Fibroblast Growth Factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung

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SUMMARY

During mouse lung morphogenesis, the distal mesenchyme regulates the growth and branching of adjacent endoderm. We report here that *fibroblast growth factor 10 (Fgf10)* is expressed dynamically in the mesenchyme adjacent to the distal buds from the earliest stages of lung development. The temporal and spatial pattern of gene expression suggests that *Fgf10* plays a role in directional outgrowth and possibly induction of epithelial buds, and that positive and negative regulators of *Fgf10* are produced by the endoderm. In transgenic lungs overexpressing *Shh* in the endoderm, *Fgf10* transcription is reduced, suggesting that high levels of SHH downregulate *Fgf10*.

Addition of FGF10 to embryonic day 11.5 lung tissue (endoderm plus mesenchyme) in Matrigel or collagen gel culture elicits a cyst-like expansion of the endoderm after 24 hours. In Matrigel, but not collagen, this is followed by extensive budding after 48-60 hours. This response involves an increase in the rate of endodermal cell proliferation. The activity of FGF1, FGF7 and FGF10 was also tested directly on isolated endoderm in Matrigel culture. Under these conditions, FGF1 elicits immediate endodermal budding, while FGF7 and FGF10 initially induce expansion of the endoderm. However, within 24 hours, samples treated with FGF10 give rise to multiple buds, while FGF7-treated endoderm never progresses to bud formation, at all concentrations of factor tested. Although exogenous FGF1, FGF7 and FGF10 have overlapping activities in vitro, their in vivo expression patterns are quite distinct in relation to early branching events. We conclude that, during early lung development, localized sources of FGF10 in the mesoderm regulate endoderm proliferation and bud outgrowth.

Key words: FGF10, epithelial-mesenchymal interactions, branching morphogenesis, lung development, mouse

INTRODUCTION

The embryonic mouse lung is an important model system in which to study the molecular mechanisms controlling branching morphogenesis. This process involves a dynamic interplay between intercellular signaling molecules, downstream signal transduction pathways, cell proliferation and cell-cell and cell-matrix interactions. Since branching morphogenesis occurs in the embryonic development of many organs, it is likely that the rules governing the growth and branching of the lung can be generalized to other systems such as the pancreas, mammary gland and kidney.

Lung development starts at embryonic day 9.5 (E9.5), with an outpocketing of the ventral foregut endoderm surrounded by splanchnic mesenchyme. Subsequently, the two primary buds elongate and undergo further branching in a highly reproducible pattern, giving rise, in the mouse embryo, to five lobes, four on the right and one on the left. Grafting experiments have shown that interactions between the epithelium and the mesenchyme drive the branching process. For example, distal mesenchyme induces ectopic budding and branching when grafted adjacent to the tracheal endoderm denuded of mesenchyme (Alescio and Cassini, 1962). Conversely, tracheal mesenchyme inhibits branching when grafted next to distal epithelium (Wessels, 1970).

Transfilter experiments demonstrate that the effect of distal lung mesenchyme is mediated by secreted factors (Taderera, 1967). Over the past few years, studies have shown that factors belonging to several different families of secreted signaling molecules and their receptors are expressed in the developing lung. For example both *Bone morphogenetic protein 4* (*Bmp4*) and *Sonic hedgehog* (*Shh*) are expressed at high levels in the distal epithelium, whereas *Wnt2* and the gene encoding a SHH receptor, *patched* (*Ptc*), are expressed in the distal mesenchyme (Levay-Young and Navre, 1992; Bellusci et al., 1996, 1997; Marigo et al., 1996; Urase et al., 1996).

Several members of the fibroblast growth factor (FGF) family and their receptors have also been implicated in mouse lung development. First, three of the four known FGF receptor genes are expressed in the endoderm. *FgfR2* transcripts are detected throughout the epithelium at early stages, but are later restricted to the distal epithelium (Peters et al., 1994), and *FgfR3* and *FgfR4*

are expressed at lower levels in the distal epithelium (Cardoso et al., 1997 and Dr Michael Weinstein, personal communication). Transgenic mouse embryos expressing a dominant negative *FgfR2 IIIb* splice variant *(FgfR2 IIIb)* in the distal lung epithelium under the control of the *Surfactant Protein C* (*SP-C*) enhancer/promoter exhibit a severe defect in lung branching (Peters et al., 1994). At birth, the lung consists only of the trachea and two main bronchi, without any lateral branches, thus providing the strongest evidence to date for a role for FGFs in lung branching morphogenesis. Less is known about the temporal and spatial expression of the ligands so far known to act through FGFR2 with high efficiency, namely FGF1 and FGF7 (Ornitz et al., 1996). FGF1 has been detected by immunohistochemistry uniformly throughout the mesenchyme of the E13 rat lung (Fu et al., 1991). Using radioactive in situ hybridization, *Fgf7* transcripts are only detected in the mouse lung from E14 onward (Mason et al., 1994). However, *Fgf7* expression is detectable by RT-PCR in the E12 rat lung, equivalent to E11 in the mouse (Post et al., 1996). The biological activity of recombinant human FGF1 and FGF7 has been tested on isolated endoderm in vitro; FGF1 induces bud formation from mesenchymal-free endoderm cultured in Matrigel, while FGF7 promotes epithelial proliferation and expansion, leading to the formation of cyst-like structures (Nogawa and Ito, 1995; Cardoso et al., 1997). Consistent with its in vitro activity, transgenic misexpression of *Fgf7* in the distal mouse epithelium using the *SP-C* enhancer/promoter leads to the formation of abnormal lungs composed of numerous dilated saccules made up of undifferentiated columnar epithelial cells (Simonet et al., 1995). This latter result suggests that FGFs affect differentiation, and this is supported by alterations in surfactant protein gene expression following treatment of lung cultures with FGF1 or FGF7 (Cardoso et al., 1997). In spite of the evidence for a role for FGF7 in lung development, mice homozygous for a null mutation in the *Fgf7* gene exhibit no obvious pulmonary abnormalities (Guo et al., 1996). This suggests that other FGF family members function in the lung in vivo and can compensate for the absence of FGF7.

Here, we report the expression and biological activity in the developing lung of a new member of the FGF family, FGF10. *Fgf10* was originally isolated from the rat and transcripts were detected at high level in the mesenchyme of the E14 lung (Yamasaki et al., 1996). However, no information was available about their localization in relation to morphogenesis. As the result of such an analysis in the embryonic mouse lung, and of studies on the effect of FGF10 on embryonic lung tissue in culture, we conclude that localized FGF10 production by distal mesenchyme is involved in the directional outgrowth of endodermal buds, and in the control of endodermal cell proliferation. Finally, we observe that, in transgenic lungs expressing high levels of SHH in the endoderm, *Fgf10* is downregulated, and we propose a model integrating *Fgf10* into the network of genes regulating branching morphogenesis.

MATERIALS AND METHODS

Murine *Fgf10* cDNA

Murine *Fgf10* was cloned by RT-PCR from E11.5 mouse lung cDNA. The forward primer was +11 to +34 in the rat sequence (5′ GGAT-ACTGACACATTGTGCCTCAG 3′) and the reverse primer was +574 to +597 (5′ TGTTTTTTGTCCTCTCCTGGGAG 3′) (Yamasaki et al.,

1996). After 30 cycles of amplification with a 55°C annealing temperature, a ~580 bp fragment containing most of the coding region was purified from low-melting agarose gel and cloned into Bluescript (pSK II +, Stratagene).

Production of FGF 10 in insect cells

The human *FGF10* cDNA (accession number AB002097 in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases), with a DNA fragment encoding an E-tag (GAPVPYPDPLEPR) and a hexameric His tag (HHHHHH) at the 3′ terminus of the coding region, was constructed in the transfer vector pBacPAK9 (Clontech). Recombinant baculovirus containing the *FGF10* cDNA was obtained by cotransfection of Sf9 cells with the recombinant pBacPAK9 and a *Bsu 36 I*-digested expression vector, BacPAK6 (Clontech).

High Five cells (approximately 2×10^6 cells/ml) in serum-free medium (EX-CELL 400, JRH Bioscience) were infected with the recombinant baculovirus. The infected cells were cultured in a spinner flask at 27°C for 65 hours before harvesting and dialysis of the medium against phosphate-buffered saline (PBS). The concentration of FGF10 in the culture medium was estimated to be approximately 1 μg/ml by dot blotting using an anti-E tag antibody (Pharmacia Biotech).

In situ hybridization

The murine cDNAs used as templates for riboprobes were the 584 bp *Fgf10* fragment described above, a 622 bp *Fgf7* clone (provided by Dr Ivor Mason), a 642 bp *Shh* clone (provided by Dr Andrew McMahon), a 475 bp *Fgf1* cDNA (provided by Dr Gail Martin) and a 387 bp *Fgf1* cDNA fragment (provided by Drs Toru Imamura and Kazuo Ozawa). The whole-mount and radioactive in situ hybridization protocols have been described (Winnier et al., 1997). To compare gene expression between lungs at different ages by section in situ hybridization, six to nine sections (7 μm thick) of E11.5 and E14.5 lungs fixed and processed under the same conditions were placed on the same microscope slide. This allowed the sections to be given exactly the same treatments. Photomicrographs were taken with the same exposure time.

