Evidence for the involvement of fibroblast growth factor 10 in lipofibroblast formation during embryonic lung development

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ABSTRACT
Lipid-containing alveolar interstitial fibroblasts (lipofibroblasts) are increasingly recognized as an important component of the epithelial stem cell niche in the rodent lung. Although lipofibroblasts were initially believed merely to assist type 2 alveolar epithelial cells in surfactant production during neonatal life, recent evidence suggests that these cells are indispensable for survival and growth of epithelial stem cells during adulthood. Despite increasing interest in lipofibroblast biology, little is known about their cellular origin or the molecular pathways controlling their formation during embryonic development. Here, we show that a population of lipid-droplet-containing stromal cells emerges in the developing mouse lung between E15.5 and E16.5. This is accompanied by significant upregulation, in the lung mesenchyme, of peroxisome proliferator-activated receptor gamma (master switch of lipogenesis), adipose differentiation-related protein (marker of mature lipofibroblasts) and fibroblast growth factor 10 (previously shown to identify a subpopulation of lipofibroblast progenitors). We also demonstrate that although only a subpopulation of total embryonic lipofibroblasts derives from Fgf10+ progenitor cells, in vivo knockdown of Fgfr2b ligand activity and reduction in Fgf10 expression lead to global reduction in the expression levels of lipofibroblast markers at E18.5. Constitutive Fgfr1b knockouts and mutants with conditional partial inactivation of Fgfr2b in the lung mesenchyme reveal the involvement of both receptors in lipofibroblast formation and suggest a possible compensation between the two receptors. We also provide data from human fetal lungs to demonstrate the relevance of our discoveries to humans. Our results reveal an essential role for Fgf10 signaling in the formation of lipofibroblasts during late lung development.

KEY WORDS: Fgf10, Lipofibroblasts, Lung, Mesenchyme

INTRODUCTION
Lipofibroblasts (LIFs) are lipid-droplet-containing interstitial fibroblasts that are located within close proximity to type 2 alveolar epithelial cells (AECIIs; McGowan and Torday, 1997; O’Hare and Sheridan, 1970). LIFs contribute to the production of pulmonary surfactant by assimilating neutral lipids and transferring them to AECIIs for final processing of the surfactant. They also contain retinoid acids that are essential for the promotion of postnatal alveolar septation, a crucial process that increases blood-gas exchange surface (Simon and Mariani, 2007). LIFs are detected in the embryonic rat lung starting at embryonic day (E) 16; thereafter their numbers increase, peaking during the second postnatal week, when alveologenesis is at its peak (Tordet et al., 1981). Although LIFs have been studied in postnatal lungs, their exact cellular origin and mechanism of differentiation remain unknown.

LIFs are present in adult human and mouse lungs (Kaplan et al., 1985; Rehan et al., 2006). In vitro models of LIF differentiation from mesenchymal progenitors, using either the human embryonic lung fibroblast cell line (WI-38) or neonatal and adult human lung biopsies, have helped to establish some of the crucial regulators of LIF differentiation (Rehan et al., 2006). Previous reports have shown that Thyl+ but not Thyl− cells are able to acquire LIF characteristics, such as lipid-droplet accumulation and collagen formation, shortly after initiation of in vitro culture (Penney et al., 1992). Recently, it has been proposed that LIFs could contribute to the AECII stem-cell niche in the adult lung (Barkauskas et al., 2013).

LIFs share common characteristics with adipocytes, and it is already established that peroxisome proliferator-activated receptor gamma (Pparg), the master regulator of adipogenesis, is also required for the maintenance of the LIF phenotype (Torday et al., 2003). In LIFs, Pparg is downstream of parathyroid hormone-related protein (Pthrp; Pthlh – Mouse Genome Informatics) signaling, and it has been shown that in vivo inactivation of the Pthrp pathway leads to abnormal alveolarization with defective surfactant synthesis (Rubin et al., 2004). After Pparg activation, LIFs express adipose differentiation-related protein (Adrp; Plin2 – Mouse Genome Informatics), a trafficking protein that escorts lipid substrates within the LIF cytosol and delivers them to adjacent AECIIIs (Schultz et al., 2002).

