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FGF-10 induces *SP-C* and *Bmp4* and regulates proximal-distal patterning in embryonic tracheal epithelium

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Hyatt, Brian A., Xiaofei Shangguan, and John M. Shannon. FGF-10 induces *SP-C* and *Bmp4* and regulates proximal-distal patterning in embryonic tracheal epithelium. *Am J Physiol Lung Cell Mol Physiol* 287: L1116–L1126, 2004; doi:10.1152/ajplung.00033.2004.—The induction, growth, and differentiation of epithelial lung buds are regulated by the interaction of signals between the lung epithelium and its surrounding mesenchyme. Fibroblast growth factor-10 (FGF-10), which is expressed in the mesenchyme near the distal tips, and bone morphogenetic protein 4 (BMP4), which is expressed in the most distal regions of the epithelium, are important molecules in lung morphogenesis. In the present study, we used two in vitro systems to examine the induction, growth, and differentiation of lung epithelium. Transfilter cultures were used to determine the effect of diffusible factors from the distal lung mesenchyme (LgM) on epithelial branching, and FGF-10 bead cultures were used to ascertain the effect of a high local concentration of a single diffusible molecule on the epithelium. Embryonic tracheal epithelium (TrE) was induced to grow in both culture systems and to express the distal epithelial marker surfactant protein C at the tips nearest the diffusible protein source. TrE cultured on the opposite side of a filter to LgM branched in a pattern resembling intact lungs, whereas TrE cultured in apposition to an FGF-10 bead resembled a single elongating epithelial bud. Examination of the role of BMP4 on lung bud morphogenesis revealed that BMP4 signaling suppressed expression of the proximal epithelial genes *Ccsp* and *Foxj1* in both types of culture and upregulated the expression of *Sprouty 2* in TrE cultured with an FGF-10 bead. Antagonizing BMP signaling with Noggin, however, increased expression of both *Ccsp* and *Foxj1*.

pulmonary; fibroblast growth factor; bone morphogenetic protein; surfactant protein C; sonic hedgehog; Sprouty

THE EMBRYONIC MOUSE LUNG BEGINS on embryonic (E) *day 9.5* as a ventral outpocketing of two primary buds from the foregut endoderm into the surrounding splanchnic mesoderm. Elongation of these primary buds followed by several rounds of dichotomous and lateral branching gives rise to the patterned embryonic lung. Cells along the proximal-distal axis of the developing lung subsequently differentiate and express markers related to their functional roles in the mature lung. It is well established that interaction of the epithelium and its surrounding mesenchyme is necessary for the proper growth, patterning, and cellular differentiation of the lung. Much recent research has focused on identifying molecules with specific patterns of expression in lung epithelium (LgE) and mesenchyme and on elucidating how their actions and interactions regulate normal lung development (9, 23, 53, 62).

The importance of mesenchymal signaling to the epithelium for patterning and induction of gene expression cannot be overstated. Lung mesenchyme (LgM) taken from the distal tips of the embryonic lung is able to induce buds in early foregut endoderm (58), to support lung branching when recombined with distal LgE (36, 56), and to induce embryonic tracheal epithelium (TrE) to branch in a lung-like pattern (1, 52, 56). The signaling molecules responsible for these inductions are conserved across species (22). In addition to patterning the LgE, distal LgM is able to support the differentiation of alveolar type II cells, as evidenced by its ability to both induce and maintain expression of the distal lung marker *surfactant protein C (SP-C)* in embryonic tracheal and LgE, respectively (52, 56). Direct physical interaction is not necessary for the effects of mesenchyme on epithelium, since diffusible factors from the mesenchyme are able to support epithelial branching in transfilter cultures (60).

Investigation of the mesenchymal factors that affect cell differentiation in the epithelium has revealed a primary role for fibroblast growth factors (FGFs). Of the six FGF family members that are expressed in the lung, *Fgf1*, *Fgf2*, *Fgf7*, *Fgf10*, and *Fgf18* are expressed in the mesenchyme (3, 14, 16, 17, 21, 24, 35, 42). *Fgf9* is expressed in both the LgE and mesothelium on *day E10.5* and is restricted to mesothelium by *day E12.5* (11).

With the exception of *Fgf18*, all of the FGFs expressed in the lung have been shown to promote a branching/budding type of growth in mesenchyme-free cultures of embryonic lung or TrE (3, 10, 40, 41, 44, 46, 63). We have previously shown that FGF-1, FGF-2, FGF-7, and FGF-9 can induce TrE to undergo distal epithelial cell differentiation as assessed by the expression of *SP-C* (25, 54).

Of the FGFs expressed in the lung, however, only *Fgf10* has been shown to be absolutely necessary for initiation of lung development; ablation of the mouse *Fgf10* gene results in the complete absence of lung development (38, 51). Conversely, ablation of the genes for *Fgf1*, *Fgf2*, *Fgf7*, or *Fgf18* has no effect on lung development (20, 37, 43). Deletion of *Fgf9* results in neonatal death due to lung hypoplasia secondary to reduced mesenchymal growth/survival (12). Initial branching occurs in *Fgf9* null mice, and epithelial cell differentiation appears qualitatively unaffected.

Because *Fgf10* null mice lack lungs, its role in epithelial cell differentiation in vivo is not known. Previous in vitro studies in our laboratory (25, 54) showed that, in contrast to FGF-1, FGF-2, and FGF-7, addition of FGF-10 to the medium of cultures of embryonic TrE did not support the induction of *SP-C*. Similarly, a recent study using mesenchyme-free culture of embryonic LgE showed that FGF-10 was ineffective in maintaining expression of *SP-C* (31). These results present a quandary, because *SP-C* is expressed in the lung endoderm on

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day E10.5 (65), before the expression of *Fgf1*, *Fgf2*, or *Fgf7* can be detected (3). *Fgf10*, however, can be detected at this point in development.

A number of other genes have been localized to the distal epithelium during lung development (8, 30). *Bone morphogenetic protein 4 (BMP4), Sonic hedgehog (Shh),* and *Sprouty 2 (Spry2)* all exhibit high expression in the distal tip epithelium (4, 5, 61, 67), suggesting roles in lung development. Furthermore, these three proteins have been shown to interact with FGFs expressed in LgM. *Bmp4* is induced in the epithelium by FGF-10 (63) as well as other FGFs (25), SHH has been reported to regulate the expression of *Fgf10* in LgM (2, 29, 47), and expression of *Spry2* is upregulated in LgE by FGF-10 (33). Together, these results indicate likely roles for BMP4, SHH, and SPRY2 in the morphogenesis and/or differentiation of the distal LgE.

In the present study, we used two in vitro systems to examine specific epithelial responses to mesenchymal signals during bud formation and elongation as well as epithelial cell differentiation. Transfilter recombination cultures were used to investigate how diffusible signals from distal LgM reprogrammed competent TrE to adopt a lung phenotype. Because of the dramatic chemoattractive response of lung endoderm toward beads soaked in FGF-10 (46, 63), we investigated how a high local concentration of this signaling molecule affected tracheal epithelial growth and differentiation. Finally, we have examined the effects of BMP4 on both of these processes.

MATERIALS AND METHODS

Isolation of tracheal and lung tissues. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Children's Hospital Research Foundation. Isolation of mouse TrE and tracheal mesenchyme (TrM) was performed as described previously (25). Briefly, tracheae were dissected from *day E12.5* or *day E13.5* FVB/N embryos (Taconic, Germantown, NY; day of plug = *day E0.5*) with Moria microsurgery knives (Fine Science Tools, Foster City, CA). Tracheae were divided into halves, treated with HBSS containing 0.05% collagenase (Sigma Chemical, St. Louis, MO) and 1% FBS for 45 min at 37°C, rinsed with ice-cold HBSS plus 10% FBS (Sigma), and treated with 1 mg/ml DNase I (Sigma), and then the epithelium was separated from mesenchyme with fine tungsten needles. TrM was stored in HBSS plus 10% FBS on ice until needed for culture. To ensure that tracheal epithelia were pure, any traces of contamination by TrM were removed by treatment with dispase (BD Biosciences, Bedford, MA).

