Exogenous Fibroblast Growth Factor-10 Induces Cystic Lung Development with Altered Target Gene Expression in the Presence of Heparin in Cultures of Embryonic Rat Lung

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Key Words

Cystic lung development • Microarray • Fibroblast growth factor-10 • Heparin • Keratinocyte growth factor

Abstract

Objectives: Signaling by fibroblast growth factor (FGF) receptor (FGFR) 2IIIb regulates branching morphogenesis in the mammalian lung. FGFR2IIIb is primarily expressed in epithelial cells, whereas its ligands, FGF-10 and keratinocyte growth factor (KGF; FGF-7), are expressed in mesenchymal cells. FGF-10 null mice lack lungs, whereas KGF null animals have normal lung development, indicating that FGF-10 regulates lung branching morphogenesis. In this study, we determined the effects of FGF-10 on lung branching morphogenesis and accompanying gene expression in cultures of embryonic rat lungs. **Methods:** Embryonic day 14 rat lungs were cultured with FGF-10 (0–250 ng/ml) in the absence or presence of heparin (30 ng/ml) for 4 days. Gene expression profiles were analyzed by Affymetrix microchip array including pathway analysis. Some of these genes, functionally important in FGF-10 signaling, were further analyzed by Northern blot, real-time PCR, in situ hybridization and immunohistochemistry. **Results:** Exogenous FGF-10 inhibited branching and induced cystic lung growth only in cultures containing heparin. In total, 252 upregulated genes and 164 downregulated genes were identified, and these included *Spry1*

(*Sprouty-1*), *Spry2* (*Sprouty-2*), *Spred-1*, *Bmp4* (bone morphogenetic protein-4, BMP-4), *Shh* (sonic hedgehog, SHH), *Pthlh* (parathyroid hormone-related protein, PTHrP), *Dusp6* (MAP kinase phosphatase-3, MKP-3) and *Clc4* (chloride intracellular channel-4, CLIC-4) among the upregulated genes and *Igf1* (insulin-like growth factor-1, IGF-1), *Tcf21* (POD), *Gyg1* (glycogenin 1), *Sparc* (secreted protein acidic and rich in cysteine, SPARC), *Pcolce* (procollagen C-endopeptidase enhancer protein, Pro CEP) and *Lox* (lysyl oxidase) among the downregulated genes. *Gsk3 β* and *Wnt2*, which are involved in canonical Wnt signaling, were up- and downregulated, respectively. **Conclusions:** Unlike FGF-7, FGF-10 effects on lung branching morphogenesis are heparin-dependent. *Sprouty-2*, BMP-4, SHH, IGF-1, SPARC and POD are known to regulate branching morphogenesis; however, potential roles of CLIC-4 and MKP-3 in lung branching morphogenesis remain to be investigated. FGF-10 may also function in regulating branching morphogenesis or inducing cystic lung growth by inhibiting Wnt2/ β -catenin signaling.

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Introduction

Lung development begins as an outpouching of the foregut endoderm into the surrounding mesoderm. The initial bud undergoes repetitive branching (branching morphogenesis), a process regulated by mesenchymal-

endodermal interactions through the release of a variety of growth factors and other signaling molecules [1, 2]. Abundant evidence indicates that signaling by the fibroblast growth factor (FGF) receptor (FGFR) 2IIIb regulates branching morphogenesis in the mammalian lung. FGFR2IIIb is primarily expressed in epithelial cells, whereas its ligands, FGF-10 and keratinocyte growth factor (KGF; FGF-7), are expressed in mesenchymal cells [3]. FGF-10, in particular, controls lung branching morphogenesis [3]. *FGF-10* null mice lack lungs, whereas *KGF* gene knockout animals have normal lung development [4, 5]. Distinct differences were also observed between FGF-10 and KGF on lung development in explant cultures. While FGF-10 exhibits chemoattractant properties toward epithelial cells, KGF lacks such an effect [3, 6]. Addition of exogenous KGF to cultures of lung explants results in inhibition of lung branching and causes cystic lung development [7, 8]. Pulmonary overexpression of KGF in transgenic mice causes pulmonary cysts and is lethal to the fetus by 15–16 days of gestation [9]. Overexpression of FGF-10, on the other hand, causes adenomatous changes with mild cyst formation [10, 11].

FGF-10 expression in the mesenchyme and FGF-10 signaling in the epithelium are both downregulated in the region of a newly formed bud [3]. FGF-10 signaling is likely to regulate expression of gene products that participate in epithelial-mesenchymal interactions and those that downregulate FGF-10 signaling during branching morphogenesis. Sprouty, Sprd (Sprouty-related protein with EVH-1 domain) and MAP kinase phosphatase (MKP)-3 are known inhibitors of FGF signaling [12–21]. MKP-3 is a cytoplasmic dual-specificity phosphatase that specifically inactivates phosphorylated forms of ERK [22–24]. Feedback modulation by MKP-3 of FGF signaling has been described in several developmental studies in chicken and zebrafish [24–26]. Sprouty, an antagonist of FGF signaling, is a key player regulating tracheal branching and eye development in *Drosophila* [14, 16] and has been shown to regulate organogenesis in vertebrates [17, 18, 21].

In this study, we determined the effects of FGF-10 on lung branching morphogenesis and gene expression in cultures of embryonic rat lungs. Similar to the effects of KGF [7], FGF-10 treatment results in inhibition of branching morphogenesis and induces cystic lung growth. However, unlike KGF, FGF-10 effects are heparin-dependent. These effects of FGF-10 were accompanied by changes in the expression of several genes, some of which have previously been shown to regulate branch-

ing morphogenesis. Affymetrix microchip array and real-time PCR analyses revealed changes in the expression of *Sproutys*, *Dusp6* (MKP-3), *Sparc* [secreted protein acidic and rich in cysteine (SPARC), a matricellular protein], *Pthlh* (parathyroid hormone-related protein, PTHrP), *Clic4* (chloride intracellular channel-4, CLIC-4), *Bmp4* (bone morphogenetic protein-4, BMP-4), *Shh* (sonic hedgehog, SHH), *Igf* (insulin-like growth factor, IGF), *Gyg* (glycogenin) and *Pod/Tcf21*. Many of these proteins have previously been shown to regulate organogenesis in general and lung development [17, 21, 27–35]. Furthermore, we identified upregulation of *Gsk3 β* and downregulation of *Wnt2*. Both genes are involved in Wnt2/ β -catenin signaling, which is crucially important for lung organogenesis [36].

Materials and Methods

Animals

Timed-pregnant rats of the Sprague-Dawley strain were obtained from Hilltop (Scottsdale, Pa., USA) and housed under pathogen-free conditions in accordance with institutional guidelines.

