Abrogation of Smad3 and Smad2 or of Smad4 Gene Expression Positively Regulates Murine Embryonic Lung Branching Morphogenesis in Culture

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Smad genes are recently identified intracellular effectors for receptor signaling in the BMP/activin/TGF-β pathway. Since TGF-β ligands are known to inhibit embryonic lung branching morphogenesis, we tested the hypothesis that Smad genes negatively regulate lung organogenesis. Antisense oligodeoxynucleotides were designed to attenuate Smad3 and Smad2 gene expression in embryonic (E11) mouse lungs over 4 days in culture. Endogenous Smad3 and Smad2 mRNA levels were suppressed by 97 and 91%, respectively, in cultured embryonic lungs when antisense oligodeoxynucleotide (40 μM) to Smad was added, compared to scrambled and sense sequence controls. The corresponding Smad3 and Smad2 protein amounts were also decreased respectively by 86 and 90% in lungs treated with Smad3 and Smad2 antisense oligodeoxynucleotide. Phenotypically, Smad antisense oligodeoxynucleotides resulted in a concentration-dependent increase in lung branching: embryonic lung branching was stimulated by up to 53% in culture with 40 μM antisense oligodeoxynucleotide, whereas both scrambled and sense controls showed no stimulatory effect. Thus, inhibition of endogenous Smad3 and Smad2 gene expression resulted in stimulation of embryonic lung branching similar to that caused by inhibition of TGF-β type II receptor expression and signaling (J. Zhao et al., 1996, Dev. Biol. 180, 242–257). Abrogation of Smad4 (DPC4), the downstream mediator of Smad3 and Smad2 proteins, with antisense oligodeoxynucleotide, also resulted in increased branching morphogenesis. Furthermore, while TGF-β alone inhibited lung branching morphogenesis in culture, addition of exogenous TGF-β1 could not overcome the stimulatory effect on lung branching of Smad antisense oligodeoxynucleotide treatment. By immunohistochemistry, Smad proteins were localized mainly to the epithelial cells lining the branching distal airways, indicating that Smad genes could regulate lung morphogenesis through mesoderm–endoderm interaction. Our results demonstrate, for the first time, that abrogation of Smad2 and Smad3 or of Smad4 gene expression stimulated early mouse embryonic lung branching morphogenesis in culture, possibly through reversing the negative influence of endogenous TGF-β signaling upon lung branching morphogenesis.

Key Words: Smad; antisense ODN; lung culture; branching morphogenesis; TGF-β; competitive RT-PCR.

INTRODUCTION

Epithelial–mesenchymal interactions have been implicated in branching morphogenesis of embryonic lung. Lung development begins with the outgrowth of paired lung buds from the foregut endoderm that invade the surrounding mesodermal mesenchyme in a genetically predetermined pattern of branching morphogenesis and lung-specific cell differentiation (reviewed by Hilfer, 1996). Secreted signaling molecules such as transforming growth factor-β (TGF-β) family peptides regulate cell growth, differentiation, and tissue morphogenesis through autocrine/paracrine signaling mechanisms. TGF-β induces growth arrest in various cell types, including lung epithelial cells, and expression of a wide array of proteins, some of which are deposited in the lung extracellular matrix.

TGF-β belongs to a superfamily including activin, bone morphogenetic proteins (BMP), and TGF-β1, 2, and 3. TGF-β ligands bind to TGF-β type II receptor and this triggers heteromerization with TGF-β type I receptors (M. A. Assaguer et al., 1990; Roberts et al., 1990). Following heteromerization,
the TGF-β type II receptor serine/threonine kinase transphosphorylates the type I receptor, resulting in the propagation of a phosphorylation signal to downstream substrates (Wrena et al., 1994).

Until recently, little was known about the signaling molecules and intracellular substrate proteins that mediate the TGF-β type I/type II receptor heterocomplex responses to extracellular TGF-β ligand binding. Genetic studies in Drosophila uncovered the mothers against dpp (mad) gene as a maternal effector that constitutes a downstream component for signaling by decapentaplegic (dpp), the fruitfly homologue of mammalian BMPs (Raftery et al., 1995; Sekelsky et al., 1995). Overexpression of Mad protein was found to complement Dpp deficiency, while Dpp failed to induce biological responses in Mad-mutant flies (Wiersforff et al., 1996; Newfeld et al., 1996). Three Smad2, Smad3, and Smad4 proteins structurally related to Drosophila Mad were also found in parallel studies of Caenorhabditis elegans development (Savage et al., 1996). These intracellular proteins are required for the function of Daf-4, a serine/threonine kinase receptor which responds to C. elegans homologues of BMP-2 and BMP-4 (Estevez et al., 1993).

Nine vertebrate Mad gene counterparts have been identified and are referred to as Smad1 through Smad8 (Derynck et al., 1996; Chen et al., 1997; Nakao et al., 1997; Suzuki et al., 1997; Topper et al., 1997). All the Smad proteins share two highly conserved amino- and carboxy-terminal domains separated by a more diverse proline-rich region (Derynck and Zhang, 1996). Further studies reveal a selectivity in different Smad proteins to mediate distinct responses to receptor signaling. Smad1 (also previously referred to as Mad1 and Dwarfin-A) is the intracellular effector for BMP-2/4 signaling (Liu et al., 1996; Hoodless et al., 1996; Yingling et al., 1996; Kretzschmar et al., 1997). Smad2 (also previously referred to as Mad2 and JV8-1) appears to respond to both activin and TGF-β (Zhang et al., 1996). Smad2 is phosphorylated by the TGF-β receptor complex (Macias-Silva et al., 1996). Overexpression of Smad3 induces strong ligand-independent TGF-β-like activity, while a truncated Smad3 mutant acts as a dominant negative inhibitor of the natural TGF-β response (Zhang et al., 1996), which strongly indicates that Smad3 is a key intracellular mediator in the TGF-β signaling pathway. On the other hand, Smad4 (DPC4) binds to and thus cooperates synergistically with Smad1, Smad2, or Smad3 to serve as a common mediator in the BMP, activin, and TGF-β signaling pathways, respectively (Lagna et al., 1996; de Caestecker et al., 1997; Zhang et al., 1997).