Northern blot analysis

Poly(A) RNA was isolated from lungs grown in culture in the presence or absence of retinoic acid (see below) using the Micro-Fast-Track™ kit (Invitrogen). Alternatively, total RNA from normal and transgenic lungs overexpressing *Shh* and from freshly dissected lungs at different gestational stages was isolated using guanidine thiocyanate extraction and cesium chloride ultracentrifugation (Chirgwin et al., 1979). 10 μg of total RNA or 500 ng of poly(A) RNA were analyzed on a 2% formaldehyde-1.2% agarose gel and blotted onto a Magna Graph Nylon transfer membrane (Micron Separations Inc., Westborough, MA). The same filters were hybridized successively with radiolabeled (^{32}P) cDNA probes for mouse *Fgf10*, *Shh* and *β-actin* (Stratagene Prime-It kit, Stratagene, La Jolla, CA). The filter was hybridized for 16 hours at 65°C in Quikhyb buffer (Stratagene) and then washed twice for 30 minutes at 65°C in 2× SSC (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7). The filter was exposed to Kodak XAR-5 film (Eastman Kodak Company, Rochester, NY) in the presence of an intensifying screen at −70°C for different times in order to obtain a signal under the saturation level of the film. The autoradiograms were scanned using a laser densitometer (BioRad Laboratories, Richmond, CA). The levels of *Shh* and *Fgf10* mRNA were normalized to the level of *β-actin* to correct for differences in RNA loaded.

Biological assays

Two different biological assays were carried out. In the first assay, lungs were dissected from E11.5 embryos and the most distal tips (approximately 0.3 mm) of the left, caudal and accessory lobes were removed using fine tungsten needles. The 'amputated' lungs (first test

tissue) and isolated distal buds (second test tissue) were then embedded either in Matrigel or rat tail type I collagen (both products obtained from Collaborative Biomedical Products, Bedford, MA). Matrigel was diluted 1:1 in the corresponding culture medium (see below) and polymerized by incubation at 37°C for 15-20 minutes. Collagen gels (4.4 mg/ml of type I collagen in DMEM) were polymerized by the addition of 0.03 M (final) $NaHCO_3$. In the second assay, lungs from E11 embryos were isolated and incubated for 10 minutes in trypsin/pancreatin solution (0.5% and 2.5%, respectively, in Ca^{2+}/Mg^{2+}-free Tyrode Ringer's saline, Hogan et al., 1994) at 4°C and then transferred to ice-cold DMEM:F12 with 10% heat inactivated fetal bovine serum to block the enzymatic reaction. The epithelial buds were separated from mesenchyme using tungsten needles and embedded in 200 µl of Matrigel diluted 1:1 with the culture medium (Nogawa and Ito, 1995). In some experiments, the purified endoderm was first placed on top of nucleopore filters (pore size 8 µm, Costar) and then covered with Matrigel.

Different concentrations of recombinant human FGFs were added both to the culture medium (200 µl/well) and to the collagen or Matrigel at the desired concentration. The following concentrations were used; FGF1 (Sigma), 30, 250, 500 and 1000 ng/ml; FGF7 (Sigma), 5, 15, 30, 60, 120, 250 and 500 ng/ml; FGF10, 5, 15, 30, 50, 250 and 500 ng/ml. Explants were grown for 1 to 3 days in DMEM:F12 medium (1:1) containing freshly added glutamine, penicillin/streptomycin and 10 µg/ml of serum albumin.

In some experiments with collagen gels, basement membrane heparan suphate proteoglycan (HSPG) (Sigma H-4777) isolated from the same source as Matrigel (Engelbreth-Holm-Swarm mouse sarcoma) was added to the culture medium.

Analysis of lung proliferation in vitro

E11.5 'amputated' lungs and their corresponding end buds were cultured in Matrigel as described above. The final volume for the culture was 400 µl/well (200 µl of Matrigel and 200 µl of medium). The explants were incubated for 24 hours with either FGF10 (250 ng/ml), FGF7 (250 ng/ml) or FGF1 (500 ng/ml) before labeling for 3 hours with the Cell Proliferation Labeling Reagent (Amersham) diluted 1:1000. Sections of BrdU-labeled explants (7 µm) were processed for immunohistochemical detection of BrdU incorporation as described (Sakai et al., 1994), using an anti-BrdU rat antibody (Accurate Chemicals, Westbury, New York) diluted 1:400 in PBS. The secondary antibody was a peroxidase-coupled rabbit anti-rat IgG (Vector) diluted 1:100 in PBS and peroxidase activity was revealed using 3-amino, 9 ethyl carbazole as the chromogen. Epithelial cells in 13 photomicrographs (at 60× magnification) of random distal portions of a section of FGF-treated and control lungs were counted and scored as labeled or unlabeled (total number of cells counted: 1124 (control); 1378 (FGF10-treated lungs)). The data were analyzed by Student's *t*-test. Results were determined to be significant if $P<0.05$.

Retinoic acid treatment

Two sets of 14 lung explants isolated from E11.5 mouse embryos were cultured for 4 days in either DMEM:F12 (1:1) medium containing glutamine, penicillin/streptomycin, human apo-transferrin (10 µg/ml) and serum albumin (1 µg/ml) or the same medium containing all-*trans*-retinoic acid (Sigma, St Louis, MO), diluted in DMSO at 10^{-6} M. Fresh retinoic acid was added every 24 hours.

RESULTS

Fgf10 mRNA expression in the embryonic lung

Yamasaki et al. (1996) reported that *Fgf10* is expressed in the mesenchyme of the E14.5 rat lung. We therefore isolated a murine *Fgf10* clone and studied the localization of transcripts during lung development by whole-mount and section in situ hybridization (Figs 1, 2, 9).

At E9.75, *Fgf10* RNA is already present around the two small lung buds growing out from the ventral foregut. Interestingly, transcript levels are significantly higher around the slightly larger right bud than the left (Fig. 1A). By E10.5, *Fgf10* expression is restricted to the distal mesenchyme of the two main bronchi (Fig. 1B).

As development proceeds, buds emerge laterally from the main bronchi, elongate and begin to branch. To determine how the dynamic pattern of *Fgf10* expression is related to the increasing complexity of the bronchial tree, and to the expression of genes such as *Shh* (Fig. 1G), we collected duplicate sets of lungs and performed the whole-mount in situ hybridization alkaline phosphatase color reaction for 6 hours or overnight. At E11.5, a short color reaction reveals that particularly high levels of *Fgf10* mRNA are present in the mesenchyme at the very distal tips of the accessory, right medial, right caudal and left lobes (lobes 1, 2, 3 and 4, respectively in Fig. 1C). Not visible on these views are two discrete regions of high expression, one anterior and one posterior, in the distal mesenchyme of the developing right cranial lobe. Lungs incubated overnight with the color substrate (Fig. 1D) show additional areas of lower *Fgf10* expression along the lateral edges of the distal mesenchyme of all lobes (arrowheads), associated with all the epithelial buds. Lateral lobe expression is more readily apparent at E12.5 when high levels of *Fgf10* expression are detected in the mesenchyme between buds (arrowheads in Fig. 1E,F). Other sites of high expression at E12.5, such as the tip of the accessory lobe (arrow in Fig. 1E), reflect the maintenance of high levels of expression from earlier stages (arrowhead 1 in Fig. 1C).