Embryonic Day 14 Rat Lung Culture

Rat embryos were dissected from the uterine decidua on day 14 of gestation. Lung primordia were dissected from embryos and cultured at the air-liquid interface on the upper surface of polycarbonate isopore membranes in Millicell-PCF assemblies (Millicore, Bedford, Mass., USA). The culture medium consisted of DMEM with 10% fetal bovine serum, streptomycin/penicillin G (100 μ g/ml), gentamicin (10 μ g/ml) and Fungizone (2.5 μ g/ml). Cultures were maintained at 37°C in 5% CO₂/95% air for 4 days, fed daily with a complete change of medium and photographed daily. Recombinant FGF-10 was obtained from two sources, namely as a gift from Dr. Ito [37] and also purchased from Collaborative Biomedical (Bedford, Mass., USA). FGF-10 was added to cultures at 0–250 ng/ml in the absence (no growth factor added) or presence of heparin at 30 μ g/ml.

Immunohistochemistry

Lung tissue was fixed in 4% paraformaldehyde, embedded in OCT compound and frozen at –80°C. Frozen sections (4 μ m thick) were stained with antibody using the EnVision System (Dako, Carpinteria, Calif., USA) and counterstained with hematoxylin. Sprouty-1 and -2 antibodies and their immunostaining method were described in a previous report [38].

In situ Hybridization

The procedure using nonradioisotopic probes is detailed in our earlier publication [39]. Briefly, frozen sections (8 μ m thick) on silylated slides were fixed in 4% paraformaldehyde. After blocking the endogenous peroxidase and alkaline phosphatase, the sections were hybridized with the digoxigenin-labeled riboprobes. The hybridized probes were amplified using a DNP Plus Tyramide System (NEN Life Sciences, Boston Mass., USA). The

sections were then treated with secondary alkaline phosphatase- or horseradish peroxidase-conjugated antibodies. Alkaline phosphatase and peroxidase substrates were Fast Red and diaminobenzidine, respectively.

Affymetrix Chip Analysis

The Affymetrix oligonucleotide chips specific for the rat (U34A, U34B and U34C) were used. RNA was extracted and purified with a Qiagen RNeasy kit (Qiagen, San Diego, Calif., USA) from pools of 4–6 cultured lungs. Five micrograms of total RNA was used for cDNA synthesis by the procedure described by Luo et al. [40]. cRNA labeled with biotin was prepared using the MEGA script system (Ambion, Austin, Tex., USA). The chips were hybridized with the labeled cRNA and stained with streptavidin phycoerythrin as described elsewhere [40]. The chips were scanned in an HP ChipScanner (Affymetrix, Santa Clara, Calif., USA) to detect the hybridization signal. Data analysis was performed with the Affymetrix microarray suite 5.0 software. Data from text files were imported to Microsoft Excel. Samples were two separate pools of lung explants which were cultured for 4 days in the presence of heparin (30 µg/ml), without (control group) or with FGF-10 (250 ng/ml). To identify genes upregulated by FGF-10 treatment, we (1) excluded transcripts depicted as decreased by the Affymetrix change algorithm, (2) included transcripts showing significant increases ($p < 0.05$) and (3) excluded transcripts detected as absent by the Affymetrix algorithm in the FGF-10-treated group. To identify genes downregulated by FGF-10, we (1) excluded transcripts depicted as increased by the Affymetrix change algorithm, (2) included transcripts showing significant decreases ($p < 0.05$) and (3) excluded transcripts detected as absent by the Affymetrix algorithm in the control group (without FGF-10). Two sets of control and FGF-treated samples were analyzed, and signals obtained from these two sets were averaged. For each transcript, the fold change was calculated by dividing the signal from the experimental group by that from the control group. In addition, we also analyzed pathways related to each up- and downregulated gene using the database at the NetAffx™ analysis center on the Affymetrix website.

Real-Time PCR

PCR primers, FAM dye-labeled probes and Taqman Universal PCR master mix were obtained from Applied Biosystems. Assays were carried out according to the manufacturer's protocol. Rat lung cDNA was synthesized from RNA as described earlier [7]. PCR was performed in an ABM Prism 7000 Sequence Detection System (Applied Biosystems, USA). The PCR reaction mixture (25 µl) consisted of master mix (12.5 µl), Assay-on-Demand gene expression assay mix (1.25 µl), rat lung cDNA (5 µl) and RNase-free water. The thermal cycler conditions were as follows: a 10-min setup at 95°C, 40 cycles of 15 s at 95°C, followed by 1 min at 60°C. Each reaction was carried out in duplicate in a 96-well plate. Endogenous control genes included with each assay were β-glucuronidase or β-2-microglobulin. The 1-sample t test (Instat statistical software) was used to compare experimental (250 ng/ml FGF-10 + 30 µg/ml heparin) and control (no FGF-10 + 30 µg/ml heparin) rates of expression.

Northern Blot Analysis

Five micrograms of lung total RNA were subjected to RT-PCR according to a protocol described elsewhere [7]. The primers for

PCR amplification of rat Sprouty-1 and -2 were derived from human sequences in the National Center for Biotechnology Information dbEST database and a publication by Hacohen et al. [14]. The primers were as follows: Sprouty-1, 5'-TCT ACC ACT GCT CCA ATG ACG-3' (sense) and 5'-CAA CCC ACC TCC AAA ATT CAT-3' (antisense), and Sprouty-2, 5'-ATC AGA GCC ATC CGA AAC ACC-3' (sense) and 5'-AAA TCT TCC TTG CTC AGT GGC-3' (antisense). On the basis of human Sprouty sequences, the predicted sizes of PCR-amplified products were 292 bp for Sprouty-1 and 377 bp for Sprouty-2.

Total RNA was fractionated in 1% agarose gels containing 0.66 M formaldehyde. Gels with ethidium bromide-stained bands were photographed under UV light and treated with 0.05 N NaOH prior to transfer to GeneScreen Plus (NEN Research Products, Boston, Mass., USA) with a Transvac Blotting System (Hoefer Scientific, San Francisco, Calif., USA). Blots were exposed to UV light in a UV Stratalinker (Stratagene, San Diego, Calif., USA) and sequentially hybridized with different probes as previously described [7]. DNA inserts were excised from plasmids containing Sprouty-1 and Sprouty-2 cDNAs and used as probes. ³²P-labeled probes were prepared using a Random Primer DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind., USA). Before hybridization with a new probe, the blots were stripped of radioactivity by boiling in 0.1 × SSC and 1% SDS. Densitometric analyses of the autoradiograms were performed on an AlphaEase Digital Imaging and Analytical System (Alpha Innotech, San Leandro, Calif., USA).

