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Discordant roles for FGF ligands in lung branching morphogenesis between human and mouse

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Abstract

Fibroblast Growth Factor (FGF) signaling plays an important role in lung organogenesis. Over recent decades, FGF signaling in lung development has been extensively studied in animal models. However, little is known about the expression, localization and functional roles of FGF ligands during human fetal lung development. Therefore, we aimed to determine the expression and function of several FGF ligands and receptors in human lung development. Using *in situ* hybridization (ISH) and RNA-sequencing, we assessed their expression and distribution in native human fetal lung. Human fetal lung explants were treated with recombinant FGF7, FGF9 or FGF10 in air-liquid interface culture. Explants were analyzed grossly, to observe differences in branching pattern, as well as at the cellular and molecular level. ISH demonstrated that *FGF7* is expressed in both the epithelium and mesenchyme; *FGF9* is mainly localized in the distal epithelium, whereas *FGF10* demonstrated diffuse expression throughout the parenchyma with some expression in the smooth muscle cells (SMCs). *FGFR2* expression was high in both proximal and distal epithelial cells as well as the SMCs. *FGFR3* was expressed mostly in the epithelial cells, with lower expression in the mesenchyme, while *FGFR4* was highly expressed throughout the mesenchyme and in the distal epithelium. Using recombinant FGFs, we demonstrated that FGF7 and FGF9 had similar effects on human fetal lung as on mouse; however, FGF10 caused the human explants to expand and form cysts as opposed to inducing epithelial

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Author contributions statement

S.D., S.B., M.R.F., D.W., and D.A.A. conceived and designed research; S.D., M.T., B.G., and D.A.A. performed experiments; S.D., M.R.F., and D.A.A. analyzed data; S.D., S.B., D.W., and D.A.A. interpreted results of experiments; S.D. and D.A.A. prepared figures; S.D. and D.A.A. drafted manuscript; S.D., M.T., B.G., S.B., D.W., M.R.F., and D.A.A. edited and revised manuscript; S.D., M.T., B.G., S.B., M.R.F., D.W., and D.A.A. approved final version of manuscript.

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branching as seen in the mouse. In conjunction with decreased branching, treatment with recombinant FGF7, FGF9 and FGF10 also resulted in decreased double-positive SOX2/SOX9 progenitor cells, which are exclusively present in the distal epithelial tips in early human fetal lung. Although FGF ligand localization may be somewhat comparable between developing mouse and human lungs, their functional roles may differ substantially.

Keywords

Human; Lung; development; FGF

Introduction

Mammalian lungs are complex organs that undergo a series of epithelial branching events, resulting in a very organized stereotypical structure containing 23 generations of branches in humans. The achievement of this outcome requires a series of strictly regulated cellular and molecular events, which include cell migration, changes in cell shape, directed cell division, proximal-distal patterning and epithelial-mesenchymal crosstalk. Several signaling pathways and transcription factors control these events in mouse lung development, including the WNT, BMP, HH, RA and HIPPO pathways. However, in murine lung branching morphogenesis the Fgf signaling pathway is one of the most critical regulators.

Several FGF ligands and all four Fgf receptors (Fgfr1–4), alongside splice variants, have been reported in the developing rodent lung [1,2]. Studies in mouse have shown that perturbation of one or more FGF ligands and/or receptors, particularly Fgf9 and Fgf10, can lead to branching defects. For example, deletion of *Fgf10*, located within the mesoderm, or its receptor *Fgfr2IIIb*, located within the epithelium, in mouse lung results in complete distal lung agenesis [3,4]. This highlights the role of Fgf10 as a main regulator of murine lung branching morphogenesis. Moreover, Fgf10 controls proximal-distal patterning in the mouse lung by preventing the differentiation of epithelial Sox9 progenitors into Sox2-expressing airway cells [5,6]. Fgf7 is also produced in the mesenchyme, and acts on epithelial Fgfr2IIIb. Fgf10 induces epithelial branching and increases cell migration, whereas Fgf7 induces cell proliferation *in vitro* causing the epithelium to form cysts but not branch [7]. Deletion or overexpression of *Fgf9* in the developing mouse lung results in branching defects, accompanied by perturbation of the HH and Wnt-beta-catenin pathways [8] and the expression of *Fgf10* and *Bmp4* [9,10]. Furthermore, deletion of either Fgfr3 or Fgfr4 results in reduced formation of alveoli, and deletion of both yields hypoplastic lungs and impaired alveologenesis in the postnatal mouse lung [11]. FGF signaling is implicated in many lung diseases and improves disease in many animal models [12–14]. However, to date, there have been no successful FGF targeting therapies used to treat human lung disease. Differences have been reported during development and disease between mouse and human models [15], suggesting that alternative supplemental sources to animal models should be sought, to achieve translational potential. In particular, human and mouse lungs are different in scale, timing and structure of conducting airway formation. More recently, we started to uncover other molecular differences between human and mouse lung [16].