Fibroblast growth factor 10 (Fgf10) is expressed by both pre- and mature adipocytes and plays an important role in the differentiation of pre-adipocytes in the white adipose tissue via activation of the Pparg pathway (Sakaue et al., 2002). Interestingly, Fgf10 also stimulates cell proliferation in white adipose tissue (Konishi et al., 2006), and Fgf10-null embryos display impaired white adipose tissue formation (Asaki et al., 2004; Mailleux et al., 2002). A recent study demonstrated that postnatal day (P) 8 mouse lipofibroblasts...
expressing low levels of platelet derived growth factor receptor, alpha polypeptide (Pdgfra) express high levels of Fgf10 and its receptors Fgfr1b and Fgfr2b (McGowan and McCoy, 2015). Using the Fgf10Cre lineage-tracing tool (El Agha et al., 2012), we have shown that Fgf10+ cells are progenitors for LIFs, among other lineages, in the developing mouse lung (El Agha et al., 2014). Based on that and extrapolating our knowledge about the role of Fgf10 in adipocytes to pulmonary LIFs, we hypothesized that Fgf10 signaling plays an important role in the formation of pulmonary LIFs. To our knowledge, there are currently no reported studies on the role of Fgf10 signaling in the formation of LIFs.

To test our hypothesis, enhancer-trap, transgenic and knock-in mouse lines were used to identify the Fgf10-expressing lipofibroblast subpopulation and to study the consequences of disrupting Fgf10 signaling for overall LIF formation in the developing mouse lung. The impact of mesenchymal Fgfr2b deletion and of ubiquitous Fgfr1b deletion on that and extrapolating our knowledge about the role of Fgf10 in adipocytes to pulmonary LIFs, we hypothesized that Fgf10 signaling plays an important role in the formation of pulmonary LIFs. To our knowledge, there are currently no reported studies on the role of Fgf10 signaling in the formation of LIFs.

RESULTS

Lipofibroblast formation increases progressively during embryonic lung development

Given that the emergence of LIFs in the embryonic mouse lung was unexplored, we first quantified the relative number of lipid-droplet-containing cells between E13.5 and E18.5 by LipidTOX staining followed by fluorescence activated cell sorting (FACS). LipidTOX is a dye that labels neutral lipids that are abundantly present in LIFs. Our results indicated that LipidTOX+ cells emerged between E15.5 and E16.5, and they represented up to 30% of the total cell count in the developing lung (Fig. 1A,B). Next, the expression levels of Adrp, Pparg and Fgf10 were examined throughout lung development by qPCR (Fig. 1C). Adrp expression showed very low levels between E11.5 and E15.5 and was upregulated beginning at E16.5, peaking at E18.5. Pparg expression was first detected at E15.5 and increased progressively up to E18.5. Fgf10 expression increased steadily from E11.5 to E18.5.

Following intraperitoneal (IP) injection of tamoxifen into Fgf10Cre/+;tomatolox/+ mice at E11.5 or E15.5, ~30 and 40% of labeled cells, respectively, trace to Adrp+ LIFs when quantified at E18.5 (El Agha et al., 2014). In the present study, IP injection of tamoxifen was carried out twice, at E11.5 and E14.5, in order to maximize the labeling of LIFs derived from Fgf10+ cells (Fig. 1D). Immunofluorescence (IF) staining for Adrp in the resulting E18.5 lungs showed that only a small proportion of Adrp+ cells are also RFP+ (Fig. 1E). However, quantification of IP indicated that ~65% of the RFP+ cells are Adrp+ (Fig. 1F), demonstrating the increased efficiency of our double tamoxifen injection approach to mark Fgf10+ LIFs. LipidTOX staining followed by FACS analysis was also used to confirm these data. Our data indicated that RFP+ cells represent ~4% of the total lung cells, of which 66.5% are also LipidTOX+ (Fig. 1H). The same analysis showed that 8.37% of total LipidTOX+ cells are RFP+ at E18.5 (Fig. 1G).

Next, we used the previously reported Fgf10-lacZ enhancer-trap mouse line to monitor Fgf10 expression during neonatal life, and showed that a subset of Fgf10+ cells displays LIF characteristics at P5 [Adrp+, Oil Red O (ORO)++; Fig. S1]. The Adrp/β-gal double-positive cells represented ~27% of total LIFs at this stage. In conclusion, Fgf10-expressing cells and their descendants represent only a fraction of the total LIF population in the developing mouse lung. In order to test whether Fgf10 signaling is involved in LIF formation, we used two independent but complementary approaches: attenuation of Fgfr2b signaling and generation of Fgf10 hypomorphic lungs.

Attenuation of Fgfr2b ligand activity leads to impaired lipofibroblast formation

To attenuate Fgfr2b ligand activity, we used our previously reported and validated double-transgenic (DTG) system to overexpress a soluble form of Fgfr2b in a ubiquitous fashion in the embryo upon doxycycline exposure (Parsa et al., 2010, 2008; Volckaert et al., 2011). Soluble Fgfr2b acts as a decoy receptor to block all Fgfr2b ligands, including Fgf10, potentially expressed at the time of doxycycline administration. Based on the expression pattern of LIF markers and the LipidTOX staining during lung development (Fig. 1B,C), doxycycline was administered between E14.5 and E18.5 to attenuate Fgfr2b ligand activity (Fig. 2A). Compared with control lungs (n=4), DTG lungs (n=4) were smaller (Fig. 2C versus B) and displayed dilated distal airways and thickened mesenchyme (Fig. 2E versus D), indicating an arrest in overall lung development. Adrp IF and ORO staining showed a decrease in Adrp expression (Fig. 2H,I versus F,G) and lipid-droplet content (Fig. 2L,M versus J,K) in DTG compared with control lungs. The observed decrease in Adrp expression and ORO staining was validated by qPCR. DTG lungs displayed a clear reduction in the expression levels of Pparg (Fig. 2N; P<0.01) and Adrp (Fig. 2O; P<0.001) compared with control lungs. The decrease in Pparg expression was also observed at the protein level as shown by western blotting (Fig. 2P).