Results

Exogenous FGF-10 Inhibits Branching of Fetal Lung Explants in Culture

Branching was examined in cultures of fetal lung explants treated with or without exogenous FGF-10. FGF-10 in the presence of heparin inhibited branching and promoted the formation of a cystic lung with increasing concentrations of FGF-10 in culture media (fig. 1g–l). These FGF-10-inhibiting effects on branching were not seen in the culture condition without heparin (fig. 1a–f). To see more clearly if the effects of inhibited branching and cystic lung development were specifically induced by the exogenous FGF-10 in culture, we omitted or added FGF-10 in the middle of the culture. The inhibition of branching and the cystic lung development caused by exogenous FGF-10 was already apparent at culture day 2, especially with 250 ng/ml FGF-10 (fig. 2, compare f, j and n with b). Cessation of FGF-10 treatment of lung explants after 2 days of culture with FGF-10 and heparin resulted in rebranching (fig. 2, compare m–p with i–l). On the other hand, adding FGF-10 on culture day 3 followed by a 24-hour culture with FGF-10 and heparin after 3 days of normal branching in culture with heparin alone resulted in inhibited branching and cystic lung development (data

Fig. 1. Effect of FGF-10 on branching morphogenesis. Embryonic day 14 fetal rat lung explants were cultured for 4 days in the presence of varying concentrations of FGF-10 (0–250 ng/ml), with or without heparin (30 µg/ml). FGF-10 in the presence of heparin inhibited branching and promoted the formation of a cystic lung with increasing concentrations of FGF-10 in culture media (**g–l**). These effects are not seen in the absence of heparin (**a–f**). Scale bars = 1 mm.

not shown). In light of these findings, the inhibited branching and cystic lung development in our culture system were considered to be specifically induced by exogenous FGF-10.

Affymetrix Microarray Analysis

To better understand the relationship between gene expression and FGF-10-induced inhibition of branching and induction of cystic development and to identify genes regulated by FGF signaling, we performed Affymetrix microarray analysis. We compared the expression profiles of explants cultured for 4 days in the presence of heparin (30 µg/ml), without (control group) and with FGF-10 (250 ng/ml), using Affymetrix gene chips. We identified 252 upregulated genes (fold change ≥ 1.5) and 164 downregulated genes (fold change ≤ 0.66) in the FGF-10-treated lung explants (online suppl. tables S1 and S2; for all online suppl. material, see www.karger.com/doi/10.1159/000334839). Some selected upregulated and downregulated genes that were considered to be important for branching morphogenesis and lung epithelial differentiation or are involved in the FGF-10 pathway, serving a variety of cellular functions, are presented in tables 1 and 2, respectively. Expression of several of the genes showing changes according to this analysis was further examined by real-time PCR. In addition, together with the pathway analysis in each of the up- and downregulated genes, we identified upregulation of the *Gsk3β* gene and downregulation of the *Wnt2* gene (table 1 and online suppl. table S1, and table 2 and online suppl. table S2, respectively), both of which are involved in the Wnt/β-catenin pathway.

Real-Time PCR Analysis of Gene Expression in Cultured Lung Explants

Lung explants obtained from rats at gestational day 14 were cultured for 4 days in the presence of heparin (30 µg/ml), without or with FGF-10 (250 ng/ml). For validation of selected FGF-10-induced genes identified by microarray analysis, real-time RT-PCR was performed on these FGF-10-nontreated and -treated groups. Many of these gene products have been described as potential regulators of lung patterning. Expression of several genes was upregulated or downregulated by treatment of explants with FGF-10 (table 3). Genes upregulated by treatment with FGF-10 included *Spry1* (Sprouty-1), *Bmp4*, *Pthlh* (PTHrP), *Dusp6* (MKP-3) and *Clic4* ($p < 0.05$). *Spry2* (Sprouty-2), *Spred-1* and *Shh* also showed increases, but these were not statistically significant ($p > 0.05$). Genes downregulated by treatment with FGF-10 included *Scgb1a1* (CC10), *Igf1* and *Gygl* ($p < 0.05$). *Sftpc* (SP-C), *Tcf21* (POD), *Sparc*, *Pcolce* (Pro CEP) and *Lox* (lysyl oxidase) were also downregulated, but the differences were not statistically significant ($p > 0.05$). There was good agreement between the data obtained

Fig. 2. Rebranching due to cessation of FGF-10 treatment in the middle of the culture. **a–l** Embryonic day 14 fetal rat lung explants were cultured for 4 days in the presence of heparin (30 μ g/ml), with 0, 125 or 250 ng/ml FGF-10. The inhibition of branching and the cystic development of lung explants with FGF-10 and heparin treatment are already apparent on culture day 2 (compare **f, j** and **n** with **b**). **m–p** Cessation of FGF-10 treatment of lung explants after 2 days of culture with FGF-10 and heparin resulted in rebranching (compare with **i–l**). Scale bars = 1 mm.

by microarray analysis and real-time PCR quantification.

Sprouty-1 and Sprouty-2 mRNA Expression by Northern Blot Analysis

The developmental changes induced by FGF-10 and heparin were accompanied by an increase in Sprouty-1 and Sprouty-2 mRNAs as shown by microarray chip and real-time PCR analyses (table 1, 3). Analysis by Northern blot also confirmed FGF-10-induced increases in the relative expression of Sprouty-1 and Sprouty-2 mRNAs in lung explants treated with FGF-10 and heparin for 4 days (fig. 3). In the absence of heparin, FGF-10 neither inhibited branching (fig. 1a–f) nor stimulated the expression of Sprouty-1 or Sprouty-2 mRNAs (fig. 3).

The differences in Sprouty-1 and Sprouty-2 mRNA expression between control (0 ng/ml FGF-10 + 30 μ g/ml heparin) and FGF-10-treated groups (125 ng/ml FGF-10 + 30 μ g/ml heparin) were also compared by densitomet-

ric analyses of the autoradiograms (fig. 4), and a significant increase in Sprouty-1 mRNA expression in the FGF-10-treated group was recognized. Sprouty-2 mRNA expression was also increased in the FGF-10-treated group, but the difference was not statistically significant.

