

Fachbereich Medizin der Justus-Liebig-Universität Gießen

Zentrum für Kinderheilkunde und Jugendmedizin

Abteilung für Allgemeine Pädiatrie und Neonatologie

Leiter: Univ.-Professor Dr. med. Klaus-Peter Zimmer

**„Fgf10 and epigenetic regulations in lung development and
Bronchopulmonary dysplasia“**

**„Fgf10 und epigenetische Regulationen während der Lungenentwicklung
und bei der Bronchopulmonalen Dysplasie“**

Habilitationsschrift

zur Erlangung der Venia legendi des Fachbereichs Medizin
der Justus-Liebig-Universität Gießen

für das Fach

„Pädiatrie“

vorgelegt von

Cho-Ming Chao

Gießen 2019

Dedicated to my family!

Table of Contents

Abbreviations and Acronyms.....	3
1. Introduction.....	5
1.1 Lung development	5
1.2 Bronchopulmonary dysplasia – the most common incurable chronic lung disease of preterm infants.....	9
1.3 Fgf10 in lung development and BPD	12
1.4 Epigenetic regulations in lung development and BPD.....	17
1.4.1 MicroRNA in lung development and BPD.....	18
1.4.2 Histone modification in lung modelling and remodelling	26
1.5 Purpose of the study.....	28
2. Results – Original publications	29
2.1 Fgf10 in lung development.....	29
2.1.1 Appendix A	29
2.1.2 Appendix B	30
2.1.3 Appendix C	30
2.1.4 Appendix D	31
2.2 Fgf10 in BPD and lung regeneration.....	32
2.2.1 Appendix E	32
2.2.2 Appendix F	32
2.2.3 Appendix G.....	33
2.2.4 Appendix H.....	34
2.3 Epigenetic regulation in lung development and BPD.....	36

2.3.1 Appendix I.....	36
2.3.2 Appendix J.....	36
2.3.3 Appendix K.....	37
2.3.4 Appendix L.....	38
3. Discussion.....	39
3.1 Transgenic mouse lines: advantages and limitations.....	39
3.2 Limitations of the single-hit hyperoxia-based BPD mouse model.....	44
3.3 Clinically relevant aspects about FGF10 in human BPD.....	46
4. Future perspectives.....	48
5. Summary	50
6. References	52
7. Acknowledgements	73
8. Declaration	74
9. Bibliography	75
10. Appendix	86

Abbreviations and Acronyms

α -SMA	α -Smooth Muscle Actin
ADRP	Adipose differentiation-related protein
AT I	Alveolar epithelial cell type I
AT II	Alveolar epithelial cell type II
ALSG	Aplasia of lacrimal and salivary glands
BADJ	Broncho-alveolar duct junction
BMP 4	Bone morphogenetic protein 4
BPD	Bronchopulmonary dysplasia
BSA	Bovine serum albumin
CCSP	Club cell secretory protein
COPD	Chronic obstructive pulmonary disease
E	Embryonic
ETV5	Ets Variant 5
FEV1	Forced expiratory volume in one second
FGF10	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GFP	Green fluorescent protein
GLI1	Glioma-Associated Oncogene 1
HBSS	Hanks balanced salt solution
HOX	Hyperoxia
HRP	Horseradish peroxidase
ID2	Inhibitor of differentiation 2
ISL1	Insulin gene enhancer protein ISL-1
IVC	Inspiratory vital capacity
LADD	Lacrimo-auriculo-dento-digital syndrome
LIF	Lipofibroblast
MYF	Myofibroblast
NF- κ B	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NOX	Normoxia
P	Postnatal
PBS	Phosphate-buffered saline
PDGF	Platelet derived growth factor

PDPN	Podoplanin
PECAM	Platelet endothelial cell adhesion molecule
PFA	Paraformaldehyde
PPAR γ	Peroxisome proliferator-activated receptor gamma
PTHrP	Parathyroid hormone-related protein
RA	Retinoid acid
SCGB1A1	Secretoglobin, Family1A, Member 1
SDS	Sodium dodecyl sulfate
SEM	Subepithelial mesenchyme
SFTPC	Surfactant protein C
SFTPB	Surfactant protein B
SHH	Sonic hedgehog
SMM	Submesothelial mesenchyme
SOX 9	SRY (sex determining region Y)-box 9
SP1	Specificity Protein 1
SPRY 2	Sprouty 2
TAE	TRIS-Acetate-EDTA
TBS-T	TRIS-buffered saline with Tween 20
TGF- β 1	Transforming growth factor β -1
TLR	Toll-like receptor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WNT	Wingless and int

1. Introduction

1.1 Lung development

The human lung develops in four histologically distinguishable stages termed pseudoglandular (week 4-17), canalicular (week 17-26), saccular (week 26-36) and alveolar (week 36-8 years) stage (Figure 1).

The main event occurring during the pseudoglandular stage is branching morphogenesis which leads to the formation of a tree-like tubular structure of the lung, thereby establishing the conducting airway. On the cellular level, many progenitor cells differentiate and give rise to different cell populations (e.g. lymphatic cells, smooth muscle cells, nerve cells, endothelial cells, ciliated cells, chondrocytes, basal cells, neuroendocrine cells and secretory cells).

The canalicular stage is characterized by the formation of a double layer pulmonary vasculature throughout the embryonic lung. This is also the first time when alveolar bipotential progenitor cells differentiate into alveolar epithelial cell type I (AEC I) and type II (AEC II) [1], leading to the first primitive respiratory epithelium capable of gas exchange. Further, lipofibroblast (lipid-droplet-containing interstitial fibroblast or LIF) emerges in close proximity to AEC II in order to support them in the production of surfactant by providing them with triglycerides [2].

During the saccular stage, the lung is undergoing a massive expansion of the alveolar sacs, also termed primitive alveoli, and the capillary and lymphatic networks. Another essential event taking place at this stage is the thinning of the mesenchyme via apoptosis to allow proper oxygen diffusion from the alveolar epithelial surface towards the blood vessels located in the mesenchyme [3].

The alveolar stage is the last stage in lung development, and starts in utero and

continues up to five to eight years of age [4]. This stage is indispensable for the formation and enormous expansion of gas-exchanging units called alveoli via secondary septa formation. This results in a total alveolar surface area of approximately 75 m² consisting of about 300 Mio. Alveoli [5]. Up to date, the cell type considered to be most important and responsible for secondary septa formation is the alveolar myofibroblast. It is located at the tip of secondary septa and deposits elastin [6][7]. In order to facilitate more efficient oxygen diffusion and carbon dioxide release, the previous double layer capillary network undergoes microvascular maturation, giving rise to a delicate single capillary network.

Of note is the fact that humans are born during the alveolar stage but mice are born during the saccular stage of lung development. Therefore, the experimental mouse model using hyperoxia to induce a BPD-like lung phenotype to study BPD is a convenient animal model to simulate injurious events in a developmental stage of the lung in which usually preterm infants are born.

Further differences between human and mouse lung development are the number of lung lobes and the number of airway generations. While human lungs consist of three lobes (cranial, medial, caudal) on the right and two lobes (cranial and caudal) on the left, the mouse has 4 lobes (cranial, medial, caudal, accessory) on the right and one lobe on the left. Concerning the airway generations, 12 have been identified in the mouse lung compared to 23 in the human lung.

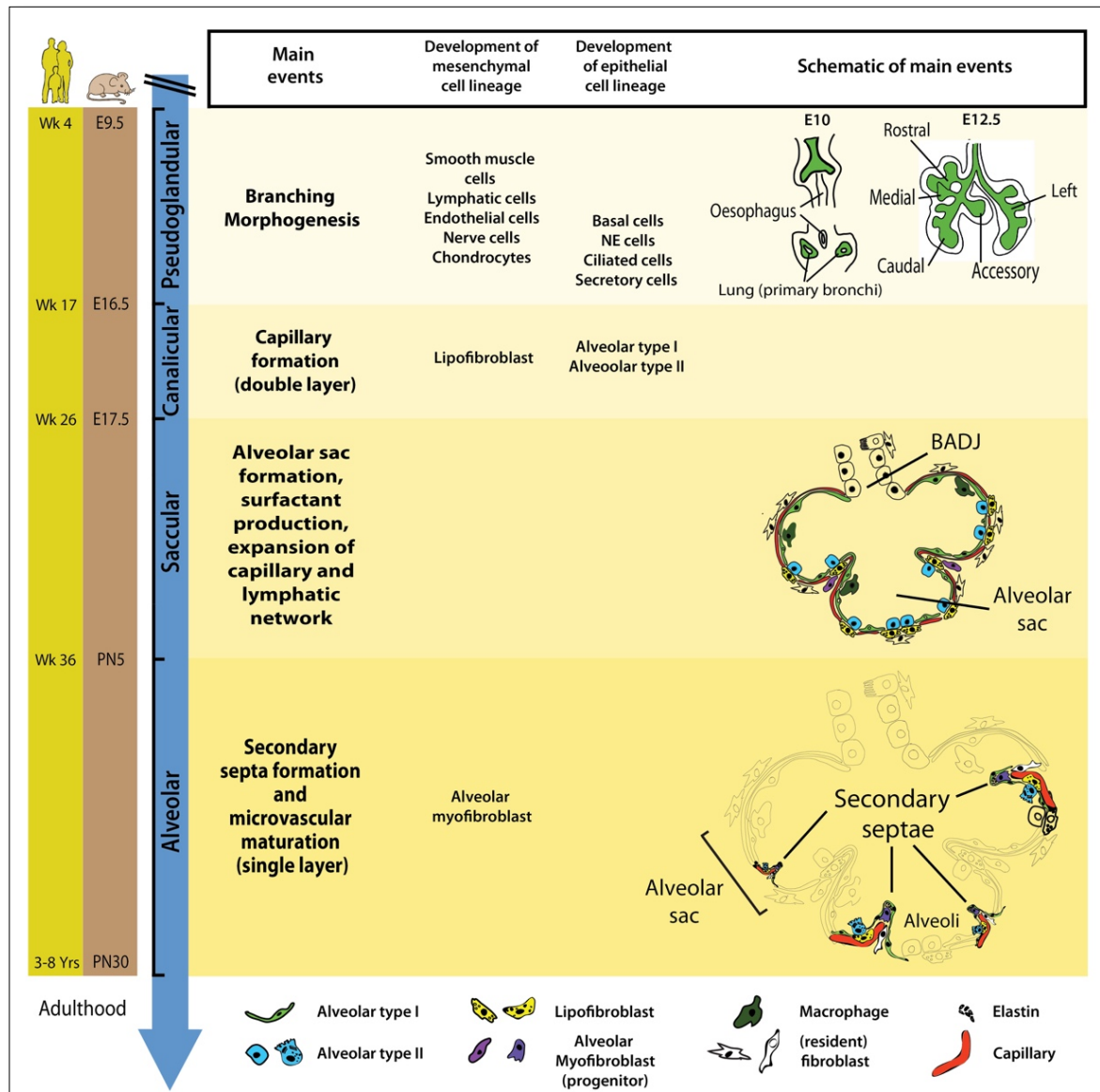


Figure 1. Stages of lung development in humans versus mice [8].

The schematic is illustrating the four stages of lung development in humans and mice including the main morphological changes and cell populations involved in each stage.

Wk: week; E: embryonic; PN: postnatal; NE: neuroendocrine

However, some authors suggest a division of lung development into two main programs: the branching morphogenesis program and the alveolar epithelial program [9][10].

During the branching morphogenesis program the distal epithelial progenitors give rise to several differentiated epithelial cell types of the conducting airway [10]. During the alveolar epithelial differentiation program the epithelial progenitors give rise to the AEC I/AEC II cells [1].

While in earlier times, scientists focused mainly on the lung epithelium, increasing attention has recently been paid to the lung mesenchymal compartment. Despite the tremendous efforts of developmental biologists in lung research, the complex crosstalk between mesenchymal and epithelial cell lineages during lung development still remains elusive. Furthermore, they assume that many of the progenitor cell populations/ sub-populations are still not yet defined.

Especially, a better understanding of the cellular and molecular processes underlying the formation of secondary septa is critical for the development of new therapies to protect or regenerate the alveoli – the crucial part of the lung which is affected in many lung diseases (e.g. bronchopulmonary dysplasia (BPD), chronic obstructive pulmonary disease (COPD), emphysema, and lung fibrosis).

1.2 Bronchopulmonary dysplasia – the most common incurable chronic lung disease of preterm infants

Bronchopulmonary dysplasia (BPD) is a chronic lung disease affecting about 40% of premature infants born before 28 weeks of gestational age [11][12][13]. The incidence of BPD correlates inversely with the birth weight and the gestational age. Since the first description of bronchopulmonary dysplasia by Northway more than 50 years ago [14], the number of survivors among very prematurely born infants has increased significantly due to improvement of the strategies in neonatal care. Despite this progress in the treatment of prematurely born infants, BPD incidence has not decreased and remains a common cause of morbidity and mortality. Apart from longterm sequelae causing lifelong morbidity, one common and potentially fatal complication during the neonatal period is pulmonary hypertension [15]. The longterm sequelae of BPD consist of neurodevelopmental and respiratory impairment, such as susceptibility to respiratory infections and pulmonary hypertension [16][17]. Indeed, prematurely born infants have a 3.6 times higher risk of being hospitalized due to respiratory infections [18][19]. One of the molecular mechanisms which has been proposed is the decreased expression of Ear1 in the airway epithelium leading to susceptibility to influenza A virus infection [20]. The cost for the treatment of survivors carrying longterm sequelae (e.g. recurrent pulmonary infections) of BPD represents a high burden for the health care system [21].

BPD is a disease caused by multiple factors, including mechanical ventilation, oxygen toxicity, pre- and postnatal infections, and immaturity of the lung. These deleterious factors lead to imbalance of pro- and anti-inflammatory mechanisms in favour of a continuous inflammation of the lung. Finally, this injurious process causes an impaired postnatal development of the lung parenchyma and pulmonary vasculature

leading to the growth arrest of the premature lung. Using genome-wide association studies, polymorphisms (MMP16, SPOCK2, FGFR-4) have been identified leading to the assumption that BPD might have a genetic component [22][23]. Due to significant differences of the histopathological findings in the lungs of infants who died from BPD, the “new” BPD (simplification of alveolar structures, dysmorphic pulmonary vasculature) is considered as a growth arrest of the lung compared to the “old” BPD (interstitial fibrosis and metaplasia of the airways) which had been described mostly as an airway disease. Due to wide variations of the current existing BPD definitions the presence and severity of BPD has not been defined properly yet [13]. The lack of reliable diagnostic criteria represents a barrier for further investigations concerning the causes of BPD and the development of new therapies. Thus, efforts have been made to identify novel biomarkers for the early diagnostic of BPD [24].

To date, there are very few therapies to prevent and treat BPD, with no curative approach available [25]. Results of preclinical studies using stem cells (e.g. mesenchymal stromal cells) to treat BPD are promising and encourage the emerging field of stem cell therapy to further validate and clinically translate these approaches [26,27].