Fgf10 hypomorphs display impaired lipofibroblast formation

As a second approach, the impact of decreased Fgf10 expression on LIF formation during lung development was investigated. Given that the complete deletion of Fgf10 leads to lung agenesis (Sekine et al., 1999), the Fgf10 hypomorphic allele was used (Mailleux et al., 2005; Ramasamy et al., 2007) to generate E18.5 Fgf10Cre/+;tomatolox (control) and Fgf10Cre/lacZ,tomatolox (hypomorphic) lungs (Fig. 3A). The Fgf10Cre+ allele is phenotypically equivalent to the Fgf10-null allele (El Agha et al., 2012). This experimental set-up allows investigation of the impact of decreased Fgf10 expression on global LIF formation (paracrine and autocrine effects of Fgf10) and the impact of decreased Fgf10 expression on the contribution of Fgf10-expressing cells to total LIFs (autocrine effect of Fgf10).

Impaired hindlimb development was observed in Fgf10Cre/lacZ;tomatolox embryos, thus validating phenotypically the reduced Fgf10 expression (data not shown). The Fgf10Cre/lacZ experimental lungs were smaller, lacked the accessory lobe (arrow in Fig. 3C versus B) and displayed thicker intersaccular walls and hemorrhagic areas upon histological analysis (data not shown). Gene expression analysis revealed a significant decrease in Fgf10 expression (Fig. 3F; P<0.01) in hypomorphic lungs (n=3) compared with littermate controls (n=3). qPCR also showed a significant reduction in the expression levels of Pparg (Fig. 3G; P<0.05) and Adrp (Fig. 3H; P<0.05) in hypomorphic lungs compared with littermate controls.

Immunofluorescence for Adrp showed that hypomorphic lungs exhibit a global decrease in Adrp expression compared with controls (Fig. 3K,L versus J). Quantification of the Adrp signal using MetaMorph software revealed a significant decrease in the number of Adrp (Fig. 3M; P<0.05) and area (Fig. 3N; P<0.05) of Adrp...
immunoreactive spots in hypomorphic lungs (n=3) compared with littermate controls (n=3).

The Tomato reporter allele was introduced in the background of Fgf10Cre/+ (control) and Fgf10Cre/lacZ (hypomorph) embryos to label Fgf10+ cells at E14.5 with a single IP injection of tamoxifen (Fig. 3A,D,E), allowing investigation of the autocrine effect of Fgf10. Lineage-labeled cells in the background of Adrp IF do not display any significant difference between control and hypomorphic lungs in terms of Adrp spot number (Fig. 3S; P=0.2568). However, we observed a significant decrease in the area of Adrp immunoreactive spots (Fig. 3T; P<0.05) in the hypomorphs (n=3) compared with controls (n=3; Fig. 3Q,R versus O,P). These data suggest a defect in lipid-droplet formation that results in impaired differentiation of LIFs.

Fgf10 acts on the mesenchyme to induce lipofibroblast formation

In order to rule out any contribution from the epithelium to LIF formation and to demonstrate a direct effect of Fgf10 on mesenchymal cells, primary lung mesenchyme was cultured at different embryonic stages (E14.5-E18.5). Following differential adhesion, unattached cells were washed and mesenchymal cells allowed to grow for 24 h before RNA isolation (Fig. 4A). The cells remaining in the primary culture consisted of a heterogeneous population of mesenchymal cells. In cultures derived from E18.5 lungs, lipofibroblasts represented only about one-third of the total adherent cells (Fig. S2). Fig. 4B shows representative images for Cdh1 IF performed on primary mesenchymal cells, indicating minimal epithelial contamination. qPCR revealed low expression...
levels for Pparg (Fig. 4D) and Adrp (Fig. 4E) at E14.5 and E15.5 with increased expression levels between E16.5 and E18.5. This is consistent with the data obtained with lung homogenates (Fig. 1C), thus indicating upregulation of Pparg and Adrp in the lung mesenchyme during late embryonic stages. Parallel to the increase in LIF markers, Fgf10 expression was also increased in late embryonic stages (Fig. 4C). Strikingly, Fgfr2b and Fgfr1b, the genes encoding the two Fgf10 receptors, were upregulated in late embryonic lung mesenchyme, with the increase in Fgfr2b expression preceding that observed for Fgfr1b (Fig. 4F,G).