In situ Hybridization

Expression of *CC10*, *SP-C*, *Sprouty-1*, *Sprouty-2* and *Spred-1* transcripts was analyzed by in situ hybridization on the embryonic day 14 lung explants cultured for 4 days with FGF-10 (0 or 250 ng/ml) and heparin (30 μ g/ml; fig. 5). In control lung (0 ng/ml FGF-10) and the region without dilatation in the FGF-10-treated lung (250 ng/ml), the proximal tubular epithelium shows *CC10* but not *SP-C* transcripts (fig. 5, compare arrowhead in a and d, or in b and e). Conversely, the distal tubular epithelium reveals *SP-C* but not *CC10* transcripts (fig. 5, compare arrow in d and a). However, in some portions of distal tubular epithelium, both *SP-C* and *CC10* transcripts can be

Table 1. Genes upregulated by FGF-10 and heparin treatment on Affymetrix microarray analysis

Gene	Gene title	Fold change	Probe set ID	Pathway
Id4	inhibitor of DNA binding 4	3.83	rc_AI072481_at	
Sp1	Sp1 transcription factor	2.46	D12768_at	Oxidative_Stress Smooth_muscle_contraction
Col9a1	procollagen, type IX, α 1	2.39	rc_AA955974_at	
Dusp6	dual-specificity phosphatase 6	2.35	U42627_at	
Casp6	caspase 6	2.25	AF025670_at	Apoptosis Apoptosis_GenMAPP Apoptosis_KEGG HSP70_and_Apoptosis
Cdh2	cadherin 2	2.24	rc_AI044325_at	
Pthlh	parathyroid hormone-like peptide	2.21	M34112_s_at	
Spred1	Sprouty protein with EVH-1 domain 1, related sequence	2.12	rc_AI045704_at	
Igfbp5	insulin-like growth factor binding protein 5	2.11	M62781_at	Smooth_muscle_contraction
Eef2	eukaryotic translation elongation factor 2	1.98	rc_AA892801_at	Translation_Factors
Col3a1	procollagen, type III, α 1	1.95	rc_AI236452_at	Inflammatory_Response_Pathway
Col2a1	procollagen, type II, α 1	1.89	AJ224879_at	
Scd2	stearoyl-coenzyme A desaturase 2	1.88	S75730_at	Fatty_Acid_Synthesis
Hbegf	heparin-binding EGF-like growth factor	1.88	rc_AA818658_at	
Fgfl	fibroblast growth factor 1	1.86	X14232_at	
Gsk3b	glycogen synthase kinase 3 beta	1.72	X53428cds_s_at	Cell_cycle_KEGG Glycogen_Metabolism Wnt_Signaling
Fgfr2	fibroblast growth factor receptor 2	1.7	L19107_s_at	
Spry2	Sprouty homolog 2 (<i>Drosophila</i>)	1.67	rc_AA850753_at	
Bmp4	bone morphogenetic protein 4	1.61	Z22607_at	TGF_Beta_Signaling_Pathway
Dusp5	dual-specificity phosphatase 5	1.56	AF013144_at	
Fasn	fatty acid synthase	1.55	X13527cds_s_at	Fatty_Acid_Synthesis
Sftpb	surfactant-associated protein B	1.52	rc_AI170380_g_at	

We identified 252 upregulated genes (fold change ≥ 1.5) by microarray analysis. The upregulated genes listed here serve a variety of cellular functions involved in branching morphogenesis or the FGF-10 signaling pathway.

seen (fig. 5, curved arrow in a and d, or in b and e). Expression of *Sprouty-1* and *Sprouty-2* transcripts in control lung had already been shown in our previous report [39]. Briefly, *Sprouty-1* transcripts were expressed in both distal tubular epithelium and the mesenchymal cells beneath the tubular epithelium. *Sprouty-2* transcripts were predominantly expressed in the distal tubular epithelium and were barely detectable in the mesenchymal cells.

In the presence of FGF-10 (250 ng/ml) and heparin (30 μ g/ml), the epithelium lining the dilated region partly showed *CC10* and/or *SP-C* transcripts (fig. 5c, f, arrows); however, in parts of the epithelium lining the dilated region, no *CC10* or *SP-C* transcripts were seen (fig. 5c, f, ar-

rowheads). *Sprouty-1* and *Sprouty-2* transcripts were seen in the distal tubular epithelium and in parts of the epithelium in the dilated region (fig. 5g, h, arrows). In addition, *Sprouty-1* transcripts were also present in the mesenchymal cells beneath the epithelium lining the dilated region (fig. 5g, arrowhead). *Spred-1* transcripts were widely seen in the mesenchymal cells as well as in the epithelium lining the dilated region (fig. 5i).

Immunohistochemistry

Expression of the genes listed in table 3 was further investigated by immunohistochemistry. For these analyses, embryonic day 14 lung explants were cultured for 4 days

Table 2. Genes downregulated by FGF-10 and heparin treatment on Affymetrix microarray analysis

Gene	Gene title	Fold change	Probe set ID	Pathway
Ogn_predicted	osteoglycin (predicted)	0.16	rc_AA997590_at	
Igf1	insulin-like growth factor 1	0.3	rc_AI112964_at	
Vcam1	vascular cell adhesion molecule 1	0.34	M84488_at	
Gyg1	glycogenin 1	0.42	U96130_at	Glycogen_Metabolism
Fgf13	fibroblast growth factor 13	0.43	rc_AI112097_at	
Lrat	lecithin-retinol acyltransferase (phosphatidylcholine-retinol-O-acyltransferase)	0.47	rc_AA899076_at	
Anxa1	annexin A1	0.48	rc_AI171962_s_at	Prostaglandin_synthesis_regulation
Sparc	secreted acidic cysteine-rich glycoprotein	0.49	R47150_f_at	
Igfbp7	insulin-like growth factor binding protein 7	0.55	rc_AI233246_at	
Col1a2	procollagen, type I, $\alpha 2$	0.56	rc_AI101443_i_at	Inflammatory_Response_Pathway
Lox	lysyl oxidase	0.56	S66184_s_at	
Tcf21	transcription factor 21	0.57	rc_AA800748_at	
Ppap2a	phosphatidic acid phosphatase 2a	0.59	rc_AA818593_g_at	
Fgf10	fibroblast growth factor 10	0.6	D79215_at	
Col3a1	procollagen, type III, $\alpha 1$	0.61	X70369_s_at	Inflammatory_Response_Pathway
Prkar2b	protein kinase, cAMP-dependent regulatory, type II β	0.61	rc_AI235758_s_at	Calcium_regulation_in_cardiac_cells G_Protein_Signaling Smooth_muscle_contraction
Col5a2	procollagen, type V, $\alpha 2$	0.62	rc_AI172281_at	
Sfrp2	secreted frizzled-related protein 2	0.63	rc_AA944317_at	
Lyz	lysozyme	0.63	rc_AA892775_at	
Fgfr1	fibroblast growth factor receptor 1	0.64	S54008_i_at	
Smad1	MAD homolog 1 (<i>Drosophila</i>)	0.65	U66478_at	TGF_Beta_Signaling_Pathway
Wnt2	wingless-related MMTV integration site 2	0.66	rc_AA997477_at	Wnt_Signaling
Cav	caveolin	0.66	Z46614cds_at	