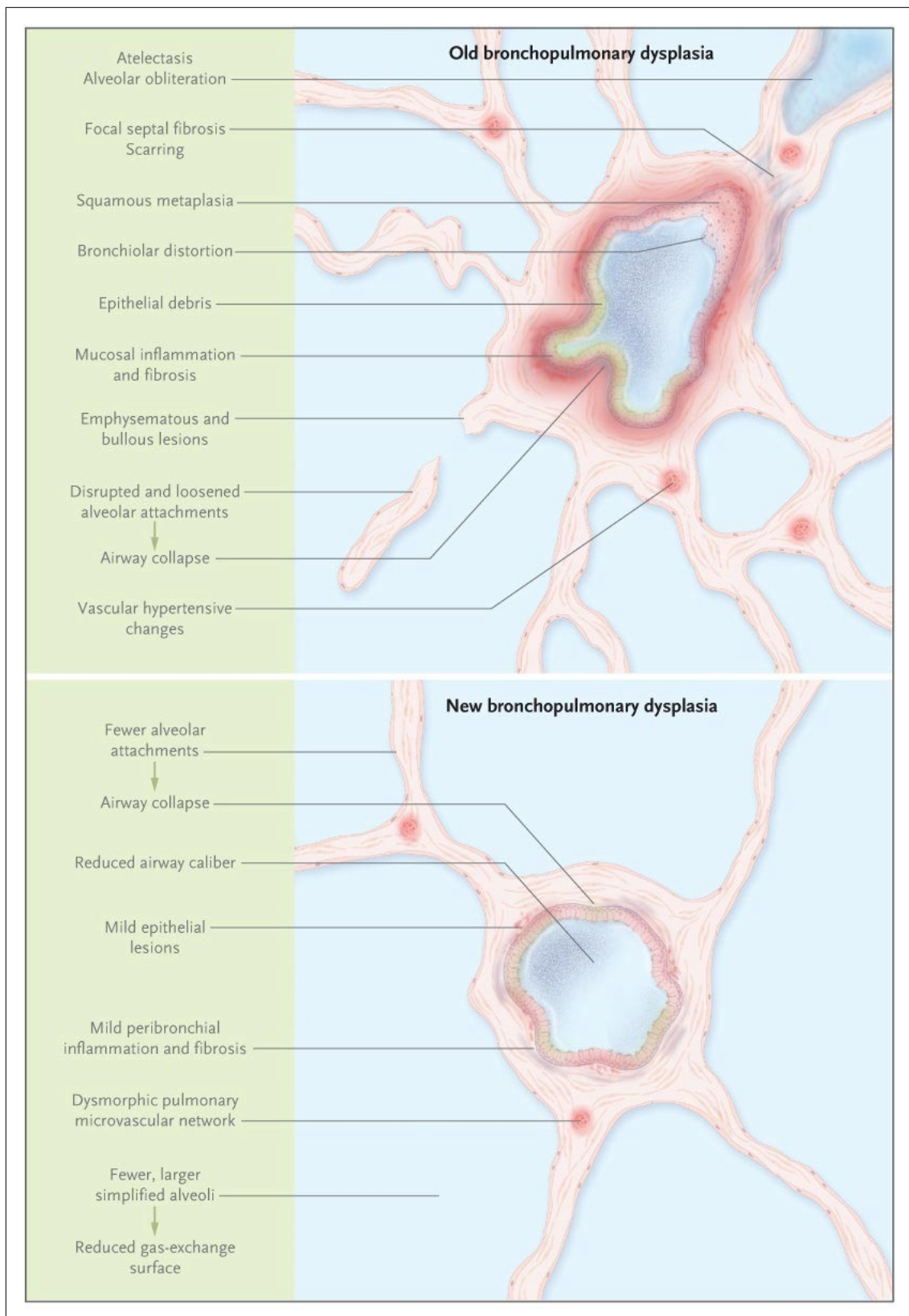


Figure 2. Schematic showing the airway and parenchymal damage in old and new Bronchopulmonary dysplasia [28]

1.3 Fgf10 in lung development and BPD

FGF10 belongs to a family of fibroblast growth factors (FGFs) in which 22 members have been identified in the human genome. According to phylogenetic analyses, the whole family can be arranged into seven subfamilies [29]. Fibroblast growth factors have endocrine, paracrine or intracrine functions by signalling through a fibroblast growth factor receptor (FGFR). Due to alternative splicing, four genes named *Fgfr1*, *Fgfr2*, *Fgfr3* and *Fgfr4* encode for seven receptors (FGFR 1b, 1c, 2b, 2c, 3b, 3c and 4) with different ligand-binding specificity. Each receptor consists of an extracellular ligand-binding domain with three immunoglobulin-like loops (D I, D II, D III), a transmembrane domain, and an intracellular tyrosine kinase domain.

FGF signalling occurs both in the epithelium and mesenchyme of the lung and thereby orchestrates epithelial–mesenchymal interactions taking place during the different stages of lung development and homeostasis. Further, FGF signalling has been shown to be critical for the formation of multiple epithelial and mesenchymal lineages in the lung and is also re-engaged during the repair process following injury [30][31][32][33][34][35][36]. Such signalling can also be impaired or abnormally activated in pathological processes such as cancer or fibrosis [30][37].

Especially, fibroblast growth factor 10 (FGF10), which is a diffusible small (20kDa) heparin-binding molecule, has been shown to play a pivotal role during embryonic lung development [38][39]. During the early pseudoglandular stage (E12.5) of mouse lung development *Fgf10* is expressed by cells located in the sub-mesothelial mesenchyme adjacent to the mesothelium of the distal developing embryonic lung buds. Among other *Fgfs*, *Fgf10* is the only gene which is expressed during the early pseudoglandular stage. Once secreted, it mainly acts in a paracrine fashion on the fibroblast growth factor receptor 2-IIIb (FGFR2b) receptor which is located on the

epithelium of the forming lung buds, thereby controlling branching morphogenesis and proliferation/differentiation of the epithelium and the mesenchyme. Among others, Wnt (Wingless and INT-1) and Fgf9 are considered as positive regulators maintaining *Fgf10* expressions in these cells. *Fgf9* is expressed in the mesothelium and the epithelium and acts through FGFR2c and FGFR1c located on mesenchymal cells. A further function of *Fgf9* is to maintain mesenchymal progenitor cells in a proliferative and undifferentiated status [40].

Two decades ago, *Fgf10* was demonstrated by using genetic deletion in the mouse to be one of the most essential key molecules for embryonic lung development. Sekine and colleagues demonstrated convincingly that the deletion of *Fgf10* led to agenesis of the lung, limbs and other organs in mice [38][39][41]. Genetic deletion of *Fgfr2b* in mice led to a similar phenotype. The mutant pups die shortly after birth due to lung agenesis and multiple defects in other organs such as the salivary gland, limb, inner ear, teeth, skin, pancreas, kidney, thyroid, pituitary gland, mammary gland [41][42][43][44][45]. However, the exact mechanism of action remains unclear.

As mentioned above, the lipofibroblast represents an important cell population supporting AECII in their function pre- and postnatally. Our group found that a subpopulation of lipofibroblast expresses Fgf10 [46].

By treating mesenchyme-free epithelium with recombinant human FGF10, Lu and colleagues identified epithelial-specific gene expressions during bud morphogenesis [47]. Using microarray analyses they identified a panel of transcriptional *Fgf10* targets, which are associated with cell rearrangement, migration, inflammatory processes, lipid metabolism, cell cycle and tumor invasion. The so-called *Fgf10* hypomorphic mouse line (*Fgf10^{lacZ/-}*), which had only approximately 20% *Fgf10* expression level compared to wild type (WT), displayed lung defects at birth. Morphologic characteristics consisted of decreased branching, increased primary

septa thickness, and disturbed pulmonary vasculature leading to intrapulmonary haemorrhages. These malformations led to dyspnea postnatally and death within 24-48 hours after birth. Similarly, constitutive *Fgf10* deficiency led to changes in lung morphology and gene expression at E18.5. Upon postnatal hyperoxic lung injury (BPD mouse model), all of the *Fgf10* deficient pups died due to quantitative and qualitative malformation of the AECII, while all wildtype pups survived [48]. In addition, a quantitative and qualitative analysis of the pulmonary vasculature revealed a decrease in the total amount of vessels and a more immature vascular muscularization (increase of non-muscularized vessels) in the mutant pups compared to the wildtype pups. Interestingly, a postnatal attenuation of *Fgfr2b* ligands results in more muscularized vessels resembling a phenotype seen in pulmonary hypertension [49]. *Fgf10* also affected the formation of mesenchymal lineages and the amplification of distal epithelial progenitor cells. Thus, *Fgf10* deficiency led to decrease in *Nkx2.1*- and *Sftpb*-expressing cells and diminished PECAM and α SMA-positive cells in mesenchymal cell lineages [50].

Interestingly, infants who died from severe BPD showed decreased FGF10-positive cells in their lung tissues [51]. Carver and colleagues show a downregulation of FGF10 through the activation of NF κ B signalling associated with inflammation in newborn lungs suffering from BPD [52].

In humans, heterozygous mutations in the *FGF10* or *FGFR2B* gene lead to the syndromes ALSG (aplasia of lacrimal and salivary glands) and LADD (lacrimo-auriculo-dento-digital), respectively [53][54][55]. Adult patients suffering from ALSG or LADD syndromes developed a significant decrease in lung function parameters (IVC, FEV1 and FEV1/IVC ratio) compared to non-carrier siblings, thereby resembling patients with chronic obstructive pulmonary disease (COPD) [56]. COPD patients have been shown to express higher levels of *FGF1*, *FGF2* and *FGFR1*

leading to the assumption that these ligands and receptor are involved in the remodelling process [57][58]. Interestingly, obvious lung defects have not been reported yet in infants with ALSG or LADD syndromes. According to our study using *Fgf10* heterozygous (*Fgf10*^{+/-}) mice we assume that these patients might suffer from quantitative and qualitative congenital defects of the AECI and AECII cells due to *Fgf10* deficiency [48]. As mentioned before, one of the main features of BPD is the simplification of alveoli due to disturbed alveologenesis. In this context, it has been shown that blockade of FGFR2b ligands during embryonic but not postnatal lung development leads to emphysema in mice. Also, Hokuto and colleagues show that FGFR2b is needed for recapitulation of alveoli after postnatal hyperoxic lung injury. Without FGFR2b signalling they saw increased inflammation, capillary leakage and higher mortality [59]. Another study showed a positive effect of FGFR2b in promoting compensatory lung growth in a pneumonectomy mouse model [60]. The regeneration process after pneumonectomy has also been associated with vascular endothelial growth factor receptor 2 (VEGFR2)/FGFR1 activation in pulmonary endothelial cells. Upon activation, the endothelial cells produce MMP14 that unmasks epidermal growth factor (EGF)-like ectodomains leading to further activation of epidermal growth factor receptor (EGFR) and expansion of epithelial progenitors [61].

Apart from *FGF10*, other aberration *FGF* and *FGFR* are also associated with diseases. For instance, *Fgfr3* and *Fgfr4* deficiency in mice results in arrested alveologenesis. The underlying mechanism is still elusive but one possible hypothesis is the disturbed epithelial-mesenchymal crosstalk leading to impaired alveolar elastogenesis [62][63]. Moreover, reports about humans carrying homozygous mutation of *FGF3* described deafness due to complete labyrinthine aplasia (Michel aplasia), microtia, and microdontia (LAMM syndrome) [64]. Further, a hereditary disease termed “autosomal dominant hypophosphatemic rickets” (ADHR)

is caused by mutations of *FGF23* which lead to polypeptides less sensitive to protease cleavage compared to healthy controls resulting in excessive loss of phosphate in the urine [65].

1.4 Epigenetic regulations in lung development and BPD

While a genetic component seems to play a role in BPD, less is known about the role of the epigenetic landscape following exogenous triggers contributing to disease development. Epigenetics is defined as the science of heritable changes in gene expression caused by mechanisms which do not modify the original nucleotide sequence. Three main epigenetic mechanisms have been discovered – noncoding RNAs (microRNA), histone modification, and DNA methylation. Growing evidence suggests that epigenetic mechanisms are importantly involved in the pathogenesis of lung diseases [66]. Here, we would like to focus on the effects of microRNA and modifications of histone tails on gene expressions in the context of lung development and BPD.

1.4.1 MicroRNA in lung development and BPD

Approximately 1% of the genome encodes for microRNAs (miRs). MicroRNAs are small regulatory RNAs, which are single-stranded, non-coding and about 20-25 nucleotides in size. They bind to the 3' untranslated region (UTR) of the target mRNA, leading to translational repression or degradation of the RNA [67][68][69]. Through these mechanisms they regulate the post-transcriptional gene expression of about 60% of protein-coding genes in mammals [70]. Thus, nowadays miRs are considered as major regulators of many biological processes during development, cell differentiation and proliferation, as well as in regeneration processes after injury [71].

After transcription, the pri-miRNA undergo a maturation process leading to a 5p strand and a 3p strand, which is complementary to the 5p strand (Fig. 3). Previously, the 3p strand was believed to be non-functional, being simply degraded. Now, it has been shown that both strands are able to modify specific gene expressions through base pairing [72]. Each miR is able to regulate different mRNAs, and, in turn, a specific mRNA can be regulated by many miRs [73]. MiRs are also key regulators of stem cells during development [74]. Today, more than two thousand miRs have been identified (miRBase.org).

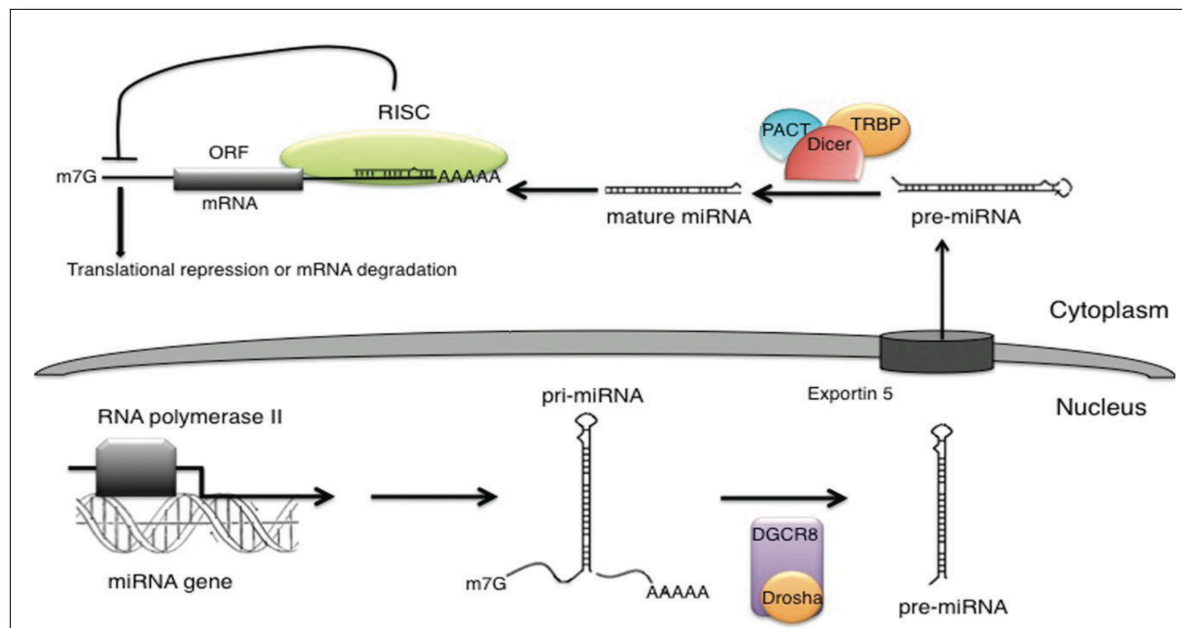


Figure 3. Schematic showing the biogenesis of miRNA [75]

During lung development, many signalling pathways, e.g. Fgf, Wnt, Tgfb, Shh, are switched on and off [76][77]. Similarly, the expression of miRs are constantly changing during different stages of lung development [78][79]. Bhaskaran and colleagues have reported that various miRs have been shown to regulate multiple gene expressions which are involved in signalling pathways during lung development [78][80]. By overexpressing miR-127 during the pseudoglandular stage they observed changes in the lung morphology in terms of inhibited branching and enlarged terminal buds. The expression of miR-127 and miR-351 increased during the saccular and alveolar stages. Most interestingly, during the time course of lung development, the mesenchyme loses the expression of the two miRs while increasing expression has been observed in the lung epithelium, suggesting their roles in orchestrating mesenchymal to epithelial transition [78]. Furthermore, the cell-specific genetic deletion of Dicer in the lung epithelium, one of the most important enzyme during miR maturation, leads to disturbed branching morphogenesis during

embryonic lung development [81]. Another research group confirmed the importance of miRs during lung development by deletion of members of the miR-17-92 cluster in mice. They observed drastic defects in lung morphogenesis leading to fatal lung hypoplasia without obvious branching defects. Interestingly, the conditional overexpression revealed impaired formation of lung epithelial progenitor cells and disturbed differentiation [82][83][84]. Further, our group showed that miR-142-3p is specifically upregulated in the lung mesenchyme during embryonic lung development. Using an in-vitro culture of embryonic lung explants, we demonstrated that miR-142-3p contributed to the proliferation and differentiation of the mesenchymal progenitor cells by modulating the WNT signalling pathway via inhibition of adenomatous polyposis coli (APC), which can directly bind to β -catenin (CTNNB1). These findings were confirmed by generation and validation of miR-142 knock-out mice [85][86]. Mir-326 was identified to play a crucial role controlling development of the lung mesenchyme. It is selectively enriched in embryonic lung mesenchymal cells and is specifically modulated by Shh activity, an essential pathway during early mouse lung development [87]. Attention was also paid to the endoderm. Using gain-of-function and loss-of-function approaches, mir302/367 was implicated in the proliferation and differentiation of the endodermal progenitor cells during early lung development [88]. Benlhabib and colleagues performed miRNA microarray studies using epithelial cells of human fetal lungs to show the importance of miR-200 and ZEB1 in controlling alveolar epithelial cell type II (AECII) maturation [89]. Another study confirmed the critical role of miR-124 in lung epithelial maturation by using rat fetal lung culture [90]. MiR-221 (anti-angiogenic) and miR-130a (pro-angiogenic) are two miRs with opposite impact on airway and pulmonary vascular morphogenesis during mouse lung development [91].

However, miRs are not only playing an essential in lung development but also in lung diseases. For instances, in BPD [92][93][94], Asthma [95], Cystic fibrosis [96], Chronic obstructive pulmonary disease (COPD) [97], Idiopathic pulmonary fibrosis (IPF) [98], Lung cancer [99][100][101], Pulmonary arterial hypertension (PAH) [102], Pneumonia [103], Pulmonary embolism [104], Pulmonary sarcoidosis [105] and Acute lung injury [117].

