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Divergent fibroblast growth factor signaling pathways in lung fibroblast subsets: where do we go from here?

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**Running head:** FGF signaling in lung fibroblasts
ABSTRACT

Lung fibroblasts play a key role in post-natal lung development, namely, the formation of the alveolar gas exchange units, through the process of secondary septation. While evidence initially highlighted roles for fibroblasts in the production and remodeling of the lung extracellular matrix, more recent studies have described the presence of different fibroblast subsets in the developing lung. These subsets include myofibroblasts and lipofibroblasts, and their precursors. These cells are believed to play different roles in alveologenesis and are localized to different regions of the developing septa. The precise roles played by these different fibroblast subsets remain unclear. Understanding the signaling pathways that control the discrete functions of these fibroblast subsets would help to clarify the roles and the regulation of lung fibroblasts during lung development. Here, we critically evaluate a recent report that described divergent fibroblast growth factor (FGF) signaling pathways in two different subsets of lung fibroblasts that express different levels of green fluorescent protein (GFP) driven by the platelet-derived growth factor receptor (PDGFR)-α promoter. The GFP expression was used as a surrogate for lipofibroblasts (GFP\textsuperscript{low}) and myofibroblasts (GFP\textsuperscript{high}). It was suggested that Fgf10/Fgf1 and Fgf18/Fgfr3 autocrine pathways may be operative in GFP\textsuperscript{low} and GFP\textsuperscript{high} cells, respectively; and that these pathways might regulate the proliferation and migration of different fibroblast subsets during alveologenesis. These observations lay important groundwork for the further exploration of FGF function during normal lung development, as well as in aberrant lung development associated with bronchopulmonary dysplasia.

Keywords: fibroblast, alveolarization, lipofibroblast, myofibroblast, FGF, PDGF
Fibroblasts have long been acknowledged to play a critical role in all stages of lung development, including the development of the alveolar gas exchange units (46). Many studies have addressed roles for fibroblasts in the production of components of the extracellular matrix (ECM), and the production of ECM remodeling enzymes in the developing lung (24, 25). The ECM framework helps to direct lung alveologenesis, acting in a coordinated manner with genetic programs, cell-cell communication, and physical forces, such as those generated by breathing motions (19, 25, 32). More recently, fibroblasts have been credited with additional roles in alveologenesis, including regulatory fibroblast-epithelial cell cross-talk (44, 47), and this idea has gained prominence with the delineation of multiple lung fibroblast subsets, including lipofibroblasts (31) and myofibroblasts (8, 46), and their respective precursor cells (29, 33). While the discrimination between – and markers of – these fibroblast subsets is complicated and not yet fully resolved, the platelet-derived growth factor (PDGF) receptor (PDGFR)-α is believed to mark a population of fibroblasts that plays a key role in post-natal lung maturation (7, 26, 28). This is in line with the critical role of PDGF/PDGFR-α signaling in lung development, where inactivation of PDGF-A or PDGFR-α in mice (6, 7) blocks alveolarization. This idea is strengthened by the observed reduction in PDGFR-α expression in the lungs of preterm infants with bronchopulmonary dysplasia (BPD), where lung development is blunted (36). Fibroblast subsets are thought to be located in different regions of the developing alveolar septa, and the existence of some of these subsets in all developing mammalian lungs is currently a matter of debate (1, 43, 50). For example, one recent report did not detect lipofibroblasts in developing human lung, based on an electron microscopy approach (43); which contrasted with an earlier report which did demonstrate the presence of lipofibroblasts in human lungs by other approaches (39). While it has been proposed that different analytical approaches may underlie the conflicting conclusions of the two studies (1), this paradox highlights the complexity of this dynamic area.
of lung development biology. Indeed, the complex interactions between fibroblast subsets during lung alveolarization, and the physiological roles played by these fibroblast subsets is currently a subject of intense experimental efforts (30). Of particular interest are the stimuli that regulate fibroblast differentiation and the discrete functions of these cells in the developing septa. Amongst the stimuli currently being investigated are growth factors of the fibroblast growth factor (FGF) family.