Thus, it appears that the TGF-β signal can be transmitted via receptor-associated Smad3 and/or Smad2 to their downstream mediator Smad4, whereas Smad1 turns out to be involved in the BMP rather than the TGF-β signaling pathway. Both exogenous TGF-β1 and TGF-β2 exert negative regulation on embryonic lung branching morphogenesis in culture (Serra et al., 1994; Zhao et al., 1996), while TGF-β3 null-mutant mice display an abnormal pattern of embryonic lung development (Kaartinen et al., 1995). On the other hand, abrogation of TGF-β type II receptor expression and/or signaling stimulated embryonic lung branching morphogenesis in culture and prevented TGF-β-induced down-regulation of cyclin A expression (Zhang et al., 1996), indicating that endogenous TGF-β also negatively regulates lung morphogenesis. However, how intracellular effector proteins in the TGF-β signaling pathway such as the Smads regulate lung organogenesis remains to be elucidated. Therefore, to investigate the functional role of Smad genes during embryonic lung branching morphogenesis, we designed antisense oligodeoxynucleotides (ODN) to attenuate Smad gene expression as a loss-of-function strategy. Due to extremely high homology between mammalian Smad3 and Smad2 (Zhang et al., 1996), the antisense ODN used in the current study was able to efficiently inhibit both Smad3 and Smad2 gene expression. We found that abrogation of Smad3 and Smad2 or of Smad4 gene expression resulted in significant stimulation of embryonic mouse lung branching morphogenesis in culture.

**MATERIALS AND METHODS**

**Organ culture.** Timed-pregnant Swiss-Webster mice were sacrificed on postcoitum day 11 (E11) and the embryos were removed. Lung primordia in early pseudoglandular stage were isolated from embryos by microdissection using micro-ophthalmic surgical instruments and a dissecting microscope under sterile conditions. Embryonic lungs were cultured under serum-free, chemically defined conditions as reported previously (Warburton et al., 1992; Zhao et al., 1996). E11 lung explants were placed on 0.80-μm MF-Millipore filters (Millipore, Bedford, MA) supported by stainless steel grids in Grobstein culture dishes (Falcon, Lincoln Park, NJ) and cultured in BGlb medium (Gibco, Grand Island, NY) supplemented with 0.1 mg/ml ascorbic acid and 50 units/ml penicillin/streptomycin. About 900 μl medium was added to each dish (approximately 10 explants per dish) to establish an air-fluid interface at the level of the explants. The cultures were maintained in 100% humidity with an atmosphere of 95% air and 5% CO₂ for 4 days. The medium was changed every 2 days.

**Quantification of terminal branches.** Branching morphogenesis was expressed as the number of epithelial sacs visible around the periphery of the lung explants. The analyses were performed (i) on whole mounts of lung explants, using transillumination to visualize structures and photomicrography to record permanent images, and (ii) on paraffin histological sections of fixed material. In either case, both lungs were counted.

**Smad antisense oligodeoxynucleotide inhibition.** Smad antisense ODN was designed to surround the translational initiation site, a place known empirically to be most effective for inhibition of gene expression. Smad antisense ODN 5’GCGAGATGGCGACAT3’ was synthesized based on mammalian Smad3 (perfect match for both rat and human Smad3 DNA; Y. Chen et al., 1996; Zhang et al., 1996) and Smad2 (only one mismatch at underlined nucleotide “G” for murine Smad2 cDNA; Baker and Harland, 1996). Two same-length control ODNs to Smad were also synthesized: (i) 5’TGTCGACCATGAGAC3’ (same nucleotides but randomly scrambled sequence) and (ii) 5’ATGCTGCACTCCATG3’ (sense sequence).

For Smad4 (DPC4), antisense ODN 5’TAGACATATGGT-
CCAT3’ was designed based on the murine cDNA sequence (Yingling et al., 1996). Same-length scrambled and sense control ODNs for Smad4 were also synthesized. The first and last three bases on each ODN sequence were modified by phosphorothioation to improve stability against nuclease degradation in culture. The ODNs were purified by OPC column before use (USC MicroCore Lab, Los Angeles, CA). Antisense, scrambled, and sense ODNs were added to embryonic mouse lung culture in aqueous solution to achieve final concentrations of 20 or 40 µM.

**Smad competitive PCR.** Smad genes are highly conserved and close homology exists among species (Derynck and Zhang, 1996). A cDNA fragment of murine Smad3 was obtained from reverse-transcribed embryonic lung total RNA by degenerate PCR and subsequently sequenced. This murine Smad3 fragment has about 95% nucleotide sequence homology to either rat or human Smad3 (Zhao and Warburton, unpublished data). Thus, a pair of PCR primers was synthesized based on the murine Smad3 cDNA sequence: primer 1 (upstream), 5’GAGTAGAGGCAGTTCTACCC3’; and primer 2 (downstream), 5’GGTTTGGGAGAACTCGGTCC3’. When primers 1 and 2 were used, the resultant PCR product from reverse-transcribed embryonic mouse lung total RNA was 234 bp in length. Construction of Smad3 competitor was done as described previously (Zhao et al., 1996). Briefly, two composite primers were used for Smad3 competitor construction (Fig. 3): each composite primer had the target Smad3 gene primer sequence (black box) attached to a short stretch of sequence (hatched box) designed to hybridize to opposite strands of a heterogeneous DNA fragment. The desired primer sequences were thus incorporated into the heterogeneous fragment during the PCR. This ensured that all Smad3 competitor molecules had the same gene-specific primer sequences as the Smad3 cDNA. The heterogeneous DNA was derived from a piece of v-erbB DNA. Smad3 competitor using primers 1 and 2 is 344 bp in length. The identities of the Smad3 and its competitor were both confirmed by DNA sequencing, proving that they both utilize the same set of PCR primers (Sanger et al., 1977).

**RNA extraction and reverse transcription.** Total RNA from each individual cultured lung explant was extracted by guanidinium thiocyanate following homogenization utilizing the Total RNA Isolation kit from 5’Prime-3’Prime (West Chester, PA). Extracted total RNA was reverse transcribed as described previously (Zhao et al., 1995). Samples were incubated at 37°C for 1 h in 20 µl of 10 mM Tris (pH 8.4), 50 mM KCl, 1 mM MgCl2, 1 mM dithiothreitol, 5 units ribonuclease inhibitor, 0.5 mM dNTP, 100 pmol oligo(dT)12-18, and 200 units M-MLV reverse transcriptase (USB, Cleveland, OH). The reaction was terminated by heating for 5 min at 95°C. Reverse-transcribed products were then used for competitive PCR assay.