Isolation of mouse LgE and LgM was performed as described for the rat (13). Briefly, distal tips from *day E12.5* embyros were removed with Moria knives, treated with dispase for 15–20 min at 37°C, rinsed with ice-cold HBSS plus 10% FBS, and treated with 1 mg/ml DNase I, and then the epithelium was separated from mesenchyme with fine tungsten needles. LgM was stored in HBSS plus 10% FBS on ice until needed, whereas LgE was treated briefly with dispase to remove any adhering mesenchymal cells.

FGF bead culture. Heparin-coated beads (Sigma) were soaked in HBSS containing 100 ng/μl of FGF-10 or 25 ng/μl of FGF-7 (both from R&D Systems, Minneapolis, MN) for 2–4 h before culture. TrE and FGF beads were gently mixed into growth factor-reduced Matrigel (BD Biosciences) and placed approximately one bead diameter apart (100–200 μm) from each other. The Matrigel was gelled at 37°C, and then 2.5–3.0 ml of DMEM/F-12 medium (D/F12; GIBCO/Invitrogen, Carlsbad, CA) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B (all from GIBCO), and 5% FBS (D/F12 + 5% FBS) were added. Some cultures were treated with 50 ng/ml of BMP4 or 500 ng/ml of Noggin/Fc (both from R&D Systems) at either 0, 1, or 2 days. The day of rudiment isolation was considered *day 0*, and cultures were maintained for several hours to 5 days.

Transfilter culture. TrEs for transfilter cultures were gently mixed into 200 μl of growth factor-reduced Matrigel on the bottom side of a 0.4-μm Millicell-CM Culture Plate Insert filter (Millipore, Bedford, MA). Rudiments were positioned as close to the filter as possible. After gelation of the Matrigel, the filter was inverted and placed in a six-well culture dish, and 1 ml of DMEM plus 3% FBS was added outside of the insert. Eight pieces of mesenchyme (either lung or trachea) were placed on the inside of the insert directly above the epithelium. In some experiments, 200 ng/ml of BMP4 or 500–1,000 ng/ml of Noggin/Fc (R&D Systems) were added to some of the cultures. Cultures were maintained for several hours to 5 days.

Lung culture. Intact lung tips were cultured on D/F12 containing 0.5% agarose and 5% FBS, with or without the addition of 200 ng/ml of BMP4. Culture medium was added to maintain explants at an air-liquid interface. Cultures were maintained for 3 days, fixed, and saved for whole mount in situ hybridization. The left proximal piece of *day E11.5* lung consisted of the region caudal to the tracheal-bronchial bifurcation and cranial to the distal tip. Explants were cultured on Millicell-CM inserts in D/F12 plus 5% FBS, with or without 200 ng/ml of BMP4. Cultures were maintained for 3 days, tips were counted, and RNA was isolated for RT-PCR.

Real-time PCR and statistical analysis. Gene expression was quantitated by real-time PCR using a Smart Cycler (Cepheid, Sunnyvale, CA). Poly(A)$^+$ RNA was isolated from pooled epithelia (Micro-FastTrack kit, Invitrogen), and cDNA templates were made by reverse transcription (cDNA Cycle kit, Invitrogen or Improm-II Reverse Transcriptase; Promega, Madison, WI). PCR reaction mixes (with final concentrations listed) were assembled with the addition of template, 0.5 μM each primer (except *SP-C*, 1.0 μM each primer), 1.5 mM $MgCl_2$, 2.5 units *Taq* polymerase (Fisher), 200 μM dNTP (Roche), 1:30,000 diluted SYBR Green dye (Molecular Bioprobes), and buffer (50 mM Tris, pH 8.3, 20 mM KCl, 1 mM $MgCl_2$, 50 ng/μl BSA, and 0.01% Triton X-100). Reaction conditions differed slightly depending on the primers used, but generally were 95°C for 150 s followed by 30–40 cycles of amplification at 95°C for 6–10 s, 53–61°C for 6–20 s, and 72°C for 10–25 s. Amplicon sizes and forward and reverse primer sequences for β-actin, L32, *SP-C*, *Bmp4*, *Shh*, Clara cell secretory protein (*Ccsp*), and *Foxj1* were as previously published (25). Real-time PCR for *Spry2* gave a 173-bp amplicon with the following forward and reverse primers: 5′-CCAATGAGCAAGGATGATTTGG-3′, 5′-ACACAGCACACACAAGTCCCATAG-3′. Measurement of amplified product was made for 6 s every cycle at a temperature above that of the melting temperature of possible nonspecific products (e.g., primer-dimers) and 1–2°C below the melting temperature of the specific product. Melt curve analyses were performed after every run to assure that a single amplified product was produced. Relative quantitation was obtained by measuring the cycle at which the greatest accumulation of product occurred (cycle threshold) and plotting that against the cycle thresholds of a dilution series of positive control samples. Only experiments in which the regression analysis of the dilution series gave an r^2 value ≥ 0.98 were used to determine quantitation. Because of the small size of the tissue samples, input levels of cDNA were too low to accurately assess by optical density readings. Therefore, all gene expression levels were normalized to L32 and β-actin.

Because quantitation was relative, levels of gene expression in the samples were calculated as fold differences to one another. Statistical analysis of these values was performed using InStat version 3.0a. The one-sample *t*-test using 1 as the theoretical value was used when comparing numbers pairwise, and $P < 0.05$ was considered significant. Fold differences were converted to percentage differences for some of the tables. Control cultures of TrE were given a value of 100% for Tables 1 and 2. In some experiments, such as comparing

Table 1. *Relative gene expression in TrE cultured transfilter to LgM*

Conditions	*SP-C*	*Bmp4*	*Spry2*	*Shh*	*Ccsp*	*Foxj1*
Control†	100% (*3*)	100% (*4*)	100% (*4*)	100% (*4*)	100% (*4*)	100% (*4*)
+BMP4	6±*2%**(*3*)	61±*25%* (*4*)	92±*40%* (*4*)	55±*10%**(*4*)	4±*3%**(*4*)	18±*16%**(*4*)
+Noggin	89±*17%* (*4*)	77±*15%* (*4*)	95±*25%* (*4*)	48±*12%**(*4*)	660±*348%*‡ (*3*)	246±*27%* (*2*)

†Levels of expression were normalized to control conditions set to 100%. *Statistically significant difference compared with control, $P < 0.05$. ‡High variance in data (see MATERIALS AND METHODS). Number in parentheses, number of independent experiments. TrE, tracheal epithelium; LgM, lung mesenchyme. SP, surfactant protein C; BMP, bone morphogenetic protein; Spry, Sprouty; Shh, sonic hedgehog; Ccsp, Clara cell secretory protein.

gene expression levels between TrE cultured transfilter to LgM vs. TrM, variances in data were so high that statistical significance could not be determined. This was normally due to one sample displaying a very large variation in gene expression. Clear differences in amplicon intensity would be seen if standard RT-PCR and gel analysis were done on these samples. Because several samples were run to completion on the Smart Cycler, small changes in gene expression could be measured, but samples having large changes in gene expression showed high variability.