Because of the highly stereotyped pattern of budding and branching in each lobe, individual buds can be followed through development by observing lungs from a series of embryos at different stages. Thus, to study the relationship between *Fgf10* expression and branching morphogenesis in more detail, lungs collected every 4 hours, between E11.5 and E12.5, were subjected to whole-mount in situ hybridization. The expression patterns observed during this time in the caudal part of the right caudal lobe (Fig. 2), are typical of those in the rest of the lung, in relation to both the formation of buds laterally from the main bronchus and the dichotomous branching of existing buds. At E11.5 (Fig. 2A), there is only one well-developed lateral bud in this lobe (bud 1). A slight lateral swelling of the endoderm of the main bronchus prefigures the outgrowth of a second bud (bud 2) more caudally. *Fgf10* expression is detected on the lateral edge of the mesenchyme overlying developing bud 2, and between bud 1 and bud 2. About 4 hours later, as bud 2 grows out, the level of *Fgf10* expression in the vicinity of this bud increases (Fig. 2B). Later still, the *Fgf10* expression domain on the lateral edge of the lobe spreads more caudally (Fig. 2C). During these stages, *Fgf10* expression is predominantly on the caudal side of bud 1 (Fig. 2A-C). As bud 1 starts to branch (Fig. 2D), the larger of the two branches grows caudally towards the domain of *Fgf10* expression. Bud 3 has yet to form, but, as with bud 2, *Fgf10* expression is already present on the lateral edges of the lobe overlying the region where bud 3 will grow out (Fig. 2D). By E12-E12.5, bud 3 has grown out and branching has begun at the tip of the main bronchus, forming
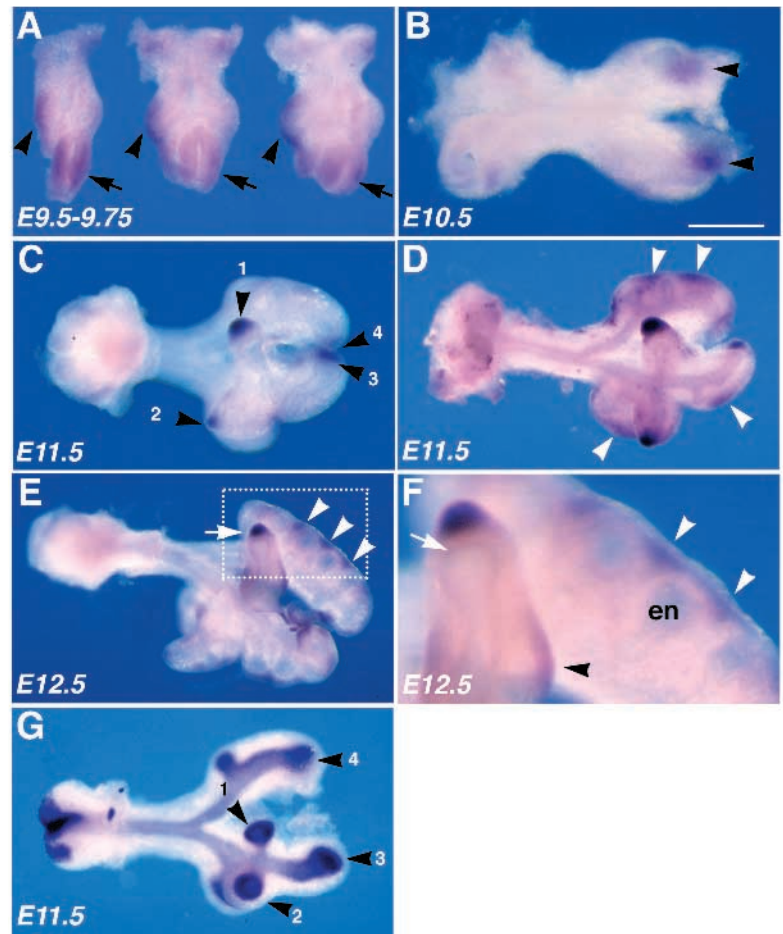
Fig. 1. *Fgf10* expression in mouse embryonic lung detected by whole-mount in situ hybridization at E9.5-9.75, E10.5, E11.5 and E12.5. (A) *Fgf10* expression at E9.5-9.75. Note higher expression around the right lung bud (arrowhead) compared with the left, and around the developing stomach (arrow). (B) *Fgf10* expression at E10.5. Note expression in distal mesenchyme of both buds (arrowheads). (C) High levels of *Fgf10* expression at E11.5 in the distal tip mesenchyme of the prospective lobes (arrowheads). (D) Overnight incubation reveals areas of lower *Fgf10* expression at E11.5. Note expression in lateral lobe mesoderm, above and in between the lateral buds (arrowheads). (E) *Fgf10* expression at E12.5. Note continued high expression at the tips of the lobes (arrow indicates accessory lobe). Note also expression the lateral interbud mesenchyme (arrowheads). (F) High magnification of E. Note absence *Fgf10* expression from zones of mesenchyme immediately adjacent to the endoderm of the lateral buds (en) and the accessory lobe (arrow). As in E, arrowheads mark *Fgf10* expression in the lateral mesoderm between the end buds. (G) *Shh* expression at E11.5. Lobes indicated by: 1, accessory; 2, right medial; 3, right caudal; 4, left. Scale bar, A,E 330 μm; B, 190 μm; C, D,G, 250 μm; F, 110 μm.

bud 4 (Fig. 2E,F). By this time, *Fgf10* expression is detected all along the edge of the lobe, with the highest levels found between buds.

Two features of *Fgf10* expression, that are common to many buds, provide insight into the regulation of *Fgf10* expression. First, as buds grow out into the lateral mesenchyme, the level of *Fgf10* expression in the vicinity of the bud increases. This is accompanied by an expansion of the *Fgf10* expression domain to overlie the region where the next bud will form.

This suggests that there is some positive regulation of *Fgf10* expression by signals from the ingrowing distal endoderm. Second, there is frequently, but not always, a zone of mesenchymal cells immediately adjacent to the distal bud that does not express *Fgf10*. The presence of this zone varies with the location and behavior of the bud. In the case of lateral buds, the zone is always present. Thus, when lateral buds first form, the *Fgf10* expression overlying the site of bud formation is separated from the endoderm by non-expressing mes-

Fig. 2. Dynamic pattern of expression of *Fgf10* between E11.5 and E12.5. Whole-mount in situ hybridization showing *Fgf10* expression at representative stages between E11.5 and E12.5 with A being the youngest and F the oldest. (A) At E11.5, *Fgf10* is expressed between bud 1 and 2 (arrowhead), and at high levels at the distal tip of the lobe. (B) *Fgf10* levels increase in the lateral mesoderm and expand caudally as bud 2 grows. (C,D) *Fgf10* expression is restricted to the mesenchyme in between bud 1 and 2, and between caudal of bud 2, overlying the region where buds have yet to form. Note that in D the main branch of bud 1 is growing toward the high *Fgf10* expression domain. (E,F) Branching of lateral buds is observed and the bronchus undergoes bifurcation at the distal tip. Note

that *Fgf10* expression is downregulated in the mesenchyme immediately adjacent to the distal endoderm (compare bud 2 in C-E). Scale bar, A-F, 90 μm.
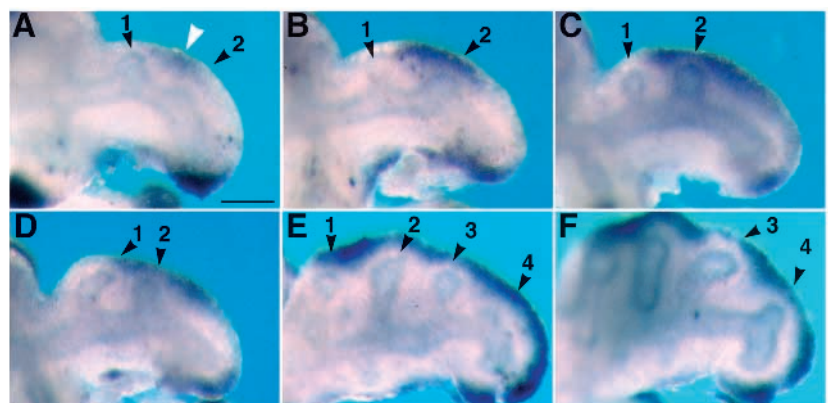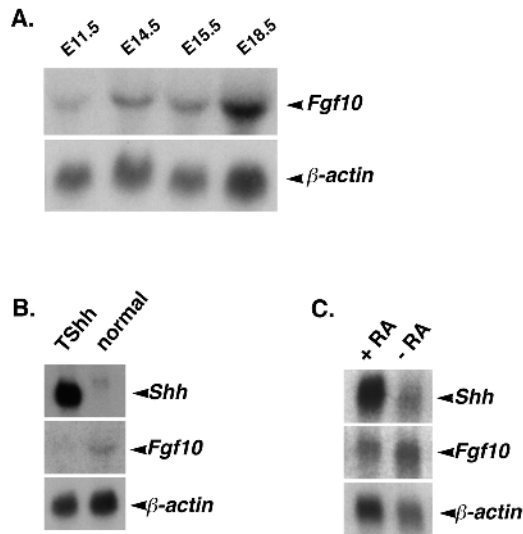
Fig. 3. *Fgf10* expression levels in normal, SP-C-*Shh* transgenic and RA treated lungs. (A) Levels of *Fgf10* RNA in the normal lung (4 kb band), revealed by northern hybridization using total lung RNA at E11.5, E14.5, E15.5 and E18.5. *Fgf10* signal levels (normalized according to a *β-actin* loading control) increase by a 70% between E11.5 and 14.5 and by 60% between E15.5 and 18.5. (B) Expression of *Fgf10* and *Shh* in normal and *SP-C-Shh* transgenic lungs at E19. Note the increase in *Shh* expression in the transgenic (40 times the control) and the decrease in *Fgf10* levels (0.5 times the control). (C) *Shh* and *Fgf10* expression in control and retinoic acid-treated lungs. Note the increase in *Shh* expression (60%) and the corresponding decrease in *Fgf10* levels (60%) in the retinoic acid-treated lungs. Exposure times: (A)*Fgf10*, 2 days;*β-actin,* 16 hours; (B) *Shh*, 16 hours; *Fgf10*, 24 hours; *β-actin*, 6 hours; (C) *Shh* and *Fgf10*, 3 days; *β-actin*, 1 day.