In order to demonstrate a direct effect for Fgf10 on mesenchymal cells, primary mesenchymal cultures of E19 rat lungs were treated with recombinant human (rh)FGF10. ORO staining after 24 h revealed a significant increase in the number of lipid-droplet-containing cells upon rhFGF10 treatment (Fig. 4I versus H; J; P<0.05). When treated with increasing concentrations of rhFGF10, these cells showed a dose-dependent increase in Pparγ as detected by western blotting (Fig. 4K,L; P<0.05).

Analysis of Fgfr1b-null lungs suggests a compensatory role for Fgfr2b in lipofibroblast formation

Given the observed sequential expression of Fgfr2b and Fgfr1b in the lung mesenchyme (Fig. 4F,G), we tested whether Fgf10 signaling in the mesenchyme via these receptors is involved in LIF formation. First, the phenotype of E18.5 Fgfr1b knockout lungs was examined, and analysis by qPCR confirmed the absence of Fgfr1b expression in knockout lungs (n=4) compared with control lungs (n=7; Fig. 5A; P<0.01). Hematoxylin and Eosin (H&E) staining did not show any apparent phenotype in knockout lungs compared with control lungs (Fig. 5H,I versus F,G). Interestingly, qPCR analysis showed that Pparg was significantly increased in the knockouts compared with controls (Fig. 5C; P<0.01), and this was accompanied by a modest increase in Adrp expression (Fig. 5D; P=0.4762). Surprisingly, Fgfr2b expression was increased (Fig. 5E; P<0.01), whereas Fgf10 expression levels remained unchanged (Fig. 5B; P=0.4082).

The expression levels of Adrp were also investigated at the protein level by IF. The staining revealed a slight increase in Adrp immunoreactivity in the knockouts (Fig. 5J,K) compared with controls (Fig. 5F,I). Quantification of the Adrp signal using MetaMorph software showed a trend towards an increase in the number (Fig. 5N; P=0.0629) and area (Fig. 5O; P=0.2921) of Adrp immunoreactive spots in knockout lungs (n=3) compared with control lungs (n=3).

Our results therefore indicate that ubiquitous deletion of Fgfr1b in the lung does not compromise LIF formation during embryonic development; instead, there is a trend towards increasing LIF formation. Interestingly, the elevated levels of Fgfr2b in Fgfr1b knockout lungs suggest that Fgfr2b could be compensating for the loss of Fgfr1b, thereby allowing normal and even enhanced LIF formation.

Fig. 2. Attenuation of Fgfr2b ligand activity leads to impaired LIF formation. (A) Inducible mouse model used to overexpress the soluble form of Fgfr2b receptor acting as a decoy receptor for Fgfr2b ligands between E14.5 and E18.5. (B,C) Whole-mount views of control (B) and DTG (C) lungs. (D,E) H&E staining of control (D) and DTG (E) lung sections. (F) IF for Adrp in control lungs. (G) High magnification of F. The inset in G shows a higher magnification. (H) IF for Adrp in DTG lungs. (I) High magnification of H. The inset in I shows a higher magnification. (J,K) ORO staining of frozen sections from control lungs. (L) High magnification of J. The inset in K shows a higher magnification. (M) ORO staining of frozen sections from DTG lungs. (N) qPCR for Pparg and Adrp showing decreased expression levels in DTG lungs compared with control lungs. (P) Western blot showing reduction in Pparg expression in DTG lungs compared with control lungs. Scale bars: 50 µm in D,J,L; 100 µm in F,H. n=4 per group. **P<0.01, ***P<0.001. DTG, double transgenic.
Partial loss of mesenchymal Fgfr2b expression impairs lipofibroblast formation

So far, we have shown that Fgfr2b is expressed in mesenchymal cells and that Fgf10 acts directly on mesenchymal cells to promote LIF formation. To test whether mesenchymal Fgfr2b signaling plays a role in LIF formation in vivo, we carried out a partial loss of Fgfr2b expression in the lung mesenchyme. Conditional genetic deletion of Fgfr2b in the lung mesenchyme was achieved by IP injection of female mice carrying Tg(Tbx4-LMECreERT2)/+; fgfr2bflox/+ (control) and Tg(Tbx4-LMECreERT2)/+; fgfr2bflox/lox (mutant) embryos with tamoxifen at E14.5. The lungs from mutant embryos are therefore expected to exhibit a 50% decrease in Fgfr2b expression in the mesenchyme only. The recombination of the Fgfr2bflox allele in E18.5 mutant lungs was validated by PCR (Fig. 6A). No major phenotypic differences were observed between mutant and control lungs at E18.5 (Fig. 6D,E versus B,C). However, qPCR analysis indicated a trend towards decreased Fgfr2b expression (Fig. 6F; \(P = 0.0995\)). A statistically significant reduction in Adrp expression was demonstrated (Fig. 6G; \(P < 0.05\)), suggesting defective LIF formation in mutant lungs (\(n = 5\)) compared with control lungs (\(n = 3\)). However, no significant differences were observed in the expression levels of Pparg (Fig. 6H; \(P = 0.2591\)) or Spry4 (as a readout for mesenchymal Fgf signaling; Fig. 6I; \(P = 0.9291\)).