Table 1. Overview of microRNAs involved in lung development and lung diseases [75][106][107].

Lung development/ lung disease	Involved microRNAs	References
Lung development	miR-127, miR-351, miR-17-92 cluster, miR-154, miRNA-146b, miRNA-29a, miRNA-503, miRNA-411, miRNA-214, miRNA-130b, miRNA-382, miRNA-181a-1*, miR-302, miR-367, miR-200, miR-375, miR-124, miR-142, miR-326, miR-221, miR130a	[78][79][80][81][82][83] [84][85][86][87][88][89][90] [91][108]

Bronchopulmonary dysplasia (BPD)	miR-154, miR-421, miRNA-21, miRNA-34a, miRNA-431, and Let-7f, miRNA-335, miRNA-146b, miRNA-29a, miR-29c, miRNA-503, miRNA-411, miRNA-214, miRNA-130b, miRNA-382, and miRNA-181a-1*, miRNA-21 and miRNA-141, miR-342, miR-335, miR-150, miR-126*, miR-151*, miR-21, miR-34a	[92][93][94] [109][110] [111]
Cystic fibrosis (CF)	miR-101, miR-126, miR-138, miR-155, miR-494	[96]
Asthma	miR-20b, miR-21, let-7,	[95]

	miR-106a, miR-126, miR-155, miR-146b, miR-133a, miR-145a, miR-146a, miR-181, miR-221, miR-222	
Pneumonia	miR-34a, miR-455, let-7f-1, miR-200b	[103]
Lung cancer	miR-495, miR-154, miR-93, miR-132, miR-17-92 cluster, miR-146b, miR218, miR-31, miR-1, miR-133b, miR-218, miR-34, miR-200 family, let-7, miR-155, miR-218	[99][100][101][112]
Idiopathic pulmonary fibrosis (IPF)	miR-29, miR-154, miR-17, miR125b, miR-200, miR-2	[98]
Chronic obstructive pulmonary disease (COPD)	miRNA-149, miR-17-92 cluster, , miR-143, miR-21, miR-204, miR-424, miR-150, miR-503, miR-210, miR-27a, miR-145, miR-206	[97]
Pulmonary arterial	miR-135a, miR-17-92	[102]

hypertension (PAH)	cluster, miR-206, miR-143, miR-150, miR-503, miR-424, miR-210, miR-145, miR-21, miR-27a, miR-204	
Pulmonary embolism	miR-28, miR-221, miR-27a/b, miR-1233, miR-134	[104][113] [114][115] [116]
Pulmonary sarcoidosis	MiR-27b, miR-192 and miR-221, miR-15b, miR-222, miR-let7f and miR-130a, miR-16, miR-130a miR-20a	[105]
Acute lung injury	miR-27a, miR-466, miR-32, miR146, miR-127, miR-155, miR-21, miR-10a/b, miR-144, miR-16, miR-223, miR-30b	[117][118][119][120][121][122]

Growing evidence coming mainly from animal studies showed that expression of numerous miRs was dysregulated in BPD. This suggested that miRs are powerful regulators in the pathogenesis of BPD.

For instance, the expression of *miR-154-5p* was decreased at day 14 in a rat BPD model using hyperoxia. Although other miRs were found to be deregulated, *miR-154-5p* was not changed at day 3 and 7 [92]. Wu et al. found an upregulation of miR-133b

and miR-7 but a downregulation of miR-152 and miR-30a-3p in the peripheral blood of infants with BPD [123]. Using miRNA microarray and PCR several miRs were identified to be deregulated in neonatal rat lungs exposed to hyperoxia: miR-342, miR-335, miR-150, miR-126*, miR-151*, miR-21 and miR-34a [124]. However, only several studies have been carried out to validate the differential expressed miR to demonstrate causality [94]. For example, miR-150 was identified as being reduced upon hyperoxia-induced lung injury. Its target protein glycoprotein nonmetastatic melanoma protein b (Gpnmb) was confirmed to be increased in the miR-150 Knock-out mouse. However, miR-150^{-/-} mice exposed to hyperoxia only showed moderate protection of lung structure at P9 but no changes at P13 [124]. Most recently, it was demonstrated that miR-421 inhibition alleviated BPD via targeting *Fgf10* in a mouse model [93]. Another miR which has been validated is miR-489 and its targets insulin-like growth factor 1 (IGF-1) and tenascin C (TNC). MiR-489 was downregulated in experimental and human BPD. Inhibition of miR-489 attenuated hyperoxia-induced inhibition of lung development. In contrast, overexpression of miR-489 reduced IGF-1 and TNC, and induced alveolar simplification and impaired lung compliance [125].

1.4.2 Histone modification in lung modelling and remodelling

In the nucleus of a eukaryotic cell, the three-dimensional structure of chromatin is a complex of DNA and specific proteins forming the chromosomes. The majority of these specific proteins are called histones. Histones are basic proteins which condense the DNA into units named nucleosomes. Histone modifications regulate transcriptional activity by modifying biochemical composition and physical structure of the chromatin [66]. For instance, the modification of NH₂-terminal tails of histones leads to changes in chromatin density and activity of the transcription. In euchromatin, histones are hyperacetylated and hypomethylated resulting in increased access to the transcriptional machinery. In contrast, Hypoacetylation and Hypermethylation of histones attenuate gene transcription. Further, histones can also be phosphorylated, ubiquitinated and nitrated. Chromatin remodeling complexes regulates chromatin structures to alter the access of the transcriptional machinery to specific gene loci. For example, the catalytic subunit Brahma (Brm) and Brahma-related gene-1 (Brg1) has been shown to interact with Nkx2.1 which is an essential regulator in early lung development. Genetic deletion of *Brg1* in lung epithelial cells leads to tumor growth [126]. Expanding literature suggest that epigenetic mechanisms are playing a role in BDP pathogenesis. Londhe et al. showed involvement of epigenetic changes through decreased expression of histone deacetylase 1 (HDAC1) and HDAC2 during hyperoxia-induced lung injury leading to alveolar simplification [127]. Another group demonstrated that lipopolysaccharide (LPS) disrupts proper alveolar development via suppression of HDAC1 and HDAC2 activity and activation of TGF- α [128]. Investigations in experimental mouse model of BPD and infants with BPD revealed that expression of miR17~92 cluster correlates

inversely with the expression of DNA methyltransferase and miR17~92 cluster promoter methylation in the pathogenesis of severe BPD [129].

1.5 Purpose of the study

Almost two decades after the discovery of FGF10 as a major regulator of branching morphogenesis, the underlying mechanisms on molecular and cellular level are still elusive [130]. The crosstalk among essential signalling pathways, such as fibroblast growth factors (FGF), wingless/integrase 1 (WNT), bone morphogenic proteins (BMP) and sonic hedgehog (SHH) needs to be understood better. Further, the cell population expressing FGF10 and those targeted by FGF10 need to be identified in lung development, BPD and lung regeneration. The understanding of cellular processes controlled by FGF10 is essential to develop new therapeutic strategies for the treatment of BPD. Finally, epigenetic regulation is an emerging topic in the field of lung development but its causative role in the pathogenesis of BPD is poorly understood and need to be thoroughly studied.

This study addresses the above mentioned points using a comprehensive set of cutting-edge methods including genetic modified mice (gain-of-function and loss-of-function), the mouse model of BPD, in-vivo lineage tracing and a broad spectrum of molecular biological and histological methods, such as RT-qPCR, gene array, in-situ-hybridization (ISH), fluorescence-activated cell sorting (FACS), in-vitro lung culture, cell culture, miRNA pull-down assay, luciferase reporter assay, western blotting, immunohistochemistry (IHC), immunofluorescence (IF), confocal microscopy, transmission electron microscopy).

2. Results – Original publications

2.1 Fgf10 in lung development

2.1.1 Appendix A

Jones, M., Lingampally, A., Dilai, S., Shrestha, A., Stripp, A., Helmbacher, F., **Chao, CM.***, Bellusci, S.*:

“Characterization of Etv4GFP and Etv5RFP reporter lines in the context of fibroblast growth factor 10 signalling during mouse embryonic lung development.”

Front Genet. 2019 Mar 14;10:178 | doi: 10.3389/fgene.2019.00178

***equally contributed.**

Summary of the findings:

The two members of the PEA3 transcription factors, ETV4 and ETV5 have been proposed to mediate FGF10/FGFR2b signalling. Using *Tg(Etv4-GFP)* and *Etv5^{CreERT2-RFP}* fluorescent reporter mouse lines, ETV4 and ETV5 have been evaluated according to their expression patterns in the lung epithelium and mesenchyme during early lung development (E12.5, E14.5). The expression patterns are in line with the known regions of FGF10 signalling. Further, *in vivo* and *in vitro* inhibition of FGF10/FGFR2b confirmed that ETV4 and ETV5 are acting downstream of FGF10 signalling.

2.1.2 Appendix B

Jones, M.[#], Dilai, S.[#], Lingampalli, A., **Chao, CM.**, Danopoulos, S., Carraro, G., Mukhametshina, R., Wilhem, J., Baumgart-Vogt, E., Al Alam, D., Chen, C., Minoo, P., Zhang, J.S.^{*}, Bellusci, S. ^{*}:

“A comprehensive analysis of fibroblast growth factor receptor 2b signalling on epithelial tip progenitor cells during early mouse lung branching morphogenesis.”

Front Genet. 2019 Jan 23;9:746 | doi:10.3389/fgene.2018.00746

***equally contributed.**

Summary of the findings:

Using the inducible transgenic mouse line *Rosa26rtTA;tet(o)sFgfr2b* to express dominant negative *Fgfr2b*, this study demonstrates that blocking FGFR2b ligands at E12.5 impacts epithelial branching, bud lumen formation and cellular adhesion. In vitro experiments using IQ1, a pharmacological inhibitor of the β -catenin/EP300 interactions, revealed that FGF10 signalling on distal epithelial progenitors is mainly mediated through β -catenin/EP300.

2.1.3 Appendix C

El Agha E, Herold S, Al Alam D, Quantius J, MacKenzie B, Carraro G, Moiseenko A, **Chao CM**, Minoo P, Seeger W, Bellusci S.:

“Fgf10-positive cells represent a progenitor cell population during lung development and postnatally.”

Development. 2014 Jan;141(2):296-306 | doi:10.1242/dev.099747

Summary of the findings:

This study demonstrates that during early (E11.5) and late (E15.5) pseudoglandular stage of embryonic lung development two populations of *Fgf10*-expressing progenitor cells emerge in the lung mesenchyme. Using a lineage-tracing approach (*Fgf10*^{iCer/+}; *tomato*^{flox/+}), we showed that *Fgf10*-positive cells give rise to different mesenchymal cell types pre- and postnatally, e.g. parabronchial/ vascular smooth muscle cell and lipofibroblast.

2.1.4 Appendix D

Al Alam D, El Agha E, Sakurai R, Kheirollahi V, Moiseenko A, Danopoulos S, Shrestha A, Schmoldt C, Quantius J, Herold S, **Chao CM**, Tiozzo C, De Langhe S, Plikus MV, Thornton M, Grubbs B, Minoo P, Rehan VK, Bellusci S.:

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

Development. 2015 Dec 1;142(23):4139-50 | doi: 10.1242/dev.109173

Summary of the findings:

Lipid-containing alveolar interstitial fibroblasts, also called lipofibroblasts (LIF), are considered as a crucial cell type of the epithelial stem cell niche for development, homeostasis and regeneration. They emerge during late pseudoglandular stage (E15.5, E16.5) of mouse lung development. In contrast, in human lipofibroblast formation seems to occur in the canalicular stage beyond 22 weeks of gestational age. FGF10/FGFR2b signalling is essential for the formation of lipofibroblast in mouse and human fetal lungs.

2.2 Fgf10 in BPD and lung regeneration

2.2.1 Appendix E

Chao CM, Yahya F, Moiseenko A, Tiozzo C, Shrestha A, Ahmadvand N, El Agha E, Quantius J, Dilai S, Kheirollahi V, Jones M, Wilhem J, Carraro G, Ehrhardt H, Zimmer KP, Barreto G, Ahlbrecht K, Morty RE, Herold S, Abellar RG, Seeger W, Schermuly RT, Zhang JS, Minoo P and Bellusci S.:

“Fgf10 deficiency is causative for lethality in a mouse model of bronchopulmonary dysplasia.”

J Pathol. 2017 Jan;241(1):91-103 | doi:10.1002/path.4834

Summary of the findings:

Constitutive *Fgf10* deficiency (*Fgf10*^{+/-}) leads to disturbed embryonic lung development (increased mean linear intercept and airspace) and impaired gene expression (increased TGFβ signalling) in mice. Neonatal hyperoxia exposure leads to failure of lung function and mortality due to increased lung injury. Further investigations revealed that *Fgf10* deficiency affects quantitatively and qualitatively the proper formation of alveolar type II cells (ATII) which is compatible with postnatal survival but not with hyperoxic lung injury (BPD mouse model).

2.2.2 Appendix F

Chao CM, Moiseenko A, Kosanovic D, Rivetti S, El Agha E, Wilhelm J, Kampschulte M, Yahya F, Ehrhardt H, Zimmer KP, Barreto G, Rizvanov AA, Schermuly RT, Reiss I, Morty RE, Rottier RJ, Bellusci S and Zhang JS.:

“Impact of Fgf10 deficiency on pulmonary vasculature formation in a mouse model of bronchopulmonary dysplasia.”

Hum Mol Genet 2018 Dec 19 | doi: 10.1093/hmg/ddy439

Summary of the findings:

FGF10 and FGFR2 are expressed in human BPD lungs and immunohistochemistry confirmed decreased expression of FGF10. Further analysis revealed a decreased expression of PECAM suggesting a dysmorphic pulmonary vasculature. Mice deficient of *Fgf10* expression (*Fgf10*^{+/-}) showed a greater loss of blood vessels associated with increase of poorly muscularized vessels compared to the controls upon hyperoxic lung injury postnatally (BPD mouse model). Interestingly, the conditional inhibition of Fgfr2b ligands (*Rosa26rtTA;tet(o)sFgfr2b*) during hyperoxia exposure postnatally leads to a pulmonary hypertension (PH) phenotype with increased vascular muscularization. Transcriptomic analysis supports the correlation between decrease of *Fgf10* expression and vascular defects.

2.2.3 Appendix G

Volckaert T*, Yuan TT*, **Chao CM**, Bell H, Sitaula A, Szimtenings L, El Agha E, Chanda D, Majka S, Bellusci S, Thannickal VJ, Faessler R, de Langhe SP.:

“Fgf10-Hippo epithelial-mesenchymal crosstalk maintains and recruits lung basal stem cells.”

Dev Cell. 2017 Oct 9;43(1):48-59.e5 | doi: 10.1016/j.devcel.2017.09.003

*equally contributed

Summary of the findings:

In this study, we first showed that tracheal basal stem/progenitor cells maintain and restrict their cell pool during homeostasis by downregulating Hippo pathway thereby generating a localized *Fgf10*-expressing stromal niche. Loss of FGFR2b signalling results in loss of basal stem cells. Interestingly, during regeneration after naphthalene-mediated epithelial injury, Hippo signalling is also downregulated associated with nuclear Yap localization and elevated expression of *Fgf10* in the airway smooth muscle cell niche. *Fgf10* overexpression recruits basal cell to the area of injury. This Yap-Wnt7b-Fgf10 regenerative pathway is activated in chronic obstructive pulmonary disease (COPD) to induce basal stem cell amplification and regeneration.

2.2.4 Appendix H

Yuan, T.[#], Volckaert, T.[#], Redente, E.F., Yuan, J., **Chao, C.M.**, Wasnick, R., Yao, C., Majka, S., Stripp, B., Günther, A., Riches, D.W.H., Bellusci, S., Thannickal, V., De Langhe, S.P.:

“Fgf10-Fgfr2b signaling generates basal cells and drives alveolar epithelial regeneration by bronchial epithelial stem cells after lung injury.”

Stem Cell Reports 2019 Apr 16. pii: S2213-6711(19)30125-0 | doi:10.1016/j.stemcr. 2019. 04. 003.