Fig. 4. Biological assays used to investigate FGF activity in lung tissue. Two assays were performed (left and right). In the first (left) lungs were dissected from E11.5 mouse embryos, the distal tips (approximately 0.3 mm) of the left and caudal lobes were removed. Distal buds and 'amputated' lungs were cultured in collagen or in Matrigel. In the second assay (right), lungs from E11-E11.25 embryos were incubated in trypsin/pancreatin. Endoderm, separated from mesenchyme using tungsten needles, was embedded in Matrigel. See Materials and Methods for a fuller description of the assays.

enchyme (see bud 2 in Fig 2A); as the buds grow out laterally into the *Fgf10*-expressing domain, the mesenchyme within a few cell diameters of the endoderm still appears not to express *Fgf10* (see buds 2 and 3 in Fig. 2E,F and lateral buds in Fig. 9A). The formation of a non-expressing zone is quite different in the case of the elongating terminal buds that generate the main bronchi of each lobe. For these buds, high levels of *Fgf10* expression are found immediately adjacent to the endoderm throughout the elongation process (see Figs 1C,E, 2A, and the bud at the distal tip of the right lobe in Fig. 9A). A zone of cells lacking *Fgf10* expression only develops later as the terminal bud prepares to undergo dichotomous branching (see Fig. 2E,F).

Downregulation of *Fgf10* in lungs overexpressing *Sonic hedgehog* in the endoderm

The signaling molecule, SHH, positively regulates *FGF4*, *FGF8* and *FGF10* expression in the developing chick limb (Laufer et al., 1994; Ohuchi et al., 1997). *Shh* is expressed at high levels in the distal endoderm of the mouse lung (Fig. 1G; and Bellusci et al., 1997), where it appears to act as a positive regulator of cell proliferation and of *Patched* expression in the mesenchyme (Bellusci et al., 1997). To test whether *Fgf10* is regulated by SHH, we examined *Fgf10* expression by northern blot analysis of RNA from normal lungs and from transgenic lungs overexpressing *Shh* in the epithelium (Bellusci et al., 1997). In normal lungs, *Fgf10* RNA levels increase through
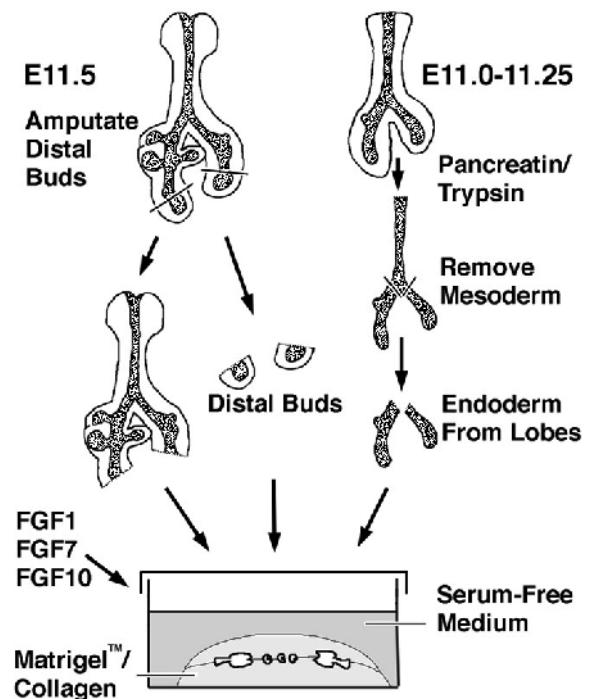
embryonic development and are higher at the end of the pseudoglandular stage (E15.5) than at E11.5, and higher still just before birth (Fig. 3A). This contrasts with *Shh* RNA levels, which decrease steadily from the end of the pseudo-glandular stage and then decrease again before birth (Bellusci et al., 1997). In E19.0 SP-C-*Shh* transgenic lungs (n=2), the levels of *Fgf10* RNA are lower than in non-transgenic lungs. In the example shown in Fig. 3B, where *Shh* levels are forty times normal, *Fgf10* transcript levels are reduced by 45%. We conclude that the production of high levels of SHH by the endoderm leads to a reduction of *Fgf10* expression in the mesenchyme.

In the chick limb, *Sonic hedgehog* expression is ectopically activated by the localized addition of retinoic acid (RA) and SHH, in turn, regulates FGFs (Riddle et al., 1993; Laufer et al., 1994). We thus studied the relationship between retinoids and the expression of *Shh* and *Fgf10* in the lung where retinoic acid (RA) treatment leads to an upregulation of *Shh* expression (Cardoso et al., 1996; Rush and Perkins, 1997). In lungs cultured for 4 days in vitro in the presence of 10^{-6} M all-*trans*-RA, there is a 60% increase in *Shh* RNA levels and a 60% decrease *Fgf10* expression, compared with controls (Fig. 3C). We conclude that the increase in *Shh* expression following RA treatment is accompanied by a decrease in *Fgf10* expression.

FGF10 induces expansion and budding of lung endoderm

To study the biological activity of recombinant human FGF10, and compare it with FGF1 and FGF7, we designed two different assays, shown schematically in Fig. 4. The first assay was designed to test the response of endoderm and mesenchyme together. The second assay, which is essentially the same as one previously described (Nogawa and Itoh, 1995; Cardoso et al., 1997), was used to test whether FGF10 affects isolated endoderm in the absence of mesoderm.

For the first assay, E11.5 lungs were isolated and distal buds (endoderm plus mesenchyme) were removed from the left, right caudal and accessory lobes. The distal buds, and the lungs from which they were removed (which we term 'amputated' lungs), were cultured in collagen or Matrigel, in the absence of serum and with, or without, added recombinant FGFs. There are a number of differences between the two test samples. The distal bud mesenchyme already expresses high levels of *Fgf10*, and so the endoderm in the bud might be expected to respond to endogenous FGF10. Moreover, distal endoderm already expresses high levels of other genes, such as *Shh* and *Bmp4*, which may be important for bud outgrowth and/or branching (Bellusci et al., 1996, 1997). In the amputated lungs, however, local sources of high levels of endogenous FGF10 have been removed, and the remaining proximal endoderm expresses much lower levels of signaling molecules than distal endoderm.

The results of the first assay with 'amputated' lungs cultured in Matrigel are shown in Fig. 5. For orientation, Fig. 5A-D show low-power views of amputated lungs after 48 hours. Fig. 5E-L are higher-power views of the truncated ends of the lobes (at the amputation stump). When FGF10 (5-500 ng/ml) was added, doses of 15 ng/ml or less had no detectable effect. Doses of 30 ng/ml or greater produced, within 24 hours, a swelling of the endoderm throughout the lung and a prominent cyst-like swelling at the truncated end of each lobe (Fig. 5E). If the culture period was extended buds appeared on the surface of the terminal cyst-like swellings (Fig. 5D,F,G). In some cases, considerable budding from the truncated ends of the lobes had already occurred by 48 hours in culture (Fig. 5D) but, more typically, extensive budding at these sites was only observed after 60 hours of culture (Fig. 5H). In control lungs (with no added FGF), some branching of the endoderm occurred within the lung during culture (Fig. 5A), but there

was no swelling of the endoderm at the site of truncation and buds do not grow out beyond the mesenchyme boundary (Fig. 5I).

In contrast to FGF10, recombinant FGF1 (250 or 500 ng/ml) elicited budding of endoderm from the site of truncation after 24 hours (Fig. 5B), with extensive budding after 48 hours (Fig. 5J). With more prolonged culture there was some secondary branching of buds (not shown). 60 ng/ml of FGF1 produced no discernible response. Recombinant FGF7 (at all doses from 5-500 ng/ml) produced swelling of the endoderm and cyst-like structures at the sites of truncation (Fig. 5C,K), essentially identical to those first observed with FGF10. As with FGF10, these cyst-like structures also progressed to extensive budding (Fig. 5L). The time of transition to budding depended on the concentration used; at low concentrations (e.g. 15 and 30 ng/ml) more extensive budding was seen earlier than with high concentrations (60, 120 and 250 ng/ml).
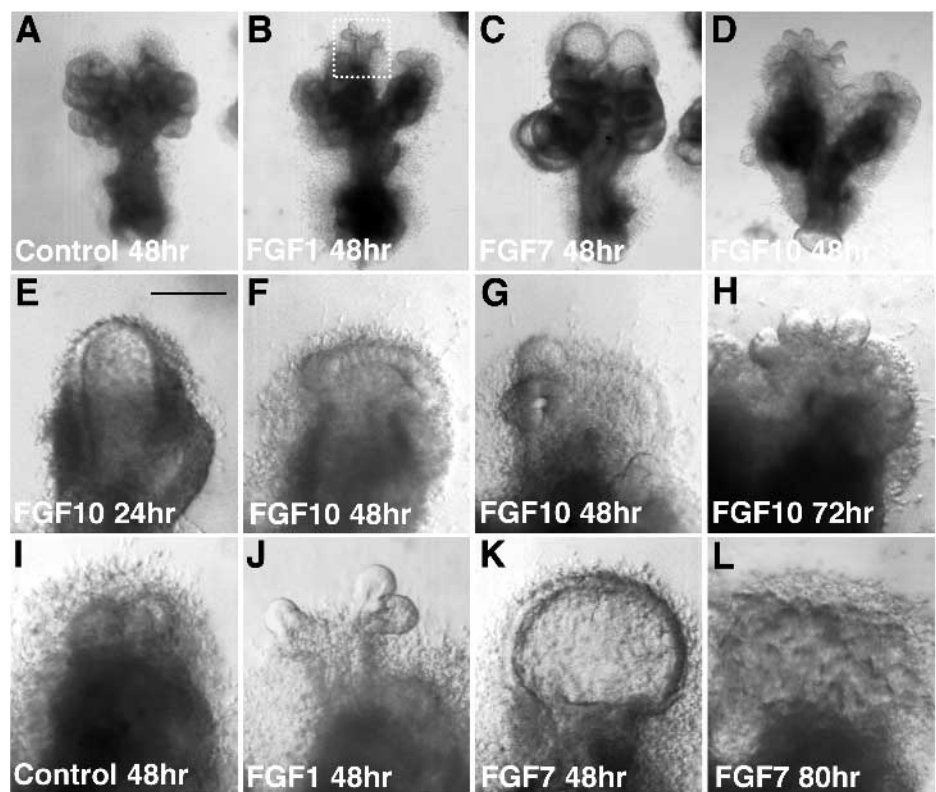