Fgf signaling is both important and necessary in rodent lung branching morphogenesis, whereas the molecular and spatial expression of the different FGFs and their receptors in the developing human lung have not been reported. Consequently, the roles of these signaling pathways in human lung development are unclear. In this study, we aimed to determine the spatial and temporal expression of the different FGF ligands and receptors in the human developing lung, as well as the effect of FGF7, FGF9 and FGF10 on human lung branching *in vitro*.

Methods

Ethics statement

After informed consent was given, de-identified human fetal samples were collected under Institutional Review Board approval (USC-HS-13-0399 and CHLA-14-2211). The only information collected was gestational age and whether there were any known genetic or structural abnormalities.

Animal experiments were performed under the research protocols (#31-17) approved by the Animal Research Committee at Children's Hospital Los Angeles and in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Explant cultures

Human fetal lung explants between 10 and 12 weeks gestation, or C57BL6 embryonic day 12.5 mouse lungs, were cultured on an air-liquid interface as previously described [17] and treated with either FGF ligands or SU5402 (details are provided in supplementary material, Supplementary materials and methods).

Immunohistochemistry

Human lungs or explants were fixed overnight in 4% PFA at 4 °C and processed for staining as previously described [16,17]. Antibodies and procedures are detailed in supplementary material, Table S1.

In situ hybridization

Fluorescence *in situ* hybridization was conducted using the Advanced Cell Diagnostics RNAScope Fluorescent Multiplex Assay (Newark, CA, USA) following the manufacturer's instructions (a detailed protocol is provided in supplementary material, Supplementary materials and methods).

Primary human fibroblast cultures

Fresh human fetal tissue (16–18 weeks gestation) was brought to a single cell suspension by manually chopping the tissue and digesting it in a mixture of DNase (Cat# DN25-1G, Sigma-Aldrich, St. Louis, MO, USA) and Collagenase (Cat# 10103586001, Roche, Indianapolis, IN, USA) in HBSS, for 30 min at 37 °C. The enzymatic activity was stopped using 10%FBS in DMEM/F12. Cells were further dissociated using a series of pipettes, syringes, and strainers, treated with red blood cell lysis buffer, washed and plated on

chamber slides or 6-well plates. Cells were allowed to adhere for 45 min, after which they were washed. Adherent cells were then treated with 200 ng/ml of FGF9 or FGF10 for 48 h. Post treatment, fibroblasts on chamber slides were washed, fixed with 4% PFA and stained with anti-CDH1 antibody and Cy3-ACTA2 antibody (C6198; Sigma-Aldrich). Fibroblasts in 6-well plates were scraped for RNA extraction.

RT-qPCR analyses

RNA was extracted using the iNtRon Biotechnology, Inc. Easy-Spin™ Total RNA Extraction Kit (Burlington, MA, USA). RNA used was reverse-transcribed into cDNA using the Tetro cDNA Synthesis Kit (Bioline, Taunton, MA, USA) according to the manufacturer's instructions. Dual color Hydrolysis Probe – Universal probe library-based qPCR, using the LightCycler 480 from Roche Applied Science (Indianapolis, IN, USA) was used with human *GAPDH* as the reference transcript, and specific human primer sequences (listed in supplementary material, Table S2).

RNA sequencing

The RNAseq data was processed using the ENCODE best practice run through software packages: FastQC, Cutadapt, Trimmomatic, STAR with either the latest gencode genome or the UCSC GRC38. Counts were obtained with htseq-count, (gencode only) and statistics generated with edgeR. The output from the statistical analysis was annotated with raw counts, reads per Kilobase of transcript per million mapped reads (RPKM), and database identifiers using Ensembl BiomaRt. RNA sequencing data can be found using the GEO accession [GSE121238](#). Detailed methods are provided in supplementary materials, Supplementary materials and methods.