Competitive PCR amplification was carried out in a Robocycler (Stratagene, La Jolla, CA) using a modification of a previously described assay for TGF-β type II receptor (Zhao et al., 1996). Thirty-five cycles of denaturation at 94°C for 2 min, annealing at 62°C for 2 min, and extension at 72°C for 2 min were routinely performed after an initial 3-min denaturation at 94°C. The final cycle included a 5-min extension step. The reaction mixture contained 10 mM Tris (pH 9.4), 50 mM KCl, 1 mM MgCl2 (optimized), 0.01% gelatin, 0.1% Triton X-100, 20 pmol primer sets, 100 µM dNTP, and 0.5 unit Advantage Klentech DNA polymerase (Clontech, Palo Alto, CA). Reaction mixture containing a known amount of competitor was added to reverse-transcribed samples derived from 20 ng total RNA or to dilutions of standard cDNA templates in a total volume of 50 µl. Concentrations of cDNA standard solutions were determined spectrophotometrically upon absorbance at 260 nm using 1 A260 = 50 µg/ml. Only 20 ng reverse-transcribed total RNA was used in respective Smad competitive PCR. β-Actin competitive PCR as an internal control was performed on the same samples along with the above genes.

**Denisometric analysis.** Electrophoresis was performed on 3% agarose gels (3:1 mixture of NuSieve and Seakem; FM C, Rockland, ME) in a submarine gel unit (C.B.S. Scientific Co., Del Mar, CA), in which target and competitor PCR products were separated by size. Gels were stained with 5 µg/ml ethidium bromide and photographed with Polaroid 667 films. The intensity was determined by densitometric analysis using ImageQuan band-analyzing software (Molecular Dynamics, Sunnyvale, CA). Calculation and statistical analysis were performed utilizing Microsoft Excel 5.0. A P < 0.05 was accepted as statistically significant. All the organ culture experiments in the present study were repeated at least three times, with identical results obtained.

**Immunohistochemistry.** Cultured lung explants were fixed with paraformaldehyde upon harvest and subsequently embedded into paraffin. Embryonic lung sections (5 µm thick) on poly-L-lysine-coated slides were used for immunohistochemical study. Smad antibody (goat polyclonal IgG; Santa Cruz Biotechnologies) was used at a concentration of 10 µg/ml for Smad protein immunostaining. Biotinylated second antibody and streptavidin–peroxidase conjugate were used to detect bound antibody. Subsequent addition of aminoethyl carbazole chromogen generated a reddish precipitate surrounding the Smad antigen (Zymed, South San Francisco, CA). Three negative controls were run in parallel with Smad antibody to ensure specificity: (1) polyclonal IgG preabsorbed with blocking peptide, (2) bovine serum albumin, and (3) water control.

**Western analysis.** Individual lung explants were harvested after culture and placed in a lysis buffer containing 1% Triton X-100, 0.5% NP-40, and protease inhibitors. The total protein concentration in each lung sample was determined using the Micro BCA Protein Assay Reagent kit (Pierce, Rockland, IL). Fifty micrograms of total protein from each sample was electrophoresed on a 7.5% Tris–Glycine polyacrylamide gel. Protein on the gel was then transferred to a Millipore Immobilon-P membrane by using a Bio-Rad Mini Trans-Blot electrophoretic transfer cell. Equal transfer efficiency for each sample was confirmed by Coomassie blue stain of the membrane after Western blot. The subsequent Western blot was performed utilizing the Chemiluminescence Western Blotting kit (Boehringer Mannheim, Indianapolis, IN). A final concentration of 200 ng/ml Smad antibody (goat polyclonal IgG; Santa Cruz Biotechnologies) was incubated with the membrane at 4°C overnight. POD (horseradish peroxidase)-labeled secondary antibody was added to incubate with the membrane for 1 h. The chemiluminescent light was captured by Kodak X-Omat AR film for a permanent record.

**RESULTS**

**Antisense ODN to Smad3 and Smad2 stimulated embryonic mouse lung branching morphogenesis in culture.** E11 lung explants (Fig. 1A) underwent extensive branching morphogenesis in BGJb media and displayed a characteristic branched pattern (Fig. 1B) after 4 days in culture. A clear stimulation of lung branching was observed when antisense ODN to Smad3 and Smad2 genes at both 20 and 40 µM was added directly to culture media (Figs. 1D and 1F), whereas parallel experiments with scrambled (Figs. 1C and 1E) or
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sense (not shown) ODN to Smad3 and Smad2 did not show any stimulatory effect. After quantification of terminal branches, Smad3 and Smad2 antisense ODN-treated lung explants resulted in a concentration-dependent stimulation on branching morphogenesis (Fig. 2, left): antisense ODN to Smad3 and Smad2 increased embryonic lung branching morphogenesis by 24 or 53% at 20 or 40 μM, respectively (P < 0.05). However, both scrambled control ODN (105 or 109% of media control at 20 or 40 μM, respectively) and sense control ODN to Smad3 and Smad2 (105 or 95% of media control at 20 or 40 μM, respectively) resulted in unchanged levels of lung branching morphogenesis in comparison to media control (Fig. 2, left).

Measurement of Smad mRNA levels by competitive RT-PCR. To evaluate the consequences of Smad antisense ODN treatment in cultured embryonic lung, a highly sensitive and accurate assay to measure specific Smad mRNA amount was developed based on competitive RT-PCR methodology. For Smad3, the same pair of PCR primers was designed and used for amplification of both Smad3 cDNA and Smad3 competitor (Fig. 3A) as described under Materials and Methods. When a constant amount of Smad3 competitor was used to coamplify with a 1:2 dilutional series of Smad3 cDNA, an electrophoretic pattern showing an inverse relationship in band intensities between Smad3 cDNA and its competitor was obtained (Fig. 3B). Thus, the ratio of Smad3 cDNA to Smad3 competitor PCR products reflected the initial amounts of Smad3 cDNA and Smad3 competitor included in the competitive PCR amplification. Following densitometric scanning, a regression line (R² > 0.95) for Smad3 competitive PCR was acquired by plotting the logarithm of band intensities of Smad3 cDNA over its competitor against the initial amounts of Smad3 cDNA added in the Smad3 competitive PCR (Fig. 3C, open circles).