Fig. 5. Effect of recombinant human FGFs on 'amputated' E11.5 lungs in Matrigel. Low power views (A-D), and high magnification views (E-L), of the truncated distal region. (A) Control lung after 48 hours of culture showing limited branching within the lung but no outgrowth from the truncated lobes. (B) Lung treated with FGF1 (500 ng/ml) clearly showing budding from the truncated end of the lobes. The area enclosed by a white dotted box is magnified in J. (C) Lung cultured with 250 ng/ml of FGF7 showing remarkable expansion of the distal endoderm throughout the lung, but particularly from the distal truncated region. (D) Lung cultured with 250 ng/ml of FGF10 showing striking budding of the distal endoderm. (E-H) Truncated end of a lobe exposed to FGF10 showing, after 24 hours, an expansion of the endoderm followed, after 48-72 hours, by a modification of the surface of the endoderm, leading to the formation of buds. (I) Truncated end of a control lobe There is limited branching within the distal mesenchyme, which has invaded the Matrigel. (J) Distal endoderm of lung treated with FGF1, showing primary and secondary budding. (K-L) Truncated end of lobes treated with FGF7 showing a expansion of the distal endoderm (K) followed, after 80 hours, by the appearance of several small buds (L). Scale bar A-D, 330 μm; E-L, 90 μm.

At the start of the cultures, the two test tissue samples express different combinations, and different levels, of regulatory molecules. Despite this, when the first assay was performed with isolated distal buds, they responded in essentially the same way as amputated stumps and the lowest doses of FGFs required to produce a clear response (30 ng/ml of FGF10; 250 ng/ml of FGF1; 5 ng/ml of FGF7) were identical. This is illustrated in Fig. 6, which shows the results of the distal bud cultures, performed in Matrigel, after 24, 48 and 72 hours. The control samples grow and bud internally, but the endoderm neither extends beyond the mesenchyme nor swells up. (Fig. 6A-C). Addition of FGF10 (30-500 ng/ml) initially produced swelling of the endoderm, giving an enlarged lumen (compare Fig. 6A and J). After 48 hours, the previously spherical endoderm had a more irregular outline, perhaps in anticipation of bud formation (Fig. 6K). After 72 hours in culture, very extensive budding of the endoderm had occurred (Fig. 6L).

In contrast to the effect of FGF10 treatment, addition of FGF1 (250-500 ng/ml) elicited the emergence of buds of endoderm within 24 hours (Fig. 6D). Initially one or two buds appeared, but then progressively more buds formed (Fig. 6E) and very extensive budding was present after 72 hours (Fig. 6F). The activity of FGF7 (5-500 ng) in this assay was almost identical to that seen with 'amputated' lungs, namely expansion followed by budding. In the example shown in Fig. 6I (250 ng/ml FGF7 for 72 hours), the swollen endoderm is still in the process of forming buds and its surface has an irregular outline.

Collagen matrix fails to support endoderm budding

We also cultured test tissues in collagen gels (data not shown). However, in contrast to the results using Matrigel, this matrix did not support endoderm budding. Both FGF10 and FGF7 produced swelling of the endoderm that was equivalent to that observed in Matrigel, at the same doses and over the same time frame. However, the cyst-like swellings never progressed to bud formation. FGF1 produced no response at all in test tissues cultured in collagen, even at doses of 500 ng/ml. One significant difference between collagen and Matrigel is that the latter contains heparan sulphate proteoglycan (HSPG), that may potentiate the biological activity of FGFs (e.g. Nurcombe et al., 1993). We therefore tested the possibility that addition of HSPG to collagen would be permissive for bud outgrowth in response to FGF. Distal endbuds and amputated lungs were cultured in collagen in the presence of 0.01, 0.1, 0.5 and 2.0 µg/ml HSPG and either 60 ng/ml FGF1 or 250 ng/ml FGF7. Under these conditions, no effect was seen with FGF1. With FGF7, 0.1 and 0.5 µg/ml HSPG slightly stimulated the expansion of endoderm, while 2 µg/ml inhibited the response. However, at no concentration of HSPG did the endoderm grow out and bud into collagen.

Endodermal expansion in response to FGF10 involves increased cell proliferation

To investigate the role of cell proliferation in the

response to FGF10, BrdU incorporation in cultures of amputated lungs was assayed by immunohistochemistry of tissue sections (Fig. 7). In the control, small end buds surrounded by multiple layers of mesenchymal cells are observed (compare Fig. 7A,E with Fig. 5I). By contrast, the FGF10-treated lung has expanded end buds, overlaid by a thin layer of mesenchymal cells (compare Fig. 7B,F with Fig. 5E). Interestingly, the most proximal endoderm of the trachea is also expanded (arrowhead in Fig. 7B). A similar effect was observed with FGF7 (compare Fig. 7C,G with Fig. 5C,K). In the presence of FGF1, a single endodermal bud without any surrounding mesenchyme emerges from the truncated end of the lung (compare Fig. 7D with Fig. 5J). In the control, a basal level of proliferation is observed in the pseudostratified distal
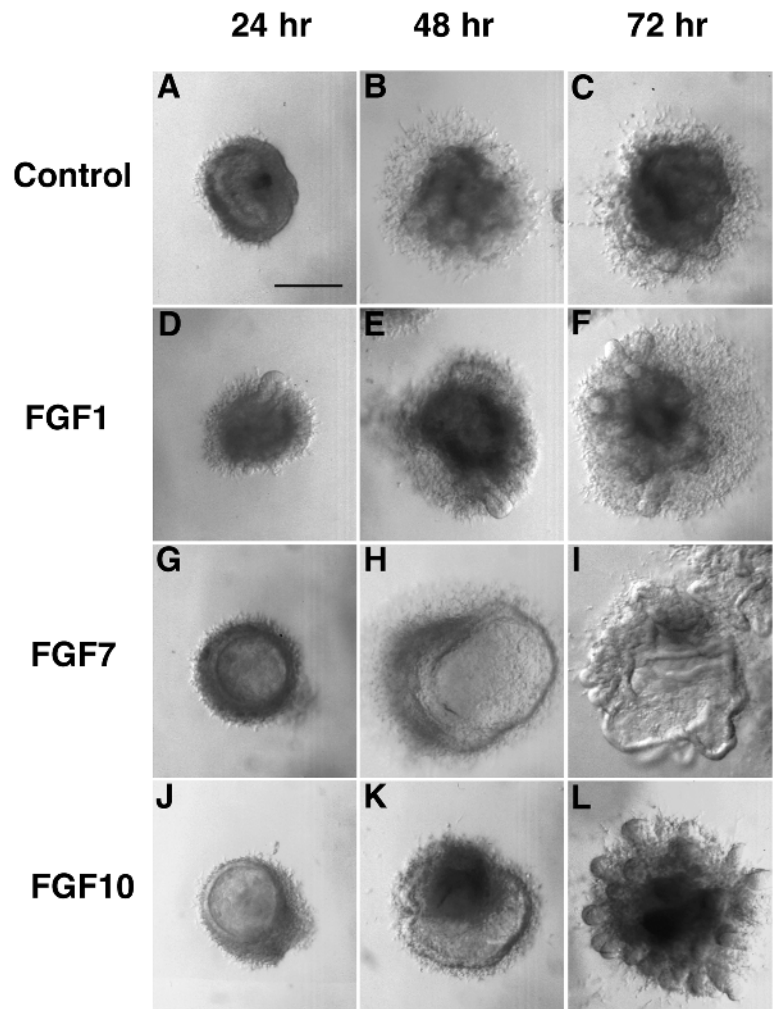

Fig. 6. Effect of recombinant human FGFs on E11.5 distal buds in Matrigel. (A-C) Isolated end bud cultured in Matrigel in medium without FGF showing after 48 and 72 hours a limited branching of the endoderm surrounded by mesoderm. Some mesenchymal cells invade the Matrigel. (D-F) After 24 hours, 500 ng/ml of FGF1 induces budding and outgrowth of the endoderm. By 48-72, multiple buds with branches have appeared. (G-I) 250 ng/ml of FGF7 induces expansion of the endoderm. A clear difference with the control is already observed at 24 hours (G). After 72 hours, the surface of the endoderm is irregular, and bud-like structures are visible. (J-L) FGF10 (250 ng/ml) induces expansion of the endoderm (J). This is followed, after 48-72 hours, by extensive budding (K,L). Scale bar, 90 µm.