Summary of the findings:

FGF10/FGFR2b signalling is required for maintenance of alveolar type II (AT2) during homeostasis postnatally. After bleomycin injury (mouse model of idiopathic

pulmonary fibrosis, IPF) FGF10/FGFR2b signalling is needed for the regeneration of the alveolar epithelium and recruitment of basal stem cell at site of injury. Cell-specific overexpression of Fgf10 in Sox2-positive bronchial epithelial cells promotes the generation of AT2 cells at the expense of new basal cells. Single-cell transcriptome analyses of new basal cells isolated from IPF lungs revealed an “indeterminate” state of differentiation by co-expressing AT1, AT2, basal cell markers. Finally, we found that new basal cells are capable of differentiating into AT2 cells during resolution of IPF in mice and that this process requires FGF10/FGFR2b signalling

2.3 Epigenetic regulation in lung development and BPD

2.3.1 Appendix I

Carraro G, Shrestha A, Rostkovius J, Contreras A, **Chao CM**, El Agha E, Mackenzie B, Dilai S, Guidolin D, Taketo MM, Günther A, Kumar ME, Seeger W, De Langhe S, Barreto G, Bellusci S.:

“MiR-142-3p balances proliferation and differentiation of mesenchymal cells during lung development.”

Development. 2014 Mar;141(6):1272-81 | doi:10.1242/dev.105908

Summary of the findings:

In this study, we determined that *miR-142-3p* orchestrates the proper proliferation and differentiation of mesenchymal progenitors by regulating the level of WNT signalling through direct interaction with adenomatous polyposis coli (*Apc*). In embryonic lung mesenchyme, WNT and FGF signalling creates a positive feedback loop and is impaired upon inhibition of *miR-142-3p*. The *miR-142-3p-Apc-Wnt/Ctnnb1* pathway has been confirmed using rescue experiments.

2.3.2 Appendix J

Shrestha A, Carraro G, El Agha E, Mukhametshina R, **Chao CM**, Rizvanov A, Barreto G, Bellusci S.:

“Generation and Validation of miR-142 Knock Out Mice.”

PLOSone. 2015 Sep 1;10(9):e0136913 | doi: 10.1371/journal.pone.0136913

Summary of the findings:

MiR-142-knockout mice were generated using homologous recombination. They are viable postnatally without sign of embryonic lethality and exhibit normal appearance and physical activity. However, they display impaired hematopoietic lineage formation including decrease in white blood cells, lymphocytes eosinophils, monocytes and platelets. *MiR-142*-knockout mice showed increased Wnt signalling associated with upregulation of Apc and p300.

2.3.3 Appendix K

Shrestha, A., Carraro, G., Mari, B., Nottet, N., Vasquez-Armendariz, A.I., Herold, S., Cordero, J., Singh, I., Barreto, G., Wilhelm, J., Gunther, S., El Agha, E., Morty, R., Zhang, J.S., **Chao, C.M.** *, Bellusci, S.*:

“A critical role for miR-142 in alveolar epithelial lineage formation during lung development.”

Cell Mol Life Sci. 2019 Mar 18 | doi: 10.1007/s00018-019-03067-8

*equally contributed.

Summary of the findings:

MiR-142-3p and *-5p* are expressed in AT1 and AT2 lineages during lung development. Activation of glucocorticoid receptor pathway in alveolar lineage formation leads to decreased *miR-142* expression. *MiR-142*-knockout mice does not display branching defects at E12.5 but show an increase in AT2 cells associated with a decrease in AT1 cells and disturbed gene expression at E18.5. In contrast, gain of

function experiments in alveolar epithelial cells leads to the opposite phenotype with decreased AT2/AT1 ratio. Ex-vivo lung culture experiments combined with loss-of-function (morpholino) and rescue approach (inhibition of Ep300/ β -catenin interaction) confirmed the role of *miR-142-3p*-Ep300/ β -catenin pathway in alveolar lineage formation.

2.3.4 Appendix L

Chao CM, van den Bruck R, Lork S, Merkle J, Krampen L, Weil P, Aydin M, Bellusci S, Jenke AC, Postberg J.:

“Neonatal exposure to hyperoxia leads to persistent disturbances in pulmonary histone signatures associated with NOS3 and STAT3 in a mouse model.”

Clinical Epigenetics. 2018 Mar 20;10:37 | doi:10.1186/s13148-018-0469-0

Summary of the findings:

Early exposure to hyperoxia postnatally (BPD mouse model) leads to significant increase in NOS3 and STAT3 expression in pulmonary endothelial cells with respective changes in histone modification patterns such as H2aZac and H3K9ac hyperacetylation. These changes in epigenetic signature at NOS3 locus remains for longer time and is associated with pathological expression of eNOS upon second exposure to oxygen later in life. Using an in vitro cell culture model, we confirmed the importance of the observed histone modification for the regulation of NOS3 expression. The long-term relevance of persisting changes in epigenetic signatures of early life events could explain pathological vascular endothelial response later in life, potentially explaining disease condition such as pulmonary hypertension in BPD.

3. Discussion

3.1 Transgenic mouse lines: advantages and limitations

FGF10 is a growth factor which is essential for the embryonic development of many organs including the lung, gut, limbs, mammary glands and pancreas.

In this study, we showed that FGF10-FGFR2b signalling is a major signalling pathway regulating proliferation and differentiation of lineage-restricted as well as progenitor cell populations during lung development. Further, our data suggests an important role for FGF10 in the pathogenesis of BPD and for the regeneration of the lung after epithelial injury.

In this context, transgenic mice are important “tools” to investigate the intricate biological processes controlled by *Fgf10* in an in-vivo “whole lung system” in order to decipher cell-cell-interactions as well as interactions between different compartments of the lung (epithelial-mesenchymal, endothelial-mesenchymal). In this study, transgenic mice have been used extensively.

First, we validated the *Tg(Etv4-GFP)* and *Etv5^{CreERT2-RFP}* fluorescent reporter mouse lines, which allow us to confirm ETV4 and ETV5 as downstream transcription factors of FGF10 signalling and to visualize their expression patterns during early lung development at E12.5 and E14.5 (**Appendix A**). Further, *Fgf10^{iCre/+};tomato^{flox/+}* has been generated and validated. Used as a lineage tracing approach, this site-specific recombinase system is a valuable “tool” to identify the *Fgf10*-expressing progenitor cells and to follow up on their cell fate during lung development (**Appendix C, D**). Using this strategy, our results showed that FGF10 is involved in differentiation of multiple mesenchymal cell types including parabronchial smooth muscle cell,

vascular smooth muscle cell and lipofibroblast. The latest is believed to play an important role in normal alveologenesis and repair after injury to the aveoli.

Fgf10 knock out mice are not viable due to agenesis of multiple organs including the lung, thus limiting their use to understand the function of *Fgf10* on cellular and molecular levels. In order to overcome this obstacle, we generated and used several mouse lines which exhibit different levels of decreased *Fgf10* expression, e.g. *Fgf10*^{+/-} (approximately 50% of *Fgf10* expression), *Fgf10-lacZ*⁻ (hypomorph, approximately 25% of *Fgf10* expression) and *Fgf10iCre/lacZ;tomatoflox/+* (hypomorph, approximately 25% of *Fgf10* expression) (**Appendix D, E, F, G**). The latest also allow the conditional labelling and thereby a temporospatial tracking of *Fgf10*-expressing cells upon administration of tamoxifen (orally or intraperitoneally) to activate the Cre recombinase. In contrast to the above-mentioned mouse lines which are constitutively deficient in *Fgf10* expression, we also made use of *Rosa26rtTA;tet(o)sFgfr2b*, which allows a conditional overexpression of a soluble form of FGFR2b receptor (dominant negative receptor) ubiquitously (Rosa26 locus) upon administration of doxycycline (orally or intraperitoneally) in order to prevent FGFR2b ligands to bind to the membrane bound FGFR2b receptor (**Appendic B, D, E**). In order to investigate the effect of *Fgf10* overexpression, *Rosa26rtTA;tet(o)Fgf10* has been generated. Ubiquitous *Fgf10* overexpression can be induced by administration of doxycycline orally or intraperitoneally (**Appendix, G, H**).

Apart from transgenic mouse lines for the loss-of-function of *Fgf10*, we also tested mouse lines owning the capability to overexpress *Fgf10* (gain-of-function) upon induction. For example, we overexpress *Fgf10* using *Rosa26rtTA;tet(o)Fgf10* mouse line after hyperoxic lung injury (BPD mouse model) and observed an improvement in lung morphology compared to the control group. Further experiments will be carried

out to investigate the lung function and the cells involved in this context (unpublished data). Furthermore, we used the same mouse line to overexpress *Fgf10* for two weeks in adult mice. Immunofluorescent staining showed a significant expansion of basal stem/progenitor cells (BSC) in the trachea indicating that *Fgf10* is required to maintain BSCs in postnatal life. This data was reinforced by a loss-of-function experiment using *Sox2-CreERT2;Fgfr2b^{flox/flox}*, where depletion of BSCs was observed (**Appendix G**). Another mouse line for the gain-of-function of *Fgf10* is the *Sox2-CreERT2;Rosa-LSL-rtTA;tetOFgf10* mouse line, which allow the specific overexpression of *Fgf10* in bronchial epithelial cells. The results show that *Fgf10* is required for the differentiation of the bronchial epithelial stem cell (BESC) population towards the ATII cell lineage to generate ATII cells after bleomycin injury of the lung (**Appendix H**).

In summary, by using transgenic mice we gained new insights into the temporospatial regulation of *Fgf10* during developmental and regenerative processes of the lung.

However, before use of the transgenic mice, they need to be validated thoroughly regarding the reliability and reproducibility of the transgene. Some of the drawbacks must be considered while interpreting the results to be able to make the right conclusions. For example, *Fgf10^{iCre/+};tomato^{flox/+}* exhibit partial *Fgf10* loss of function and express only 50% of *Fgf10* due to insertion of the Cre-ERT2 cassette in exon 1 of the *Fgf10*. Further, there is a mismatch between *Cre* and *Fgf10* expression levels, especially in the postnatal period. The low expression of *Cre* is still sufficient to induce recombination till P60, but efficient recombination in later postnatal stages still needs to be validated [131].

Another major limitation using *Rosa26rtTA;tet(o)sFgfr2b* - a doxycycline-based tetracycline-controlled transactivator system - is that the expression of the dominant negative receptor soluble FGFR2b is globally. Thus, potential secondary effects cannot be completely excluded. Especially, the blockade of FGFR2b ligands in the mesenchyme, which has been shown to be able to act directly on the mesenchyme, could cause indirect effects on the epithelium due to potential epithelial-mesenchymal-interactions [132](Al Alam et al., 2015).

Furthermore, the dominant negative receptor is scavenging FGF1, 3, 7, 10 and 22. Although, we could show that during early pseudoglandular stage (E12.5) of lung development, *Fgf10* is solely expressed, *Fgf1* and *Fgf7* are emerging FGFs in the mesenchyme during late pseudoglandular stages at E14.5 and E15.5 (**Figure 1A, Appendix B**).

In this context, it is important to mention that the P-ERK on primary culture of lung mesenchyme is not significantly increased at E14.5 [133]. To show that the impact seen after the blockade of FGFR2b signalling is epithelial-specific, we showed by gene array that the “FGF10 signature” (genes changed upon inhibition of FGFR2b signalling) is only changed in the epithelium versus the mesenchyme (**Figure S11, Appendix B**). The inhibition of FGF10 signalling is further validated using the FGF10-SHH feedback loop via ETV4/ETV5 (**Figure 4H,I, Appendix B**) [134].

The lethality observed in *Fgf10*^{+/-} mice upon hyperoxic lung injury is due to increased damages of the lungs (**Figure 1F,G, Appendix E**). Since the decreased expression of *Fgf10* is constitutive, we cannot conclude that a proper *Fgf10* expression level is needed to cope with the toxicity of hyperoxia due to potential built-in defects which already occurred during embryonic development. Indeed, further investigations revealed a quantitative and qualitative malformation of the ATII cells associated with surfactant protein C (SFTPC) deficiency in the normoxia and hyperoxia group. In

order to test the potential protective effect of Fgf10/FGFR2b ligands during hyperoxic lung injury, we performed the same experiment using *Rosa26rtTA;tet(o)sFgfr2b*. There, we also observed lethality but with less severity suggesting that FGF10/FGFR2b ligands are indeed playing a protective role during hyperoxic lung injury (**Figure 5F,G, Appendix E**). As mentioned above, FGFR2b is attenuating FGF1, 3, 7, 10 and 22, thus the effect seen cannot be solely attributed to the loss-of-function of FGF10. In order to overcome this drawback, we generated a conditional *Fgf10* knock-out mouse line driven by the ubiquitous promotor *Rosa26* based on a tamoxifen-estrogen receptor systems (*Rosa26-CreERT2; Fgf10^{flox/flox}*). Unfortunately, the validation revealed that the conditional deletion of *Fgf10* on cellular level was quite heterogenous and only targets around 20% of the cells in the lung (unpublished data).

Despite an existing variety of transgenic mouse lines (mostly epithelial-specific, e.g. *Sftpc*, *Hopx*, *Scgb1a1* etc.) being used to decipher the functional role of *Fgf10* in lung injury and regeneration, the progress is still hampered by a lack of more cell-specific mouse driver lines allowing an inducible genetic deletion or overexpression of *Fgf10* in a temporospatial fashion. Especially, cell-specific driver lines which target mesenchymal cell populations are not available, since so far research has been focussed mainly on the lung epithelium. However, growing evidence confirmed the importance of epithelial-mesenchymal interactions in lung development and regeneration after lung injury. The lung mesenchyme is populated by a highly diverse and heterogenous cell population which is largely understudied. The identification of molecular markers of lung mesenchymal cells and the generation of the according mouse lines to label mesenchymal-specific cell population in-vivo will allow progress in the understanding of lung mesenchymal biology during development and regeneration. In particular, it will promote the understanding of FGF10 in this context

since FGF10 is expressed by a cell population located in the submesothelial mesenchymal compartment.

Consequently, much effort has been spent by other groups to establish new cell-specific driver lines to target mesenchymal cell populations. Once validated, these driver lines could be used for further generation of mouse lines for the inducible genetic deletion of *Fgf10* in the mesenchyme. For instance, Ntokou and colleagues generated an inducible mouse Cre-driver line to target mesenchymal cells of the adipose-differentiation related protein (ADRP) lineage, including the lipofibroblasts [135]. Other cellular markers have been discovered to target mesenchymal cell populations in pre- and postnatal lung mesenchyme. Most recently, transcription factor 21 (Tcf21) has been shown to give rise to a subpopulation of fibroblasts and lipofibroblasts as well as smooth muscle cells during embryogenesis at E11.5 [136]. T-box transcription factor Tbx4 (Tbx4)-lung enhancer driven Tet-on system have been established to target lung mesenchyme pre- and postnatally [137]. Also, platelet-derived growth factor receptor alpha ($Pdgfr\alpha$) are expressed in the mesenchyme of multiple organs, including the lung, during embryonic development. In the context of the lung, they have been shown to be expressed in myofibroblasts, a cell type critical for alveologenesis in the lung [138][139][140][141]. All these driver lines targeting mesenchymal cell population could be crossed with mouse lines which allow labelling or modification of the FGF10 level, such as *Fgf10*^{flox/flox}; *tomato*^{flox/+}.

3.2 Limitations of the single-hit hyperoxia-based BPD mouse model

The BPD mouse model is a well-established and widely accepted model to mimic the lung phenotype of BPD (alveolar simplification, dysmorphic lung vasculature)

seen in preterm infants. However, one major critique of this mouse model of BPD is the use of high level of oxygen (85% oxygen from postnatal day 0 till postnatal day 8) to induce disruption of proper alveologenesis. This level of oxygen supply is rarely used to achieve a proper oxygen saturation in prematurely born infants in neonatal intensive care units (NICU). This drawback could be addressed by using a lower level of oxygen proposed by different authors, e.g. 40% [142], 50% [143], 60% [144][145][146], 70% [147] and 75% [148][149][150][151][152]. However, one should consider that lower oxygen level usually requires a longer time period of oxygen exposure of up to 3-fold.

Another limitation of this “one-hit” mouse model of BPD is the lack a mechanical injury of the lung tissue by mechanical ventilation. Despite the increasing use of non-invasive approaches such as continuous positive airway pressure (CPAP) and nasal intermittent positive pressure ventilation (NIPPV) to replace invasive mechanical ventilation (IMV) in the airway management of preterm infants suffering respiratory distress syndrome (RDS) thereby avoiding barotrauma, some studies failed to show a significant beneficial effect on the reduction of the rate of BPD [153]. Nevertheless, barotrauma is still considered as a major injurious stimuli in the pathogenesis of BPD [154]. It has been shown that a harsh ventilation regime disrupts lung alveolarization whereas a mild ventilation protocol does not lead to pathological changes in alveolarization [155].

Lastly, inflammation pre- or postnatally is believed to play an important role in the perturbation of normal alveologenesis and preterm infants suffering sepsis are at higher risk to develop BPD [156][157][158]. Consistent with this notion, some animal model of BPD combine hyperoxia exposure and bacterial lipopolysaccharide (LPS) or exposure to *Ureaplasma* [159][160][161].