Immunofluorescence for Adrp was also carried out, and the results indicated a decrease in Adrp staining in mutant lungs.
Fig. 6L,M) compared with control lungs (Fig. 6J,K). Quantification of the Adrp signal using MetaMorph software revealed a trend towards a decreased number (Fig. 6N; P=0.1037) and area (Fig. 6O; P=0.1716) of Adrp immunoreactive spots in mutant lungs (n=5) compared with control lungs (n=3).

Our results therefore indicate that deletion of one Fgfr2b allele in the lung mesenchyme starting at E14.5 impairs the LIF status at E18.5 at the RNA level and to a lesser extent at the protein level.

Relevance of lipofibroblast formation in mice to human lungs

In order to demonstrate the relevance of our findings to human lungs, the expression levels of FGF10 and ADRP were analyzed in human fetal lung homogenates between weeks 10 and 21 of gestation (3≤n≤6 for each time point). Our human data show a significant increase in FGF10 only between 10 and 18 weeks of gestation (Fig. 7A), whereas in the mouse Fgf10 expression rises constantly throughout lung development (Fig. 1C). Our results also indicate that ADRP expression is unchanged between 10 and 21 weeks of gestation (Fig. 7B). Interestingly, the start of Adrp expression in the mouse was seen between E15.5 and E16.5 (Fig. 1C), corresponding to weeks 20 and 22 of human gestation. These data suggest that LIF formation in human lungs is likely to occur past week 22, corresponding to the mid-canalicular stage of lung development.

Using the human embryonic lung fibroblast cell line (WI-38), we showed that treatment with rhFGF10 led to a significant increase in the number of lipid-droplet-containing cells (Fig. 7D versus C; E; n=3; P<0.05). The triolein uptake assay demonstrated increased lipogenesis in WI-38 cells treated with increasing concentrations of rhFGF10 (Fig. 7F; P<0.05). Treatment of WI-38 cells with
increasing concentrations of rhFGF10 also led to a dose-dependent increase in PPARg (Fig. 7G), thus reinforcing the proposed role of mesenchymal FGF10 signaling in LIF formation. Lastly, epithelial and mesenchymal cells were isolated from fetal human lungs at 16 weeks gestational age and RNA was isolated. Gene expression analysis showed an enrichment of FGF10 and FGFR1b expression in the mesenchymal fraction, whereas FGFR2b was enriched in the epithelial fraction (Fig. 7H-J; \( n = 2 \)).

**DISCUSSION**

Although pulmonary LIFs were first described in the 1970s and extensively studied in rodents, their lineage origin and molecular pathways leading to their formation are still unknown. Previously, we used Fgf10-lacZ mice to show that during early embryonic lung development (E12.5-E13.5) expression of Fgf10 identifies airway smooth muscle cell progenitors (Mailleux et al., 2005). More recently, using Fgf10\(^{Cre}\) knock-in mice, we showed that during late pseudoglandular and early saccular stages (E15.5-E18.5) Fgf10 is predominantly expressed in cells of the lung parenchyma compared with the airway smooth muscle cells, and these cells are mostly identified as LIFs (El Agha et al., 2014). Here, we provide evidence that Fgf10 plays a functional role in LIF formation.

LIFs and adipocytes share many aspects of their terminal differentiation program, known as lipogenesis. However, it is currently unknown whether the similarities between LIFs and adipocytes extend into earlier stages of lineage formation. The present findings provide new evidence that LIF formation depends on Fgf10 signaling, and this parallels the Fgf10 dependence of white adipose tissue formation reported earlier (Asaki et al., 2004; Sakaue et al., 2002). Interestingly, the role of the autocrine or...
paracrine effect of Fgf10, acting through Fgfr2b, in the development of the white adipose tissue has already been suggested (Ohta and Itoh, 2014). Here, we demonstrate that Fgf10 also signals in an autocrine or paracrine fashion in another mesenchymal and adipocyte-like cell type, the pulmonary LIF.

**Fgf10 expression identifies a distinct subset of pulmonary lipofibroblasts**

Our data identify a previously unappreciated heterogeneity of pulmonary LIFs. Based on our results, LIFs consist of Fgf10+ and Fgf10− subpopulations.