Whole mount in situ hybridization. Cultured TrE were treated with 2.5 ml of BD Cell Recovery Solution (BD Biosciences) for 1 h at 4°C to release the tissue from Matrigel and then fixed in 4% paraformaldehyde in PBS overnight at 4°C. Digoxigenin-labeled sense and antisense riboprobes were synthesized (Riboprobe System, Promega) from the rat cDNA for *SP-C* (15) and murine cDNAs for *Bmp4* (a gift from Dr. Brigid Hogan, Duke Univ.), *Shh* (a gift from Dr. Andrew McMahon, Harvard Univ.), *Spry2* (26), and *Nkx2.1* (19). Whole mount in situ hybridization was carried out as described by Wilkinson (66), with slight variations.

RESULTS

Embryonic TrE does not normally branch or express markers of distal lung differentiation. TrE can, however, be induced to branch identically to LgE and express the alveolar type II cell phenotype when recombined with embryonic distal LgM (56). Culture of purified embryonic TrE in a complex medium containing FGFs results in dramatic rudiment growth, although it does not recapitulate the lung branching that occurs when it is recombined with LgM (25, 54). Importantly, embryonic TrE in these cultures is induced to progress through a program of morphological and molecular type II cell differentiation, including the generation of lamellar bodies and the initiation of *SP-C* expression (54). We therefore chose embryonic TrE for the present studies as a competent nonlung tissue in which we could examine changes in gene expression during the induction of the lung phenotype.

Embryonic TrE cultured transfilter to embryonic LgM expresses lung differentiation genes. To determine the effects of diffusible factors produced by distal LgM on a competent, *day E12.5* non-LgE, TrE was cultured on one side of a Teflon filter in apposition to eight pieces of *day E12.5* LgM (Fig. 1*A*). The design of the filter allowed diffusion of macromolecules but did not allow the passage of cellular processes. After 1 day, the cut ends of the TrE sealed and the rudiments formed cysts (Fig. 1*B*). After 2 days, the rudiments continued to grow and began to bud (Fig. 1*C*). Rudiments cultured for 3 days continued to expand and showed clear branch points (Fig. 1*D*), a process that continued until the termination of the cultures at the end of the fourth day (Fig. 1*E*). In our initial experiments, we used four to six pieces of LgM but found that eight pieces gave a more consistent response of the TrE. In addition to branching, the cultured TrE was expressing *SP-C* at its distal tips (Fig. 1*H*), indicating induction of distal lung epithelial differentiation. Real-time RT-PCR of TrE cultured transfilter to LgM confirmed the induction of *SP-C* we observed by in situ hybridization. In addition, real-time PCR also showed that LgM-induced TrE expressed other markers associated with the developing lung, including *Ccsp, Foxj1 (Hfh4), Bmp4*, *Spry2,* and *Shh* (data not shown). Although TrE cultured opposite TrM grew and formed a large epithelial cyst (Fig. 1*G*), it did not branch or express *SP-C*. TrE cultured without any mesenchyme on the other side of the filter formed a small cyst that did not grow or branch (Fig. 1*F*).

Bmp4, which is expressed in distal lung epithelial cells (4), can be induced in TrE by LgM (44) and in bronchial epithelium by FGF-10 (63). Whether this induced *Bmp4* expression reflects acquisition of a distal lung epithelial phenotype or is generally associated with increased epithelial cell proliferation is not known. *Bmp4* is not expressed in distal tips until after budding occurs (63), and distal tips have increased cell proliferation after, but not before, budding occurs (41). We therefore compared *Bmp4* expression in *SP-C*-positive branching TrE/LgM transfilter cultures to *SP-C*-negative nonbranching TrE/TrM transfilter cultures. *Bmp4* expression was on average 17-fold higher ($n = 6$, range 2- to 60-fold) in TrE cultured opposite LgM than in TrE cultured opposite TrM. In these same experiments, *SP-C* expression was 3,030-fold higher ($n = 5$, range 15- to 6,600-fold) in branching TrE than in nonbranching TrE. It should be noted that the *SP-C* signal in TrE cultured transfilter to TrM was essentially at background levels, which accounts for the wide range of the observed increase.

Three observations from experiments in which TrE was cultured transfilter to LgM demonstrated that short-range dif-

Table 2. *Relative gene expression in TrE cultured with FGF-10 beads*

Conditions	*SP-C*	*Bmp4*	*Spry2*	*Shh*	*Ccsp*	*Foxj1*
Control†	100% (*6*)	100% (*5*)	100% (*4*)	100% (*6*)	100% (*6*)	100% (*6*)
+BMP4	26±*12%**(*6*)	224±*43%**(*5*)	199±*30%**(*4*)	55±*12%**(*6*)	17±*7%**(*6*)	39±*13%**(*6*)
+Noggin	24±*9%**(*4*)	60±*12%**(*4*)	153±*39%* (*4*)	84±*25%* (*4*)	885±*379%*‡ (*3*)	594±*298%*‡ (*3*)

†Levels of expression were normalized to control conditions set to 100%. *Statistically significant difference compared with control, $P < 0.05$. ‡High variance in data (see MATERIALS AND METHODS). Number in parentheses, number of independent experiments.

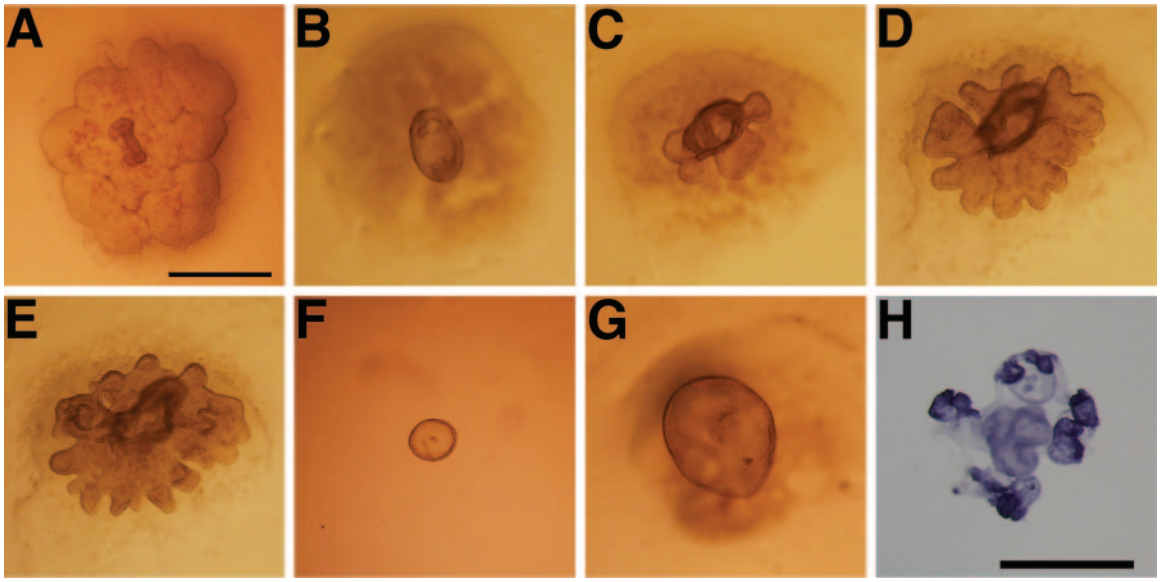

Fig. 1. Tracheal epithelium (TrE) branches and expresses surfactant protein C (*SP-C*) in response to soluble signals from lung mesenchyme (LgM). Embryonic (E) *day 12.5* TrE was cultured on one side of a 0.4-μm-pore-size membrane directly across from 8 pieces of *day E12.5* distal tip LgM on the other side (*A*). After 1 day of culture, the epithelium cysts and the mesenchyme coalesce and spreads slightly (*B*). Over 2 (*C*), 3 (*D*), and 4 (*E*) days, the TrE grows and branches in a lung-like manner. Culture of TrE alone results in the formation of a small cyst after 5 days (*F*), whereas TrE cultured across from 8 pieces of tracheal mesenchyme for 4 days results in the formation of a large cyst (*G*). Whole mount in situ hybridization shows that TrE cultured transfilter to LgM for 5 days expresses *SP-C* at the tips of the branched epithelium but not in the rest of the epithelium (*H*). *B–E* are micrographs of the same culture. Scale bar = 500 μm.

fusible signals from the LgM were able to correctly pattern lung epithelial growth. First, the induced TrE branched in a fashion similar to that seen in intact lungs in culture (Fig. 1, *D* and *E*). Second, *SP-C* expression was correctly localized at the distal end of growing tips and absent from proximal regions of the epithelium, as seen in the developing lung in vivo (Fig. 1*H*). This occurred even though the entire epithelium was initially the same distance from the LgM mass (Fig. 1*A*). Third, the induced TrE never grew beyond the boundaries of the LgM transfilter to it, demonstrating that the range of these signals was limited.