In summary, despite the high diversity of animal models of BPD including mice, rats, baboons, sheep, rabbits, for the purpose of this study, the single-hit hyperoxia-based BPD mouse model represents a highly reliable and reproducible model mimicking alveolar simplification and vascular remodeling. Furthermore, the availability of transgenic mice is a major advantage allowing new insights into biological processes on molecular and cellular levels. Last but not least, with respect to issues concerning cost for husbandry and sample analyses as well as animal welfare ethics, the mouse model of BPD will remain the method of choice.

However, for further drug validation (e.g. intratracheal administration or inhalation of recombinant FGF10) the neonatal pups represent a handling problem due to the very small sizes of the pups. The analyses of physiological parameters such as lung function, echocardiography (assessment of pulmonary hypertension) and imaging of the lung vacuature using computed tomography would be a technical limitation in a mouse model of BPD.

3.3 Clinically relevant aspects about FGF10 in human BPD

Besides the studies mentioned in the introduction (Chapter 1.3., [51][158]), there are only several other studies showing that FGF10 signalling is involved in human BPD. FGF10 level has been measured in cord blood of 269 preterm infants of whom 76 infants developed BPD subsequently. Analyses revealed that low cord blood FGF10 levels is associated with the development of severe BPD and could be used as a biological predictor [162]. Ambalavanan et al showed in a cohort of 29 preterm and mechanically ventilated infants (<34 week gestation) that elevated FGF2 (bFGF) in tracheal aspirates correlates with the development of BPD [163]. Using next

generation sequencing techniques rare heterozygous variant deletions or single-nucleotide variants (SNVs) involving TBX4 or FGF10 have been identified to be involved in neonatal lethal pulmonary hypoplasias with primary defects in branching morphogenesis suggesting a disruption of the TBX4-FGF10-FGFR2 epithelial-mesenchymal signalling pathway [164][165]. Investigations of this signalling pathway in autopsy samples from infants suffering BPD is lacking. One preclinical validation of recombinant FGF10 using a hyperoxia-based BPD mouse model combined with an in-vitro approach showed that exogenous recombinant FGF10 treatment was able to promote the growth of pulmonary mesenchymal stem cells, ATII cells and to reduce the expression of proinflammatory cytokines [166]. So far, no clinical trials have been carried out to test the effect of recombinant FGF10 in BPD. The only clinical phase 2 trial involving Keratinocyte growth factor (KGF, FGF7) for the treatment of acute respiratory distress syndrome (KARE) in adults did not show improvement of physiological or clinical outcomes and might even worsen clinical outcomes due to adverse events [167].

4. Future perspectives

FGF10 and FGFR2b signalling is believed to be essential in lung development and during regeneration in lung diseases, such like BPD.

Clinical-grade recombinant FGF10 might have therapeutic potential for the treatment of BPD. However, based on the facts that other FGFs (e.g. FGF1 and FGF2) have been shown to be involved in the remodelling process of bronchial airways in COPD, thus, potentially detrimental [58], the therapeutic use of FGF10 needs to be thoroughly validated. Also, potential adverse side effects need to be evaluated [167].

In collaboration with a pharmaceutical company and as a first translational step, we will validate the therapeutic effect of recombinant FGF10 in a mouse model of BPD.

In brief, neonatal pups after hyperoxia-induced perturbed alveolarization from postnatal day zero till postnatal day eight will be administered recombinant FGF10 intraperitoneally in three episodes (to minimize FGF10-related adverse side effects) until time of analyses at postnatal day 45. The primary read outs include lung function, lung morphometry, lung vascular morphometry and echocardiography. Lung tissues will be stored for further analyses. This study will be performed as a proof-of-concept and will lead to follow-up studies using large animal models of BPD in case of positive results. Large animal models will allow the intratracheal or inhalative administration of recombinant FGF10 which will be much more favourable and clinically relevant.

Apart from the above-mentioned translational approaches, FGF10 expression (gene and protein levels) needs to be better clinically characterized within the heterogeneous cohort of preterm infants at risk of developing BPD (gestation age, risk factors for BPD, postnatal ventilation regimes, genetic predisposition etc.). Thus,

future studies including the measurement, e.g. using tracheal aspirates or exhaled breath condensate (EBC), of the expression of FGF10 in preterm infants is highly desirable.

The establishment of a centralized biobank and databank would promote collection and characterization of biomaterials, provide organized availability and facilitate accessibility for research.

In parallel to efforts invested in the clinical setting, more basic research needs to be done in order to gain deeper insight into the cellular and molecular mechanisms of FGF signalling governing alveologenesis and regeneration processes in BPD.

The combination of both clinical and basic research will allow a better design of therapeutic strategies to prevent and to treat BPD in the future.

5. Summary

Increasing evidence is confirming the key role of FGF10 in lung developmental processes pre- and postnatally as well as in lung regeneration after injury, such like in Bronchopulmonary dysplasia (BPD).

With regard to the role of *Fgf10* in lung development, we first confirmed using transgenic reporter mouse line that the two transcription factors, ETV4 and ETV5, indeed acted downstream of FGF10/FGFR2b signalling. Further, we showed that FGF10 signalling on distal epithelial progenitors was essential during early lung development and was mainly mediated through β -catenin/EP300. Paying attention to the lung mesenchyme, we were able to identify and lineage-trace two populations of *Fgf10*-expressing progenitor cells which gave rise to different mesenchymal cell types pre- and postnatally, e.g. lipofibroblast.

In the next chapter, we provided evidence that *Fgf10* was crucial for proper development of the alveolar epithelial cells type II (ATII), the pulmonary vasculature and to cope with hyperoxic lung injury using a mouse model for BPD. Perturbation of FGF10/FGFR2b signalling led to a phenotype similar to pulmonary hypertension. During regeneration after naphthalene-mediated epithelial injury, the Yap-Wnt7b-*Fgf10* regenerative pathway is activated in the airway smooth muscle cell niche to recruit basal cell to the area of injury. Similarly, we have shown that FGF10/FGFR2b signalling was needed for the regeneration of the alveolar epithelium after bleomycin injury (mouse model of idiopathic pulmonary fibrosis, IPF). Finally, in the last chapter we emphasized the critical role of epigenetic regulations in lung development and BPD. We identified *miR-142-3p* as a major epigenetic regulator for the proliferation and differentiation of mesenchymal cells and for the alveolar lineage formation during

lung development. Also, early exposure to high oxygen levels was associated with persisting changes in histone modifications in pulmonary endothelial cells, which might be accountable for the development of pulmonary hypertension in the pathogenesis of BPD.

6. References

1. Treutlein, B.; Brownfield, D.G.; Wu, A.R.; Neff, N.F.; Mantalas, G.L.; Espinoza, F.H.; Desai, T.J.; Krasnow, M.A.; Quake, S.R. Reconstructing lineage hierarchies of the distal lung epithelium using single-cell RNA-seq. *Nature* **2014**, *509*, 371–375.
2. Torday, J.S.; Rehan, V.K. On the evolution of the pulmonary alveolar lipofibroblast. *Exp. Cell Res.* 2016.
3. Kresch, M.J.; Christian, C.; Wu, F.; Hussain, N. Ontogeny of apoptosis during lung development. *Pediatr. Res.* **1998**.
4. Burri, P.H. Structural aspects of postnatal lung development - alveolar formation and growth. *Biol. Neonate* **2006**, *89*, 313–322.
5. Ochs, M.; Nyengaard, J.R.; Jung, A.; Knudsen, L.; Voigt, M.; Wahlers, T.; Richter, J.; Gundersen, H.J. The number of alveoli in the human lung. *Am J Respir Crit Care Med* **2004**, *169*, 120–124.
6. Noguchi, A.; Reddy, R.; Kursar, J.D.; Parks, W.C.; Mecham, R.P. Smooth muscle isoactin and elastin in fetal bovine lung. *Exp. Lung Res.* **1989**, *15*, 537–552.
7. Vaccaro, C.; Brody, J.S. Ultrastructure of developing alveoli. I. The role of the interstitial fibroblast. *Anat. Rec.* **1978**, *192*, 467–479.
8. Chao, C.M.; El Agha, E.; Tiozzo, C.; Minoo, P.; Bellusci, S. A breath of fresh air on the mesenchyme: impact of impaired mesenchymal development on the pathogenesis of bronchopulmonary dysplasia. *Front Med* **2015**, *2*, 27.
9. Volckaert, T.; De Langhe, S.P. Wnt and FGF mediated epithelial-mesenchymal crosstalk during lung development. *Dev Dyn* **2015**, *244*, 342–366.

10. Chang, D.R.; Martinez Alanis, D.; Miller, R.K.; Ji, H.; Akiyama, H.; McCrea, P.D.; Chen, J. Lung epithelial branching program antagonizes alveolar differentiation. *Proc Natl Acad Sci U S A* **2013**, *110*, 18042–18051.
11. Stoll, B.J.; Hansen, N.I.; Bell, E.F.; Shankaran, S.; Laptook, A.R.; Walsh, M.C.; Hale, E.C.; Newman, N.S.; Schibler, K.; Carlo, W.A.; et al. Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. *Pediatrics* **2010**, *126*, 443–456.
12. Stoll, B.J.; Hansen, N.I.; Bell, E.F.; Walsh, M.C.; Carlo, W.A.; Shankaran, S.; Laptook, A.R.; Sánchez, P.J.; Van Meurs, K.P.; Wyckoff, M.; et al. Trends in care practices, morbidity, and mortality of extremely preterm Neonates, 1993-2012. *JAMA - J. Am. Med. Assoc.* 2015.
13. Jensen, E.A.; Dysart, K.; Gantz, M.G.; McDonald, S.; Bamat, N.A.; Keszler, M.; Kirpalani, H.; Laughon, M.M.; Poindexter, B.B.; et al. The Diagnosis of Bronchopulmonary Dysplasia in Very Preterm Infants: An Evidence-Based Approach. *Am. J. Respir. Crit. Care Med.* **2019**.
14. Northway Jr., W.H.; Rosan, R.C.; Porter, D.Y. Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. *N. Engl. J. Med.* **1967**, *276*, 357–368.
15. Mourani, P.M.; Sontag, M.K.; Younoszai, A.; Miller, J.I.; Kinsella, J.P.; Baker, C.D.; Poindexter, B.B.; Ingram, D.A.; Abman, S.H. Early pulmonary vascular disease in preterm infants at risk for bronchopulmonary dysplasia. *Am. J. Respir. Crit. Care Med.* **2015**.
16. Simpson, S.J.; Hall, G.L.; Wilson, A.C. Lung function following very preterm birth in the era of “new” bronchopulmonary dysplasia. *Respirology* 2015.
17. Rossor, T.; Greenough, A. Advances in paediatric pulmonary vascular disease associated with bronchopulmonary dysplasia. *Expert Rev. Respir. Med.* 2015.

18. Domm, W.; Misra, R.S.; O'Reilly, M.A. Affect of Early Life Oxygen Exposure on Proper Lung Development and Response to Respiratory Viral Infections. *Front. Med.* **2015**.
19. Domm, W.; Yee, M.; Misra, R.S.; Gelein, R.; Nogales, A.; Martinez-Sobrido, L.; O'Reilly, M.A. Oxygen-dependent changes in lung development do not affect epithelial infection with influenza A virus. *Am. J. Physiol. Cell. Mol. Physiol.* **2017**.
20. O'Reilly, M.A.; Yee, M.; Buczynski, B.W.; Vitiello, P.F.; Keng, P.C.; Welle, S.L.; Finkelstein, J.N.; Dean, D.A.; Lawrence, B.P. Neonatal oxygen increases sensitivity to influenza A virus infection in adult mice by suppressing epithelial expression of Ear1. *Am. J. Pathol.* **2012**.
21. Álvarez-Fuente, M.; Arruza, L.; Muro, M.; Zozaya, C.; Avila, A.; López-Ortego, P.; González-Armengod, C.; Torrent, A.; Gavilán, J.L.; del Cerro, M.J. The economic impact of prematurity and bronchopulmonary dysplasia. *Eur. J. Pediatr.* **2017**.
22. Shaw, G.M.; O'Brodovich, H.M. Progress in understanding the genetics of bronchopulmonary dysplasia. *Semin. Perinatol.* **2013**, *37*, 85–93.
23. Rezvani, M.; Wilde, J.; Vitt, P.; Mailaparambil, B.; Grychtol, R.; Krueger, M.; Heinzmann, A. Association of a FGFR-4 gene polymorphism with bronchopulmonary dysplasia and neonatal respiratory distress. *Dis. Markers* **2013**.
24. F rster, K.; Sass, S.; Ehrhardt, H.; Mous, D.S.; Rottier, R.J.; Oak, P.; Schulze, A.; Flemmer, A.W.; Gronbach, J.; H bener, C.; et al. Early identification of bronchopulmonary dysplasia using novel biomarkers by proteomic screening. *Am. J. Respir. Crit. Care Med.* **2018**.
25. Poets, C.F.; Lorenz, L. Prevention of bronchopulmonary dysplasia in extremely

- low gestational age neonates: current evidence. *Arch. Dis. Child. Fetal Neonatal Ed.* 2018.
26. Gronbach, J.; Shahzad, T.; Radajewski, S.; Chao, C.M.; Bellusci, S.; Morty, R.E.; Reicherzer, T.; Ehrhardt, H. The potentials and caveats of mesenchymal stromal cell-based therapies in the preterm infant. *Stem Cells Int.* 2018.
27. Nitkin, C.R.; Rajasingh, J.; Pisano, C.; Besner, G.E.; Thébaud, B.; Sampath, V. Stem cell therapy for preventing neonatal diseases in the 21st century: Current understanding and challenges. *Pediatr. Res.* 2019.
28. Baraldi, E.; Filippone, M. Chronic lung disease after premature birth. *N. Engl. J. Med.* **2007**, *357*, 1946–1955.
29. Itoh, N.; Ornitz, D.M. Fibroblast growth factors: From molecular evolution to roles in development, metabolism and disease. *J. Biochem.* 2011.
30. El Agha, E.; Kosanovic, D.; Schermuly, R.T.; Bellusci, S. Role of fibroblast growth factors in organ regeneration and repair. *Semin. Cell Dev. Biol.* 2016.
31. Yuan, T.; Volckaert, T.; Redente, E.F.; Hopkins, S.; Klinkhammer, K.; Wasnick, R.; Chao, C.M.; Yuan, J.; Zhang, J.S.; Yao, C.; et al. FGF10-FGFR2B Signaling Generates Basal Cells and Drives Alveolar Epithelial Regeneration by Bronchial Epithelial Stem Cells after Lung Injury. *Stem Cell Reports* **2019**.
32. Yuan, T.; Volckaert, T.; Chanda, D.; Thannickal, V.J.; De Langhe, S.P. Fgf10 Signaling in Lung Development, Homeostasis, Disease, and Repair After Injury. *Front. Genet.* **2018**.
33. Volckaert, T.; Yuan, T.; Chao, C.M.; Bell, H.; Sitaula, A.; Szimtenings, L.; El Agha, E.; Chanda, D.; Majka, S.; Bellusci, S.; et al. Fgf10-Hippo Epithelial-Mesenchymal Crosstalk Maintains and Recruits Lung Basal Stem Cells. *Dev. Cell* **2017**.
34. Volckaert, T.; Dill, E.; Campbell, A.; Tiozzo, C.; Majka, S.; Bellusci, S.; De

- Langhe, S.P. Parabronchial smooth muscle constitutes an airway epithelial stem cell niche in the mouse lung after injury. *J. Clin. Invest.* **2011**, *121*, 4409–4419.
35. Quantius, J.; Schmoldt, C.; Vazquez-Armendariz, A.I.; Becker, C.; El Agha, E.; Wilhelm, J.; Morty, R.E.; Vadász, I.; Mayer, K.; Gattenloehner, S.; et al. Influenza Virus Infects Epithelial Stem/Progenitor Cells of the Distal Lung: Impact on Fgfr2b-Driven Epithelial Repair. *PLoS Pathog.* **2016**.
36. Warburton, D.; Perin, L.; Defilippo, R.; Bellusci, S.; Shi, W.; Driscoll, B. Stem/progenitor cells in lung development, injury repair, and regeneration. *Proc Am Thorac Soc* **2008**, *5*, 703–706.
37. Dianat-Moghadam, H.; Teimoori-Toolabi, L. Implications of Fibroblast Growth Factors (FGFs) in Cancer: From Prognostic to Therapeutic Applications. *Curr. Drug Targets* **2019**.
38. Bellusci, S.; Grindley, J.; Emoto, H.; Itoh, N.; Hogan, B.L. Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. *Development* **1997**, *124*, 4867–4878.
39. Bellusci, S.; Furuta, Y.; Rush, M.G.; Henderson, R.; Winnier, G.; Hogan, B.L. Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. *Development* **1997**, *124*, 53–63.
40. del Moral, P.M.; De Langhe, S.P.; Sala, F.G.; Veltmaat, J.M.; Tefft, D.; Wang, K.; Warburton, D.; Bellusci, S. Differential role of FGF9 on epithelium and mesenchyme in mouse embryonic lung. *Dev. Biol.* **2006**, *293*, 77–89.
41. Sekine, K.; Ohuchi, H.; Fujiwara, M.; Yamasaki, M.; Yoshizawa, T.; Sato, T.; Yagishita, N.; Matsui, D.; Koga, Y.; Itoh, N.; et al. Fgf10 is essential for limb and lung formation. *Nat. Genet.* **1999**, *21*, 138–141.
42. Arman, E.; Haffner-Krausz, R.; Gorivodsky, M.; Lonai, P. Fgfr2 is required for