In a recent report in the *Journal*, McGowan and McCoy (27) employ the expression levels of green fluorescent protein (GFP) driven by the *Pdgfra* promoter (which drives PDGFR-α expression) to discriminate between fibroblast subsets. In this system, GFP expression was used as a surrogate for lipofibroblasts (defined as GFP<sub>low</sub>) and myofibroblasts (defined as GFP<sub>high</sub>). This approach was based on prior work by their group that associated a higher abundance of lipid droplets in GFP<sub>low</sub> cells, and increased expression of smooth muscle actin in GFP<sub>high</sub> cells (26). Using these tools, the investigators described temporal changes in the proliferation index of GFP<sub>low</sub> versus GFP<sub>high</sub> cells, where GFP<sub>low</sub> cells exhibited a peak in proliferation (assessed by Ki67 expression using flow cytometry) at post-natal day (P)4, while GFP<sub>high</sub> cells exhibited a stable proliferation rate over the period P2 to P4, which then dropped significantly by P8. This builds on earlier work by the investigators which documented changes in the abundance and proliferation indices of GFP<sub>low</sub> versus GFP<sub>high</sub> cells during post-natal lung development (23). Given the acknowledged roles of fibroblast growth factor (FGF) in mediating transdifferentiation of lipofibroblasts and myofibroblasts during post-natal lung development (35), McGowan and McCoy (27) turned their attention to FGF receptor abundance and the FGF signaling pathways in GFP<sub>low</sub> versus GFP<sub>high</sub> cells. The investigators described different expression profiles of selected components of the FGF signaling machinery in GFP<sub>low</sub> versus GFP<sub>high</sub> cells. Notable amongst these observations was that *Fgfr3* and *Fgfr4* mRNA levels were elevated in GFP<sub>low</sub> cells, compared with GFP<sub>high</sub> cells; however, in contrast, the cell-surface abundance of Fgfr3 protein was increased on
GFP\textsuperscript{high} cells. Additionally, the cell-surface abundance of Fgfr4 was not different between GFP\textsuperscript{low} and GFP\textsuperscript{high} cells, but the relative proportion of Fgfr4 on the cell-surface (comparing total to cell surface-bound) was higher in GFP\textsuperscript{high} cells. This led the authors to suggest that Fgfr3 is a mediator of GFP\textsuperscript{high} cell behavior. Furthermore, \textit{Fgf18}, which encodes Fgf18, a ligand for Fgfr3, was highly expressed in GFP\textsuperscript{high} cells, but \textit{Fgf18} expression was barely detected in GFP\textsuperscript{low} cells. This observation suggested to the authors that autocrine signaling via the Fgf18/Fgfr3 axis may modulate GFP\textsuperscript{high} cell (myofibroblast) behavior. In contrast, \textit{Fgf10} mRNA was enriched in the GFP\textsuperscript{low} cells, as was type IIIb splice-variant of \textit{Fgfr1} mRNA (\textit{Fgfr1-IIIb}) suggesting to the authors that the Fgf10/Fgfr1 axis was operative in GFP\textsuperscript{low} cells.

These data, presented schematically in Fig. 1, contribute to a growing body of evidence that FGF signaling mediates the complex roles and interactions of lipofibroblasts and myofibroblasts in the developing lung. The investigators are to be commended for quantitatively monitoring FGF signaling dynamics in fibroblast subsets from developing mouse lungs that could be discriminated from one another \textit{ex vivo}.

In pioneering work addressing Fgf1 effects on lung fibroblasts, Moisés Selman’s group demonstrated that Fgf1 antagonized transforming growth factor (TGF)-\(\beta\) signaling in lung fibroblasts, and that Fgf1 was anti-fibrogenic and antagonized TGF-\(\beta\)-driven fibroblast-to-myofibroblast transdifferentiation (37, 38). Since then, much work has addressed FGF signaling in lung physiology and disease, including lung development. Several FGF ligands and receptors have validated roles in lung development that were uncovered in gene ablation studies; notable amongst these are the Fgf10 (42) and Fgf18 (45) ligands, and the Fgfr2b receptor (2, 13), where knockout animals exhibit abnormal lung phenotypes. These data emerge as all the more important given a recent report of the single nucleotide polymorphism (SNP) rs1966265 in \textit{FGFR4} being associated with BPD (40). Together, these reports provide compelling evidence for a role of FGF signaling in normal and aberrant lung
alveologenesis. The study by McGowan and McCoy (27) under discussion here addresses this
idea, in the context of the regulation of fibroblast subset function during alveolar formation.