Statistical analyses

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc. La Jolla, CA, USA). Student's *t*-test, or paired Student's *t*-test when appropriate, was used to compare two groups. Data are presented as mean ± SEM. The results were considered significant if $P \leq 0.05$.

Results

FGFs ligands and receptors are widely expressed in the developing human lung

We sought first to determine which *FGFs* and *FGFRs* are expressed in the developing human lung. RNA sequencing on native human fetal lung was performed at different periods of gestational development (10.5 weeks=mid-pseudoglandular; 14 weeks=late-pseudoglandular; 20 weeks= mid-canalicular) (Figure 1A). Human lung developmental stages are similar to those in mouse, although timing scale is different. The pseudoglandular stage spans 5–17 weeks gestation, corresponding to murine embryonic days E12–15; whereas the human canalicular stage is 16–26 weeks gestation, the equivalent of E15–17 in mouse [18]. Our data showed that the canonical *FGF* ligands *FGF9*, *FGF10*, *FGF18*, and *FGF20* were expressed at a higher level in the developing human lung, whereas *FGF2*, *FGF7*, *FGF16*, *FGF17*, and *FGF22* levels were significantly lower (Figure 1A). The intracellular *FGF* ligands *FGF12*, *FGF13*, and *FGF14* also demonstrated low expression

levels throughout human fetal lung development. All of the FGF receptors (*FGFR1–4*) were strongly expressed throughout the pseudoglandular and canalicular stages, with *FGFR4* displaying the highest expression of the four (Figure 1A).

Next, we wanted to establish the spatial localization of some of the expressed ligands and receptors, especially those that are most commonly studied in the mouse lung. Using fluorescence *in situ* hybridization on human fetal lung sections, at different gestational stages (11, 14, 16 and 18 weeks), we found that *FGF7* was expressed throughout the tissue in both epithelium and mesenchyme at each gestational age (Figure 1B,C,H,I); whereas *FGF9* was mostly expressed in the distal epithelial tips and mesothelial cells at each stage, with minimal expression in the proximal epithelium (Figure 1D,E,J,K). *FGF10* was predominantly expressed in the mesenchyme, both distally and proximally (Figure 1F,G,L,M). *FGFR4* was expressed in the mesenchyme and the distal epithelium, with an increase in expression from 11 to 14 weeks, as predicted by RNA sequencing (Figure 1A), but absent in the proximal epithelium (Figure 1B,C,H,I). *FGFR3* was strongly expressed in the proximal and distal epithelium, with lower expression in the mesenchyme (Figure 1D,E,J,K). Furthermore, *FGFR2* was highly expressed in the distal epithelium with low levels in the proximal epithelium (Figure 1F,G,L,M). Localization of *FGFR2* (Figure 1N–Q) and *FGFR1* (Figure 1R–U) was also validated using IHC at 11, 14, 16 and 18 weeks. A strong presence of both receptors in the proximal and distal epithelium was seen throughout each stage, but expression in the mesenchyme appeared to decrease in the canalicular stage when compared to the pseudoglandular stage.

FGF7 and FGF9 induce cyst formation in human fetal lung explants, similar to mouse

Next, we tested the effect of exogenous FGF ligands on human fetal lung explants and compare this to their described effects on mouse explants. Human fetal lung explants between 10–12 weeks gestational age and E12 mouse lung explants were cultured on air-liquid interface as described in the methods. Human explants treated with recombinant human (rh) FGF7 (200 ng/ml) showed enlarged distal buds (Figure 2D) as compared to control (Figure 2B), consistent with cyst formation previously described in mouse and shown in Figure 2D' versus 2B'. Similarly, rhFGF9 (200 ng/ml) treatment resulted in cyst formation and thickened mesenchyme (Figure 2F) as compared to control (Figure 2B). This effect was similar to what is seen in mouse explants (Figure 2F' versus 2B'). Quantification of the cystic areas in the human explants confirmed that FGF7 induced a significant increase in cyst area compared to control (**p*=0.0197, Figure 2I), while FGF9 had a non-significant trend towards increased cyst area (*p*=0.0662, Figure 2I). These results are comparable to what is observed in the mouse explants where quantification of the cystic areas after treatment with FGF7 or FGF9 demonstrated a significant increase in cyst area as compared to control (*p*=0.0121 and 0.0085 respectively, Figure 2J).