- limb outgrowth and lung-branching morphogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, 96, 11895–11899.
43. De Moerlooze, L.; Spencer-Dene, B.; Revest, J.M.; Hajihosseini, M.; Rosewell, I.; Dickson, C. An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. *Development* **2000**, 127, 483–492.
44. Peters, K.; Werner, S.; Liao, X.; Wert, S.; Whitsett, J.; Williams, L. Targeted expression of a dominant negative FGF receptor blocks branching morphogenesis and epithelial differentiation of the mouse lung. *EMBO J.* **1994**, 13, 3296–3301.
45. Parsa, S.; Ramasamy, S.K.; De Langhe, S.; Gupte, V. V.; Haigh, J.J.; Medina, D.; Bellusci, S. Terminal end bud maintenance in mammary gland is dependent upon FGFR2b signaling. *Dev. Biol.* **2008**.
46. El Agha, E.; Herold, S.; Al Alam, D.; Quantius, J.; MacKenzie, B.; Carraro, G.; Moiseenko, A.; Chao, C.M.; Minoo, P.; Seeger, W.; et al. Fgf10-positive cells represent a progenitor cell population during lung development and postnatally. *Development* **2014**, 141, 296–306.
47. Lu, J.; Izvolsky, K.I.; Qian, J.; Cardoso, W. V Identification of FGF10 targets in the embryonic lung epithelium during bud morphogenesis. *J. Biol. Chem.* **2005**, 280, 4834–4841.
48. Chao, C.M.; Yahya, F.; Moiseenko, A.; Tiozzo, C.; Shrestha, A.; Ahmadvand, N.; El Agha, E.; Quantius, J.; Dilai, S.; Kheirollahi, V.; et al. Fgf10 deficiency is causative for lethality in a mouse model of bronchopulmonary dysplasia. *J. Pathol.* **2017**.
49. Chao, C.M.; Moiseenko, A.; Kosanovic, D.; Rivetti, S.; El Agha, E.; Wilhelm, J.; Kampschulte, M.; Yahya, F.; Ehrhardt, H.; Zimmer, K.P.; et al. Impact of Fgf10

- deficiency on pulmonary vasculature formation in a mouse model of bronchopulmonary dysplasia. *Hum. Mol. Genet.* **2019**.
50. Ramasamy, S.K.; Mailleux, A.A.; Gupte, V. V; Mata, F.; Sala, F.G.; Veltmaat, J.M.; Del Moral, P.M.; De Langhe, S.; Parsa, S.; Kelly, L.K.; et al. Fgf10 dosage is critical for the amplification of epithelial cell progenitors and for the formation of multiple mesenchymal lineages during lung development. *Dev. Biol.* **2007**, *307*, 237–247.
51. Benjamin, J.T.; Smith, R.J.; Halloran, B.A.; Day, T.J.; Kelly, D.R.; Prince, L.S. FGF-10 is decreased in bronchopulmonary dysplasia and suppressed by Toll-like receptor activation. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2007**, *292*, L550-8.
52. Carver, B.J.; Plosa, E.J.; Stinnett, A.M.; Blackwell, T.S.; Prince, L.S. Interactions between NF-kappaB and SP3 connect inflammatory signaling with reduced FGF-10 expression. *J. Biol. Chem.* **2013**, *288*, 15318–15325.
53. Entesarian, M.; Matsson, H.; Klar, J.; Bergendal, B.; Olson, L.; Arakaki, R.; Hayashi, Y.; Ohuchi, H.; Falahat, B.; Bolstad, A.I.; et al. Mutations in the gene encoding fibroblast growth factor 10 are associated with aplasia of lacrimal and salivary glands. *Nat Genet* **2005**, *37*, 125–127.
54. Rohmann, E.; Brunner, H.G.; Kayserili, H.; Uyguner, O.; Nurnberg, G.; Lew, E.D.; Dobbie, A.; Eswarakumar, V.P.; Uzumcu, A.; Ulubil-Emeroglu, M.; et al. Mutations in different components of FGF signaling in LADD syndrome. *Nat Genet* **2006**, *38*, 414–417.
55. Milunsky, J.M.; Zhao, G.; Maher, T.A.; Colby, R.; Everman, D.B. LADD syndrome is caused by FGF10 mutations. *Clin Genet* **2006**, *69*, 349–354.
56. Klar, J.; Blomstrand, P.; Brunmark, C.; Badhai, J.; Hakansson, H.F.; Brange, C.S.; Bergendal, B.; Dahl, N. Fibroblast growth factor 10 haploinsufficiency

- causes chronic obstructive pulmonary disease. *J Med Genet* **2011**, *48*, 705–709.
57. Kranenburg, A.R.; De Boer, W.I.; Van Krieken, J.H.J.M.; Mooi, W.J.; Walters, J.E.; Saxena, P.R.; Sterk, P.J.; Sharma, H.S. Enhanced expression of fibroblast growth factors and receptor FGFR-1 during vascular remodeling in chronic obstructive pulmonary disease. *Am. J. Respir. Cell Mol. Biol.* **2002**.
58. Kranenburg, A.R.; Willems-Widyastuti, A.; Mooi, W.J.; Saxena, P.R.; Sterk, P.J.; de Boer, W.I.; Sharma, H.S. Chronic obstructive pulmonary disease is associated with enhanced bronchial expression of FGF-1, FGF-2, and FGFR-1. *J. Pathol.* **2005**.
59. Hokuto, I.; Perl, A.K.; Whitsett, J.A. FGF signaling is required for pulmonary homeostasis following hyperoxia. *Am J Physiol Lung Cell Mol Physiol* **2004**, *286*, L580-7.
60. Chen, L.; Acciani, T.; Le Cras, T.; Lutzko, C.; Perl, A.K. Dynamic regulation of platelet-derived growth factor receptor alpha expression in alveolar fibroblasts during realveolarization. *Am J Respir Cell Mol Biol* **2012**, *47*, 517–527.
61. Ding, B.S.; Nolan, D.J.; Guo, P.; Babazadeh, A.O.; Cao, Z.; Rosenwaks, Z.; Crystal, R.G.; Simons, M.; Sato, T.N.; Worgall, S.; et al. Endothelial-derived angiocrine signals induce and sustain regenerative lung alveolarization. *Cell* **2011**, *147*, 539–553.
62. Srisuma, S.; Bhattacharya, S.; Simon, D.M.; Solleti, S.K.; Tyagi, S.; Starcher, B.; Mariani, T.J. Fibroblast growth factor receptors control epithelial-mesenchymal interactions necessary for alveolar elastogenesis. *Am. J. Respir. Crit. Care Med.* **2010**.
63. Weinstein, M.; Xu, X.; Ohyama, K.; Deng, C.X. FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung. *Development* **1998**.

64. Tekin, M.; Öztürkmen Akay, H.; Fitoz, S.; Birnbaum, S.; Cengiz, F.B.; Sennaroğlu, L.; Incesulu, A.; Yüksel, K.; Hasanefendioğlu Bayrak, A.; Şentürk, S.; et al. Homozygous FGF3 mutations result in congenital deafness with inner ear agenesis, microtia, and microdontia. *Clin. Genet.* **2008**.
65. White, K.E.; Carn, G.; Lorenz-Depiereux, B.; Benet-Pages, A.; Strom, T.M.; Econs, M.J. Autosomal-dominant hypophosphatemic rickets (ADHR) mutations stabilize FGF-23. *Kidney Int.* **2001**.
66. Hagood, J.S. Beyond the Genome: Epigenetic Mechanisms in Lung Remodeling. *Physiology* **2014**.
67. Ambros, V. The functions of animal microRNAs. *Nature* **2004**.
68. Bartel, D.P. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell* **2004**.
69. Huntzinger, E.; Izaurralde, E. Gene silencing by microRNAs: Contributions of translational repression and mRNA decay. *Nat. Rev. Genet.* **2011**.
70. Esteller, M. Non-coding RNAs in human disease. *Nat. Rev. Genet.* **2011**.
71. Ameres, S.L.; Zamore, P.D. Diversifying microRNA sequence and function. *Nat. Rev. Mol. Cell Biol.* **2013**.
72. Ambros, V.; Lee, R.C. Identification of microRNAs and Other Tiny Noncoding RNAs by cDNA Cloning. In *RNA Interference, Editing, and Modification*; **2009**.
73. Friedman, R.C.; Farh, K.K.H.; Burge, C.B.; Bartel, D.P. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* **2009**.
74. Gangaraju, V.K.; Lin, H. MicroRNAs: Key regulators of stem cells. *Nat. Rev. Mol. Cell Biol.* **2009**.
75. Sessa, R.; Hata, A. Role of microRNAs in Lung Development and Pulmonary Diseases. *Pulm. Circ.* **2013**.
76. Cardoso, W. V. Regulation of early lung morphogenesis: questions, facts and

- controversies. *Development* **2006**.
77. Warburton, D.; El-Hashash, A.; Carraro, G.; Tiozzo, C.; Sala, F.; Rogers, O.; De Langhe, S.; Kemp, P.J.; Riccardi, D.; Torday, J.; et al. Lung organogenesis. *Curr Top Dev Biol* **2010**, *90*, 73–158.
78. Bhaskaran, M.; Wang, Y.; Zhang, H.; Weng, T.; Baviskar, P.; Guo, Y.; Gou, D.; Liu, L. MicroRNA-127 modulates fetal lung development. *Physiol. Genomics* **2009**.
79. Williams, A.E.; Moschos, S.A.; Perry, M.M.; Barnes, P.J.; Lindsay, M.A. Maternally imprinted microRNAs are differentially expressed during mouse and human lung development. *Dev. Dyn.* **2007**.
80. Ornitz, D.M.; Yin, Y. Signaling networks regulating development of the lower respiratory tract. *Cold Spring Harb. Perspect. Biol.* **2012**.
81. Harris, K.S.; Zhang, Z.; McManus, M.T.; Harfe, B.D.; Sun, X. Dicer function is essential for lung epithelium morphogenesis. *Proc. Natl. Acad. Sci.* **2006**.
82. Carraro, G.; El-Hashash, A.; Guidolin, D.; Tiozzo, C.; Turcatel, G.; Young, B.M.; De Langhe, S.P.; Bellusci, S.; Shi, W.; Parnigotto, P.P.; et al. miR-17 family of microRNAs controls FGF10-mediated embryonic lung epithelial branching morphogenesis through MAPK14 and STAT3 regulation of E-Cadherin distribution. *Dev Biol* **2009**, *333*, 238–250.
83. Lu, Y.; Thomson, J.M.; Wong, H.Y.F.; Hammond, S.M.; Hogan, B.L.M. Transgenic over-expression of the microRNA miR-17-92 cluster promotes proliferation and inhibits differentiation of lung epithelial progenitor cells. *Dev. Biol.* **2007**.
84. Ventura, A.; Young, A.G.; Winslow, M.M.; Lintault, L.; Meissner, A.; Erkeland, S.J.; Newman, J.; Bronson, R.T.; Crowley, D.; Stone, J.R.; et al. Targeted Deletion Reveals Essential and Overlapping Functions of the miR-17~92

- Family of miRNA Clusters. *Cell* **2008**.
85. Carraro, G.; Shrestha, A.; Rostkovius, J.; Contreras, A.; Chao, C.M.; El Agha, E.; Mackenzie, B.; Dilai, S.; Guidolin, D.; Taketo, M.M.; et al. miR-142-3p balances proliferation and differentiation of mesenchymal cells during lung development. *Development* **2014**, *141*, 1272–1281.
86. Shrestha, A.; Carraro, G.; El Agha, E.; Mukhametshina, R.; Chao, C.M.; Rizvanov, A.; Barreto, G.; Bellusci, S.; Kalinichenko, V. V. Generation and validation of MIR-142 knock out mice. *PLoS One* **2015**.
87. Jiang, Z.; Cushing, L.; Ai, X.; Lü, J. miR-326 is downstream of sonic hedgehog signaling and regulates the expression of Gli2 and Smoothened. *Am. J. Respir. Cell Mol. Biol.* **2014**.
88. Tian, Y.; Zhang, Y.; Hurd, L.; Hannenhalli, S.; Liu, F.; Lu, M.M.; Morrissey, E.E. Regulation of lung endoderm progenitor cell behavior by miR302/367. *Development* **2011**.
89. Benlhabib, H.; Guo, W.; Pierce, B.M.; Mendelson, C.R. The miR-200 family and its targets regulate type II cell differentiation in human fetal lung. *J. Biol. Chem.* **2015**.
90. Wang, Y.; Huang, C.; Chintagari, N.R.; Xi, D.; Weng, T.; Liu, L. miR-124 regulates fetal pulmonary epithelial cell maturation. *Am. J. Physiol. Cell. Mol. Physiol.* **2015**.
91. Mujahid, S.; Nielsen, H.C.; Volpe, M.A. V MiR-221 and miR-130a Regulate Lung Airway and Vascular Development. *PLoS One* **2013**.
92. Xing, Y.; Fu, J.; Yang, H.; Yao, L.; Qiao, L.; Du, Y.; Xue, X. MicroRNA expression profiles and target prediction in neonatal Wistar rat lungs during the development of bronchopulmonary dysplasia. *Int. J. Mol. Med.* **2015**.
93. Yuan, H.; Xiong, D.; Huang, F.; Cui, J.; Luo, H. MicroRNA-421 inhibition

- alleviates bronchopulmonary dysplasia in a mouse model via targeting Fgf10. *J. Cell. Biochem.* **2019**.
94. Nardiello, C.; Morty, R.E. MicroRNA in late lung development and bronchopulmonary dysplasia: the need to demonstrate causality. *Mol. Cell. Pediatr.* **2016**.
95. Zhang, Y.; Sun, E.; Li, X.; Zhang, M.; Tang, Z.; He, L.; Lv, K. miR-155 contributes to Df1-induced asthma by increasing the proliferative response of Th cells via CTLA-4 downregulation. *Cell. Immunol.* **2017**.
96. Oglesby, I.K.; Agrawal, R.; Mall, M.A.; McElvaney, N.G.; Greene, C.M. miRNA-221 is elevated in cystic fibrosis airway epithelial cells and regulates expression of ATF6. *Mol. Cell. Pediatr.* **2015**.
97. Shen, W.; Liu, J.; Zhao, G.; Fan, M.; Song, G.; Zhang, Y.; Weng, Z.; Zhang, Y. Repression of toll-like receptor-4 by microRNA-149-3p is associated with smoking-related COPD. *Int. J. COPD* **2017**.
98. Milosevic, J.; Pandit, K.; Magister, M.; Rabinovich, E.; Ellwanger, D.C.; Yu, G.; Vuga, L.J.; Weksler, B.; Benos, P. V.; Gibson, K.F.; et al. Profibrotic role of miR-154 in pulmonary fibrosis. *Am. J. Respir. Cell Mol. Biol.* **2012**.
99. Li, S.; Meng, H.; Zhou, F.; Zhai, L.; Zhang, L.; Gu, F.; Fan, Y.; Lang, R.; Fu, L.; Gu, L.; et al. MicroRNA-132 is frequently down-regulated in ductal carcinoma in situ (DCIS) of breast and acts as a tumor suppressor by inhibiting cell proliferation. *Pathol. Res. Pract.* **2013**.
100. Wei, T.; Zhu, W.; Fang, S.; Zeng, X.; Huang, J.; Yang, J.; Zhang, J.; Guo, L. miR-495 promotes the chemoresistance of SCLC through the epithelial-mesenchymal transition via Etk/BMX. *Am. J. Cancer Res.* **2017**.
101. Lin, X.; Yang, Z.; Zhang, P.; Shao, G. MiR-154 suppresses non-small cell lung cancer growth in vitro and in vivo. *Oncol. Rep.* **2015**.