In the next step, different amounts of reverse-transcribed total RNA from cultured embryonic lungs were used to coamplify with the same amount of Smad3 competitor, and a similar regression line (R² > 0.95) with the same slope value was obtained (Fig. 3C, closed circles). The same slope values as above indicated that recombinant Smad3 behaved identically to the Smad3 cDNA derived from reverse-transcribed products. Thus, it is possible to accurately measure Smad3 mRNA levels as its cDNA equivalent using the linear equation derived from recombinant Smad3 standards. The detection limit for Smad3 competitive RT-PCR is 0.0625 fg Smad3 cDNA or 10 ng reverse-transcribed total RNA (Fig. 3C).

Competitive PCR methodology was developed for other murine Smad mRNA quantification, including Smad1, Smad2, and Smad4 (DPC4), in embryonic mouse lung, using a similar strategy as for Smad3 (standard curve not shown). Because of high homology between mammalian Smad genes (especially between Smad3 and Smad2, >95%), PCR primers were carefully chosen and resultant PCR products were sequenced or digested with restriction enzymes to ensure specificity.

FIG. 1. Smad gene expression modulated embryonic murine lung branching morphogenesis. E11 embryonic mouse lung dissected from embryo (A) underwent branching morphogenesis to develop a characteristic branching pattern (B) after 4 days in B6jb medium culture. While antisense ODN to Smad3 and Smad2 at 20 or 40 μM demonstrated a concentration-dependent stimulation of lung branching morphogenesis (D and F), scrambled ODN to Smad3 and Smad2 at the same concentrations showed no effect (C for 20 μM and E for 40 μM). The stimulatory effect on branching could not be overcome by addition of exogenous TGF-β1 in the presence of Smad antisense ODN (H), while coadministration of both Smad scrambled ODN and TGF-β1 resulted in inhibition of branching morphogenesis (G). Bar represents 150 μm.
FIG. 2. Antisense ODN to Smad3 and Smad2 or to Smad4 (DPC4) gene stimulated embryonic mouse lung branching morphogenesis in a concentration-dependent manner. E11 lung explants were cultured in media control (MC), scrambled (SR), sense, (SE), or antisense ODN to Smad3 and Smad2 (left) or Smad4 (middle) for 4 days and the number of terminal branches were quantitated after culture. Error bars indicate standard deviation from at least 10 lungs for each assayed condition. While two control ODNs (SR and SE) yielded numbers of terminal branches at both 20 and 40 μM similar to those of MC, antisense ODN to Smad3 and Smad2 resulted in a concentration-dependent stimulation of embryonic lung terminal branches (124 and 153% compared to MC at 20 and 40 μM, respectively). A similar dose-dependent stimulation of branching morphogenesis was also observed when antisense ODN to Smad4 was used in embryonic lung explant culture (middle). When antisense ODNs to both Smad3 or Smad2 and Smad4 were used at 40 μM in lung explant culture, branching morphogenesis was increased by 59% (right).

Attenuation of endogenous Smad gene expression by antisense ODN in embryonic mouse lung culture. Embryonic lungs were extracted for total RNA after culture, and reverse-transcribed total RNA was then subjected to competitive PCR assay for both Smad3 and Smad2 mRNA determination. Embryonic lungs cultured in various concentrations (20 and 40 μM) of scrambled and sense control ODNs to Smad3 and Smad2 yielded amounts of Smad3 mRNA comparable to media control, as shown by Smad3 competitive PCR electrophoretic pattern (Fig. 4A). In contrast, lungs treated with antisense ODN to Smad3 and Smad2 showed decreased amounts of endogenous Smad3 mRNA, indicating that Smad3 and Smad2 antisense ODN, not scrambled or sense control ODN, efficiently inhibited Smad3 gene expression. Due to the high linearity of Smad3 competitive PCR standard curve (Fig. 3), amounts of Smad3 mRNA in different samples can be visually compared based on the electrophoretic pattern (Fig. 4A). However, accurate measurement was obtained after densitometric analysis and subsequent calculation (Fig. 4B, left). Antisense ODN to Smad3 and Smad2 inhibited endogenous Smad3 mRNA levels in a dose-dependent manner, 20 μM Smad antisense ODN decreased Smad3 mRNA levels by 41%, and 40 μM to a remnant 3% of basal level (P < 0.05). In contrast, none of the control ODN (scrambled and sense) to Smad3 and Smad2 at either of the above concentrations significantly changed Smad3 mRNA levels in cultured lungs.

Smad3 and Smad2 antisense ODN not only efficiently abrogated endogenous Smad3 gene expression in embryonic mouse lung culture, but also negatively regulated endogenous Smad2 mRNA levels in the same assayed lung samples (Fig. 4A). Similar to Smad3 inhibition, Smad3 and Smad2 antisense ODN attenuated endogenous Smad2 gene in a dose-dependent fashion: 20 and 40 μM antisense ODN to Smad3 and Smad2 significantly down-regulated Smad2 mRNA levels to 64 and 9%, respectively, in comparison to media control (Fig. 4B). Thus, the Smad3 and Smad2 antisense ODN we used in the current study was able to efficiently and simultaneously inhibit mRNA expression of both Smad3 and Smad2 genes.

Although antisense ODN to Smad3 and Smad2 attenuated both Smad3 and Smad2 mRNA levels in the embryonic lung culture, endogenous Smad1 and Smad4 levels were unaffected by Smad3 and Smad2 antisense ODN (Fig. 4A), indicating that Smad3/2 antisense ODN only inhibited Smad3 and Smad2 gene expression with high efficiency and specificity. Thus, the observed stimulation of embryonic lung branching morphogenesis could be attributed to the abrogation of Smad3 and Smad2, but not Smad1 and Smad4, gene expression.