Fig. 7. Cell proliferation in control and FGF-treated 'amputated' lungs. BrdU incorporation (3 hour labeling) following lung explant culture (for 24 hours in Matrigel) without added FGFs (A,E) or in the presence of FGF10 (250 ng/ml) (B,F), FGF7 (250 ng/ml) (C,G) or FGF1 (500 ng/ml) (D,H). (A-D) Low-power photomicrographs and (E-H) high-power magnifications of the boxed regions. (A) Control lung showing distal epithelium surrounded by several layers of mesenchymal cells. (B) FGF10-treated lung showing an expansion of the distal endoderm and also of the proximal endoderm of the trachea (arrowhead). (C) FGF7-treated lung exhibits a clear expansion of the distal endoderm. (D) FGF1-treated lung showing a single budding of endoderm from the truncated end of the lung. (E) Labeled cells are present in both the endoderm and mesenchyme. (F) Highly labeled endoderm with a thin mesenchymal layer (arrowhead). (G) Expanded endoderm with numerous labeled cells. Note the presence of a thin mesenchymal layer overlying the bud (arrowhead). (H) Epithelial layer without surrounding mesenchyme shows a high level of BrdU staining. Scale bar, A-D, 200 μm; E-H, 40 μm.
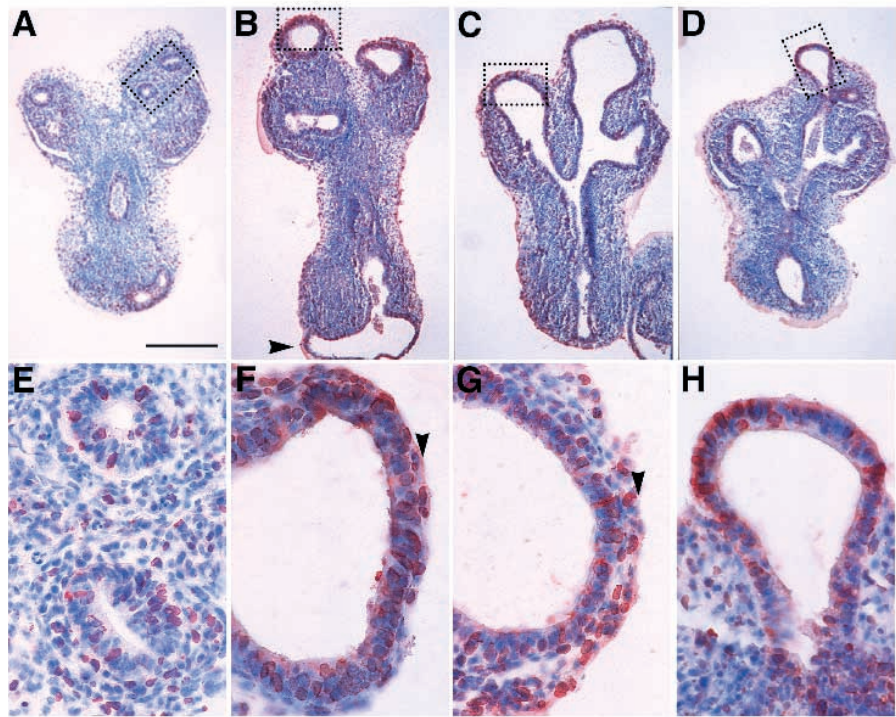
endoderm (proportion of labeled cells 29.5±5.7%) (Fig. 7E). This is significantly (*P*<0.05) lower than in lungs treated with FGF10 (56.6±4.3%) (Fig. 7F). Although not quantified, a higher proportion of labeled epithelial cells was also seen in the lungs treated with FGF7 or FGF1 (Fig. 7G,H). We conclude that cell proliferation plays an important role in the expansion of the distal endoderm observed after treatment with FGF10.

The response of mesenchyme-free endoderm to FGF10

In the second of our culture assays, we tested whether FGF10 can act directly on the endoderm in the absence of mesenchyme. Mesenchyme-free endoderm was isolated from E11.5 lungs and cultured in Matrigel without serum (Fig. 4). In the absence of exogenous FGF, the endoderm failed to proliferate and died (Cardoso et al., 1997) (Fig. 8A). With 250 or 500 ng/ml of recombinant FGF10 the survival of the endoderm was greatly enhanced and over the first 24 hours of culture the tissue expanded to form a cyst-like structure. In most cases, this phase was followed by the appearance of long bud-like extensions. Fig. 8D shows an example that produced primary buds after 24 hours and secondary buds (branches of the primary buds) after 48 hours.

Previous studies using Matrigel cultures reported that FGF1 elicits elongated bud formation from mesenchyme-free endoderm, whereas FGF7 treatment leads to the formation of cyst-like structures (Nogawa and Itoh, 1995; Cardoso et al., 1997). We successfully reproduced these results with FGF1 (500 ng/ml) (Fig. 8B) and FGF7 (15, 30, 60, 250 and 500 ng/ml) (Fig. 8C). FGF7 produced expansion of the endoderm and the formation of hollow spheroidal structures with an irregular surface. However, in contrast to the results with distal end buds and 'amputated' lungs, these never progressed to definitive bud formation, even after 5 days and at all concen-

trations tested. Isolated endoderm in Matrigel culture therefore shows a distinctly different response to FGF7 compared with FGF10 that does not seem to depend upon the concentration of FGF7 added.

FGFs have different spatiotemporal expression patterns of expression

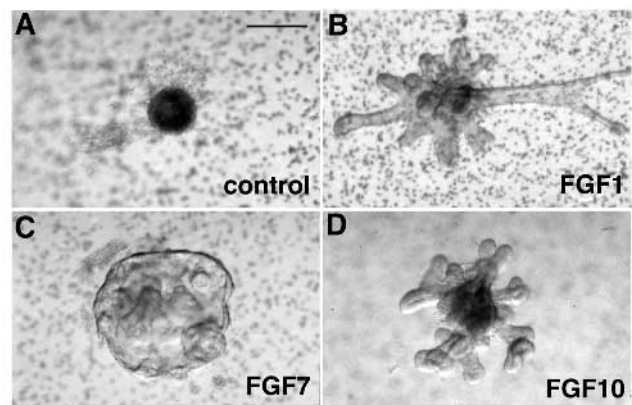In the assays described above there is a considerable overlap

Fig. 8. Activity of FGF1, FGF7 and FGF10 in cultures of mesenchyme-free endoderm (A) Endoderm grown for 48 hours in control medium, without FGFs. Most cells have died, but a few remain as a dense aggregate. (B) Culture with 500 ng/ml of FGF1. Growth and budding of the endoderm has occurred, with some buds being very elongated. (C) Culture with 250 ng/ml of FGF7. Endoderm has grown and expanded to form a cyst-like structure with an irregular surface, without obvious bud formation. (D) Culture with 250 ng/ml of FGF10. Endoderm has grown and formed primary and secondary buds. Scale bar, A-D, 250 μm.
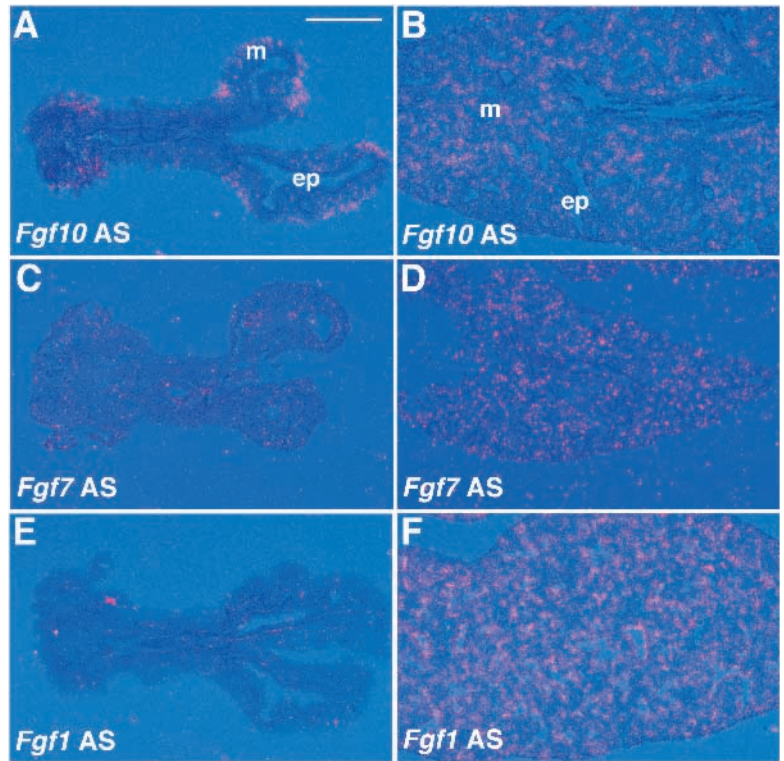
Fig. 9. Distinct expression of *Fgf7* and *Fgf10*. Section in situ hybridization using ^{35}S-labeled antisense riboprobes for *Fgf10* (A,B), *Fgf7* (C,D) and *Fgf1* (E,F) at E11.5 (A,C,E), E13.5 (B,F) and E14.5 (D). Photomicrographs at each stage are taken with the same exposure time. At E11.5, note that high levels of *Fgf10* transcript (A) are present in the mesenchyme immediately adjacent to the endoderm of the terminal bud of the primary bronchus but are absent from the mesenchyme above lateral buds. Note also the absence of *Fgf7* (C) and *Fgf1* (E) expression. At E13. 5 and 14.5, *Fgf10* (B) and *Fgf7* (D) are both expressed in lung mesenchyme. At E13.5, *Fgf1* is also expressed in the epithelium (F). m, mesenchyme; ep, distal epithelium. Scale bar: A-F, 250 μm.