Previously, we reported that in Fgf10Cre mice, the partial deletion of transcription factor binding sites located in intron 1 of the Fgf10 gene led to decreased Cre expression compared with the endogenous Fgf10 expression (El Agha et al., 2012). Thus, the possibility that Fgf10− LIFs reflect inefficient labeling of Fgf10+ cells cannot be excluded. However, as lacZ− LIFs are also found in Fgf10-lacZ lungs, we deduce that there are at least two sources of LIFs in the embryonic lung, partly distinguishable by Fgf10 expression.

The difference between these two pools (Fgf10+ and Fgf10− LIFs) in terms of origin and fate is unclear. In addition, a crucial question remains open: what are the other endogenous factors contributing to LIF lineage specification and maturation? For example, it has been shown that lung Thy1+ cells differentiate into LIFs through activation of Pparg, whereas Thy1− cells differentiate into myofibroblasts (McIntosh et al., 1994; Phipps et al., 1990; Varisco et al., 2012). Pparg signaling is one of the major pathways described to date that is involved in the differentiation of LIFs (Ferguson et al., 2009; McGowan and Torday, 1997). In addition, ex vivo studies of human and mouse tissues suggest that endothelial cells within the developing adipose tissue can give rise to mature adipocytes (Tran et al., 2012), although this has been contested recently (Berry and Rodeheffer, 2013). The use of lineage-specific labeling for Thy1 and endothelial cells in the background of Fgf10Cre+;tomatofox/+
would help to identify whether these are distinct cell populations capable of giving rise to LIFs.

**Fgf10 plays a crucial role in lipofibroblast formation**

The overexpression of a dominant negative decoy receptor for Fgfr2b ligands demonstrated that Fgfr2b ligands are crucial for LIF formation during late lung development. In addition to Fgf10, Fgf1 and Fgf7 are the two other Fgfr2b ligands expressed in the developing lung (Bellusci et al., 1997). Contrary to Fgf10, which binds Fgfr2b and Fgfr1b, Fgf7 binds only to Fgfr2b, whereas Fgf1 binds to all Fgf receptors (Ornitz et al., 1996). Fgf7-null mice are viable and display impaired hair and kidney development (Guo et al., 1996; Qiao et al., 1999), whereas Fgf1-null mice develop enlarged adipocytes and inflammation of the adipose tissue only after a high-fat diet (Jonker et al., 2012). It remains possible that Fgf1 and Fgf7 signaling could also contribute to LIF formation. However, the data obtained with the Fgf10 hypomorphs strongly suggest a combined autocrine and paracrine mode for Fgf10 signaling during LIF formation, because both LIF subpopulations (Fgf10<sup>+</sup> and Fgf10<sup>−</sup>) are adversely affected by decreased Fgf10 expression. A recent study suggested an autocrine role for Fgf10 signaling in promoting lipid storage in LIFs, but with no evidence of such signaling (McGowan and McCoy, 2015). Considering the many similarities between LIFs and adipocytes and given the known role of Fgf10 in adipogenesis, it is reasonable to draw parallels between adipocytes and LIFs.

**Fgfr1b and Fgfr2b play redundant roles in the mesenchyme as receptors for Fgf10**

Fgfr2b is the main Fgf10 receptor, as demonstrated by phenotypical similarities between Fgfr2b-null pups (De Moerlooze et al., 2000) and Fgf10-null pups (Sekine et al., 1999). However, Fgf10 also binds with high affinity to Fgfr1b (Ornitz et al., 1996), which is expressed by both the epithelium and the mesenchyme (Lee et al., 2008). In the early developing mouse lung, it is well accepted that mesenchymal Fgf10 acts on the epithelium through its receptor Fgfr2b, whereas a direct action on the mesenchyme does not occur until E14.5 (De Langhe et al., 2006). Our results suggest that during late lung development, Fgf10 signals to the mesenchyme through both Fgfr2b and Fgfr1b and that the two receptors play redundant
roles in terms of LIF formation. The expression of Fgfr2b in the lung mesenchyme is a novel finding that suggests a mechanism of alternative splicing to allow the expression of this receptor. In the future, it will be interesting to investigate what controls Fgfr2b expression in the mesenchyme. The generation of double conditional knockout of Fgfr1b/2b in the mesenchyme will also be important to clarify whether these two receptors are the only mediators of Fgf10 signaling in the lung mesenchyme.

Can Fgf10-regulated epithelial signals also contribute to the formation of lipofibroblasts during development?