FGF10 does not induce branching in human fetal lung explants

In the presence of rhFGF10 (200 ng/ml), human fetal explants showed enlarged buds (Figure 2H versus Figure 2B) as compared to control explants (***p*=0.0072, Figure 2I). Quantification of the number of branches between 0 and 48 h demonstrated a significant decrease in branches as compared to controls (**p*= 0.0228, Figure 2L). In contrast, a

significant increase (* $p=0.034$, Figure 3L) in branching was observed in mouse explants treated with rhFGF10 (Figure 2H') as compared to controls (Figure 2B'), with no enlargement of the distal bud area (Figure 2J), as previously described [19]. Different concentrations of rhFGF10 (400 and 800 ng/ml; Figure 2K) were tested on human fetal explants; all of which induced significantly enlarged cyst formation when compared to controls (400 ng/ml: ** $p=0.0012$, 800 ng/ml: *** $p=0.0015$; Figure 2M) and failed to induce branching post 48 h in culture (400 ng/ml: ** $p=0.0115$, 800 ng/ml: *** $p=0.0082$; Figure 2L). Later stage human lung explants (13–14 weeks) treated with FGF10 (200 ng/ml) also displayed significantly enlarged distal buds (Figure 2N–O, * $p=0.024$).

FGF ligands did not affect cell proliferation in human fetal lung explants

In mouse and rat explant models, exogenous FGF ligands induce epithelial or mesenchymal cell proliferation [20,21]. To test whether this is true for human lung explants, we assessed cell proliferation using immunostaining for Ki67. While FGF7, FGF9 and FGF10 all induced signaling in human explants as shown by western blot assessment of ERK phosphorylation ($p=0.0678$, * $p=0.0028$, ** $p=0.0266$ respectively; Figure 3F,G), they did not induce any changes in Ki67+ cell numbers (Figure 3B–D versus Figure 3A) compared to controls. Quantification of the total number of proliferating cells in the epithelium, the mesenchyme or both combined confirmed that these FGF ligands had no effect on cell proliferation (Figure 3E).

FGF treatments altered the co-expression of SOX2/SOX9 double positive cells

We showed recently that the human lung bud epithelial tips co-express SOX2 and SOX9 and that this co-expression is required for proper branching in the human fetal lung [16]. Moreover, in mouse lungs, FGF signaling alters the proximal-distal patterning [22]. We therefore assessed the co-expression of SOX2 and SOX9 in the explants treated with the FGF ligands. Following 48 h in culture, control explants displayed co-expression of SOX2 and SOX9 in the distal bud tips (Figure 4A). However, treatment with FGF7 and FGF9 resulted in expansion of SOX9 cells and a near absence in expression of SOX2 (Figure 4B and 4C respectively). Treatment with FGF10 lead to expansion of the SOX9 domain, and a decrease in SOX2 expression in the bud tips (Figure 4D). RT-qPCR analyses of *SOX2* showed decreased expression in the explants treated with FGF9 (* $p=0.026$) compared to control, while a non-significant trend towards decrease was observed in explants treated with FGF7 and FGF10 (Figure 4I). Analyses of known *FGF* targets by RT-qPCR showed that expression for each gene was significantly increased following FGF7 and FGF9 treatment (*SPRY2* * $p=0.0028$ and ** $p=0.0067$), *SPRY4* (* $p=0.0003$, ** $p=0.0030$), *ETV4* (* $p=0.0002$, ** $p=0.019$), *ETV5* (* $p=0.0085$, ** $p=0.0248$), and *DUSP6* (* $p=0.0001$, ** $p=0.0001$) [23–27] with a minimal increase by FGF10 (Figure 4J).