FGF-10 chemoattracts TrE and reprograms it to express SP-C. Having established that the signals produced by LgM required to reprogram TrE to express a lung phenotype were diffusible, we next determined whether a single diffusible molecule produced by LgM could replicate any or all of these effects. FGF-10 was of primary interest because of its expression in the distal LgM (3), its ability to chemoattract LgE in vitro (46, 63), and the complete absence of lungs when its gene is deleted (38, 51). FGF-10 beads were placed in close proximity (100–200 μm) to TrE (Fig. 2*A*) and cultured for up to 4 days (Fig. 2, *A–E*). After 1 day in culture, the TrE formed small cysts with thick walls (Fig. 2*B*), with the wall nearest the FGF-10 bead appearing thicker than that furthest from the bead. After 2 days, the TrE had noticeably migrated toward the FGF-10 bead (Fig. 2*C*), a process that continued over the next 48 h (Fig. 2, *D* and *E*), whereupon the TrE contacted and often engulfed the bead. When we placed two beads in apposition to the TrE, we observed that two elongations of the epithelium were induced (data not shown), suggesting that separate signaling centers had been established. It should be noted that although the most dramatic TrE expansion and chemoattraction was seen in the epithelium in closest proximity to the bead, the epithelium on the side away from the bead also showed expansion.

To determine whether the FGF-10-induced chemoattraction was accompanied by distal lung epithelial differentiation, cultured TrE were examined for *SP-C* expression. FGF-10-soaked beads induced *SP-C* expression in cultured TrE in a pattern similar to that seen in distal lung tips in vivo: *SP-C* was only expressed in the epithelium that was in closest proximity to the source of FGF-10 (Fig. 2*F*). The induction of *SP-C* by FGF-10 beads was identical to that seen with beads soaked in FGF-7 (Fig. 2*H*), which we have previously shown is capable of inducing *SP-C* in rat embryonic TrE (54). Direct contact with the bead was apparently not necessary for the response to either FGF-10 or FGF-7, since the domain of *SP-C* expression extended well beyond the region of contact (Fig. 2*F*). These results showed that FGF-10 was able to induce TrE to expand and migrate in a manner identical to that described for LgE (63). Importantly, these data demonstrate for the first time that FGF-10 is able to initiate a program of distal lung differentiation in a competent epithelium. Further studies revealed that FGF-10 beads had the ability to chemoattract LgE out of its own mesenchyme but could not chemoattract TrE surrounded by TrM (data not shown), in agreement with previously published results (46).

TrE cultured with FGF-10 beads were examined for the expression of distal epithelial genes by real-time PCR. *SP-C* expression was at background levels in freshly isolated TrE, but we detected signal for both *Bmp4* and *Spry2*. *SP-C* was induced in TrE as soon as 15 h after being placed in culture with an FGF-10 bead. After 2 days of culture, *SP-C* and *Bmp4* expression were increased 47.5-fold (range 19- to 84-fold, $n = 4$) and 5.8-fold (range 4- to 8-fold, $n = 3$), respectively, compared with freshly isolated TrE. *SP-C* levels increased

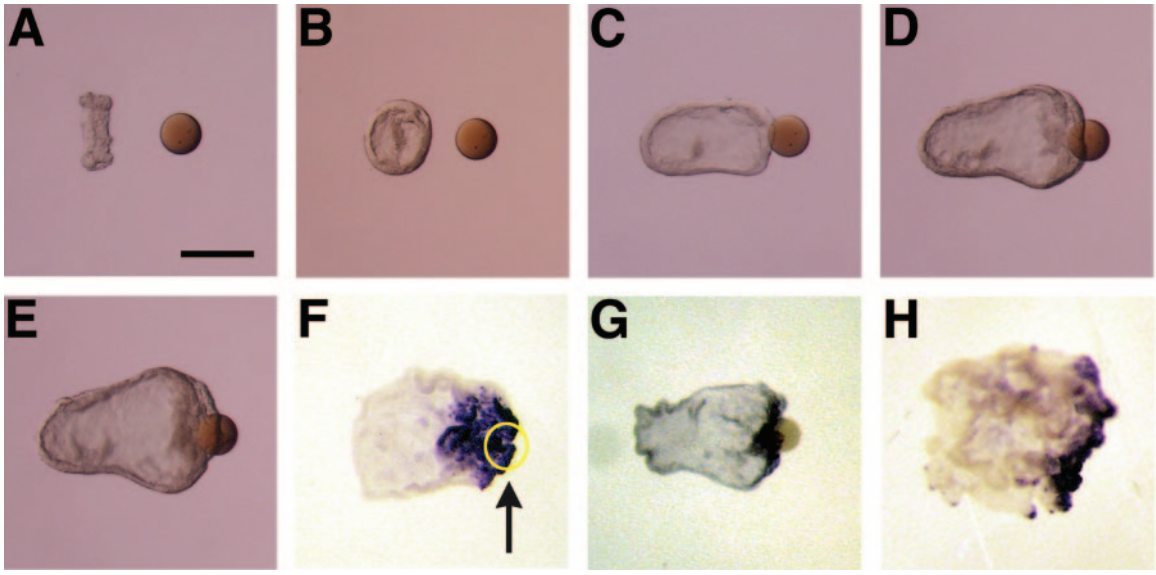

Fig. 2. TrE grows and expresses *SP-C* and bone morphogenetic protein 4 (*Bmp4*) in response to an FGF-10 bead. *Day E12.5* TrE was cultured ~100–200 μm from a heparin-coated bead soaked in FGF-10 (*A*). The TrE forms a cyst after 1 day in culture (*B*) and elongates toward the bead after 2 days (*C*). The TrE continues to move toward the bead after 3 (*D*) to 4 (*E*) days of culture, expands in size nearer the bead, and partially engulfs it. Shown is whole mount in situ hybridization performed for *SP-C* on TrE cultured for 4 days with an FGF-10 bead (*F*) and for *Bmp4* on TrE cultured with an FGF-10 bead for 5 days (*G*). Both genes are expressed in cells nearest the FGF-10 bead (the yellow circle indicates the position of the bead in culture). Whole mount in situ hybridization shows that after 4 days in culture, *SP-C* is expressed in cells closest to the FGF-7 bead (*H*). Scale bar = 250 μm.

further to 487-fold (range 95- to 1,093-fold, $n = 3$) after an additional 2 days of culture with FGF-10 beads, whereas *Bmp4* levels did not increase further. TrE cultured with FGF-10 beads showed little change in *Spry2* expression after 2 days in culture. After 4 days, however, *Spry2* increased approximately twofold, but this was not statistically significant ($n = 3$).