We identified 164 downregulated genes (fold change ≤ 0.66) by microarray analysis. The downregulated genes listed here serve a variety of cellular functions involved in branching morphogenesis or the FGF-10 signaling pathway.

with FGF-10 (0 or 250 ng/ml) and heparin (30 μ g/ml). In the absence of FGF-10, Sprouty-1 and -2 were expressed in the distal tubular epithelium (fig. 6c, g, arrows). In the presence of FGF-10 (250 ng/ml), Sprouty-1 and -2 were expressed in the distal tubular epithelium and also in the epithelium of the dilated region (fig. 6d, h, arrows). In addition, Sprouty-1 expression was also observed in the mesenchymal cells beneath the epithelial cells lining the dilated region (fig. 6d, arrowhead). These protein expression patterns were identical with those of mRNA expression analyzed by in situ hybridization (fig. 5g, h).

PTHrP, BMP-4 and MKP-3 were predominantly expressed in the distal tubular epithelium, especially in the distal-tip tubular epithelium in the absence of FGF-10 (fig. 7a, c, e, arrows). Relatively distinct expression of

these proteins was seen in the lining epithelium of the cystic region in the presence of FGF-10 (250 ng/ml; fig. 7b, d, f, arrows). PTHrP and BMP-4 expression was also seen in the periphery of the lung in the pleural or subpleural cells (fig. 7b–d, arrowheads). There was lower expression of these three proteins in mesenchymal cells, and no obvious difference was recognized by immunohistochemistry between the conditions of absence and presence of FGF-10.

SP-C, SPARC and IGF-1 were also predominantly expressed in the distal tubules in the absence of FGF-10 (fig. 8a, c, e, arrows). In the presence of FGF-10 (250 ng/ml), relatively weaker expression of these proteins was also seen in the epithelium lining the cystic region (fig. 8b, d, f, arrows).

Fig. 3. Northern blot analysis of Sprouty-1 and Sprouty-2 expression in embryonic day 14 fetal rat lung explants treated with FGF-10. Total RNA was extracted from explants after 4 days of culture in the presence of varying concentrations of FGF-10 (0–250 ng/ml), without and with heparin (30 µg/ml). Northern blot was hybridized successively with ³²P-labeled cDNA probes for Sprouty-1 and Sprouty-2. The developmental changes induced by FGF-10 and heparin were accompanied by stimulated expression of both Sprouty-1 and Sprouty-2 mRNA. In the absence of heparin, FGF-10 neither stimulated mRNA expression of Sprouty-1 and Sprouty-2 nor inhibited branching (fig. 1a–f).

Table 3. Real-time PCR to validate data from microarray analysis

Gene symbol	Gene title	Real-time PCR analysis, % (n = 3)	DNA microarray analysis	
			change, %	probe set ID
<i>Genes upregulated by FGF-10 treatment</i>				
<i>Spry1</i> (Sprouty-1)	Sprouty homolog 1 (<i>Drosophila</i>) (predicted)	215 ± 12*	140 ^a	rc_AA800572_at
<i>Spry2</i> (Sprouty-2)	Sprouty homolog 2 (<i>Drosophila</i>)	132 ± 33	167 ^a	rc_AA850753_at
<i>Spred1</i>	Sprouty protein with EVH-1 domain 1, related sequence	118 ± 65	212 ^a	rc_AI045704_at
<i>Bmp4</i>	bone morphogenetic protein 4	270 ± 28*	161 ^a	Z22607_at
<i>Shh</i>	sonic hedgehog homolog (<i>Drosophila</i>)	256 ± 108	139	L27340_at
<i>Pthlh</i> (PTHRP)	parathyroid hormone-like peptide	386 ± 98*	221 ^a	M34112_s_at
<i>Dusp6</i> (MKP-3)	dual-specificity phosphatase 6	276 ± 65*	235 ^a	U42627_at
<i>Clic4</i>	chloride intracellular channel 4	180 ± 17*	115 ^a	rc_AI030141_at
<i>Genes downregulated by FGF-10 treatment</i>				
<i>Scgb1a1</i> (CC10)	secretoglobin, family 1A, member 1 (uteroglobin)	72 ± 9*	75	rc_AI009524_at
<i>Sftpc</i> (SP-C)	surfactant-associated protein C	84 ± 18	82 ^a	X14221_at
<i>Igf1</i>	insulin-like growth factor 1	53 ± 9*	30 ^a	rc_AI112964_at
<i>Tcf21</i> (POD)	transcription factor 21	47 ± 27	57 ^a	rc_AA800748_at
<i>Gyg1</i>	glycogenin 1	46 ± 4*	42 ^a	U96130_at
<i>Sparc</i>	secreted acidic cysteine-rich glycoprotein	73 ± 16	49 ^a	R47150_f_at
<i>Pcolce</i> (Pro CEP)	procollagen C-endopeptidase enhancer protein	76 ± 13	71 ^a	AF016503_s_at
<i>Lox</i>	lysyl oxidase	69 ± 20	56 ^a	S66184_s_at

Real-time PCR was performed for validation of selected FGF-10-regulated genes identified by microarray analysis. Some of these genes have been previously described as potential regulators of lung patterning. There is good agreement between the data obtained by microarray analysis and real-time PCR quantification. Values are expressed as a percentage of the control (no FGF-10, heparin only). Data are means ± SD (n = 3). * p < 0.05.

^a Indicates fold change as defined in Materials and Methods.

Fig. 4. Densitometric analyses of the autoradiograms shown in figure 3. The developmental changes shown in figure 1 were accompanied by a statistically significant increase in Sprouty-1 mRNA. The marginal increase in Sprouty-2 mRNA was not statistically significant. In the absence of heparin, FGF-10 neither inhibited branching (fig. 1a–f) nor stimulated the expression of Sprouty-1 or Sprouty-2 mRNAs (fig. 3). Sprouty-1 mRNA expression in FGF-10-treated explants was $176.7 \pm 49.82\%$ of the control value ($p = 0.013$). Sprouty-2 mRNA expression in FGF-10-treated explants was $126.7 \pm 19.85\%$ of the control value ($p = 0.059$). The data are means $\pm$ SD.