Abrogation of Smad4 (DPC4) gene expression also resulted in increased embryonic lung branching morphogenesis. The downstream effector protein for Smad3 and Smad2 signaling in the activin/TGF-β pathway was also tested for its role during embryonic lung organogenesis. While both scrambled and sense ODN to Smad4 did not
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FIG. 3. Competitive RT-PCR for Smad3 mRNA measurement. (A) The same pair of primers (primers 1 and 2, black boxes) were used for amplification of both Smad3 cDNA and its competitor, and the resultant PCR products were 234 and 344 bp in length, respectively. (B) A constant amount of Smad3 competitor (1 fg) was coamplified with a dilutional series of Smad3 cDNA (lanes 1 to 10: 0, 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, and 16 fg) and the resultant electrophoretic pattern showed a reverse relationship of intensities between Smad3 cDNA and its competitor bands. (C) The initial amount of Smad3 cDNA was plotted against the logarithm of (Smad3/competitor) after densitometric scanning of both Smad3 and its competitor band intensities. A linear regression line (open circles) was thus obtained. A similar regression line with identical slope value was also obtained (closed circles) when different amounts of reverse-transcribed products from embryonic lung replaced Smad3 cDNA in Smad3 competitive PCR. Both regression lines have coefficient factor values ($R^2$) greater than 0.95.

change lung branching, antisense ODN to Smad4 led to stimulation of embryonic lung branching morphogenesis (Fig. 2, center). As determined by the number of terminal branches, antisense ODN to Smad4 increased embryonic lung branching by 19 and 33% at 20 and 40 μM, respectively (P < 0.05), while neither scrambled nor sense ODN to Smad4 showed any significant change in lung branching morphogenesis in culture (Fig. 2, center). Thus, Smad4 antisense ODN stimulated embryonic branching morphogenesis in cultured lungs in a concentration-dependent manner, similar to that of antisense ODN to Smad3 and Smad2.

Specificity of Smad4 antisense ODN was confirmed by competitive PCR quantification of Smad4 mRNA level in cultured embryonic lungs. As shown in Fig. 6, antisense ODN to Smad4 efficiently attenuated Smad4 mRNA expression: 20 μM Smad4 antisense ODN decreased Smad4 mRNA to 58% and 40 μM antisense ODN to Smad4 reduced Smad4 mRNA level in cultured embryonic lung to a remnant 10% in comparison to media control. However, both scrambled and sense ODN to Smad4 had no inhibitory effect on Smad4 mRNA expression. Additionally, while Smad4 antisense ODN efficiently inhibits Smad4 gene expression, other Smad gene expression, including Smad1, Smad2, and Smad3, remained unchanged regardless of Smad4 antisense treatment on embryonic lung explant in culture (Fig. 6A). Therefore, the observed stimulatory effect upon lung branching morphogenesis with Smad4 antisense-treatment resulted from abrogation of Smad4, not Smad1, Smad2, or Smad3, gene expression.

Since abrogation of either Smad3/Smad2 or Smad4 resulted in similar stimulation of embryonic lung branching, the combined effects of simultaneous inhibition of both Smad3/Smad2 and Smad4 were subsequently studied by antisense ODN inhibitory strategy. While either Smad3/Smad2 or Smad4 antisense ODN alone (40 μM) resulted in respective 53 and 33% increase of lung branching, coadministration of both Smad3/Smad2 and Smad4 antisense ODNs (40 μM) stimulated embryonic lung branching morphogenesis by 56%, comparable to that of either Smad3/Smad2 or Smad4 alone (P < 0.05) (Fig. 2, right). Such a lack of syner-
Inhibition of Smad3 and Smad2 gene mRNA by antisense ODN. (A) As shown by competitive PCR electrophoretic pattern, embryonic mouse lung explants exposed to media control (MC) or scrambled (SR) or sense (SE) ODN to Smad3 and Smad2 gene at both 20 and 40 μM yielded comparable amounts of both Smad3 and Smad2 mRNA. In contrast, lungs treated with Smad antisense (AS) ODN to Smad3 and Smad2 at above concentrations resulted in reduction of both Smad3 and Smad2 mRNA levels. (B) Quantification of both Smad3 and Smad2 mRNA levels after densitometric scanning showed a dose-dependent inhibition of both Smad3 and Smad2 mRNA by antisense ODN, while both Smad3 and Smad2 mRNA amounts in controls (media control and scrambled and sense ODN) remained unchanged. Antisense ODN to Smad3 and Smad2 showed no inhibitory effect on either Smad1 or Smad4/DPC4 mRNA level. Although only one typical lung was shown in each electrophoretic pattern, error bars (B) represent standard deviations from at least six lungs for each experimental condition.

Stimulatory effect on lung branching stimulation when both Smad3/Smad2 and Smad4 were simultaneously inhibited confirmed that these Smad genes function through an identical signaling pathway during lung organogenesis. That is, the observed stimulation of lung branching by antisense ODN to either Smad3/Smad2 or Smad4 is due to blockade of the TGF-β signaling.

Stimulatory effect on lung branching morphogenesis by Smad antisense ODN was not overridden by exogenous TGF-β. To further test the effect of abrogation of Smad genes on branching, embryonic lungs in culture were treated with exogenous TGF-β1 in the presence of Smad antisense ODN. Previous studies have shown that TGF-β1 ligand decreases embryonic lung branching morphogenesis in a dose-dependent fashion (Serra et al., 1994; Zhao et al., 1996). Herein, 10 ng/ml TGF-β1 showed an inhibitory effect on lung branching in the presence of 40 μM scrambled (Fig. 1G) or sense (not shown) control ODN to Smad3 and Smad2. When terminal branches were quantitated, 10 ng/ml TGF-β1 reduced lung branching by 30% in plain media or by 26% in 40 μM Smad3 and Smad2 scrambled ODN (Fig. 5, open bars). A similar inhibitory effect was also observed when Smad3 and Smad2 control ODN was substituted with Smad4 control ODN in the presence of TGF-β1: TGF-β1 decreased lung branching morphogenesis by 31% in the presence of 40 μM scrambled ODN to Smad4 (closed bars). However, such an inhibitory effect was abolished when antisense ODN to either Smad3 and Smad2 or Smad4 replaced respective scrambled control ODN in lung culture media. Stimulation of lung branching was observed in the presence of Smad antisense ODN, regardless of exogenously added TGF-β1 (Fig. 5). While 40 μM antisense ODN to Smad3 and Smad2 increased lung branching by 48%, a comparable 41% elevation was still observed when exogenous TGF-β1 was added in addition to antisense ODN (open bars). A similar refractoriness to exogenous TGF-β1-induced lung branching inhibition was also obtained when antisense ODN to Smad4 was used: A 30% stimulation of...
branching remained, regardless of exogenously added TGF-β1 in embryonic lung culture treated with Smad4 antisense ODN (closed bars). The above findings indicate that TGF-β failed to exert its negative effect on lung branching morphogenesis when the TGF-β signaling pathway was blocked by abrogation of Smad3 and Smad2 or their downstream Smad4 gene expression.