between the responses of samples to FGF1, FGF7 and FGF10. To address the relevance of these results to lung development in vivo, we compared the expression of *Fgf7*, *Fgf1* and *Fgf10* at two stages; E11.5 and E13.5-14.5 (Fig. 9). In radioactive section in situ hybridization assays, *Fgf10* transcripts were present at high levels in localized regions of distal mesenchyme at E11.5 (Fig.

Fig. 10. Schematic model of the expression and function of *Fgf10* in branching morphogenesis. (A) Prior to visible bud formation, *Fgf10* expression is present in mesoderm overlying the future bud site. Additional, unknown, factor(s) involved in the decision of the endoderm to form a bud are indicated by X. (B) FGF10 activity causes expansion of the endoderm at sites of bud outgrowth. Localized *Fgf10* expression (in this case lateral, rather than medial) determines the initial direction of bud outgrowth (white arrows). (C) Buds continue to grow towards regions of high *Fgf10* expression. (D) Long-range positive regulation (blue) and short-range negative regulation (yellow) of *Fgf10* expression by signals from the bud endoderm. Short-range negative regulation (yellow) may be mediated by SHH. (E) With *Fgf10* expression responding to bud outgrowth, the expression domain shifts to overlie the site where the next bud will form. A new cycle of bud induction and outgrowth continues from A. (F) Buds ultimately branch. Like the original bud, the branches grow towards regions with high levels of *Fgf10* expression.
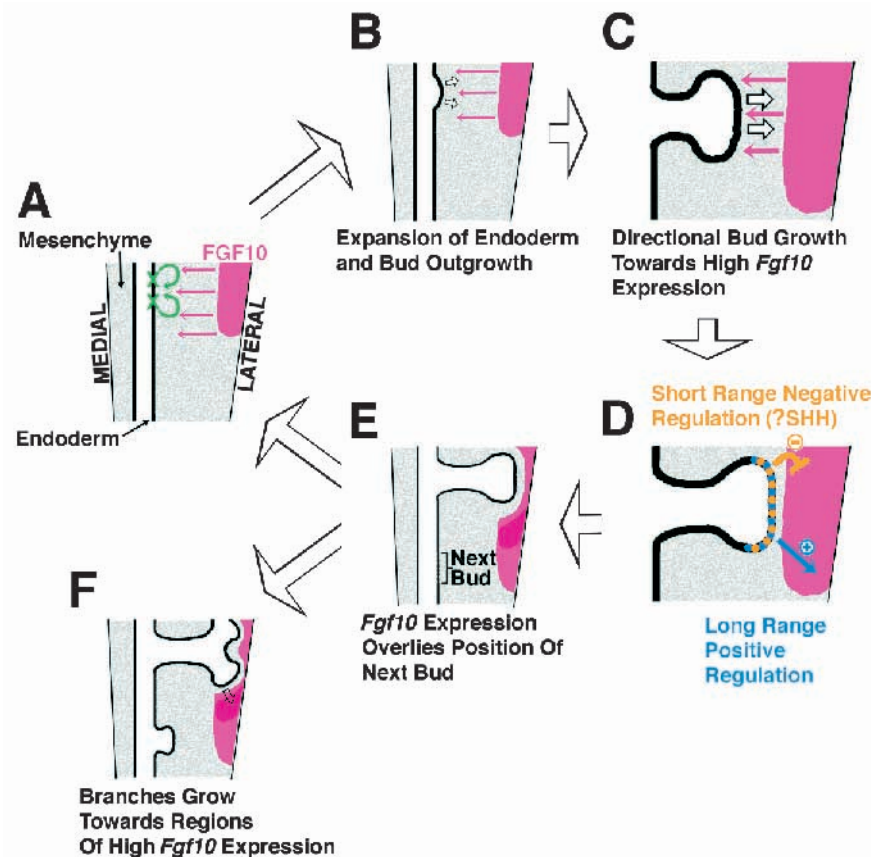
9A), consistent with the results of whole-mount in situ hybridization. Abundant *Fgf10* mRNA was also present in restricted domains of the mesenchyme at E13.5 (Fig. 9B). However, under the same conditions, no expression of either *Fgf1* or *Fgf7* was detected in lungs at E11.5 (Fig. 9C,E) although transcripts of both genes were detected at E13.5 or E14.5 (Fig. 9D). In the case of *Fgf7*, transcripts were in the mesenchyme but *Fgf1* RNA could be detected in both the endoderm and mesenchyme. These results, and those of Mason et al. (1994), suggest that neither *Fgf1* nor *Fgf7* are transcribed at significant levels at the very early stages of lung development, compared with *Fgf10*.

DISCUSSION

Fgf10 expression during early lung development

In this paper we provide evidence for an involvement of the *Fgf10* gene in branching morphogenesis. By contrast to both *Fgf1* and *Fgf7*, we show that *Fgf10* fulfills two of the criteria expected of a gene involved in establishing a branching pattern: it is expressed at high levels from the earliest stages of lung development, and in a highly localized pattern, consistent with FGF10 effecting local changes in the morphogenetic behavior of lung endoderm.

Fgf10 expression in splanchnic and distal lung mesenchyme at E9.5-9.75 and E10.5 raises the possibility that FGF10 is involved in the initial production of paired buds of endoderm from the ventral foregut and their outgrowth to form the precursors of the main bronchi. Subsequently, expression of the *Fgf10* gene is closely associated with key events in branching morphogenesis. For example, in the lateral mesenchyme of a typical lobe, *Fgf10* expression extends over future sites of endodermal bud formation prior to buds being morphologically detectable (Fig. 2), allowing FGF10 to play a role from the earliest stages of bud outgrowth. Thus, *Fgf10* could be an inducer of new bud formation. However, perhaps because of some lateral inhibition or 'counting' mechanism, buds normally form only at specific distances from each other, giving a reproducible pattern of internode distances. *Fgf10* transcripts in the lateral mesenchyme overlie not only the position of the next bud, but also all the endoderm between this site and the previous bud. This does not rule out that *Fgf10* contributes to the induction process, but implies that some other factor(s) must also be involved in determining the precise locations at which buds will form.

Once endodermal buds have formed, the growth of buds towards regions of mesoderm expressing *Fgf10* mRNA suggests that FGF10 governs the directional outgrowth of buds. Such a relationship is very reminiscent of developmental processes involved in generating the *Drosophila* respiratory system. This has been shown to involve the directed migration of tracheal cells towards a local source of an FGF-like ligand (the *branchless* gene product), in response to signal transduction through an FGF receptor (encoded by the *breathless* gene) (Glazer and Shilo, 1991; Lee et al., 1996; Sutherland et al., 1996). Thus, in both *Drosophila* and mouse the production of a branched respiratory system involves the directional movement of respiratory cell precursors towards a localized source of an FGF ligand, either by migration and elongation, or by outgrowth of epithelial buds.

For all buds, the *Fgf10* expression pattern is consistent with FGF10 governing the directed growth of endoderm. However, when considering branching morphogenesis in the lung, it is important to recognize that not all buds undergo the same program of development. For example, the first buds to appear are those that form the main bronchi of each lobe. These buds undergo an extended period of elongation before entering a more typical program of repetitive bud outgrowth and branching. Interestingly, during the elongation phase of the primary buds *Fgf10* expression is present in mesenchyme immediately adjacent to the endoderm, but later the expression around these buds resembles that around lateral buds, where mesenchyme adjacent to the endoderm does not express *Fgf10*. Thus, the regulation of mesenchymal *Fgf10* expression appears to be subtly different for buds undergoing different programs of development.