Fig. 5. In situ hybridization to demonstrate the effect of FGF-10 on the expression of *Scgb1a1* (*CC10*), *Sftpc* (*SP-C*), *Sprouty-1*, *Sprouty-2* and *Sprad-1* mRNA in rat lung explants. Embryonic day 14 rat lung explants were cultured for 4 days without FGF-10 (**a**, **d**) or with 250 ng/ml FGF-10 (**b**, **c**, **e**–**i**). Heparin (30 µg/ml) was added to all cultures. The proximal tubular epithelium shows *CC10* but not *SP-C* transcripts (compare arrowhead in **a** and **d**, or in **b** and **e**). Conversely, the distal tubular epithelium shows *SP-C* but not *CC10* transcripts (compare arrow in **d** and **a**). However, in some distal tubular epithelium, both *SP-C* and *CC10* transcripts can be seen (curved arrow in **a** and **d**, or in **b** and **e**). In the region

dilated after FGF-10 treatment, the lining epithelium partly shows *CC10* (**c**, arrow) and/or *SP-C* (**f**, arrow) transcripts. However, in parts of the epithelium, no *CC10* or *SP-C* transcripts can be seen (**c**, **f**, arrowhead). *Sprouty-1* and *-2* transcripts are seen in the distal tubular epithelium and in parts of the epithelium in the dilated region (**g**, **h**, arrows). In addition, *Sprouty-1* transcripts are also present in the mesenchymal cells beneath the epithelium lining the dilated region (**g**, arrowhead). *Sprad-1* transcripts are widely distributed in the mesenchymal cells as well as in the epithelium lining the dilated region (**i**). Scale bars = 1 mm (**a**, **b**, **d**, **e**) and 100 µm (**c**, **f**–**i**).

Fig. 6. Immunostaining for Sprouty-1 and -2 in rat lung explants treated with/without FGF-10. Embryonic day 14 rat lung explants were cultured for 4 days without FGF-10 (**a, c, e, g**) or with 250 ng/ml FGF-10 (**b, d, f, h**). Heparin (30 μ g/ml) was added to all cultures. In the absence of FGF-10, Sprouty-1 and -2 expression is seen in the distal tubular epithelium (**c, g**, arrow). In the presence of FGF-10, Sprouty-1 and -2 are expressed in the distal tubular epithelium and in the dilated epithelium (**d, h**, arrow). In addition, Sprouty-1 expression is also seen in the mesenchymal cells, especially around the dilated tubules (**d**, arrowhead). This feature is the same as the Sprouty-1 transcript expression (fig. 5g, arrowhead). The epithelium lining the dilated region with FGF-10 treatment is largely pseudostratified (**d**, inset). Scale bars = 1 mm (**a, b, e, f**) and 100 μ m (**c, d, g, h**).

Discussion

FGF-10 in the Presence of Heparin Inhibits Branching and Induces Cystic Growth in Cultured Lung Explants

FGF-10 signaling is essential for lung branching morphogenesis [3, 5, 41]. The growth factor is produced by embryonic lung mesenchymal cells and regulates epithelial budding [3]. This study demonstrates that FGF-10 mimics the previously reported effects of KGF on fetal

lung explants in culture [7] but requires the presence of heparin in the culture media. At high concentrations, FGF-10 (plus heparin) inhibited branching. However, in the absence of heparin, the FGF-10-treated fetal lung explants continued to show a branching pattern similar to control explants. It thus appears that FGF-10 activity is sensitive to heparin. The sensitivity to heparin is not unique to FGF-10; heparin is also known to modulate the activities of KGF and another FGFR2IIIB ligand, acidic

Fig. 7. Immunostaining of products of genes upregulated by FGF-10. Embryonic day 14 rat lung explants were cultured for 4 days without FGF-10 (**a, c, e**) or with 250 ng/ml FGF-10 (**b, d, f**). Heparin (30 μ g/ml) was added to all cultures. In the absence of FGF-10, PTHrP (**a**), BMP-4 (**c**) and MKP-3 (**e**) proteins are detected in the distal tubules, especially at the tubular tip (**a, c, e**, arrow). In the presence of FGF-10, PTHrP (**b**), BMP-4 (**d**) and MKP-3 (**f**) protein expression is detected in the lining epithelium of the cystic region (**b, d, f**, arrow). PTHrP and BMP-4 protein expression is also seen at the periphery of the lung, in the pleural or subpleural cells (**b–d**, arrowhead). Scale bar = 100 μ m.

Color version available online

FGF, by enhancing the binding of acidic FGF and inhibiting that of KGF to FGFR2IIIB [42]. Differential effects of heparin have been observed with regard to the mitogenic activities of FGF-7 and FGF-10; FGF-7 was inhibitory, while FGF-10 had a stimulatory effect [43]. Nogawa and Ito [44] have shown that a combination of acidic FGF and heparin increased luminal expansion and inhibited branching of embryonic lung epithelium. Like heparin, the cell-associated heparan sulfate (HS) chains also enhance the binding of acidic FGF to FGFR2IIIB [42]. FGF-10 and FGF-7 require specific HS sulfation patterns for binding and presentation to the receptor. For example 6-O-sulfation but not 2-O-sulfation of HS chains is needed for binding of FGF-10, whereas both sulfations are needed for binding FGF-7 [45]. Thus, the HS patterns associated with epithelial cells would regulate binding of FGF-10, a key regulator of the branching process, to FGFR2b and its activation. Evidence suggests that FGF-7

activity is less dependent on the presence of HS. In contrast, FGF-10 is more dependent on the availability of HS for formation of a stable complex with FGFR2b. Two recent studies demonstrated the importance of endogenous HSs in lung growth and morphogenesis and gene expression [46–48]. The exogenously added FGF-10 used in our study may also require heparin supplementation to elicit developmental and gene expression responses. Our results are in contrast to those reported by Mailleux et al. [17] using mouse lung explants with FGF-10 and without heparin. These investigators reported that in this system, FGF-10 at high concentrations (500 ng/ml) increases lung branching. This discrepancy may be due to the species difference and the composition of the culture media. We did not use such high concentrations of FGF-10 in our culture system.

Although the physiological roles of high concentrations of FGF-10 in lung branching morphogenesis are un-

Fig. 8. Immunostaining of products of genes downregulated by FGF-10. Embryonic day 14 rat lungs were cultured for 4 days without FGF-10 (**a**, **c**, **e**) or with 250 ng/ml FGF-10 (**b**, **d**, **f**). Heparin (30 μ g/ml) was added to all cultures. In the absence of FGF-10, SP-C (**a**), SPARC (**c**) and IGF-1 (**e**) proteins are detected in the epithelium of distal tubules (**a**, **c**, **e**, arrow). In the presence of FGF-10, relatively weaker expression of SP-C (**b**), SPARC (**d**) and IGF-1 (**f**) is noted in the lining epithelium of the cystic regions (**b**, **d**, **f**, arrow). Scale bar = 100 μ m.

known, the present study demonstrates the ability of FGF-10 to influence the process in vitro. FGF-10 overexpression in mice lungs leads to disruption of lung morphogenesis accompanied by pulmonary adenomas [10]. Localized expression of FGF-10 induces cystic adenomatoid malformation in fetal rat lungs [11], thus emphasizing the potential role of FGF-10 in the development of human cystic adenomatoid malformation.