The study of McGowan and McCoy (27) relies very heavily on the use of Pdgfra
promoter-driven GFP expression to discriminate between two PDGFRα+ cell populations.
Low (GFPlow) and high (GFPhigh) GFP expression was used as surrogate markers for lung
lipofibroblasts and lung myofibroblasts, respectively. While the approach is elegant, it is also
open to criticism. For example, what is the possibility that a proportion of lung lipofibroblasts
that are relevant to alveologenesis, do not express PDGFR-α? This PDGFR-α-negative
subpopulation would have been missed in this study. As such, it would be interesting to see a
parallel analysis that employs direct markers of lipofibroblasts such as Plin2 [encoding
perilipin 2, also called adipose differentiation related protein (ADRP); or lipid droplets].
Similar concerns persist with myofibroblasts, where alveolar myofibroblasts which may have
originated from PDGFR-α+ fibroblast precursors might subsequently have lost PDGFRα
expression, since in vitro the PDGF-A ligand suppresses expression of Acta2, which encodes
α-smooth muscle actin, and thus may antagonize fibroblast-to-myofibroblast differentiation
(23). Along these lines, the contentions of the investigators would be considerably
strengthened if elements of their ex vivo and in vitro analyses could be demonstrated in situ in
intact lung tissue. For example, myofibroblasts are generally localized to the tips of the septa
while the lipofibroblasts are localized to the base of the septa and alveoli walls in the
developing lung (20). Thus, it would be interesting to co-localize the expression of both FGF
ligands and FGF receptors of interest in discrete regions within the developing septa.

Given lipofibroblast and myofibroblast differentiation dynamics during post-natal lung
maturation, it is concerning that the investigators only examined the cell-surface expression,
and total-to-surface ratio of selected FGF receptors at one time-point, namely post-natal day
(P)8. In their current (and previous) reports, the investigators described dynamic changes in
both the abundance and proliferative index of GFP\textsuperscript{low} and GFP\textsuperscript{high} cells. If these changes in the abundance and the proliferative index are to be attributed to FGF signaling, then the expression of the FGF receptors and ligands on these two cell populations over the time-course of lung alveolarization would be important to document. Furthermore, these studies raise the question of the many other FGF ligands and receptors not addressed in the report. It remains important to address the relative expression levels of other FGF receptors on GFP\textsuperscript{high} versus GFP\textsuperscript{low} cells, which may transmit Fgf18 signals. This is particularly important in light of the observations that Fgf18 can also bind Fgfr1, Fgfr2 (16, 18) and Fgfr4 (16).