In mouse lung explants, *Sftpc* has been shown as one of the targets of FGF10 [28]. Since none of the downstream targets tested showed significant change in response to FGF10, we sought to determine whether SFTPC could be a target in the human explants. We demonstrated that pro-SFTPC is absent in the human lung at 11.6 weeks (Figure 4K) and begins to be expressed at week 14 (Figure 4L and M, respectively). Similarly, HTII-280 was absent at 11.6 weeks (Figure 4K), with low expression at 14 weeks (Figure 4L) and a robust

expression at 18.2 weeks (Figure 4M). HTII280 did not always co-localize with pro-SFTPC supporting previous reports that HTII-280 may also be expressed in undifferentiated epithelium [29]. Furthermore, we had previously established that smooth muscle cells (SMCs) appear to promote SOX2+ cells and suppress the SOX9+ population [16]. Given the expansion of SOX9+ cells in the FGF-treated explants, we assessed the expression of SMCs using immunostaining for ACTA2. There was an evident decrease in SMC expression in FGF7, FGF9, and FGF10 treated explants as compared to controls (Figure 4F–H versus Figure 4E).

FGF9 inhibits the differentiation of SMCs from human fetal cells, while inhibiting *FGF10* expression

In mice, FGF9 inhibits the differentiation of SMCs while inducing *Fgf10* expression [10]. Primary cultures of human fetal lung fibroblasts were obtained from lungs between 16–18 weeks gestation and cultured in the absence or presence of 200 ng/ml rhFGF7, rhFGF9 or rhFGF10. Immunofluorescence staining of treated fibroblasts demonstrated that, similar to mouse fibroblasts, FGF9 triggered a decrease in SMC differentiation, as indicated in the reduced staining for ACTA2 (Figure 5C versus 5A); whereas treatment with FGF7 and FGF10 demonstrated no apparent difference in SMC differentiation as compared to control fibroblasts (Figure 5B and 5D versus 5A respectively). These results were validated by RT-qPCR (Figure 5E), where *ACTA2* expression was significantly less following FGF9 treatment as compared to control ($p=0.0113$). However, FGF7 and FGF10 treatments did not have a significant effect on *ACTA2* expression. We investigated next whether, like in mouse, FGF9 induces the expression of *FGF10*. We demonstrated that in FGF9 treated explants, *FGF10* expression levels were significantly decreased compared to control fibroblasts ($p=0.0421$; Figure 5F). These results are the opposite of what have been described in mouse fibroblasts [10].

FGFR inhibition prevents branching of human fetal lung explants

In order to determine whether global FGF signaling inhibition impairs human fetal lung branching, we treated our explant cultures with SU5402, a tyrosine kinase inhibitor known to inhibit FGFR signaling. Human fetal lung explants (10–12 weeks gestation) were treated with 10 μ M SU5402. The explants treated with SU5402 demonstrated minimal branch formation as compared to controls (Figure 6D versus 6C). This was validated via quantification of the fold-change in number of branches between 0 and 48 h, which showed a significant decrease in the number of branches in the human fetal explants treated with SU5402 as compared to controls (Figure 6G, $p=0.0049$). Treatment with SU5402 resulted in an expansion of SOX2 expressing cells as opposed to the decrease following FGF treatment, with a near complete loss of SOX9 expression in the bud tips (Figure 6F) as compared to co-expression of SOX2/SOX9 in the control explants (Figure 6E).

Discussion

FGF signaling and its role in mouse lung development has been extensively studied; however, its role in human lung development remains unclear. Microarray studies performed on both the developing mouse and human lung reported the expression of several FGFs and

their receptors [30,31], and are in accordance with our RNA sequencing data on human lung at various gestational stages. For example, *FGF18* expression increases significantly from 11 to 14 or 20 weeks gestation. Moreover, the temporal expression of FGF10 corroborates our previously published data showing that *FGF10* expression does not increase during the pseudoglandular stage, but increases between the pseudoglandular and canalicular stages [32]. While the spatial expression of *FGF7*, *FGF9*, and FGFRs is very similar to that described in mouse; the expression of *FGF10* differed significantly. In mouse lung, a Fgf10 gradient guides branching morphogenesis, whereby *Fgf10* expression is concentrated in the distal mesenchyme near branch buds [33]. *In situ* hybridization performed on human fetal lung samples did not show a concentrated gradient of *FGF10* expression, but rather a diffuse expression throughout the mesenchyme. This difference could be a timing discrepancy, with the possibility that earlier stage human lungs may have a localized FGF10 expression. However, the 11 weeks human fetal lungs that we assessed correspond to mid-pseudoglandular stage mouse lung (around E13), a period when *Fgf10* expression is still localized to bud-neighboring domains. FGF10 expression was also detected in the airway SMCs, similar to mouse.