Gene Profiling Reveals Novel Targets of FGF-10 in Cultured Lung Explants

In this study, we used microarray analysis and real-time PCR to identify genes whose expression is regulated by FGF-10 in lung explant cultures and to provide insights into the mechanism of growth factor-induced cystic growth and inhibition of lung branching. Our results show that FGF-10 regulates the expression of several key genes known to be involved in branching morphogenesis

(*Tcf21/POD*, *Sparc*, *Igf*, *Bmp4*, *Shh*, *Spry*), type II epithelial cell differentiation and surfactant synthesis (*Pthlh/PTHrP*), fluid transfer and cyst formation (*Clic4*) and growth factor-induced signaling pathways (*Dusp6/MKP-3*, *Spry*). BMP-4, Sprouty-1, Sprouty-2 and SHH are all highly expressed in distal epithelium during lung development [27, 39, 49–54]. Upregulation of these genes by FGF-10 has also been shown in a variety of other systems [53, 55, 56]. Two additional genes expressed in embryonic lung and upregulated by FGF-10 are *Clic4* and *Pthlh/PTHrP*. In our study, *Sprouty-1* and *Sprouty-2* were both upregulated with FGF-10 treatment, and the fold change in each was 1.40 and 1.67, respectively (as the fold change in *Sprouty-1* was <1.5, it was not listed in table 1). However, upregulation of both Sprouty genes with FGF-10 treatment was also confirmed by Northern blot analysis (fig. 3), and *Sprouty-1* upregulation was statistically significant by densitometry analysis (fig. 4; $p = 0.013$). The

expression of both Sprouty mRNAs in the epithelium of the dilated region was also confirmed by in situ hybridization (fig. 5g, h). On the other hand, the lung epithelial-specific genes *Scgb1a1/CC10* and *Sftpc/SP-C* were both found to be downregulated by real-time PCR analysis (table 3), but as the fold change in each was 0.72 and 0.77 (both >0.66), respectively, neither gene was listed in table 2. Examination of the expression of both mRNAs by in situ hybridization revealed no expression in parts of the epithelium of the dilated region after FGF-10 treatment (fig. 5c, f).

Although these many important up- and downregulated genes following FGF-10 treatment were identified in our study, significant differences were also observed in FGF-10-targeted genes reported in a previous study [57] and this study. Several of the genes identified as targets were not present in the list reported by Lu et al. [57]. Likewise, several of the genes reported by Lu et al. [57] as targets of FGF-10 signaling were either poorly expressed or showed no change in expression in the present study. These were BMP1a, Tacstd2, Tm4sf3, Anxa1, Anxa3, AHNK, TIMP-3, Akap8 and β -catenin. The relevance of the genes identified in the two studies to branching morphogenesis remains unknown. The differences in gene expression in the two studies may be due to species differences (rat vs. mouse) or the tissue used, i.e. mesenchyme-free epithelium [57] versus whole lung explants (this study). There were also genes that were identified as targets in both studies, such as stearoyl-coenzyme A desaturase. The following discussion emphasizes the potential roles of the genes identified in this study in normal lung development and in FGF-10-induced cystic lung development.

Upregulated Genes – Spry, Clic4, Bmp4, Dusp6/MKP-3 and Pthlh/PTHrP

Sproutys are known to inhibit FGF signaling in a variety of systems [14, 16, 18, 58, 59], and Sprouty-1 and Sprouty-2 mRNAs and proteins are expressed at various stages of lung development and in adults [17, 18, 21, 38, 39, 54]. Inhibition of Sprouty-2 expression with antisense oligonucleotides in cultured mouse lung explants enhanced branching [17, 21], indicating that Sprouty-2 acts as an inhibitor of lung branching morphogenesis. Other investigators have also shown increased expression of Sprouty-2 following FGF-10 treatment of embryonic rat lung explants in culture [60]. However, very little information is available about the role of Sprouty-1, the expression of which is also induced by FGF-10 in lung branching morphogenesis.

The CLIC family is a highly conserved family of seven proteins that are expressed in multiple tissues, including the lung [61]. CLIC-4 is a multifunctional protein that participates in cell cycle control. It is involved in endothelial proliferation, capillary sprouting and lumen formation in vitro [62]. It has been proposed that CLIC-4 regulates tubulogenesis by interacting with the cytoskeleton. EXC-4, a member of the CLIC family, is required for the formation of the excretory canal system of *Caenorhabditis elegans* and to prevent cystic luminal expansions in the excretory cell [63, 64]. Zhou et al. [8] showed that KGF-induced lung cystadenomas and fluid accumulation are driven by cystic fibrosis transmembrane conductance regulator-independent chloride transport and are associated with decreased expression of the alpha subunit of the epithelial sodium channel. The mechanism of FGF-10-induced lung cystic adenomatoid malformation, tubulogenesis and lumen formation may involve members of the CLIC family [62–64].

BMP-4 is expressed in epithelial cells in the tips of growing lung buds [28]. Previous studies have demonstrated that BMP-4 expression is induced by FGF-10 [49, 53]. The increased BMP-4 expression in FGF-10-treated lung explants (fig. 7d, arrow) inhibits branching and induces cystadenomatous lung, changes similar to those seen in transgenic mice with lung-specific overexpression of BMP-4 in the distal epithelium under the control of the SP-C promoter [28].

MKP-3, a cytoplasmic dual-specificity phosphatase that inactivates phosphorylated forms of ERK [22–24], is expressed in lung, heart, brain and kidney [65] and also in early embryonic mouse lung (embryonic day 14.5) [66]. Our data also showed that immunohistochemical staining localized MKP-3 to the distal epithelium of embryonic rat lungs (fig. 7e, f). The inducibility of the MKP-3 gene by FGF signaling seen here has also been observed by other investigators in PC12 cells, chicken neural plate and limb bud, mouse lens explants and neural tube explants [13, 67–71]. What role MKP-3 plays in lung branching and patterning remains to be investigated.