Proximal lung epithelial cell differentiation in rudiments chemoattracted by FGF-10 was examined by measuring *Ccsp* and *Foxj1* levels by real-time RT-PCR. Both *Ccsp* and *Foxj1* expression were not above background in freshly isolated TrE. Compared with uncultured TrE, *Ccsp* expression increased 51-fold (range 8- to 180-fold, $n = 5$) after 2 days in culture, whereas *Foxj1* expression did not change. Similar to the results for *SP-C*, *Ccsp* expression increased further to 1,200-fold (range 136- to 3,012-fold, $n = 3$) after an additional 2 days of culture, and *Foxj1* increased nearly ninefold. These data indicate that FGF-10 not only induced distal differentiation in TrE but also supported proximal gene expression.

FGF-10 beads and LgM induce different levels of gene expression in TrE. TrE cultured transfilter to LgM morphologically resembled a lung in miniature, with *SP-C* expressed only at distal tips (Fig. 1). In contrast, TrE cultured with an FGF-10 bead exhibited the characteristics of a single lung epithelial bud, with *SP-C* and *Bmp4* present in those cells nearest the FGF-10 source and absent in the epithelium furthest from the bead (Fig. 2). In both experimental systems, however, the induced TrE expressed both distal and proximal lung epithelial differentiation markers, suggesting that FGF-10 by itself was as capable as the multiple diffusible factors produced by LgM in inducing lung proximal-distal patterning and differentiation. Quantitative examination of gene expression in TrE cultured with LgM or FGF-10 beads, however, revealed that there were differences in the magnitude of their responses to these different inductive sources. TrE cultured transfilter to LgM expressed *SP-C* at a significantly higher level (3.31-fold higher, $P < 0.05$, $n = 7$) than TrE cultured with an FGF-10 bead. TrE cultured with an FGF-10 bead, however, expressed significantly higher levels of *Bmp4* (3.74-fold higher, $P < 0.01$, $n = 7$) and *Ccsp* (28-fold higher, $P < 0.01$, $n = 6$) than TrE cultured transfilter to LgM. Expression of *Spry2*, *Shh,* and *Foxj1* in the TrE were statistically the same regardless of whether the TrE was induced by LgM or FGF-10.

BMP4 affects growth, branching, and the expression of proximal lung markers in transfilter cultures. The role of BMP4 in lung development has not been fully elucidated. It has been reported that adding BMP4 to explant cultures of whole mouse lungs increases branching (7, 57). Conversely, addition of BMP4 to cultures of embryonic rat distal lung tips had no effect on branching (55). Furthermore, BMP4 decreased growth of LgE cultured mesenchyme-free with FGFs (25, 63). We therefore examined the effects of BMP4 on TrE cultured transfilter to LgM. Compared with control transfilter cultures (Fig. 3, *A–C*), addition of BMP4 resulted in significantly decreased growth and branching of the epithelium (Fig. 3, *G–I*). BMP4 did not inhibit the initial budding of the epithelium but did significantly inhibit further growth. When we examined gene expression by real-time RT-PCR, we found that BMP4 decreased the expression of *SP-C*, *Ccsp*, *Foxj1,* and *Shh* but did not change levels of *Spry2* or *Bmp4* (Table 1).

To examine further the effects of BMP4 on TrE cultured transfilter to LgM, the BMP4 antagonist Noggin was added to the cultures. Inhibition of BMP4 signaling did not disrupt TrE growth and branching (Fig. 3, *D–F*), which appeared similar to controls (Fig. 3, *A–C*). Addition of both Noggin and BMP4 (Fig. 3, *J–L*) demonstrated that Noggin could antagonize exogenous BMP4 at 3 days (Fig. 3*K*). This did not persist for the entire culture period, since exogenous BMP4 still inhibited TrE expansion from 3 to 5 days (Fig. 3*L*). Although the morphol-

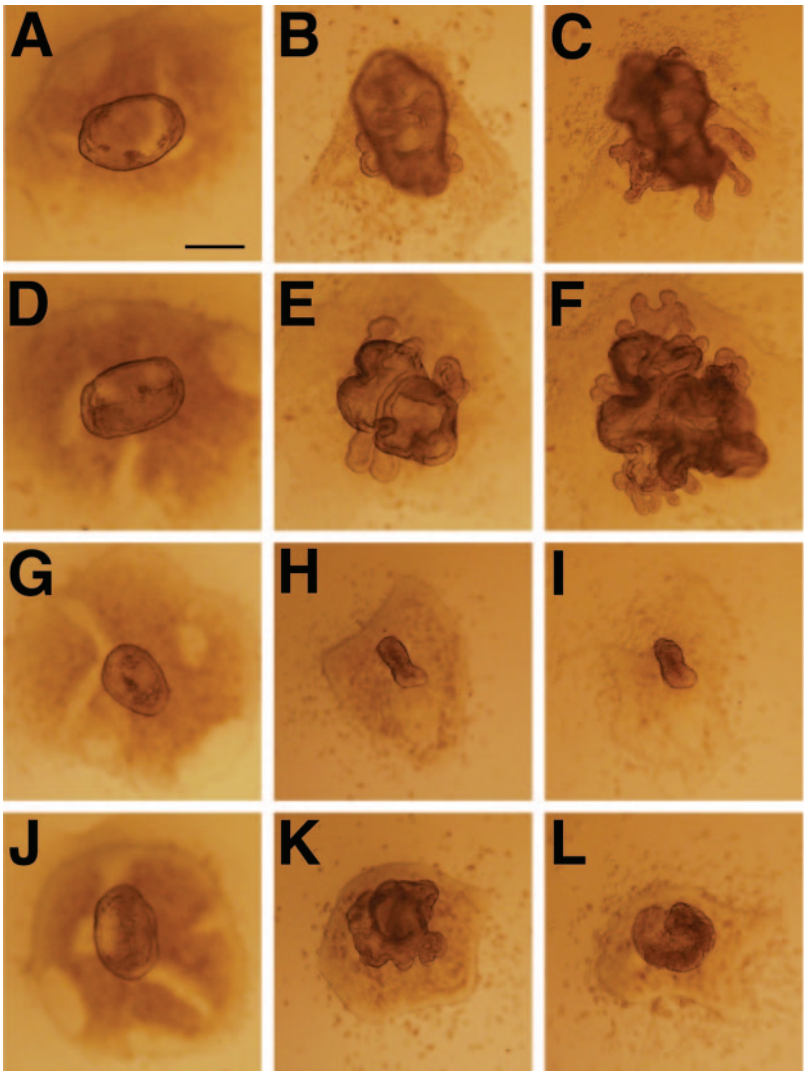

Fig. 3. Effects of altering BMP4 signaling on TrE cultured transfilter to distal LgM. *Day E12.5* TrE was cultured on one side of a 0.4-μm-pore-size membrane directly across from 8 pieces of *day E12.5* LgM for up to 5 days. Growth and branching after 1 (*A, D, G, J*), 3 (*B, E, H, K*), and 5 (*C, F, I, L*) days in culture are shown. Addition of Noggin (500–1,000 ng/ml) has no effect on TrE growth and branching (*D–F*) compared with the control (*A–C*). Addition of BMP4 (200 ng/ml) does not appear to affect the formation of a cyst at 1 day (*G*) but greatly inhibits TrE growth and branching at 3 and 5 days (*H* and *I*). Addition of both Noggin and BMP4 demonstrates that Noggin inhibits the effects of BMP4 at 3 days (*J* and *K*), but this is diminished by *day 5* (*L*), where some growth repression is apparent. Scale bar = 250 μm.

ogy of TrE cultured with and without Noggin appeared similar, Noggin caused changes in gene expression. Noggin increased expression of the proximal lung markers *Ccsp* and *Foxj1*, whereas *SP-C* levels were unchanged (Table 1).