102. Lee, H.-W.; Park, S.-H. Elevated microRNA-135a is associated with pulmonary arterial hypertension in experimental mouse model. *Oncotarget* **2015**.
103. Huang, S.; Feng, C.; Zhai, Y.Z.; Zhou, X.; Li, B.; Wang, L.L.; Chen, W.; Lv, F.Q.; Li, T.S. Identification of miRNA biomarkers of pneumonia using RNA-sequencing and bioinformatics analysis. *Exp. Ther. Med.* **2017**.
104. Zhou, X.; Wen, W.; Shan, X.; Qian, J.; Li, H.; Jiang, T.; Wang, W.; Cheng, W.; Wang, F.; Qi, L.; et al. MiR-28-3p as a potential plasma marker in diagnosis of pulmonary embolism. *Thromb. Res.* **2015**.
105. Kiszalkiewicz, J.; Piotrowski, W.J.; Pastuszek-Lewandoska, D.; Górski, P.; Antczak, A.; Górski, W.; Domańska-Senderowska, D.; Migdalska-Sek, M.; Czarnecka, K.H.; Nawrot, E.; et al. Altered miRNA expression in pulmonary sarcoidosis. *BMC Med. Genet.* **2016**.
106. Angulo, M.; Lecuona, E.; Sznajder, J.I. Role of MicroRNAs in Lung Disease. *Arch. Bronconeumol.* 2012.
107. Johar, D.; Siragam, V.; Mahood, T.H.; Keijzer, R. New insights into lung development and diseases: the role of microRNAs. *Biochem. Cell Biol.* **2014**.
108. Wang, Y.; Huang, C.; Reddy Chintagari, N.; Bhaskaran, M.; Weng, T.; Guo, Y.; Xiao, X.; Liu, L. MiR-375 regulates rat alveolar epithelial cell trans-differentiation by inhibiting Wnt/ β -catenin pathway. *Nucleic Acids Res.* **2013**.
109. Ruiz-Camp, J.; Quantius, J.; Lignelli, E.; Arndt, P.F.; Palumbo, F.; Nardiello, C.; Surate Solaligue, D.E.; Sakkas, E.; Mižíková, I.; Rodríguez-Castillo, J.A.; et al. Targeting miR-34a/ Pdgrfa interactions partially corrects alveologenesis in experimental bronchopulmonary dysplasia. *EMBO Mol. Med.* **2019**.
110. Yang, Y.; Qiu, J.; Kan, Q.; Zhou, X.G.; Zhou, X.Y. MicroRNA expression profiling studies on bronchopulmonary dysplasia: A systematic review and

- meta-analysis. *Genet. Mol. Res.* 2013.
111. Dong, J.; Carey, W.A.; Abel, S.; Collura, C.; Jiang, G.; Tomaszek, S.; Sutor, S.; Roden, A.C.; Asmann, Y.W.; Prakash, Y.S.; et al. MicroRNA-mRNA interactions in a murine model of hyperoxia-induced bronchopulmonary dysplasia. *BMC Genomics* **2012**.
112. Xin, C.; Zhang, H.; Liu, Z. MiR-154 suppresses colorectal cancer cell growth and motility by targeting TLR2. *Mol. Cell. Biochem.* **2014**.
113. Wang, Q.; Ma, J.; Jiang, Z.; Wu, F.; Ping, J.; Ming, L. Diagnostic value of circulating microRNA-27a/b in patients with acute pulmonary embolism. *Int. Angiol.* **2018**.
114. Liu, T.; Kang, J.; Liu, F. Plasma Levels of microRNA-221 (miR-221) are Increased in Patients with Acute Pulmonary Embolism. *Med. Sci. Monit.* **2018**.
115. Kessler, T.; Erdmann, J.; Vilne, B.; Bruse, P.; Kurowski, V.; Diemert, P.; Schunkert, H.; Sager, H.B. Serum microRNA-1233 is a specific biomarker for diagnosing acute pulmonary embolism. *J. Transl. Med.* **2016**.
116. Xiao, J.; Jing, Z.C.; Ellinor, P.T.; Liang, D.; Zhang, H.; Liu, Y.; Chen, X.; Pan, L.; Lyon, R.; Liu, Y.; et al. MicroRNA-134 as a potential plasma biomarker for the diagnosis of acute pulmonary embolism. *J. Transl. Med.* **2011**.
117. Wang, Y.; Zhang, X.; Tian, J.; Liu, G.; Li, X.; Shen, D. Sevoflurane alleviates LPS-induced acute lung injury via the microRNA-27a-3p/TLR4/MyD88/NF- κ B signaling pathway. *Int. J. Mol. Med.* **2019**.
118. Jin, Y.; Yang, C.; Sui, X.; Cai, Q.; Guo, L.; Liu, Z. Endothelial progenitor cell transplantation attenuates lipopolysaccharide-induced acute lung injury via regulating miR-10a/b-5p. *Lipids Health Dis.* **2019**.

119. Siddiqui, M.R.; Akhtar, S.; Shahid, M.; Tauseef, M.; McDonough, K.; Shanley, T.P. miR-144 Mediated Inhibition of ROCK1 Protects Against LPS Induced Lung Endothelial Hyperpermeability. *Am. J. Respir. Cell Mol. Biol.* **2019**.
120. Yang, Y.; Yang, F.; Yu, X.; Wang, B.; Yang, Y.; Zhou, X.; Cheng, R.; Xia, S.; Zhou, X. miR-16 inhibits NLRP3 inflammasome activation by directly targeting TLR4 in acute lung injury. *Biomed. Pharmacother.* **2019**.
121. Yan, Y.; Lu, K.; Ye, T.; Zhang, Z. MicroRNA-223 attenuates LPS-induced inflammation in an acute lung injury model via the NLRP3 inflammasome and TLR4/NF- κ B signaling pathway via RHOB. *Int. J. Mol. Med.* **2019**.
122. Zhou, T.; Chen, Y. The Functional Mechanisms of miR-30b-5p in Acute Lung Injury in Children. *Med. Sci. Monit.* **2019**.
123. Wu, Y.-T.; Chen, W.J.; Hsieh, W.-S.; Tsao, P.-N.; Yu, S.-L.; Lai, C.-Y.; Lee, W.-C.; Jeng, S.-F. MicroRNA Expression Aberration Associated with Bronchopulmonary Dysplasia in Preterm Infants: A Preliminary Study. *Respir. Care* **2013**.
124. Bhaskaran, M.; Xi, D.; Wang, Y.; Huang, C.; Narasaraju, T.; Shu, W.; Zhao, C.; Xiao, X.; More, S.; Breshears, M.; et al. Identification of microRNAs changed in the neonatal lungs in response to hyperoxia exposure. *Physiol. Genomics* **2012**.
125. Olave, N.; Lal, C. V.; Halloran, B.; Pandit, K.; Cuna, A.C.; Faye-Petersen, O.M.; Kelly, D.R.; Nicola, T.; Benos, P. V.; Kaminski, N.; et al. Regulation of alveolar septation by microRNA-489. *Am. J. Physiol. Cell. Mol. Physiol.* **2016**.
126. Cao, Y.; Vo, T.; Millien, G.; Tagne, J.B.; Kotton, D.; Mason, R.J.; Williams, M.C.; Ramirez, M.I. Epigenetic mechanisms modulate thyroid transcription factor 1-mediated transcription of the surfactant protein B gene. *J. Biol. Chem.* **2010**.

127. Londhe, V.A.; Sundar, I.K.; Lopez, B.; Maisonet, T.M.; Yu, Y.; Aghai, Z.H.; Rahman, I. Hyperoxia impairs alveolar formation and induces senescence through decreased histone deacetylase activity and up-regulation of p21 in neonatal mouse lung. *Pediatr. Res.* **2011**.
128. W., N.; N., L.; H., H.; J., Z.; Y., Z. Lipopolysaccharide induces up-regulation of TGF-(alpha) through HDAC2 in a rat model of bronchopulmonary dysplasia. *PLoS One* **2014**.
129. Robbins, M.E.; Dakhlallah, D.; Marsh, C.B.; Rogers, L.K.; Tipple, T.E. Of mice and men: correlations between microRNA-17~92 cluster expression and promoter methylation in severe bronchopulmonary dysplasia. *Am. J. Physiol. Cell. Mol. Physiol.* **2016**.
130. El Agha, E.; Bellusci, S. Walking along the Fibroblast Growth Factor 10 Route: A Key Pathway to Understand the Control and Regulation of Epithelial and Mesenchymal Cell-Lineage Formation during Lung Development and Repair after Injury. *Scientifica (Cairo)*. **2014**, 2014, 538379.
131. El Agha, E.; Al Alam, D.; Carraro, G.; MacKenzie, B.A.; Goth, K.; de Langhe, S.P.; Voswinckel, R.; Hajihosseini, M.K.; Rehan, V.K.; Bellusci, S. Characterization of a novel Fibroblast growth factor 10 (Fgf10) knock-in mouse line to target mesenchymal progenitors during embryonic development. *PLoS One* **2012**.
132. Al Alam, D.; El Agha, E.; Sakurai, R.; Kheirollahi, V.; Moiseenko, A.; Danopoulos, S.; Shrestha, A.; Schmoldt, C.; Quantius, J.; Herold, S.; et al. Evidence for the involvement of Fibroblast Growth Factor 10 in lipofibroblast formation during embryonic lung development. *Development* **2015**.
133. De Langhe, S.P.; Carraro, G.; Warburton, D.; Hajihosseini, M.K.; Bellusci, S.

- Levels of mesenchymal FGFR2 signaling modulate smooth muscle progenitor cell commitment in the lung. *Dev. Biol.* **2006**, 299, 52–62.
134. Herriges, J.C.; Verheyden, J.M.; Zhang, Z.; Sui, P.; Zhang, Y.; Anderson, M.J.; Swing, D.A.; Zhang, Y.; Lewandoski, M.; Sun, X. FGF-Regulated ETV Transcription Factors Control FGF-SHH Feedback Loop in Lung Branching. *Dev. Cell* **2015**.
135. Ntokou, A.; Szibor, M.; Rodríguez-Castillo, J.A.; Quantius, J.; Herold, S.; El Agha, E.; Bellusci, S.; Salwig, I.; Braun, T.; Voswinckel, R.; et al. A novel mouse Cre-driver line targeting Perilipin 2-expressing cells in the neonatal lung. *Genesis* **2017**.
136. Park, J.; Ivey, M.J.; Deana, Y.; Riggsbee, K.L.; Sörensen, E.; Schwabl, V.; Sjöberg, C.; Hjertberg, T.; Park, G.Y.; Swonger, J.M.; et al. The Tcf21 lineage constitutes the lung lipofibroblast population. *Am. J. Physiol. Cell. Mol. Physiol.* **2019**.
137. Zhang, W.; Menke, D.B.; Jiang, M.; Chen, H.; Warburton, D.; Turcatel, G.; Lu, C.H.; Xu, W.; Luo, Y.; Shi, W. Spatial-temporal targeting of lung-specific mesenchyme by a Tbx4 enhancer. *BMC Biol.* **2013**.
138. Bostrom, H.; Willetts, K.; Pekny, M.; Leveen, P.; Lindahl, P.; Hedstrand, H.; Pekna, M.; Hellstrom, M.; Gebre-Medhin, S.; Schalling, M.; et al. PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell* **1996**, 85, 863–873.
139. Bostrom, H.; Gritli-Linde, A.; Betsholtz, C. PDGF-A/PDGF alpha-receptor signaling is required for lung growth and the formation of alveoli but not for early lung branching morphogenesis. *Dev Dyn* **2002**, 223, 155–162.
140. Gouveia, L.; Betsholtz, C.; Andrae, J. PDGF-A signaling is required for secondary alveolar septation and controls epithelial proliferation in the

- developing lung. *Development* **2018**.
141. Oak, P.; Pritzke, T.; Thiel, I.; Koschlig, M.; Mous, D.S.; Windhorst, A.; Jain, N.; Eickelberg, O.; Foerster, K.; Schulze, A.; et al. Attenuated PDGF signaling drives alveolar and microvascular defects in neonatal chronic lung disease. *EMBO Mol. Med.* **2017**.
142. Wang, H.; Jafri, A.; Martin, R.J.; Nnanabu, J.; Farver, C.; Prakash, Y.S.; MacFarlane, P.M. Severity of neonatal hyperoxia determines structural and functional changes in developing mouse airway. *Am. J. Physiol. Cell. Mol. Physiol.* **2014**.
143. Chang, M.; Bany-Mohammed, F.; Cristina Kenney, M.; Beharry, K.D. Effects of a superoxide dismutase mimetic on biomarkers of lung angiogenesis and alveolarization during hyperoxia with intermittent hypoxia. *Am. J. Transl. Res.* **2013**.
144. Ahlfeld, S.K.; Gao, Y.; Wang, J.; Horgusluoglu, E.; Bolanis, E.; Clapp, D.W.; Conway, S.J. Periostin downregulation is an early marker of inhibited neonatal murine lung alveolar septation. *Birth Defects Res. Part A - Clin. Mol. Teratol.* **2013**.
145. Belcastro, R.; Lopez, L.; Li, J.; Masood, A.; Tanswell, A.K. Chronic lung injury in the neonatal rat: Up-regulation of TGF β 1 and nitration of IGF-R1 by peroxynitrite as likely contributors to impaired alveologenesis. *Free Radic. Biol. Med.* **2015**.
146. Masood, A.; Yi, M.; Lau, M.; Belcastro, R.; Li, J.; Kantores, C.; Pace-Asciak, C.R.; Jankov, R.P.; Tanswell, A.K. Cyclooxygenase-2 inhibition partially protects against 60% O₂-mediated lung injury in neonatal rats. *Pediatr. Pulmonol.* **2014**.
147. Bachiller, P.R.; Cornog, K.H.; Kato, R.; Buys, E.S.; Roberts, J.D. Soluble

- guanylate cyclase modulates alveolarization in the newborn lung. *Am. J. Physiol. Cell. Mol. Physiol.* **2013**.
148. Harijith, A.; Pendyala, S.; Reddy, N.M.; Bai, T.; Usatyuk, P. V.; Berdyshev, E.; Gorshkova, I.; Huang, L.S.; Mohan, V.; Garzon, S.; et al. Sphingosine kinase 1 deficiency confers protection against hyperoxia-induced bronchopulmonary dysplasia in a murine model: Role of s1p signaling and nox proteins. *Am. J. Pathol.* **2013**.
149. Monz, D.; Tutdibi, E.; Mildau, C.; Shen, J.; Kasoha, M.; Laschke, M.W.; Roofls, T.; Schmiedl, A.; Tschernig, T.; Bieback, K.; et al. Human Umbilical Cord Blood Mononuclear Cells in a Double-Hit Model of Bronchopulmonary Dysplasia in Neonatal Mice. *PLoS One* **2013**.
150. Gortner, L.; Monz, D.; Mildau, C.; Shen, J.; Kasoha, M.; Laschke, M.W.; Roofls, T.; Schmiedl, A.; Meier, C.; Tutdibi, E. Bronchopulmonary dysplasia in a double-hit mouse model induced by intrauterine hypoxia and postnatal hyperoxia: Closer to clinical features? *Ann. Anat.* **2013**.
151. Popova, A.P.; Bentley, J.K.; Cui, T.X.; Richardson, M.N.; Linn, M.J.; Lei, J.; Chen, Q.; Goldsmith, A.M.; Pryhuber, G.S.; Hershenson, M.B. Reduced platelet-derived growth factor receptor expression is a primary feature of human bronchopulmonary dysplasia. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2014**, 307, L231-9.
152. Anyanwu, A.C.; Bentley, J.K.; Popova, A.P.; Malas, O.; Alghanem, H.; Goldsmith, A.M.; Hershenson, M.B.; Pinsky, D.J. Suppression of inflammatory cell trafficking and alveolar simplification by the heme oxygenase-1 product carbon monoxide. *Am. J. Physiol. Cell. Mol. Physiol.* **2014**.
153. Behnke, J.; Lemyre, B.; Czernik, C.; Zimmer, K.-P.; Ehrhardt, H.; Waitz, M. Non-invasive ventilation in neonatology. *Dtsch. Aerzteblatt Online* **2019**.

