce a disruption of TGF-β-mediated signaling in a manner similar to that induced by overexpression of inhibitory Smad proteins and SnoN proteins or mutation of genes that encode TGF-β receptors or Smad proteins (57, 80, 81). This could be relevant to certain human cancers that, despite the presence of active TGF-β receptors and Smad proteins, are unresponsive to TGF-β (82, 83). Dominant disruption of lefty function in tumors that overexpress lefty might restore gene activation and growth inhibition by TGF-β (79). However, a separate model for the action of lefty in human cancers can be envisaged. According to this second model, lefty acts as a tumor suppressor in a fashion similar to the action of inhibitors of TGF-β signaling such as Sno. Loss of Sno has been shown to increase susceptibility to tumorigenesis in mice (87). This model is quite conceivable since overexpression of TGF-β, despite the loss of TGF-β sensitivity in cancer cells, is paradoxically associated with an aggressive and invasive tumor growth (85–87). In this scenario, TGF-β promotes tumor growth, and factors that inhibit TGF-β signaling abate the TGF-β-mediated responses and suppress tumor growth. TGF-β promotes late-stage tumor progression, tumor invasion, and metastasis in a number of model systems, including human colon (88) and breast (89) carcinomas. These actions of TGF-β in TGF-β-resistant tumors are presumably mediated by autocrine and/or paracrine mechanisms (90, 91). Blocking these actions of TGF-β by lefty overexpression in tumors might slow down the aggressive tumor growth or may lead to tumor regression. Consistent with this line of reasoning, introduction of lefty to tumor cells reduces tumor growth in nude mice.²

The lefty protein is expressed as a precursor of 42 kDa that is cleaved at Arg⁷⁷ and Arg⁸⁵ to release polypeptides of 34 and 28 kDa, respectively (71). We found the 28-kDa lefty polypeptide to induce MAPK activity, but the 34-kDa protein appears to be inactive. Surprisingly, 42-kDa lefty is also capable of inducing MAPK activity, indicating that the lefty precursor is biologically active (71). It was recently shown that activated Ras, acting via Erk MAPKs, causes phosphorylation of R-Smad proteins at specific sites in the region linking the DNA-binding domain and the transcriptional activation domain. This phosphorylation inhibits the TGF-β-induced nuclear accumulation of Smad2 and Smad3 and Smad-dependent transcription (38). These findings imply that induction of MAPK activation by lefty might modulate gene transcription by TGF-β. Treatment of P19 cells with both BMP-4 and lefty induces a more vigorous MAPK activity in 5 min, but this activity is much less than that induced by lefty alone at later time points (71). Therefore, although lefty inhibits BMP signaling along the Smad pathway, its effect on the MAPK activity of BMP appears to be complex, being both additive as well as antagonistic (71).

In summary, lefty is an inhibitory member of the TGF-β family that does not have any discernible effect on its own on Smad signaling, but blocks the TGF-β or BMP actions by preventing the phosphorylation of R-Smad proteins. This inhibition is not due to synthesis of any protein, including Smad6 and Smad7. Lefty inhibits the phosphorylation of R-Smad by a constitutively active TGF-β type I receptor, which suggests that its action is directed at an event downstream from receptor activation. This action is sufficient to inhibit TGF-β- or BMP-mediated R-Smad phosphorylation and subsequent heterodimerization of R-Smad with Smad4, nuclear translocation of R-Smad4 heterocomplexes, and TGF-β or BMP-mediated gene transcription. These findings show that growth-promoting and transcriptional responses to TGF-β or BMP actions are abated by lefty, despite the presence of phosphorylation of R-Smad proteins. The data suggest that lefty is an integral part of the mechanisms that provide negative feedback during TGF-β and BMP signaling.

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