The present work shows clearly that Fgf10 acts directly on the mesenchyme and that Fgfr1b and Fgfr2b are likely to play redundant roles as receptors for Fgf10. However, Fgf10 also acts on the distal epithelial progenitor cells expressing Fgfr2b throughout lung development. Therefore, it is possible that some of the effects observed upon reduction of Fgf10 levels or upon inhibition of the Fgfr2b ligand activity might result from the loss of epithelium-derived signals that would also impact LIF formation.

We also investigated the expression levels of Sfpdc and Scgb1a1 in the various mutants by qPCR (Fig. S3). Sfpdc expression levels were significantly reduced in Fgfr2b attenuated (Fig. S3A; P<0.001) and Fgf10 hypomorphic lungs (Fig. S3C; P<0.01) but not in Fgfr1b knockout (Fig. S3E) or Fgfr2b conditional knockout lungs (Fig. S3G) compared with littermate controls. Scgb1a1 expression levels did not display significant changes in any of these mutants (Fig. S3B,D,F,H). In the future, the possible involvement of epithelium-derived signals in LIF formation upon the manipulation of Fgf10 signaling can be tested by knocking out Fgfr1b and Fgfr2b in the lung epithelium (specifically in Sftpdc+ cells) at E14.5 and examining LIF formation at E18.5.

In conclusion, our findings identify, for the first time, the developmental heterogeneity of pulmonary LIFs and specifically describe the Fgf10+ LIF subpopulation. We demonstrate that Fgf10 signals in an autocrine or paracrine fashion to promote the formation of LIFs, probably through Pparg activation. Our data from human fetal tissues and cells suggest that the role of Fgf10 signaling in LIF formation in mice can be extrapolated to humans. Fgf10+ cells and LIFs represent important under-studied cell populations in the lung that might be crucial for the repair process after injury during adult life.

MATERIALS AND METHODS

Ethics statement

Human fetal biopsies at different gestational ages were obtained under IRB approval CHLA-14-2211. Exclusion criteria were human immunodeficiency virus and hepatitis. All the samples used in this study had no known genetic abnormalities.

Animal experiments were performed under the research protocol approved by the Animal Research Committee at Children’s Hospital Los Angeles and Labiomed at Harbor UCLA and in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The approval identification for the studies at the University Giessen (approval numbers 405_M, 437_M and 452_M). 31-08 and 253-11). Mouse protocols were also approved by Justus Liebig Children’s Hospital Los Angeles and Labiomed at Harbor UCLA and in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The approval identification for studies at the University Giessen. Children’s Hospital Los Angeles is AAALAC A3276-01 (protocols 31-08 and 253-11). Mouse protocols were also approved by Justus Liebig University Giessen (approval numbers 405_M, 437_M and 452_M).

Mice

The Rosa26Tomato/CreERT2;Fgffr2b+/- double heterozygous mice were previously described (Parsa et al., 2008). To attenuate Fgf2b ligands, pregnant females carrying double-transgenic embryos and single-transgenic littermate controls were put on a doxycycline-containing diet (normal rodent diet with 0.0625% doxycycline; Harlan Teklad). For lineage tracing and sorting of Fgf10+ cells, we used Fgf10CreERT2 mice crossed with Tomarbox/flox mice reporter mice as previously described (El Agha et al., 2012, 2014). Females carrying Fgf10CreERT2;Tomato/lox+/- embryos received two IP injections of tamoxifen (0.1 mg/g of body weight each) at E11.5 and E14.5, and lungs were collected at E18.5. In order to perform lineage tracing in the context of Fgf10 hypomorph, Fgf10CreERT2;Tomato/lox+/- mice were crossed with Fgf10 lacZ/+ mice (Kelly et al., 2001) to generate Fgf10CreERT2;Tomato/lox+/- (control) and Fgf10CreERT2;lacZ/lox+/- (hypomorph) embryos. The Fgf10CreERT2 allele corresponds to a loss-of-function allele for Fgf10 (El Agha et al., 2012), whereas the Fgf10-lacZ allele is a hypomorphic allele for Fgf10 (Ramasamy et al., 2007).

FACS sorting

Lungs from E18.5 Fgf10CreERT2;Tomato/lox+/- embryos were dissected in Hank’s balanced salt solution (HBSS) on ice, put into a mixture of 0.5% collagenase-DNase in HBSS, cut into small pieces and incubated for 30 min at 37°C with vigorous shaking. The cells were then stained through 100 μm followed by 40 μm cell strainers, washed and counted. Cells were stained with CD45 for 30 min on ice to exclude blood cells. The endogenous Tomato signal was detected using the PE channel. DAPI was used to exclude dead cells. The analyses and sorting were performed using a FACSaria III cell sorter and FACS Diva version 6.1.3 (BD Biosciences). Gates were set according to unstained controls.