The study of McGowan and McCoy discussed here (27) also identified Fgf18 mRNA to be enriched in GFP\textsuperscript{high} versus GFP\textsuperscript{low} cells. Furthermore, Fgfr3 mRNA, which encodes a receptor for Fgf18, was enriched in GFP\textsuperscript{high} versus GFP\textsuperscript{low} cells. Thus, McGowan and McCoy (27) suggested that the Fgf18/Fgfr3 axis was operative in myofibroblasts and acted in an autocrine fashion to modulate myofibroblast behavior during lung alveologenesis. Fgf18 has been discussed to be a mediator of lung alveologenesis by Jacques Bourbon and colleagues (10), since FGF-18 expression in the lung peaks between P3 and P18, which is coincident with the peak period of lung alveolarization in rodents. Furthermore, Fgf18 was demonstrated to be pro-elastogenic (promoting the expression of Eln mRNA, which encodes tropoelastin, as well as Lox, encoding lysyl oxidase). Fgf18 was also demonstrated to be pro-proliferative on lung fibroblasts \textit{in vitro}, to promote Acta2 expression, and to drive the expression of genes that regulate lung alveologenesis (17). McGowan and McCoy (27) demonstrated that Fgf18 was anti-proliferative in primary lung fibroblasts, while Bourbon and colleagues (10) documented the pro-proliferative activity of Fgf18 on primary lung fibroblasts. The reasons for these discordant observations are not clear, but might be related to the use of post-natal mouse (27) versus fetal rat fibroblasts (10), or the use of 5-ethynyl-2′-deoxyuridine (27) versus \[^{3}\text{H}]\text{thymidine} (10) incorporation to monitor cell proliferation.
The report by McGowan and McCoy (27) should be followed by the demonstration of a functional impact of the Fgf18/Fgfr3 and Fgf10/Fgfr1 axes on the properties of lung fibroblasts. While the v-akt thymoma viral proto-oncogene 1 (also called Akt) phosphorylation (and hence, activation) studies are a good starting point, Akt is broadly activated by FGF receptors, as well as PDGF receptors, and many other signaling systems in fibroblasts. Whether or not changes in Akt activation are due to either the Fgf18/Fgfr3 or Fgf10/Fgfr1 axes remains unclear. To specifically and unequivocally implicate either Fgfr3 or Fgfr1 in the modulation of Akt signaling by Fgf18 or Fgf10, respectively, a genetic ablation study would have to be undertaken. For example, using small interfering (si)RNA, where the role of Fgfr3 or Fgfr1 could be definitively attributed. Similarly, it would be important to demonstrate a functional effect of the Fgf18/Fgfr3 and Fgf10/Fgfr1 axes in lung fibroblasts on fibroblast behavior. In their study, McGowan and McCoy (27) claim that Fgf18 restricts fibroblast proliferation through Fgfr3, and that Fgf8b, but not Fgf18, promotes fibroblast migration, also via Fgfr3. However, Fgfr3 has not been causally implicated in either process. It may well be that Fgf18 and Fgf8b are acting via other FGF receptors that were not considered in the study. Ablation of Fgfr3 expression in an siRNA approach in the fibroblast proliferation and migration assays would confirm or refute whether Fgfr3 was indeed the mediator of these effects. However, FGF signaling in the GFP^low cells was hardly considered in the study, although very preliminary evidence implicating Frf10 and Fgfr1-IIIb in GFP^low cell behavior was provided. It would be interesting to know whether Fgf10 stimulation of lung fibroblasts, in the background of an Fgfr1 knockdown, also impacted proliferation and migration. How FGF signaling may impact, for example, fibroblast-to-myofibroblast differentiation would be easier to delineate once the causal FGF players had been identified. There is evidence that FGF signaling may regulate Let-7d microRNA expression in the mesenchyme (11), and considering the recent report that Let-7d regulates lung fibroblast phenotype (21), this FGF/Let-7d pathways seems worth pursuing. Along similar lines, the
ability of FGF signaling to drive the action of other newly-identified modulators of lung
alveolarization or myofibroblast differentiation, including soluble guanylate cyclase (3) and
adrenomedullin (22) highlight just some of the other candidate pathways worth exploring.

Other evidence for a role for FGF signaling in alveolar development comes from in vivo over-expression of a soluble dominant-negative (dn)Fgfr2b receptor which acted as an
FGF ligand sponge, that prevented neoalveolarization in adult mice (35). This system also
impacted the embryonic development of the lung, where the dnFgfr2b was expressed in an
inducible reverse tetracycline trans-activator (rtTA)-system under the control of the Sftpc
promoter (which normally drives surfactant-associated protein C). In these mice,
alveolarization assessed at P25 was severely blunted, but only when doxycycline was applied
during the embryonic period, and not when doxycycline was applied from the day of birth
onwards. That study opened several questions, including: (i) did the dnFgfr2 equally and
effectively neutralize all FGF ligands; and (ii) when doxycycline was applied starting in the
post-natal period, when the alveolo-capillary barrier is relatively well organized (compared
with the embryonic stages), did the Sftpc-driven dnFgfr2b reach the fibroblast subpopulations
in question, since the dnFgfr2b is known to selectively bind certain FGF ligands and not
others (9)? In the event of the autocrine Fgf18/Fgfr3 and Fgf10/Fgfr1 loops proposed in the
study of McGowan and McCoy discussed here (27) being operative during lung
alveologenesis, the close proximity of ligand to receptor in autocrine signaling may have
prevented interference by the Sftpc-driven dnFgfr2b.