Fgf10 is a critical factor in mouse lung branching morphogenesis. Deletion of *Fgf10* or its receptor *Fgfr2IIIb* in mouse lung results in complete lung agenesis [3,4]. Furthermore, rhFGF10 protein induces branching in E12 embryonic mouse lung and isolated epithelial buds (Figure 2) [19,33]). Interestingly, our data did not support a role for FGF10 in inducing branching in the human fetal lung at any of the stages studied (10–14 weeks). Instead, exposure resulted in cyst formation in pseudoglandular stage lung explants. A previous study showed that both FGF7 and FGF10 induced fluid secretion and increased intraluminal volume *in vitro* in first trimester human fetal explants cultured in Matrigel [34]. We also observed that FGF7 and FGF9 induced cyst formation in 10–12 weeks human explants, similar to mouse [7,10,33]. Although human explants had a tendency to form some cysts even in the absence of FGFs, the cysting was much more pronounced in the presence of FGF ligands and completely absent following treatment with FGF inhibitor, suggesting that the cysting effect observed is due to the FGF activity.

Fgf10 controls proximal-distal patterning in the mouse lung by preventing the differentiation of epithelial Sox9 progenitors into Sox2-expressing airway cells [5,6]. Others and we have recently shown that distal tip buds in the early human lung co-express SOX2 and SOX9 as opposed to only SOX9 in mouse [16,35,36]. Moreover, we showed that the maintenance of this double positive population is required for branching morphogenesis [16]. Herein, our results demonstrated that FGF10 impaired the balance and co-localization of SOX2/SOX9 and impaired branching in the early (10–12 weeks) human lung. A recent study found that treatment of human fetal lung bud tip organoids with FGF7/retinoic acid/CHIR-99021 was enough to support growth (budding) and maintenance of the cultures as well as their progenitor cell population co-expressing SOX2 and SOX9, suggesting that FGF10 is dispensable at this stage of development [35]. This is consistent with our study showing that FGF10 does not induce branching in explant cultures. Another study using *in vitro* human lung organoids with a combination of seven factors (FGF7, FGF10, NOGGIN, RSP01, EGF, CHIR99021 and SB431542) demonstrated that these organoids can grow, self-renew and branch following several weeks in culture. Removal of FGF10 alone from the culture

media had no effect on organoid self-renewal or the expression of *SOX2* and *SOX9* [36]. The presence of FGF7, FGF9 or FGF10 resulted in an increase of *SOX9* and a decrease in *SOX2* expression in our cultures (Figure 4B–D). In contrast, inhibition of FGF signaling resulted in increased *SOX2* and decreased *SOX9* expression. We also showed that the balance of the *SOX2*/*SOX9* double positive progenitor population appears to be controlled by the maintenance of SMCs in the cultured explants. In response to FGF7, FGF9 and FGF10, we observed a significant decrease in SMC differentiation in the explant cultures, which likely caused an imbalance in *SOX2*/*SOX9* double positive progenitors and hence impaired branching. Primary cultured fibroblasts failed to differentiate to SMCs in the presence of FGF9. While FGF9 has been shown to induce *Fgf10* expression in mouse lung cultures [10], the opposite is true in human lung explants.

FGF ligands are known mitogenic factors in several organ systems, including the lung. Previous studies have shown that FGF7 promotes proliferation of lung epithelium *in vitro* [7], whereas lack of *Fgf9* decreases cell proliferation as assessed by BrdU incorporation [21]. In our culture model, using Ki67 staining, we did not see a difference in either epithelial or mesenchymal cell proliferation in the presence of FGF7, FGF9 or FGF10; despite detectable activation of ERK MAPK. The lack of effect of the FGF ligands on proliferation is likely due to timing and scale, as the human lung requires a longer time to proliferate and grow as compared to rodent lungs. Despite this lack of proliferation, FGF7 and FGF9 were able to induce the expression of several known FGF signaling target genes (*ETV4*, *ETV5*, *SPRTY2*, *SPRTY4* and *DUSP6*). In contrast FGF10 failed to significantly modulate any of these targets. In mouse, *Fgf10* induces SFTPC expression (distal fate) in the tracheal epithelium *in vitro* [28]. We showed that SFTPC is absent in the human lung at early stages of development, which could explain the lack of modulation of other target genes. This lack of target gene modulation could also be determined by whether or not tyrosine (Y) 734 of FGFR2b is phosphorylated in response to FGF10. This in turn would respectively either cause the receptor to be endocytosed resulting in activation of the PI3K/AKT pathway or alternatively result in degradation of the receptor [37]. Moreover, the affinity of the FGFs to heparin sulfate can regulate the extent of the cell response to a specific FGF [38]. In contrast, global inhibition of FGF signaling using the SU5402 inhibitor stopped branching in the human fetal explants, and cells acquired a *SOX2*⁺ (proximal) fate. This suggests that FGF signaling is important for lung branching, perhaps in a different manner than it is in mouse. We stipulate that the preservation of the proximal-distal axis by balanced FGF signaling is also important in human lung branching; but perhaps different ligands/receptors are involved in this process in humans.