Smad proteins were localized primarily to distal epithelium in embryonic murine lung. To further test the hypothesis that Smad gene expression is involved in regulating endoderm-mesoderm interaction during embryonic mouse lung branching morphogenesis, different Smad proteins were localized in cultured embryonic lungs by immunostaining. Smad1 protein, the intracellular effector for BMP pathway, was primarily detected in the distal epithelial cells (Fig. 7A, arrowheads), while proximal epithelium was free of Smad1 staining (arrow). Under high power, Smad1 protein localized at highest intensity in epithelial cells lining the tips of branching bronchi (Fig. 7B, arrowheads), which is reminiscent of BMP localization pattern by whole-mount in situ hybridization (Bellusci et al., 1996). Both Smad2 (Fig. 7C) and Smad3 (Fig. 7D) were localized to the epithelial cells in the distal airways, especially in terminal bronchial epithelium undergoing branching. However, little or no Smad2 and Smad3 staining was detected in pulmonary mesenchyme. The overlapping pattern of Smad2 and Smad3 protein localization indicates that Smad2 and Smad3 may have or share similar functions during embryonic lung morphogenesis. Based on the immunostaining results, all the Smad proteins we tested were primarily detected in cytoplasm of epithelial cells and were concentrated along the mesenchymal side of the cytosol, while relatively less staining was found on the luminal side of cytoplasm (Figs. 7A–7D). Such a polar distribution of Smad proteins in cytoplasm suggests that Smad proteins participate in epithelial-mesenchymal interaction. For Smad1, Smad2, and Smad3, proteins were exclusively detected in the distal epithelium, whereas immunostaining was absent in the pulmonary mesenchyme (Figs. 7A–7D). However, Smad4 (DPC4) staining was observed in both lung distal epithelial and stromal cells, although lesser staining was found in mesenchyme (Fig. 7F). Like the other Smads, Smad4 protein was not found in proximal epithelial cells (Fig. 7F, arrow). Therefore, Smad proteins overlap as well as distinguish from each other in their localization pattern, indicating that they may function in a cooperative manner in the same cells as well as possibly having distinct functions in different cell populations during embryonic lung branching morphogenesis.

Antisense ODN also decreased Smad protein levels in cultured lungs. Western analysis was used to confirm the specificity of Smad antibodies and the reduction of Smad protein levels with Smad antisense ODN administration. As shown in Fig. 8A, cultured lungs treated with Smad3/Smad2 antisense ODN showed decreased levels of both Smad3 and Smad2 proteins in comparison to lungs with scrambled ODN treatment, while Smad4 protein amounts in cultured lungs remained unchanged with Smad3/Smad2 ODN administration (Fig. 8A, left). On the other hand, antisense to Smad4 specifically reduced only Smad4 protein, not Smad3 or Smad2 protein amounts, in embryonic lungs in culture (Fig. 8A, right). Smad1 protein levels in lungs were not significantly altered regardless of Smad2, Smad3, or Smad4 ODN treatment (data not shown). After densitometric analysis, Smad2, Smad3, or Smad4 protein levels were respectively decreased to only 14, 10, or 11% (P < 0.05) of media control levels with Smad antisense ODN treatment (40 μM), while either scrambled or sense Smad ODN at the same concentration did not have any inhibitory effect on Smad protein levels (Fig. 8B). Therefore, only the antisense Smad ODN, not the scrambled or the sense Smad ODN, resulted in a significant reduction in the corresponding Smad protein level, while other Smad protein amounts were not changed.

Specificity of Smad proteins was established: each Smad antibody recognized the band corresponding to the predicted size of the respective Smad protein on Western blot using lysis of cultured embryonic lungs treated with Smad ODN. In the parallel control experiments, similar Western results were obtained using Smad antibody with either embryonic lung primary mesenchymal cells or NIH 3T3 cells (data not shown), confirming the specificity of Smad antibodies used in the current study. Additionally, these specific Smad protein bands were diminished when Smad antibodies were preabsorbed with respective control peptides prior to Western analysis.

**DISCUSSION**

Morphogenetic autocrine/paracrine interactions between mesenchymal and epithelial cells, as well as cell-matrix
Antisense ODN to Smad4 (DPC4) decreased its mRNA level. Smad4 mRNA expression was inhibited in a dose-dependent manner by antisense ODN to Smad4 at both 20 and 40 μM in cultured embryonic lungs, while either scrambled or sense control ODN at the same concentrations did not affect Smad4 gene expression. (A) Smad4 antisense ODN failed to change Smad1, Smad2, or Smad3 mRNA expression. (B) Error bars represent standard deviation from at least 10 lung explants for each assayed condition, although one lung was shown in each electrophoretic pattern (A).

interactions, are implicated in the morphogenesis of branched mammalian organs, including lung, kidney, breast, pancreas, prostate, salivary gland, and sweat glands, as well as tooth buds. Early embryonic lung develops at E9.5 in the mouse as an endodermal appendage of the anteroventral pharynx, which extends into the surrounding mesenchyme in a process termed branching morphogenesis. Mesenchymal recombination studies have demonstrated that peripheral embryonic lung mesenchyme can induce ectopic branching as well as concomitant expression of peripheral lung-specific genes, when grafted adjacent to proximal lung epithelium in embryonic lung in culture, and that this effect is mediated by soluble and diffusible factors (Alescio and Cassini, 1962; Shannon, 1994).