To determine whether physical contact between LgM and LgE would alter its effects in culture, BMP4 was added to cultures of intact pieces of *day E11.5* and *day E12.5* lungs. These samples were hybridized with a probe for *Nkx2.1* to facilitate resolution of the epithelium when counting distal buds. When added to cultures of the most distal buds of the left and right lobes of *day E12.5* lungs, BMP4 had no effect on growth or the number of epithelial branches after 3 days (Fig. 4). When more proximal pieces of *day E11.5* lungs (see MATERIALS AND METHODS) were isolated and cultured with BMP4, the average number of epithelial branches increased slightly over controls (10.86 vs. 9.86, $P = 0.046$, $n = 14$). These experiments were done using D/F12 plus 5% FBS. When *day E11.5* lungs were cultured serum-free in this medium, we saw no growth or branching of *day E11.5* lungs regardless of whether BMP4 was present or absent (data not shown).

BMP4 affects growth and gene expression in TrE cultured with FGF-10 beads. We next examined the effects of BMP4 on the migration and differentiation of TrE cultured with FGF-10 beads. Addition of BMP4 to these cultures resulted in inhibition of rudiment growth (Fig. 5) and reductions in the levels of *SP-C*, *Ccsp*, *Foxj1*, and *Shh* (Table 2). Interestingly, BMP4 increased expression of *Spry2* and *Bmp4* in TrE cultured with FGF-10 beads (Table 2) but effected no change in *Spry2* or *Bmp4* when added to transfilter cultures (Table 1). Noggin antagonism of BMP4 signaling in FGF-10 bead cultures resulted in normal rudiment growth and chemoattraction toward the FGF-10 bead (data not shown) as well as an increase in the expression of *Ccsp* and *Foxj1* (Table 2). *Spry2* levels were unchanged, but *Bmp4* and *SP-C* levels were decreased (Table 2).

TrE from FGF-10 null mice grows and expresses SP-C. Whereas *Fgf10* null mice have no lungs, they do form a trachea that ends in a blind sac. We sought to determine whether the induction of lung differentiation requires that a competent epithelium receive an FGF-10 signal at some point during development. TrE was isolated from *day E12.5 Fgf10*$^{-/-}$

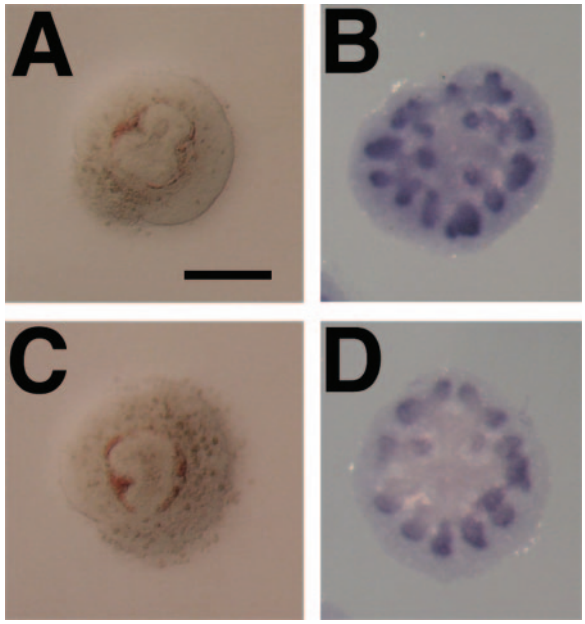

Fig. 4. Effects of BMP4 on branching of cultured distal lung tips. *Day E12.5* left and right distal lung tips were cultured for 3 days with and without the addition of BMP4 (200 ng/ml). Distal tips cultured for 1 day appear the same with (*C*) or without (*A*) the addition of BMP4. After 3 days of culture, tips were fixed, and whole mount in situ hybridization for *Nkx2.1* (*Ttf-1*) was performed to visualize the branching tips. The number of end buds in tips cultured without BMP4 (*B*) appears the same as in tips with BMP4 (*D*). This is borne out by end bud counts, with 13.1 and 13.7 buds present in tips cultured without and with BMP4, respectively ($n = 19$ cultured tips from 3 independent experiments; $P = 0.16$). Scale bar = 250 μm.

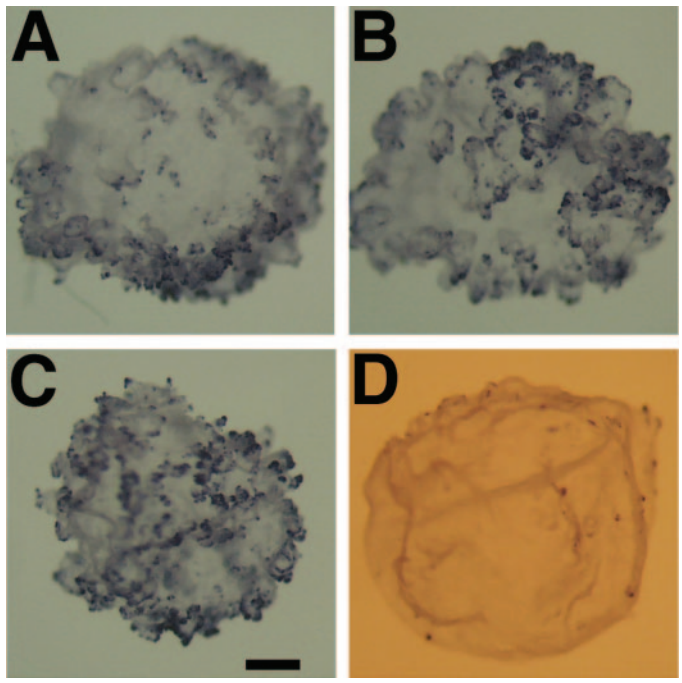

Fig. 6. FGF-1 induces *SP-C* expression in *Fgf10*$^{-/-}$ TrE. *Day E12.5* TrE rudiments were isolated and cultured for 5 days in medium containing FGF-1 (100 ng/ml) and then processed for *SP-C* whole mount in situ hybridization. *Day E12.5* TrE from *Fgf10*$^{-/-}$ mice (*B*) qualitatively express the same amount of *SP-C* as TrE taken from normal littermates (*A*). Competence to respond to lung induction is restricted by *day E13.5*. Rudiments from the cranial half of *day E13.5* TrE cultured with FGF-1 (*D*) express much less *SP-C* than do rudiments from the caudal half (*C*). Scale bar = 250 μm.

embryos and cultured mesenchyme-free in medium containing FGF-1, which we have previously shown can reprogram TrE to express *SP-C* (25). *Fgf10*$^{-/-}$ TrE (Fig. 6*B*) expanded and expressed *SP-C* at levels identical to that of TrE cultured from phenotypically normal littermates (Fig. 6*A*).

Competence of TrE to respond to induction is temporally restricted along its cranial-caudal axis. Although *day E13.5* rat TrE can be reprogrammed in culture to express type II cell differentiation, *day E16.5* TrE is not inducible (54), indicating that by *day E16.5* the rat TrE is determined and is no longer competent to respond to lung inductive signals. We examined cranial and caudal halves of mouse TrE to determine whether and when they became unresponsive to reprogramming to a distal lung epithelial phenotype. When cultured with FGF-1, the cranial and caudal halves of *day E12.5* TrE both expressed equal amounts of *SP-C* as gauged by whole mount in situ hybridization, results confirmed by real-time PCR (data not shown). The cranial (Fig. 6*D*) and caudal (Fig. 6*C*) halves of *day E13.5* TrE, however, showed clearly different *SP-C* levels by whole mount in situ hybridization. Real-time RT-PCR confirmed this: caudal *day E13.5* TrE halves expressed 900% higher levels of *SP-C* than cranial halves ($n = 2$). These data indicate that on *day E13.5*, the TrE is becoming restricted in its