This is the first comprehensive study on the role of FGF signaling in human fetal lung. A fully developed mouse lung contains 13 generations of branches, while a human lung contains 23. In addition to size and scale, a major structural difference between mouse and human airway composition has been described: cartilaginous airways are restricted in the mouse lung to the trachea and main bronchi, while in human lung they extend to the terminal bronchioles. Moreover, timing differences between the human and mouse are complex, have not fully been defined, and could contribute to the discrepant observations about the role of FGF10 in lung development. While structural differences between mouse and human together with our data suggest that observed differences in the effect of FGF10

may not be solely based upon timing, we cannot formally rule out a role for timing as well. Our results provide evidence that FGF10 has a different role in human versus mouse lung, and although some FGFs (FGF9) play a similar physiological role, the molecular pathways driving it may differ. Further studies aiming to elucidate the cellular and molecular targets of the different FGFs and their receptors in the developing human lung are needed in order to address a gap of knowledge. This in turn will accelerate the translational potential of animal studies and open the way to better therapeutic insight for the use of individual FGFs to treat human diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. FGF ligands and receptors are widely expressed in the developing human lung
 (A) RNA expression of FGF ligands and receptors expressed in RPKMs $\pm$ SEM within the developing lung (n=3 per time point). (B-M) Fluorescence *in situ* hybridization (FISH) on human fetal lung sections at 11 weeks (B,D,F), 14 weeks (C,E,G), 16 weeks (H,J,L) and 18 weeks (I,K,M) gestation showing FGF ligands (red) *FGF7* (B,C,H,I) in the mesenchyme and epithelium both distally and proximally, *FGF9* (D,E,J,K) strongly in the distal epithelial tips and mesothelium with minimal expression in the proximal epithelium, and *FGF10* (F,G,L,M) expressed mainly within the mesenchyme both distally and proximally. FISH of FGFRs (green) demonstrated that *FGFR4* (B,C,H,I) is expressed in the distal epithelial tips and mesenchyme and the proximal lung with a significant increase in expression between 11 weeks (B) to 14 weeks (C), *FGFR3* (D,E,J,K) expression in the distal and proximal compartments, and *FGFR2* (F,G,L,M) expression in the distal and proximal lung. Scale bar is 50 μ m. (N-U) IHC of FGFR2 on human fetal lung at different developmental stages: 11 wk (N), 14 wk (O), 16 wk (P) and 18 wk (Q). (R-U) IHC of FGFR1 on human fetal lung at

different developmental stages: 11 Wks (R), 14 wk (S), 16 wk (T) and 18 wk (U). Scale bar is 100 μm

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Figure 2. FGF10 did not induce branching in human fetal lung explant cultures

(A-H) Human fetal (10.3 wk) and (A'-H') mouse embryonic (E12) lung explant cultures at t=0 and t=48 h (B-H and B'-H') in the presence or absence (B, B') of 200 ng/ml rhFGF7 (D, D'), rhFGF9 (F, F'), or rhFGF10 (H, H'). (I) Graph representing area of epithelial buds post 48 h in culture of control and FGF treated human explants (FGF7, FGF9, FGF10) (n=4 for each condition; *p=0.0197; **p=0.0072). (J) Graph presenting the area of epithelial buds following 48 h in culture of control and FGF treated mouse explants (FGF7, FGF9, FGF10) (n=4 for each condition; *p=0.0121; **p=0.0085). (K) Human fetal lung explants (10 wk) treated with 400 ng/ml and 800 ng/ml of rhFGF10 for 48 h. (L) Graphs presenting the fold-increase in the number of distal branches following 48 h treatment in rhFGF10 dose treated human explants and 200 ng/ml rhFGF10 mouse explants (Human: n=9, 200 ng/ml *p=0.0228, 400 ng/ml ** p=0.0115, 800 ng/ml ***p=0.0082; Mouse: n=3, *p=0.0342). (M) Graph presenting the area of epithelial buds following 48 h in culture of control and FGF10 dose treated human explants (200 ng/ml, 400 ng/ml, 800 ng/ml) (n=5 for each condition; *p=0.0072; **p=0.0012; ***p=0.0015). (N) Human fetal (13.2 wk) lung explant cultures at t=0 and t=48 h in the presence or absence of 200 ng/ml rhFGF10 (n=3 of each). (O) Graph presenting the area of epithelial buds following 48 h in culture of 13.2–14 weeks control and FGF10 treated human explants (n=3 for each, *p=0.024)