A number of soluble peptide growth factors are potential mediators instructing primitive lung epithelial branching morphogenesis. In general, the peptide growth factors that activate cognate receptors with tyrosine kinase intracellular signaling domains such as EGFR, FGFR, c-met, IGFR, KGFR, and PDGFR exert inductive or permissive influences on lung development (Warburton et al., 1992; Miettinen et al., 1995, 1997; Souza et al., 1995; Shiratori et al., 1996), while those cognate receptors with serine/threonine kinase signaling domains, such as the TGF-β receptors, are inhibitory (Serra et al., 1994; Zhao et al., 1996).

Overexpression of Smad genes provided genetic evidence that Smads are effector proteins in BMP/activin/TGF-β receptor signaling and that different Smads induce distinct biological activities. Microinjection of Smad1 into Xenopus embryo mimics mesoderm ventralizing effects of BMP-4 (Liu et al., 1996). Smad1 moves to the nucleus from the cytoplasm when BMP-4 is expressed. BMP-2, not TGF-β, results in Smad1 phosphorylation (Hoodless et al., 1996). On the other hand, Smad2 is regulated by TGF-β, not BMP, and missense mutations of Smad2 are not only inactive, but also are found in colorectal carcinoma (Eppert et al., 1996). TGF-β stimulates the phosphorylation and nuclear translocation of Smad2 in Mv1Lu cells (Nakao et al., 1997). Smad2 transiently interacts with and is directly phosphorylated by the TGF-β receptor complex on C-terminal serines. The latter phosphorylation requires activation of TGF-β type I receptor by TGF-β type II receptor (Macias-Silva et al., 1996). Mutation of these phosphorylation sites generates a dominant negative Smad2 which blocks TGF-β-dependent responses, while Smad2 fails to accumulate in the nucleus in response to TGF-β signaling.
FIG. 7. Immunohistochemistry of Smad protein in cultured embryonic lungs. Smad protein was localized by immunostaining in cultured embryonic lung sections. (A) Smad1 protein was found primarily in distal epithelium, (B) especially in the epithelial cells lining the tips of branching airway. Both (C) Smad2 and (D) Smad3 share a similar immunostaining pattern: their proteins were detected in epithelial cells along the terminal bronchi. (E) Smad4 (DPC4) protein was found in both distal epithelial and mesenchymal cells, (F) while lung section incubated with anti-IgG preabsorbed with blocking peptide (antigen) yielded negligible background. Magnification: A, 100×; C–F, 200×; B, 400×.

Overexpression of Smad2 functions as an activin-like mesoderm inducer, and addition of activin enhances the concentration of Smad2 in the nucleus (Baker and Harland, 1996). Therefore, while Smad1 induces BMP-like responses, Smad2 seems to be involved in transduction of activin/TGF-β signaling.

It has also been shown that normal ligand-induced responses can be recapitulated or inhibited by overexpressing wild-type versus inactive Smads. C-terminal-truncated Smad3 acts as a dominant negative, overexpression of which inhibits the natural TGF-β response (Zhang et al., 1996). However, this inhibitory effect was not observed when dominant negative Smad3 was substituted with either mutant Smad1 or Smad2. Furthermore, overexpression of
FIG. 8. Western analysis of Smad proteins for cultured embryonic lungs. (A) Antisense (AS) ODN to Smad3 and Smad2 reduced both Smad3 and Smad2 protein levels relative to those of cultured embryonic lungs with scrambled (SR) ODN, while Smad4 protein amounts did not change (left). Smad4 (DPC4) antisense ODN specifically decreased Smad4 protein level in comparison to scrambled ODN-treated lungs, while both Smad3 and Smad2 protein levels remained the same regardless of the addition of Smad4 ODN (right). (B) Densitometric analysis subsequent to Western assay showed a greatly reduced level of Smad protein in cultured lungs with respective Smad antisense ODN treatment, while both SR and sense (SE) ODN controls yielded levels of Smad proteins similar to those of the media control (MC). Data are presented as means ± SD.

Smad3 stimulated basal TGF-β-responsive transcriptional activity, whereas both basal and TGF-β-induced transcriptional activity was inhibited when Smad1 was overexpressed (Y. Chen et al., 1996). Thus, Smad3 acts as an intracellular effector of the TGF-β response, indicating a selectivity in Smad activities and that different Smad proteins mediate respectively BMP, activin, and TGF-β signaling pathways.

Both Smad3 and Smad2 are capable of responding to extracellular TGF-β signals. Smad2 is phosphorylated upon association with the TGF-β receptor complex and mutation of the Smad2 phosphorylation sites functions as a dominant negative, blocking TGF-β signaling (Macas-Silva et al., 1996). However, overexpression of dominant negative Smad3, but neither Smad1 nor Smad2, inhibited the TGF-β response (Zhang et al., 1996). While the intracellular interactions between Smad3 and Smad2 remain to be clarified, comparison between Smad3 and Smad2 sequences revealed that their proteins share 97% identity, suggesting that they may have very similar functions.

Because Smad3 and Smad2 are such closely homologous proteins, we chose a Smad antisense ODN strategy in the current study based on the mammalian Smad3 sequence which has only one nucleotide mismatch with the corresponding murine Smad2 cDNA. Consequently, our Smad antisense ODN was able to down-regulate both Smad3 and Smad2 gene expression simultaneously. An alternative Smad antisense ODN was designed based on the Smad2 sequence at its translational initiation site and thus had one nucleotide mismatch with Smad3 cDNA. This alternative Smad2 antisense ODN also displayed a strong inhibitory effect on mRNA and protein expression of both Smad3 and Smad2 genes (data not shown). However, while both of the above Smad antisense ODNs efficiently attenuated Smad3 and Smad2 expression, they had little or no effect on expression of other divergent Smad genes, including Smad1 and Smad5 (data not shown), indicating that the antisense ODN used in the current study specifically inhibited expression of Smad3 and Smad2 genes, but not other Smads.