PTHrP is an autocrine/paracrine factor that is widely expressed during embryogenesis and attenuates programmed cell differentiation. This protein and parathyroid hormone share a common receptor (PTH/PTHrP receptor), which is expressed in the mesenchymal cells of many developing organs [72]. PTHrP is expressed in lung epithelium during branching morphogenesis and in type II cells later during lung maturation [31]. PTHrP stimulates surfactant phospholipid synthesis in type II cells in vitro. PTHrP^{−/−} and PTHrP receptor^{−/−} mouse embryos

did not show any significant defect in branching morphogenesis but showed arrested type II cell and mesenchymal cell differentiation and reduced surfactant production [72]. PTHrP is considered to play a key role in mesenchymal-epithelial interactions vital to the lung and other organs. However, the functional significance of early PTHrP expression and its induction by FGF-10 during branching morphogenesis is not clear. The increased expression of PTHrP induced by FGF-10 in our cultured lung explants might lead to increased differentiation of mesenchymal/epithelial cells.

Downregulated Genes – Sparc, Igf, Tcf21/POD and Glycogenin

SPARC (or osteonectin) is a secreted glycoprotein that may play a role in branching morphogenesis. SPARC is expressed by epithelial cells during the pseudoglandular stage of rat lung development. Strandjord et al. [34] used a neutralizing SPARC antibody and a synthetic peptide to block SPARC activity in cultures of rat lung explants and showed that lung explants treated with either reagent exhibit diminished branching and airway dilatation. In our study, SPARC expression was reduced by treatment of explants with FGF-10, possibly causing inhibition of branching and cystic lung development.

Signaling by IGF and its receptor, IGF-1R, appears to regulate branching morphogenesis, vasculogenesis and alveolar epithelial type I and type II cell differentiation [73–75]. Han et al. [73] observed that IGF-1/IGF-1R appeared early in human lung epithelium during gestation (4 weeks), suggesting a role of this signaling pathway in lung branching morphogenesis, and hypothesized that the high neonatal mortality in the IGF-1R knockouts was due to a defect in branching morphogenesis. They also observed that lung explants treated with anti-IGF-1R antibody developed airway collapse, large gaps in the mesenchyme compartment and an increase in the number of apoptotic mesenchymal cells [73]. These reports may support our finding that downregulation of the IGF-1 gene by FGF-10 disrupted branching morphogenesis and caused cystic lung development.

POD-1 is expressed in lung mesenchymal cells and likely participates in mesenchymal-epithelial interactions. POD-1 knockout mice die within minutes after birth as a result of respiratory stress [33]. There is evidence of failure of normal branching morphogenesis of the lung, both in the early and late pseudoglandular stages [33]. Additionally, a defect in epithelial cell differentiation and normal patterning of airway epithelium is seen [33]. Antisense inhibition of POD-1 also disrupted

branching morphogenesis in murine embryonic kidney [76]. Reduced POD expression as a result of FGF-10 (plus heparin) treatment of rat lung explants in culture may thus inhibit branching morphogenesis.

Glycogen synthesis in mammalian tissues is initiated by autoglucosylation of glycogenin. There is evidence suggesting glycogenin as a regulator of glycogen synthesis in human skeletal muscle [77]. A critical role for glycogen stored in epithelial cells is to serve as a substrate for surfactant lipids during lung maturation. FGF-7 pulmonary overexpression caused glycogen-laden, columnar epithelial cells in transgenic embryonic mouse lung [9]. However, the potential role that FGF-10 signaling may play in glycogen synthesis through suppression of glycogenin synthesis remains to be determined.

Effect of FGF-10 on the Wnt2/ β -Catenin Signaling Pathway

Together with the pathway analysis in each of the up- and downregulated genes, we identified upregulation of the *Gsk3 β* gene and downregulation of the *Wnt2* gene (table 1 and online suppl. table S1, and table 2 and online suppl. table S2, respectively). Both genes are involved in Wnt2/ β -catenin signaling, which is crucially important for lung organogenesis, as *Wnt2a/2b* knockout transgenic mice showed lung agenesis in the early embryo [36]. In the absence of canonical Wnt signaling, free cytosolic β -catenin was phosphorylated by Gsk3 β at the N terminus and ubiquitinated, then degraded by proteasome, thus inactivating the signaling pathway [78, 79]. In this way, upregulation of *Gsk3 β* and downregulation of *Wnt2* were both contributing to inhibition of the Wnt2/ β -catenin signaling pathway. On the other hand, some previous reports showed that FGF-10 and -8 were induced by Wnt/ β -catenin signaling activation and that the crosstalk loop of these factors was important for initiation of limb generation or regeneration [80, 81]. In our report, in contrast, FGF-10 might have negatively regulated Wnt2/ β -catenin signaling by upregulating *Gsk3 β* and downregulating *Wnt2a*. This is the first report that FGF-10 may also function in regulating branching morphogenesis or inducing cystic lung formation by inhibiting the Wnt2/ β -catenin signaling pathway, although the evidence of this direct regulation should be further clarified.

Protein Expression Behavior for Some Up- and Downregulated Genes

Protein expression of upregulated (Sprouty-1, Sprouty-2, PTHrP, BMP-4, MKP-3) and downregulated

genes (SP-C, SPARC, IGF-1) as assessed by real-time PCR analysis was also determined by immunohistochemistry in both FGF-10-nontreated and -treated conditions (fig. 6–8). These immunohistochemical results were relatively well coordinated with those of each gene expression pattern. Namely, the immunoreaction against the proteins expressed by the upregulated genes was recognized broadly or distinctly in the lining epithelium of the cystic region in the FGF-10-treated condition (fig. 6b, d, f, h, fig. 7b, d, f). In contrast, relatively weak immunoreaction against proteins expressed by the downregulated genes was seen in the lining epithelium of the cystic region in the FGF-10-treated condition (fig. 8b, d, f).

Conclusions

In summary, our results show that FGF-10 signaling in the presence of heparin results in cystic lung growth in rat lung explants in culture. FGF-10-induced increased epithelial cell proliferation may be a mechanism by which cystic changes occur in cultured lung explants. Additionally, the altered expression of several key genes

known to be involved in lung branching morphogenesis may play a role. These include *Bmp4*, *Spry*, *Tcf21/POD*, *Igf1*, *Sparc*, *Shh* and *Pthlh/PTHrP*. The expression of several genes whose products have previously been shown to regulate branching morphogenesis is increased (*Spry*, *Bmp4*, *Shh*) or decreased (*Igf1*, *Sparc*, *Tcf21/POD*) as a result of exogenous FGF-10 added to cultures of rat fetal lung explants. Potential roles of these and other genes such as *Clic4* and *Dusp6/MKP-3* in cystic lung development remain to be investigated. Another important finding is that FGF-10 may also regulate branching morphogenesis or induce cystic lung growth by inhibiting the Wnt2/ β -catenin signaling pathway. These results provide some important insights into clarifying the mechanisms of branching morphogenesis with reciprocal target gene expression triggered by FGF-10 in lung development.

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