Figure 3. FGF7, FGF9 and FGF10 did not alter cell proliferation in human fetal lung explant cultures

(A-D) Immunofluorescence (IF) staining for Ki67 (red) and CDH1 (green) of human fetal lung explants in absence (A) or presence of 200 ng/ml FGF7 (B), FGF9 (C) and FGF10 (D). Scale bar is 50 μ m. (E) Graphs presenting the percentage of Ki67 positive cells in control and FGF treated explants within the epithelium, mesenchyme, and throughout the entire explant (n=5 for each condition). (F) Western blots for p-ERK and β -actin in human fetal lung explants treated for 30 min in the absence or presence of 200 ng/ml FGF7, FGF9 or FGF10. (G) Graph of western blot densitometry ratios for p-ERK normalized to β -actin (n=5 for each condition; FGF7 p= 0.0678, *p=0.0028, **p=0.0266).

Figure 4. FGF7, FGF9 and FGF10 alter the double-positive SOX2/SOX9 progenitor cell population.

(A-D) Immunofluorescence (IF) staining for SOX9 (red) and SOX2 (green) in human fetal lung explants in (A) the absence or presence of 200 ng/ml (B) FGF7, or (C) FGF9 and (D) FGF10. (E-H) IF staining for ACTA2 (red) and CDH1 (green) in human fetal explants in (E) the absence or presence of 200 ng/ml of (F) FGF7, (G) FGF9, (H) FGF10. (I) RT-qPCR for *SOX2* relative to *GAPDH* (n=5 for each condition, *p=0.026). (J) RT-qPCR for FGF targets relative to *GAPDH* [n=8 for each; *SPRY2* (FGF7 *p= 0.0028, FGF9 **p=0.0067), *SPRY4* (FGF7 *p= 0.0003, FGF9 **p=0.0030), *ETV4* (FGF7 *p= 0.0002, FGF9 **p=0.019), *ETV5* (FGF7 *p= 0.0085, FGF9 **p=0.0248), *DUSP6* (FGF7 *p= 0.0001, FGF9 **p=0.0001)]. (K-M) IF staining for SPC (red) and HTII-280 (green) in native human fetal lung at (K) 11.6 wk, (L) 14.6 wk, and (M) 18.1 wk. Scale bars are 50 μ m.

Figure 5. FGF9 inhibits smooth muscle cell differentiation

(A-C) Immunofluorescence staining for ACTA2 (red) and CDH1 (green) in human fetal lung fibroblasts (HFLF) in (A) the absence or presence of 200 ng/ml of (B) FGF7, (C) FGF9 or (D) FGF10. Scale bar is 25 μ m. (E) RT-qPCR for *ACTA2* relative to *GAPDH* in HFLFs treated with FGFs (n=3; *p=0.0113). (F) RT-qPCR for *FGF10* relative to *GAPDH* in HFLFs treated with FGFs (n=3; *p=0.0421).

Figure 6. FGFR inhibition prevents branching of human fetal lung explants
 (A-D) Human fetal (11.2 wk) lung explants cultured for (C,D) 48 h in (B,D) the presence or (A,C) absence of 10 μ M SU5402. (E-F) Immunofluorescence staining for SOX2 (green), SOX9 (red) and ACTA2 (white) in (F) SU5402-treated and (E) control explants. Scale bar is 50 μ m. (G) Graph presenting the fold-increase in the number of distal branches following 48 h treatment with 10 μ M SU5402 in human fetal explants (n=3, *p=0.0002).