In the current study, we were not able to differentiate between the effects of inhibition of Smad3 and Smad2 gene expression using Smad antisense ODN, probably due to the extremely high homology between mammalian Smad3 and Smad2 sequences. As a result, the observed stimulatory effect on lung branching morphogenesis by Smad antisense ODN treatment could not be attributed specifically to Smad2 or Smad3. Nevertheless, since both Smad3 and Smad2 function in the TGF-β signaling pathway, we feel confident deducing that the stimulation of embryonic lung branching we observed following abrogation of Smad2 and/or Smad3 resulted from the release of endogenous TGF-β-mediated negative influence on morphogenesis. Therefore, our results further support that Smad2 and/or Smad3, not other Smads, are the major intracellular effectors in response to TGF-β signaling.

Smad3 and Smad2 are homologous yet distinct gene products. Thus, we were able to develop competitive PCR assays for respective quantitation of Smad3 and Smad2 mRNA levels. PCR primers were carefully chosen from the least homologous sequences to ensure specific amplification of either Smad3 or Smad2 gene (see Materials and Methods), and subsequent PCR products were sequenced or digested with appropriate restrictive enzymes to confirm identities. Competitive PCR coupled with reverse transcription is an accurate and highly sensitive method of measuring specific low-abundance gene message in limited sample material (Zhao and Nishimoto, 1995). Using conventional hybridization methods like Northern blot, 10–50 μg total RNA is routinely used for one mRNA determination (Siebert and Larrick, 1992). However, as shown in our competitive PCR
assay, only 20 ng total RNA was used to measure Smad3 or Smad2 mRNA yield in a single cultured embryonic lung. This high sensitivity has advantages for limited sample material, as is the situation in mRNA measurement in embryonic tissues. The competitive PCR method developed herein enabled us to measure Smad mRNA levels in a single embryonic mouse lung without sample pooling.

The TGF-β pathway is known to negatively regulate branching morphogenesis of mammalian organs, and TGF-β is a potent inhibitor of epithelial cell growth. Thus, several components of TGF-β signaling pathways have been studied as potential tumor suppressor genes. TGF-β type II receptor mutations are found in human colon cancer cells (Markowitz et al., 1995) and probably permit the escape of these cells from TGF-β-mediated negative growth control. Our previous studies showed that antisense ODN to TGF-β type II receptor resulted in stimulation of lung branching and prevented TGF-β-induced down-regulation of both cyclin A and PCNA (Zhao et al., 1996), supporting the inhibitory role of endogenous TGF-β signaling on organ growth. Similarly, both missense and deletional somatic mutations of Smad3 were identified in human lung cancer and colorectal carcinoma (Uchida et al., 1996; Eppert et al., 1996). Smad4 (DPC4), which associates with Smad1, Smad2, or Smad3 in response to BMP, activin, or TGF-β, respectively, was initially isolated as a mutated gene in human pancreatic carcinomas (Hahn et al., 1996; Schutte et al., 1996). Such mutations in the Smad4 C-terminal domain were later found to impair the heteromeric interaction between Smad4 and other receptor-associated Smads (Wu et al., 1997). Our finding that abrogation of Smad3 and Smad2 genes resulted in stimulation of embryonic lung branching, similar to abrogation of TGF-β type II receptor, indicates that both TGF-β receptors and the downstream Smads are important mediators of the TGF-β pathway and thus participate in TGF-β-mediated negative signaling.

Smad4 (DPC4) is a downstream mediator for both Smad3 and Smad2 (Lagna et al., 1996; Shi et al., 1997; Zhang et al., 1997). Smad4 also serves as a partner for other Smads and associates with them. Thus, it is of biological significance to test the role of Smad4 during embryonic lung branching morphogenesis. As we have shown in the current study, Smad4, like TGF-β type II receptor or Smad3/2, also stimulated branching morphogenesis when Smad4 gene expression was inhibited by its antisense ODN in culture. But, addition of antisense ODN to both Smad3/2 and Smad4 simultaneously did not result in a synergistic effect upon lung branching, indicating that both Smad3/2 and Smad4 function through the endogenous TGF-β signaling pathway to exert its negative regulation upon lung morphogenesis. Therefore, our results support the notion that TGF-β receptors and Smad genes work in tandem during endogenous TGF-β signaling: abrogation of any of their components in the TGF-β pathway resulted in disruption of TGF-β-mediated negative regulation of embryonic lung branching morphogenesis.

The activated heteromeric Smad complex may be translocated into the nucleus to function as a transcriptional activator. How the Smad complex moves into the nucleus to activate downstream genes remains to be elucidated (X. Chen et al., 1996). Very recently, Smad7 was characterized as an antagonist of endogenous TGF-β signaling, by preventing TGF-β-dependent formation of heteromeric Smad complexes, and inhibits the subsequent nuclear accumulation (Hayashi et al., 1997; Topper et al., 1997). However, activation of both TGF-β receptors and their intracellular effector Smads is clearly required for TGF-β signaling to play a role in the negative regulation of lung branching morphogenesis.

Smad proteins were detected mainly in the distal bronchial epithelium. Such localization patterns are reminiscent of genes including BMPs, FGFs, HNF, and sonic hedgehog, which have been shown to be important to the normal morphogenesis of embryonic lung (Belluscè et al., 1996). Like Smads, the above proteins have been detected in the epithelial tips of branching airways in embryonic lungs. Together with the evidence that abrogation of Smad gene expression regulates embryonic lung branching, it is likely that Smad gene expression regulates epithelial-mesenchymal interactions. Furthermore, while Smad proteins were found mainly in the epithelial cytoplasm, the highest concentration of Smad proteins was observed lining the mesenchymal side of the epithelium adjacent to the basement membrane. Such a gradient pattern of Smad protein intracellular localization further supports the notion that Smad genes function during embryonic lung branching morphogenesis through transducing endoderm-mesoderm interactions.

We conclude that endogenous TGF-β peptide signaling through not only TGF-β receptors, but also Smads, exerts negative control of the process of early embryonic mouse lung morphogenesis in culture. This negative regulation is released by abrogation of endogenous Smad3, Smad2, or Smad4 function using antisense ODN as an experimental approach. It is possible that these Smads, together with TGF-β receptors and other downstream effector genes, orchestrate TGF-β-mediated gene regulation during embryonic lung organogenesis. We speculate that endogenous TGF-β signaling through the Smads may serve to negatively modulate and therefore balance the positive functions of signaling by other peptide growth factor pathways, including EGF, FGF, and PDGF, which exert positive inductive and permissive influences on lung morphogenesis.

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