Histology

After fixation in 4% PFA, lungs were washed in PBS, dehydrated in graded ethanol solutions, cleared in xylene and embedded in paraffin. For immunostaining, 5-μm-thick sections were cleared twice with xylene and rehydrated, equilibrated in water and then washed in PBS. Antigen retrieval was performed in citrate buffer (pH 6) at 95°C for 15 min. H&E staining was done according to standard procedures. For immunostaining, slides were incubated with primary antibodies against Acta2 (Sigma, C6198; 1:200), Cdh1 (BD Biosciences, 610181; 1:200), Sfpdc (Seven Hills, WRAB-09337; 1:150) and Adrp (Abcam, ab52356; 1:150) at 4°C overnight. For Adrp immunofluorescence (IF) staining, fluorescein isothiocyanate-conjugated secondary antibodies (Jackson Laboratories, 111-095-046; 1:1000) were used. MetaMorph software (Leica Microsystems) was used for quantifying Adrp immunoreactive spots (count and size). Three to four 40x fields per sample were analyzed. Oil red O staining was performed as previously described (Rehan and Torday, 2003; Rehan et al., 2007, 2006). Immunohistochemistry was performed with Dako EnVision kit according to the manufacturer’s instructions. Fluorescence images were acquired using a Leica monochrom camera attached to a Leica DM4000B microscope. Bright-field and differential interference contrast images were acquired using an AxioCam MRc color CCD camera (Zeiss Microimaging).

Primary culture of lung fibroblasts and WI-38 cell line

Whole lungs were dissected from embryos, minced into small pieces and subjected to 0.025% trypsin (20 min at 37°C for rat lungs) or 0.5% collagenase IV (45 min at 37°C for mouse lungs) digestion to give rise to single cells. Mesenchymal cells were separated from epithelial cells by differential adhesion as described previously (Lebeche et al., 1999). Thirty minutes after plating, floating cells and media were removed and adherent cells were washed several times with PBS. Human embryonic fibroblast cell line WI-38 was cultured in the presence or absence of different concentrations of recombinant human (rh)FGF10 (0, 1, 10, 100, 250 ng/ml; R&D Systems, 345-FG-025/CF).

Isolation of fetal human lung cells

Human distal lung pieces, cut at 1 mm thickness from the edge of a specimen at gestational age 16 weeks, were placed into a Miltenyi C-tube (Kelly et al., 2001) to generate Fgf10-lacZ/+ lungs was performed as previously described (Al Alam et al., 2011).

X-gal staining

lacZ staining of postnatal Fgf10-lacZ lungs was performed as previously described (Al Alam et al., 2011).

DEVELOPMENT
the gentleMACS dissociator. After washing and red blood cell lysis, the cell suspensions were filtered through a 40 µm filter, counted and blocked with Fc fragments for 15 min. After blocking, the cells were incubated with anti-CD45 microbeads (Miltenyi Biotec, 130-045-801; 10 µl per 10^7 cells), eluted through the column to exclude the CD45+ cells. The remaining cells were incubated with anti-CD326 microbeads to select positively for epithelial cells. After elution, the CD326+ cells were flushed from the magnetic column. RNA was extracted from the enriched epithelial and mesenchymalfractions depleted of CD45.

Gene expression analyses

RNA extracted from lung tissues or cells was reverse transcribed into cDNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science) according to the manufacturer’s instructions. cDNA was used for dual color Hydrolysis Probe–Universal probe library-based quantitative real-time (qPCR), using the LightCycler 480 from Roche Applied Science. Primers were designed using the Roche Applied Science probe-based assay design center. Hprt gene or Gapdh assay, commercially available from Roche Applied Science, was used as the reference gene.

Western blotting

Whole lungs or cultured lung mesenchymal cells were disrupted immediately in RIPA buffer containing protease inhibitors. Equal amounts of total lung protein (50 µg) from each assayed sample were used for chemiluminescent western analysis (Roche Molecular Biochemicals) on immunoblot PVDF membranes (Bio-Rad). Immunoblotting was performed using antibodies against PPARγ (Santa Cruz, sc-7196; 1:200) and GAPDH (Chemicon, MAB 374; 1:1000).

Triolein uptake

Triolein uptake by the LIFs was measured as previously described (Karadag et al., 2009). Briefly, lung tissues were minced into small pieces and incubated with [3H]tri olein (5 µCi/ml) in Dulbecco’s modified Eagle’s medium supplemented with 20% rat serum. The tissue pieces were then washed twice with 1 ml of ice-cold PBS and homogenized. A portion of the homogenate was used for protein extraction and the remainder for neutral lipoid content.

Statistical analyses

Statistical analyses were performed using GraphPad Prism software. Non-parametric tests were used to compare two groups and one way-ANOVA was used to compare multiple groups. Data are presented as mean values± s.e.m. The results were considered significant if P<0.05.

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Competing interests

The authors declare no competing or financial interests.

Author contributions