Lawrence Prince and colleagues have documented that Fgf10 expression in the
developing septa is dramatically reduced in clinical BPD and that Fgf10 was required for
saccular stage branching in developing mouse lungs (4); furthermore, the localization of the
myofibroblasts in the developing lung parenchyma of BPD patients was abnormal. Thus,
these investigators suggested that perturbations to Fgf10 signaling in lung fibroblasts may
underlie aberrant migration of myofibroblasts in the lungs of BPD patients. The study of
McGowan and McCoy discussed here (27) documents that Fgf10 expression was higher in GFP\textsuperscript{low} versus GFP\textsuperscript{high} cells, suggesting that Fgf10 signaling might act in an autocrine pathway in lipofibroblasts, because Fgfr1-IIIb (an Fgf10 receptor) was also enriched in GFP\textsuperscript{low} versus GFP\textsuperscript{high} cells. A functional role of the Fgf10/Fgfr1-IIIb axis in lipofibroblasts in the developing lung awaits experimental demonstration. However, in light of the data generated by the Prince and colleagues (4), and reports that Fgf10 marked a progenitor cell population relevant to lung development (14, 15), this possibility also warrants study in the context of blunted lung alveologenesis in BPD.

The intriguing idea of autocrine Fgf18/Fgfr3 and Fgf10/Fgfr1 loops regulating fibroblast subset behavior is an exciting concept in lung alveologenesis. The increasing availability of transgenic reporter mice, such as the dual- and multi-color report systems including the brainbow and confetti reporter mice, open up new opportunities for the \textit{in vivo} demonstration of these regulatory pathways during late lung development. These reporter systems, when combined with the appropriate driver lines would allow for the definitive demonstration of the existence of autocrine loops, \textit{in situ}, in developing mouse lung. Along these lines, the conditional, inducible deletion FGF ligands in mesenchymal cells would permit validation of the autocrine loop idea for the ligand/receptor pairs \textit{in vivo}. Given the apparent importance of divergent FGF signaling pathways to alveologenesis, addressing these ideas in a pathological context is very important. In particular, to extend these studies to explore roles for the proposed Fgf18/Fgfr3 and Fgf10/Fgfr1 signaling pathways in arrested alveolarization, for example, the blunted secondary septation seen in rodent (5, 34) or other animal models (12, 41, 49) of BPD, where mesenchymal cell dynamics are altered (48).

The study by McGowan and McCoy (27) discussed here provides some insight into the roles potentially played by FGF signaling in fibroblast subset dynamics during lung alveologenesis, an area of emerging interest in normal and aberrant late lung development. These studies provide a firm basis for further work that should address functional roles for
FGF signaling both in PDGFRα\(^+\) and PDGFRα\(^-\) fibroblast subsets in normally and aberrantly developing alveoli.

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The authors have no disclosures to make.

**AUTHOR CONTRIBUTIONS**

J.R.C. and R.E.M. wrote the manuscript.

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26. McGowan SE, Grossmann RE, Kimani PW, and Holmes AJ. Platelet-derived growth factor receptor-alpha-expressing cells localize to the alveolar entry ring and have


Fig. 1. Possible interactions between constituent cells of the developing septum mediated by the fibroblast growth factor axes. The fibroblast growth factor (FGF) system interactions proposed in the report of McGowan and McCoy (27) are indicated in the background of GFP\textsuperscript{low} (dim green, believed to be lipofibroblasts) and GFP\textsuperscript{high} (bright green, believed to be myofibroblasts) stylized cells. The interactions are represented in the context of ligand and receptor elements associated with GFP\textsuperscript{negative} (brown; believed to be lung fibroblasts that do not express platelet-derived growth factor receptor-$\alpha$) stylized cells; as well as epithelial (blue) and endothelial (red) cells. Block arrows indicate interactions proposed by McGowan and McCoy. Solid arrows indicate possible interactions between septal cells based on the observations of McGowan and McCoy. Dashed arrows indicate alternative or additional FGF signaling axes that may be operative in the developing septum that have not yet been addressed experimentally. A question mark (?) indicates that the identity of the ligand or receptor has not been experimentally demonstrated. The production of different FGF ligands by the fibroblast subsets depicted has \emph{not} been considered in the scheme. Abbreviations: Adm, adrenomedullin; FGF, fibroblast growth factor; FGF-L, FGF ligand; FGF-R, FGF receptor; GFP, green fluorescent protein; sCG, soluble guanylate cyclase.
Figure 1