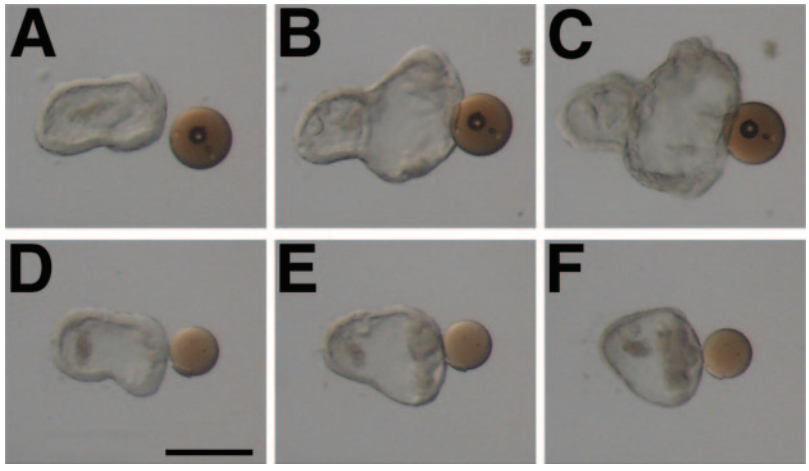

Fig. 5. BMP4 inhibits growth of TrE cultured with an FGF-10 bead. TrE were placed in culture with FGF-10 beads, and after 2 days (*A* and *D*), BMP4 (200 ng/ml) was added to the medium. Whereas TrE cultured without BMP4 continued to expand on culture at *days 3* (*B*) and *4* (*C*), addition of BMP4 inhibits further TrE growth after 3 (*E*) and 4 (*F*) days. Scale bar = 250 μm.

ability to respond to lung-inductive cues and that this restriction proceeds in a cranial-to-caudal direction.

DISCUSSION

A number of studies have documented the importance of mesenchymal-epithelial interactions in lung development. In this study, we have used two different culture systems to examine the effects of diffusible factors expressed by the LgM on the initiation of lung epithelial growth, branching, and differentiation. In these models, we used TrE instead of LgE as the responding tissue. Because early embryonic TrE can be reprogrammed to in a lung-like pattern (52, 56) and express distal lung markers and differentiate as type II cells (54), using it as the responding tissue allowed us to study the initiation of distal lung patterning and differentiation. The use of LgE as the responding tissue would have been complicated by the fact that it has already been induced as lung and therefore might be "prepatterned" to respond to signaling cues. In addition, we used *day E12.5* TrE for our studies because the competence of the TrE begins to be restricted in a cranial-to-caudal direction after that stage.

Previous studies using transfilter cultures have shown that diffusible signals from LgM induce LgE to grow and branch (60). In the present study, we used transfilter cultures of LgM plus TrE to examine specific changes in gene expression occurring with the initiation of growth, branching, and differentiation that take place when TrE is reprogrammed to a lung epithelial phenotype. We found that TrE cultured transfilter to LgM grew and branched identically to what was observed with LgE (60). In addition, we observed that the induced TrE expressed *SP-C*, demonstrating that the initiation of distal lung differentiation does not require direct contact between the epithelium and mesenchyme. This observation is significant because previous studies have shown that cellular processes from embryonic lung epithelial cells traverse the basal lamina to contact subtending fibroblasts and that the number of contacts was correlated with the extent of distal epithelial differentiation (6, 18). Furthermore, *SP-C* expression was localized to the distal tips of the epithelium, showing that proximal-distal spatial coordinates were maintained in the absence of epithelial-mesenchymal contact. These results further validate the use of transfilter cultures to study specific signaling pathways during lung development.

As described above, the diffusible factors from LgM induced *SP-C* only at the distal tips of the branching TrE. This differed from what we had observed with TrE in mesenchyme-free culture where inducing factors were present throughout the medium. The fact that *SP-C* was induced throughout the epithelium (25, 54) indicated that there is not a defined subset of cells that can respond to induction. In the transfilter cultures, we placed the TrE as close as possible to the filter below the LgM, with the entire rudiment equidistant from the mass of mesenchymes. To establish the proximal-distal patterning we observed, only specific areas of the TrE would have elongated as buds, whereas those areas destined to become proximal regions would not. How does this occur? One possibility is that the mass of mesenchyme contains discrete signaling centers that produce gradients of diffusible signals, and only those cells closest to these centers are induced to bud and elongate, leaving the rest behind to adopt a proximal fate. A second possibility is that all of the cells in the TrE receive a threshold level of budding signal, and the ones that respond by budding immediately repress the response in nearby regions. We believe that a combination of these two possibilities may be at work. We think it unlikely, however, that proximal-distal patterning is established by differential growth alone, since changes in LgE proliferation in culture do not occur until after it buds (41). Furthermore, the proximal regions of the TrE cultured transfilter to LgM continued to grow, and part of that growth was away from the mesenchyme. Finally, TrE cultured transfilter to TrM grew well, but without any discernible proximal-distal patterning or induction of *SP-C*.

Of the many diffusible factors produced by LgM, much interest has focused on FGF family members because of their ability to support growth and differentiation in a variety of tissues (49). FGF-10 has been of particular interest because it is absolutely required for the initiation of lung development (38, 51). We examined whether FGF-10 by itself could reproduce some or all of the properties of LgM by placing an FGF-10 bead close to the TrE. Identical to previously published reports using LgE (46, 63), the TrE expanded and grew toward the FGF-10 bead, which was reminiscent of the migration of *Drosophila* tracheal cells in response to the *Fgf10* ortholog *branchless* (59). The induced TrE resembled one of the elongating epithelial buds induced by LgM in transfilter culture as well as the distal tips of developing lungs in vivo. Importantly, this elongating bud expressed *SP-C* at the tip closest to the source of FGF-10. These results are the first demonstration that FGF-10 can initiate distal lung epithelial differentiation in a competent epithelium. Previous work from our laboratory showed that a seemingly high concentration (1 μg/ml) of FGF-10 added to culture medium was unable to induce *SP-C* in either rat (54) or mouse (25) TrE. Similarly, a high concentration of FGF-10 added to the medium was unable to sustain expression of *SP-C* in mesenchyme-free cultures of mouse lung endoderm (31). The only difference between our present and past experiments with FGF-10 is the manner in which the ligand is delivered. Use of an FGF-10 bead presents a high local concentration of ligand that was apparently not mimicked when we added FGF-10 to the medium. The molecular basis of how a high local concentration of FGF-10 has its effects is unknown, but recent evidence suggests a critical role for proteoglycans in FGF-10 signaling (27, 28, 55). Direct contact between the TrE and the FGF-10 bead was not necessary for this process, since *SP-C* was induced as soon as 15 h, a point when the TrE was still well removed from the FGF-10 bead. We believe that the requirement for a high local concentration of FGF-10 to induce distal epithelial budding and differentiation may be what is occurring in vivo, since this would be an effective way in which to precisely restrict the generation of lung buds and hence promote normal patterning. In any case, our observations extend the function of FGF-10 in the lung beyond its role as a chemoattractive molecule to an inducer of distal lung epithelial cell differentiation. The observation that FGF-10 can induce *SP-C* also explains why distal lung differentiation is induced when other FGFs known to be important for lung differentiation (e.g., *Fgf7*, *Fgf1*, and *Fgf2*) are ablated.

As discussed above for the transfilter data, our FGF-10 bead experiments also support the idea that the epithelium can pattern itself. A striking observation in the FGF-10 bead

experiments was that *SP-C* was induced only in that epithelium closest to the ligand source, even though the initial difference in distance to the bead between the closest and most distant TrE cells was only a few cell diameters. Although the concentration of FGF-10 seen by the most distant epithelium may have been slightly lower than that by the closer epithelium, we do not think that our observations can be explained by concentration differences alone. One reason for this is that TrE was placed at slightly different distances (100–200 μm) from the FGF-10 bead, yet all established a proximal-distal axis, with only that region closest to the bead expressing *SP-C*. If FGF-10 concentration alone were critical, then *SP-C* should have been induced more extensively in those TrE that were placed closer to the bead, but this did not occur. It should also be noted that the epithelium furthest from the bead did exhibit some growth and therefore was being influenced by FGF-10. In addition, TrE cultured with an FGF-7 bead showed a more generalized growth (i.e., was not chemotattracted), similar to what we previously observed when FGF-7 was present throughout the culture medium (25). *SP-C* expression in these cultures, however, was again observed primarily in those cells nearest the bead (Fig. 2*H*). Because the epithelium cannot signal back to the bead to alter its output of ligand, the epithelial cells that are the first to respond to FGF-10 may somehow influence or pattern their neighbors. One possible mechanism is through the induction of *Bmp4* (see below).