A key question that we have tried to answer (data not shown) is whether FGF10 is sufficient to induce ectopic buds from tracheal endoderm denuded of proximal mesenchyme, thus mimicking the effect of distal mesenchyme originally reported by Alescio and Cassini (1962). We have removed proximal mesenchyme locally from the trachea of E11.5 day lungs and then either placed COS cell expressing *Fgf10* adjacent to the denuded endoderm in collagen cultures, or incubated the modified lungs in Matrigel culture with FGF10. In neither case did ectopic buds grow out of the mesenchyme-denuded region of the trachea, although endodermal swelling was seen after 24 hours. After this time, proximal mesenchyme grew back over the denuded endoderm. We conclude that either proximal endoderm does not respond to FGF10 by budding, or that this factor is not sufficient to overcome the inhibitory influence of proximal mesenchyme on tracheal outgrowth and budding. Further experiments are in progress to address this problem.

Positive and negative regulation of *Fgf10* expression: possible involvement of SHH.

In the mouse lung, changes in *Fgf10* expression as buds grow out lead us to suggest that some signal(s) from the endoderm positively and negatively regulate *Fgf10* transcription in the mesoderm. Although we have not yet identified these signals, one possibility is that high levels of SHH downregulate *Fgf10*. As outlined below, several lines of evidence support this idea.

First, as the embryonic lung matures, the levels of *Shh* RNA decrease (Bellusci et al., 1997) but those of *Fgf10* steadily increase (Fig. 3). Second, distal endoderm expresses high levels of Shh, and all distal buds ultimately become surrounded by mesenchyme that does not express *Fgf10*. Additional evidence for a negative relation between *Shh* and *Fgf10* comes from the observation that *Fgf10* transcript levels are downregulated in SP-C-*Shh* transgenic lungs, in which the level of *Shh* is increased. Treatment of cultured lungs with 10^{-6} M retinoic acid (RA) also decreases the level of *Fgf10* expression. While this concentration of RA undoubtedly affects the expression of multiple genes in the lung (Bogue et al., 1994; Cardoso et al., 1996), it does increase the levels of *Shh* expression and has been reported to decrease the amount of distal branching (Cardoso et al., 1996; Rush and Perkins, 1997; Margaret Rush, personal communication). Thus, if we suppose that RA treatment downregulates *Fgf10* via an increase in *Shh* expression, our data from normal, SP-C-*Shh* transgenic and 10^{-6} M RA-treated lungs all support the idea that high levels of SHH negatively regulate *Fgf10*. In contrast to this finding, SHH appears to act as positive regulator of *FGF10* transcription in the chick limb (Ohuchi et al., 1997). This may reflect tissue-specific regulation of *Fgf10*, but further experimentation is needed to address this possibility.

Biological activity of FGF10 on embryonic lung tissue

When endoderm and mesoderm are cultured together in Matrigel, either as distal buds or as 'amputated' lungs, exogenous FGF10 produces cyst-like swelling of the endoderm within 24 hours, at doses as low as 30 ng/ml. This is followed, at all concentrations tested, by a budding of the endoderm after 48-60 hours. This effect is also seen in cultures of endoderm alone, indicating that FGF10 acts directly on the endoderm, without the absolute requirement for a secondary signal from the mesoderm.

The response of endoderm to FGF10 can be correlated with the known localization of FGF receptors. At E12.5, only one of the four known murine FGF receptor genes, *FgfR1*, is expressed in lung mesoderm. The others (*FgfR2*, *FgfR3* and *FgfR4*) are all expressed in the lung endoderm; *FgfR2* at high levels and *FgfR3* and *FgfR4* distally at much lower levels (Cardoso et al., 1997; Dr Michael Weinstein, personal communication). We expect that at least some of the effect of FGF10 on lung endoderm is mediated by the main FGF7 receptor, FGFR2IIIb, since expression of a dominant negative form of this receptor blocks embryonic lung branching in vivo (Peters et al., 1994).

The initial response of lung tissue to FGF10 is for the endoderm to expand and form cyst-like spheroids. There are at least three possible, non-exclusive, mechanisms that could account for this behavior; chloride-dependent fluid pumping directed towards the lumen (Souza et al, 1995; Zhou et al., 1996), changes in the organization of cells in the endoderm and differential cell proliferation. While we cannot exclude the first two mechanisms, our BrdU-labeling experiments suggest that cell proliferation is a major factor, since the expansion to a cyst-like structure is accompanied by an almost two-fold increase in endodermal cell proliferation. FGF10 can increase the rate of cell proliferation of fetal rat skin keratinocytes (an epithelial cell type), but has essentially no activity on mesenchymal NIH 3T3 fibroblast cells (Emoto et al., 1997). Thus the increased proliferation in lung endoderm is in line with the known activity of FGF10 as a growth factor, with some specificity for epithelial cells.

Biological activity of FGF10 compared with FGF1 and FGF7

In our biological assays, using both endoderm and mesenchyme combined, and endoderm alone, the responses of lung tissue to FGF10 are quite distinct from those to FGF1. Specifically, in Matrigel cultures FGF1 elicits immediate budding of the endoderm, whereas the response to FGF10 is an initial expansion of the endoderm followed later by budding.

When the activities of FGF10 are compared with those of FGF7 on distal end buds and 'amputated' lungs in Matrigel gel, the responses are quite similar. Both factors induce initial expansion of the endoderm, followed by budding. However, the transition to budding is more rapid with FGF10 than with FGF7, especially at the highest doses of FGF7 used (more than 60 ng/ml). When the factors are compared for their activity on isolated endoderm, however, distinct biological activities are observed, independent of the concentration of factor added. FGF10, at all concentrations above the minimum required to obtain a response, induces first swelling and then budding. By contrast, FGF7, at all concentrations tested, only induces expansion of the endoderm into cyst-like structures, a response reported by others in mesoderm-free cultures in Matrigel (Cardoso et al., 1997).

At present, we have no simple explanation for how distal mesoderm modifies the response of distal endoderm to FGF7 but not FGF10. Future insight into the relationships between FGF7 and FGF10 in vivo will come from biochemical studies on the receptor specificity of the two ligands, and from the study of both FGF10-deficient and FGF7;FGF10 double null mutant mice.

The role of the extracellular matrix in endodermal budding

Consistent with the findings of Nogawa and Itoh (1995) we find that culturing lung tissue in collagen matrix does not result in the outgrowth of endodermal buds, whereas Matrigel is permissive for budding. The collagen gel used mainly consists of type I collagen, whereas Matrigel is composed of several extracellular matrix components including laminin, entactin/nidogen, type IV collagen, heparan sulphate proteoglycan (HSPG) and other minor components of basement membrane matrices. Matrigel also contains trace amounts of various growth factors: TGFβ, EGF, IGF1, FGF2 and PDGF (Vukicevic et al., 1992). Budding cannot be induced by FGFs in collagen supplemented with HSPG but it is possible that some other component of Matrigel, for example laminin and/or entactin/nidogen (Kadoya et al., 1997), acts as a necessary cofactor. However, it is also possible that type I collagen also actively inhibits budding. This idea is supported by the normal localization of collagen fibers in a thick sheath around the trachea and around portions of the primary bronchi that are not actively budding (Wessels, 1970).

A model of FGF10 function and its place in the network of signaling in the embryonic lung

In branching morphogenesis buds form, grow out and ultimately branch. Buds formed at the branch points repeat this cycle. *Fgf10* expression, and the biological activity of FGF10 in lung cultures, suggests a model in which FGF10 acts at all stages of this process (illustrated schematically in Fig. 10).

We suppose that FGF10 is involved in the initial expansion of endoderm seen in the earliest formation of a bud, since the first response of endoderm cells in vitro to FGF10 is to form a cyst-like structure. Probably, some other factor(s) (depicted by X in Fig. 10A), are also necessary to define the region of endoderm that will form a bud. X is depicted as promoting bud formation, but this could be achieved by antagonizing other factors, such components of the extracellular matrix, that are inhibitory for budding. FGF10 produced in distal mesoderm then appears to be involved in the directional growth of buds (Fig. 10B,C). A complex, highly dynamic pattern of *Fgf10* expression is required for these roles, and this pattern is likely to involve positive and negative signals from the endoderm. We predict that a positive, as yet unidentified, regulator of *Fgf10* expression is produced by the endoderm and acts at a distance to increase the level and extent of *Fgf10* gene expression as the buds grow out. Mesenchymal cells in the immediate vicinity of lateral buds do not express *Fgf10*. In principle, expressing cells might be displaced by non-expressing cells accompanying the growing bud. However, if one considers the terminal buds forming the bronchi, where *Fgf10* is first expressed in the mesoderm immediately adjacent to the endoderm and then declines, the pattern is more consistent with downregulation by signals from the endoderm. We therefore suppose that distal endoderm produces a negative regulator of *Fgf10* expression and tentatively ascribe this activity to high levels of SHH.

Decreasing the proliferation of the distal tip could also be an essential part of the branching process and may involve BMP4 (Bellusci et al., 1996). With *Fgf10* expression in the mesenchyme responding to bud outgrowth, the expression domain shifts to overly the site where the next bud will form (Fig. 10E). A new cycle of bud induction and outgrowth is initiated (see arrows in Fig. 10). Like the outgrowth of buds from the main bronchus, the buds that form from branches of existing buds grow towards regions of high *Fgf10* expression (Fig. 10F).

For the future, it will be of interest to test these ideas by examining the relationship between *Fgf10* and other genes involved in lung development, both in vivo and in culture.

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