Although FGF-10 beads and LgM both induced similar growth and patterning of TrE, there were some clear differences in the expression of lung differentiation genes. LgM induced a much higher level of *SP-C* than did FGF-10 beads, indicating that whereas FGF-10 has the ability to induce *SP-C*, other factors produced by LgM increase its expression. In contrast, FGF-10 induced a higher level of *Bmp4* than LgM. The reason for this is not entirely clear. FGF-10 induces epithelial buds, and *Bmp4* is specifically expressed at the leading edge of these elongating buds (63). Perhaps compensatory signals from LgM attenuate epithelial *Bmp4* expression, thus its expression is unregulated and maximized in FGF-10 bead cultures. FGF-10 also induced a higher level of *Ccsp* in TrE than did LgM. This was somewhat surprising, given the increased expression of *Bmp4* in FGF-10 bead cultures and the potential role for BMP4 in limiting proximal gene expression (see below). One possible explanation is that the lower level of *SP-C* induced by FGF-10 beads is due to a smaller domain of expression than that induced by LgM. *Ccsp* would therefore be expressed in the relatively increased number of *SP-C*-negative cells.

Bmp4 is expressed in the distal LgE and diffusely in the LgM (4). The importance of BMP4 in lung development is illustrated by defects in lungs when BMP4 is either overexpressed (4) or inhibited (32, 64). Our observations that *Bmp4* was induced only in the leading edge of TrE chemoattracted toward an FGF-10 bead and that *Bmp4* expression was much higher (17-fold) in branching TrE induced by LgM than in nonbranching TrE cultured transfilter to TrM indicate that the induction and upregulation of *Bmp4* is associated with the formation of distal lung signaling centers. However, although *Bmp4* is coexpressed with *SP-C* in LgE, there are no data supporting a direct role for BMP4 in inducing *SP-C*. To the contrary, overexpression of BMP4 in developing LgE in vivo reduced *SP-C* (4). Furthermore, the addition of BMP4 to TrE cultured with FGF-1 (25) or transfilter to LgM (Table 1) or with an FGF-10 bead (Table 2) also decreased *SP-C*. FGF-10 induced a higher level of *Bmp4* in TrE than did LgM but induced a lower level of *SP-C*.

Although we observed that BMP4 inhibited epithelial growth and branching when added to transfilter cultures of LgM and TrE and epithelial growth when added to FGF-10 bead cultures, we saw no effect of BMP4 on branching of explanted intact distal lung tips. The basis for these disparate observations is not clear. One possible explanation comes from the observation (45) that heparan sulfate proteoglycans (HSPGs) are able to retain Noggin at the cell surface. This allows Noggin to regulate the diffusion of BMP4, thereby affecting gradient formation. The enzymes we used to isolate TrE (collagenase and dispase) may have disrupted cell surface and extracellular HSPGs, leading to a loss of Noggin and therefore local regulation of BMP4 activity.

If BMP4 is important for proximal-distal lung patterning and does not induce *SP-C*, what is its role in the distal epithelial tips? In a previous study (25), we found that although BMP4 did not induce *SP-C* in TrE, it suppressed expression of the proximal genes *Ccsp* and *Foxj1*; furthermore, addition of Noggin to these cultures increased *Ccsp* and *Foxj1*. We have extended these observations in the present study, where addition of BMP4 to both transfilter and FGF-10 bead cultures decreased *Ccsp* and *Foxj1* expression (Tables 1 and 2) and the addition of Noggin increased *Ccsp* and *Foxj1* expression (Tables 1 and 2). The addition of BMP4 to cultured proximal pieces of *day E11.5* lung also decreased *Ccsp* and *Foxj1* levels (data not shown). These observations are consistent with data in vivo, where inhibiting BMP4 by targeted expression of Noggin and Gremlin in LgE expands proximal gene expression at the expense of distal genes (32, 64). In addition, *Pod-1* null mice show decreased *Bmp4* and increased *Ccsp* expression (50), and proximalization of lungs by retinoic acid results in low levels of *Bmp4* (34). The fact that BMP4 negatively regulates proximal gene expression but does not induce *SP-C* suggests that it mediates mesenchymal signals received by the distal epithelium directing proper patterning and distal differentiation, possibly through its regulation of cell proliferation (25, 63). Noggin also inhibited *Sp-C* in FGF-10 bead cultures. One explanation for these data is that by inhibiting BMP4, Noggin causes an increase in proximal gene expression at the expense of *SP-C*-expressing cells. An alternate possibility is that Noggin may also have inhibited other endogenous BMP-signaling pathways, such as BMP7 (4), and that these are involved in inducing *SP-C*.

In addition to antagonizing proximal gene expression in induced TrE, BMP4 also increased expression of other genes. Addition of BMP4 to TrE-FGF-10 bead cultures increased the expression of both *Spry2* and *Bmp4*. A time course study showing that *Bmp4* was upregulated by 2 days, whereas *Spry2* did not increase until 4 days of culture, indicated that increased *Spry2* was not an immediate response to BMP4. Because *Sprys* are upregulated by FGFs (39) and FGF-10 specifically upregulates *Spry2* in LgE (33), it is not surprising that FGF-10 upregulated *Spry2*. That *Spry2* was also upregulated by BMP4, however, is a new finding. Although the addition of BMP4 to transfilter cultures did not increase *Spry2* or *Bmp4*, it also did not decrease their expression; *Spry2* and *Bmp4* were the only genes we examined that were not decreased by BMP4 (Table

2). That *Spry2* and *Bmp4* expression in transfilter cultures were not increased by BMP4 may be due to the cross talk between the epithelium and mesenchyme.

FGF-10 is required for lung bud formation and induces *Bmp4* in cultured bronchial epithelium (63). Those data and results presented here are consistent with a model in which FGF-10 produced in the mesenchyme initiates epithelial budding and chemoattracts it, while also inducing *Bmp4* and *SP-C* in the distal regions of the extending bud. BMP4 inhibits proliferation of the distal tip cells and increases expression of *Spry2*, which antagonizes FGF signaling. Increased *Bmp4* and/or *Spry2* expression limits proximal differentiation in the distal epithelium. These and other signals from the distal epithelium signal back to the mesenchyme to decrease the FGF-10 signal. FGF-10 expression is upregulated by an unknown mechanism at future bud positions that are far enough away from the distal tip to avoid repression by BMP4 and SPRY2. Support for this model comes from the recent observation that overexpression of SPRY4 in the distal LgE inhibits bud formation but not elongation (48). Considered with the BMP4 results discussed above, these data support the possibility that BMP4 acts to limit proliferation, whereas SPRY2 inhibits budding.

In summary, we have shown that a high local concentration of FGF-10 has the ability to chemoattract TrE in vitro, recapitulating in vivo bud formation. In addition, FGF-10 induced the expression of *SP-C* and *Bmp4* in the epithelial cells nearest the FGF-10 source and was able to support proximal-distal patterning of the induced epithelium. Finally, the upregulation of *Spry2* in the induced epithelium by BMP4 suggests a possible new genetic interaction in lung growth, branching, and differentiation.

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