154. Hussein, O.; Walters, B.; Stroetz, R.; Valencia, P.; McCall, D.; Hubmayr, R.D. Biophysical determinants of alveolar epithelial plasma membrane wounding associated with mechanical ventilation. *Am. J. Physiol. Cell. Mol. Physiol.* **2013**.
155. Ratner, V.; Sosunov, S.A.; Niatetskaya, Z. V.; Utkina-Sosunova, I. V.; Ten, V.S. Mechanical ventilation causes pulmonary mitochondrial dysfunction and delayed alveolarization in neonatal mice. *Am. J. Respir. Cell Mol. Biol.* **2013**.
156. Shahzad, T.; Radajewski, S.; Chao, C.-M.; Bellusci, S.; Ehrhardt, H. Pathogenesis of bronchopulmonary dysplasia: when inflammation meets organ development. *Mol. Cell. Pediatr.* **2016**.
157. Klinger, G.; Levy, I.; Sirota, L.; Boyko, V.; Lerner-Geva, L.; Reichman, B. Outcome of early-onset sepsis in a national cohort of very low birth weight infants. *Pediatrics* **2010**.
158. Carver, B.J.; Plosa, E.J.; Stinnett, A.M.; Blackwell, T.S.; Prince, L.S. Interactions between NF- κ B and SP3 connect inflammatory signaling with reduced FGF-10 expression. *J. Biol. Chem.* **2013**.
159. Lee, H.J.; Lee, Y.J.; Choi, C.W.; Lee, J.A.; Kim, E.K.; Kim, H.S.; Kim, B. II; Choi, J.H. Rosiglitazone, a peroxisome proliferator-activated receptor- γ agonist, restores alveolar and pulmonary vascular development in a rat model of bronchopulmonary dysplasia. *Yonsei Med. J.* **2014**.
160. Syed, M.A.; Bhandari, V. Hyperoxia exacerbates postnatal inflammation-induced lung injury in neonatal BRP-39 null mutant mice promoting the M1 macrophage phenotype. *Mediators Inflamm.* **2013**.
161. Yoder, B.A.; Coalson, J.J. Animal models of bronchopulmonary dysplasia. The preterm baboon models. *Am. J. Physiol. Cell. Mol. Physiol.* **2014**.
162. Mohamed, W.A.; Aseeri, M.A. Cord blood fibroblast growth factor-10 as a

- possible predictor of bronchopulmonary dysplasia in preterm infants. *J. Neonatal. Perinatal. Med.* **2014**.
163. Ambalavanan, N.; Novak, Z.E. Peptide growth factors in tracheal aspirates of mechanically ventilated preterm neonates. *Pediatr. Res.* **2003**.
164. Karolak, J.A.; Vincent, M.; Deutsch, G.; Gambin, T.; Cogné, B.; Pichon, O.; Vetrini, F.; Mefford, H.C.; Dines, J.N.; Golden-Grant, K.; et al. Complex Compound Inheritance of Lethal Lung Developmental Disorders Due to Disruption of the TBX-FGF Pathway. *Am. J. Hum. Genet.* **2019**.
165. Vincent, M.; Karolak, J.A.; Deutsch, G.; Gambin, T.; Popek, E.; Isidor, B.; Szafranski, P.; Le Caignec, C.; Stankiewicz, P. Clinical, Histopathological, and Molecular Diagnostics in Lethal Lung Developmental Disorders. *Am. J. Respir. Crit. Care Med.* **2019**.
166. Han, T.; Chi, M.; Wang, Y.; Mei, Y.; Li, Q.; Yu, M.; Ma, Q.; Chen, Y.; Feng, Z. Therapeutic effects of fibroblast growth factor-10 on hyperoxia-induced bronchopulmonary dysplasia in neonatal mice. *Am. J. Transl. Res.* **2017**.
167. McAuley, D.F.; Cross, L.M.; Hamid, U.; Gardner, E.; Elborn, J.S.; Cullen, K.M.; Dushianthan, A.; Grocott, M.P.; Matthay, M.A.; O’Kane, C.M. Keratinocyte growth factor for the treatment of the acute respiratory distress syndrome (KARE): a randomised, double-blind, placebo-controlled phase 2 trial. *Lancet Respir. Med.* **2017**.

7. Acknowledgements

First, I would like to thank Prof. Dr. Saverio Bellusci for giving me the opportunity to learn and do research in his laboratory. He was and will always be my highly appreciated teacher both in being a scientist and in being a leader. His scientific expertise, his positive mind set and his tolerance/endurance in training me to become a clinician scientist will stay in my mind forever.

I would like to express the same gratitude to Prof. Dr. Klaus-Peter Zimmer for being my clinical mentor and teacher from the University Childrens Hospital. Without his support and enthusiasm throughout the last ten years, the completion of this study would not be possible. Prof. Dr. Klaus-Peter Zimmer as a clinician scientist himself, was always a good example for me to pursue the path as a clinician scientist.

I would also like to acknowledge all my colleagues and collaborators as well as the animal caretakers and co-workers behind the scenes, for stimulating scientific and non-scientific discussions and for their support in making decisions in challenging situations.

Last but not least, I would like to thank my parents for their unconditional love, patience and understanding in any situations. Thank you for giving me the mental energy I sometimes desperately needed to move forward. I would also like to thank my brother and sister, who was there whenever I needed help in any regard.

8. Declaration

I, Cho-Ming Chao, born April 17th 1979 in Saigon, Vietnam, herewith declare that I have written this work with the title “Fgf10 and epigenetic regulations in lung development and Bronchopulmonary dysplasia” on my own and without illegitimate assistance and that the results and facts from all the literature that was involved in the process of creating this dissertation have been entirely indicated. Furthermore, I declare that this dissertation was neither in this version, nor in a similar form, handed in at any other department of or faculty of a university or comparable institution.

Gießen, 2019

Cho-Ming Chao

9. Bibliography

Original articles (chronological order)

1) Shrestha, A., Carraro, G., Mari, B., Nottet, N., Vasquez-Armendariz, A.I., Herold, S., Cordero, J., Singh, I., Barreto, G., Wilhelm, J., Gunther, S., El Agha, E., Morty, R., Zhang, J.S., **Chao, C.M.** *, Bellusci, S.*. *A critical role for miR-142 in alveolar epithelial lineage formation during lung development*. Cell Mol Life Sci. 2019 Mar 18. doi: 10.1007/s00018-019-03067-8, *equally contributed. **IF=7.0**

2) Yuan, T.[#], Volckaert, T.[#], Redente, E.F., Yuan, J., **Chao, C.M.**, Wasnick, R., Yao, C., Majka, S., Stripp, B., Günther, A., Riches, D.W.H., Bellusci, S., Thannickal, V., De Langhe, S.P. *Fgf10-Fgfr2b signaling generates basal cells and drives alveolar epithelial regeneration by bronchial epithelial stem cells after lung injury*. **Stem Cell Reports** 2019 Apr 16. pii: S2213-6711(19)30125-0. doi:10.1016/j.stemcr. 2019. 04. 003. **IF=5.5**

5) Jones, M., Lingampally, A., Dilai, S., Shrestha, A., Stripp, A., Helmbacher, F., **Chao, C.M.***, Bellusci, S.* *Characterization of Etv4GFP and Etv5RFP reporter lines in the context of fibroblast growth factor 10 signalling during mouse embryonic lung development*. Front Genet. 2019 Mar 14;10:178. doi: 10.3389/fgene.2019.00178, *equally contributed. **IF=3.5**

6) Rubio K, Singh I, Dobersch S, Sarvari P, Günther S, Cordero J, Mehta A, Wujak L, Cabrerias-Fuentes H, **Chao CM**, Bellusci S, Seeger S, Guenther A, Preissner KT,

Wygrecka M, Savai R, Papy-Garcia D, Dobрева G, Savai Pullamsetti S, Braun T and Barreto G. *HDAC inactivation by EP300 disrupts the MiCEE complex in Idiopathic Pulmonary Fibrosis*. **Nature Communications** <https://doi.org/10.1038/s41467-019-10066-7>. **IF=11.9**

7) Jones, M.[#], Dilai, S.[#], Lingampalli, A., **Chao, CM.**, Danopoulos, S., Carraro, G., Mukhametshina, R., Wilhem, J., Baumgart-Vogt, E., Al Alam, D., Chen, C., Minoo, P., Zhang, J.S.^{*}, Bellusci, S. ^{*} (2018) *A comprehensive analysis of fibroblast growth factor receptor 2b signalling on epithelial tip progenitor cells during early mouse lung branching morphogenesis*. **Front Genet.** 2019 Jan 23;9:746. doi:10.3389/fgene.2018.00746, ^{*}equally contributed. **IF=3.5**

8) **Chao CM**, Moiseenko A, Kosanovic D, Rivetti S, El Agha E, Wilhelm J, Kampschulte M, Yahya F, Ehrhardt H, Zimmer KP, Barreto G, Rizvanov AA, Schermuly RT, Reiss I, Morty RE, Rottier RJ, Zhang JS and Bellusci S. *Impact of Fgf10 deficiency on pulmonary vasculature formation in a mouse model of bronchopulmonary dysplasia*. **Hum Mol Genet** 2018 Dec 19. doi: 10.1093/hmg/ddy439. [Epub ahead of print]. **IF=4.5**

9) Kalymbetova TV, Selvakumar B, Rodríguez-Castillo JA, Gunjak M, Malainou C, Heindl MR, Moiseenko A, **Chao CM**, Vadász I, Mayer K, Lohmeyer J, Bellusci S, Böttcher-Friebertshäuser E, Seeger W, Herold S, Morty RE. *Resident alveolar macrophages are master regulators of arrested alveolarization in experimental bronchopulmonary dysplasia*. **J Pathol** 2018. Jun;245(2):153-159. **IF=5.9**

- 10) **Chao CM**, van den Bruck R, Lork S, Merkle J, Krampen L, Weil P, Aydin M, Bellusci S, Jenke AC, Postberg J. *Neonatal exposure to hyperoxia leads to persistent disturbances in pulmonary histone signatures associated with NOS3 and STAT3 in a mouse model. Clinical Epigenetics. 2018 Mar 20;10:37. IF=5.5*
- 11) Volckaert T*, Yuan TT*, **Chao CM**, Bell H, Sitaula A, Szimtmtenings L, El Agha E, Chanda D, Majka S, Bellusci S, Thannickal VJ, Faessler R, de Langhe SP (2017) *Fgf10-Hippo epithelial-mesenchymal crosstalk maintains and recruits lung basal stem cells. Dev Cell. 2017 Oct 9;43(1):48-59.e5. *equally contributed. IF=9.2*
- 12) Moiseenko A, Kheirollahi V, **Chao CM**, Ahmadvand N, Quantius J, Wilhem J, Herold S, Ahlbrecht K, Morty RE, Rizvanov AA, Minoo P, El Agha E, Bellusci S. *Origin and characterization of alpha smooth muscle actin-positive cells during murine lung development. Stem Cells. 2017 Apr 3. doi: 10.1002/stem.2615. IF=5.6*
- 13) **Chao CM**, Yahya F, Moiseenko A, Tiozzo C, Shrestha A, Ahmadvand N, El Agha E, Quantius J, Dilai S, Kheirollahi V, Jones M, Wilhem J, Carraro G, Ehrhardt H, Zimmer KP, Barreto G, Ahlbrecht K, Morty RE, Herold S, Abellar RG, Seeger W, Schermuly RT, Zhang JS, Minoo P and Bellusci S. *Fgf10 deficiency is causative for lethality in a mouse model of bronchopulmonary dysplasia. J Pathol. 2017 Jan;241(1):91-103. IF=5.9*
- 14) Mehta A, Cordero J, Dobersch S, Romero-Olmedo AJ, Savai R, Bodner J, **Chao CM**, Fink L, Guzman-Diaz E, Singh I, Dobрева G, Rapp UR, Günther S, Ilinskaya ON, Bellusci S, Damman H, Braun T, Seeger W, Gattenlöhner S, Tresch A, Günther A, Barreto G. *Detection of embryonic isoforms of GATA6 and NKX2-1 in exhaled*

breath condensate for lung cancer diagnosis. EMBO Mol Med. 2016 Dec 1;8(12):1380-1389. **IF=10.6**

15) Karakaya M, Heller R, Kunde V, Zimmer KP, **Chao CM**, Nürnberg P, Cirak S. *Novel Mutations in the Nonselective Sodium Leak Channel (NALCN) Lead to Distal Arthrogryposis with Increased Muscle Tone. Neuropediatrics* 2016 Aug;47(4):273-7. doi: 10.1055/s-0036-1584084. Epub 2016 May 23. **IF=1.7**

16) Hauch H, Klinkel N, **Chao CM**, Behrje J, Kreuzaler P, Bagheri A, Sibelius U, Berthold D. *Constitution of a New Specialised Pediatric Home Care Team - First Year Experience. Klin Padiatr.* 2016 Apr;228(3):145-8. doi: 10.1055/s-0042-103327. Epub 2016 May 2. **IF=0.8**

17) Al Alam D, El Agha E, Sakurai R, Kheirollahi V, Moiseenko A, Danopoulos S, Shrestha A, Schmoldt C, Quantius J, Herold S, **Chao CM**, Tiozzo C, De Langhe S, Plikus MV, Thornton M, Grubbs B, Minoo P, Rehan VK, Bellusci S. *Evidence for the involvement of fibroblast growth factor 10 in lipofibroblast formation during embryonic lung development. Development.* 2015 Dec 1;142(23):4139-50. doi: 10.1242/dev.109173. Epub 2015 Oct 28. **IF=5.8**

18) Shrestha A, Carraro G, El Agha E, Mukhametshina R, **Chao CM**, Rizvanov A, Barreto G, Bellusci S. *Generation and Validation of miR-142 Knock Out Mice. PLOSone.* 2015 Sep 1;10(9):e0136913. doi: 10.1371/journal.pone.0136913. eCollection 2015. **IF=2.8**

19) MacKenzie B, Henneke I, Hezel S, Al Alam D, El Agha E, Chao CM, Quantius J,

Wilhelm J, Jones M, Goth K, Li X, Seeger W, Koenigshoff M, Herold S, Rizvanov A, Günther A, Bellusci S. Attenuating endogenous Fgfr2b ligands during bleomycin-induced lung fibrosis does not compromise murine lung repair. *Am J Physiol Lung Cell Mol Physiol*. 2015 May 15;308(10):L1014-24. doi: 0.1152/ajplung. 00291.2014.

IF=4.1

20) Rochais F, Sturny R, Chao CM, Mesbah K, Bennett M, Mohun TJ, Bellusci S, Kelly RG. FGF10 promotes regional fetal cardiomyocyte proliferation and adult cardiomyocyte cell cycle reentry. *Cardiovascular Research*. 2014 Dec 1;104(3):432-

42. **IF=7.0**

21) Carraro G, Shrestha A, Rostkovius J, Contreras A, **Chao CM**, El Agha E, Mackenzie B, Dilai S, Guidolin D, Taketo MM, Günther A, Kumar ME, Seeger W, De Langhe S, Barreto G, Bellusci S. *MiR-142-3p balances proliferation and differentiation of mesenchymal cells during lung development*. **Development**. 2014 Mar;141(6):1272-81. **IF=5.8**

22) El Agha E, Herold S, Al Alam D, Quantius J, MacKenzie B, Carraro G, Moiseenko A, **Chao CM**, Minoo P, Seeger W, Bellusci S. *Fgf10-positive cells represent a progenitor cell population during lung development and postnatally*. **Development**. 2014 Jan;141(2):296-306. **IF=5.8**

23) Ullrich S, Berchtold S, Ranta F, Seebohm G, Henke G, Lupescu A, Mack AF, **Chao CM**, Su J, Nitschke R, Alexander D, Friedrich B, Wulff P, Kuhl D, Lang F. *SGK1 mediates glucocorticoid induced inhibition of insulin secretion*. **Diabetes**. 2005 Apr;54(4):1090-9. **IF=7.2**

24) Peterhoff M, Sieg A, Brede M, **Chao CM**, Hein L, Ullrich S. *Inhibition of insulin secretion via distinct signaling pathways in α_2 -adrenoceptor knockout mice.* **Eur J Endocrinol.** 2003 Oct;149(4):343-50. **IF=5.1**

Reviews

1) Gronbach J*, Shahzad T*, Radajewski S, **Chao CM**, Bellusci S, Morty RE, Reicherzer T, Ehrhardt H. *The potentials and caveats of mesenchymal stromal cells based therapies in the preterm infant.* **Stem Cell Int.** 2018 Apr 8;2018:9652897. **IF=3.9**

2) Naehrig S*, **Chao CM***, Naehrlich L. *Mucoviscidosis: diagnosis and therapy.* **Dtsch Arztebl Int.** 2017 Aug 21;114(33-34):564-574. *equally contributed. **IF=4.5**

3) Shahzad T, Radajewski S, **Chao CM**, Bellusci S, Ehrhardt H. *Pathogenesis of bronchopulmonary dysplasia: when inflammation meets organ development.* **Mol Cell Pediatr.** 2016 Dec;3(1):23. doi: 10.1186/s40348-016-0051-9. Epub 2016 Jun 29. **IF=0.1**

4) **Chao CM**, Moiseenko A, Zimmer KP, Bellusci S. *Alveologenesis: key cellular players and fibroblast growth factor 10 signaling.* **Mol Cell Pediatr.** 2016 Dec;3(1):17. doi: 10.1186/s40348-016-0045-7. Epub 2016 Apr 21. **IF=0.1**

5) **Chao CM**, El Agha E, Tiozzo C, Minoo P, Bellusci S. A breath of fresh air on the mesenchyme: Impact of impaired mesenchymal development on the pathogenesis of

Bronchopulmonary dysplasia. *Front Med (Lausanne)*. 2015 Apr 28;2:27. doi: 10.3389/fmed.2015.00027. eCollection 2015. **IF=3.1**

Total Impact-Factor gesamt: 136.4

H-Index (Google Scholar): 13

Published abstracts

1) **CM Chao**, G Carraro, Z Rako, J Kolck, V Zimmermann, J Wilhelm, W Seeger, RJ Rottier, RE Morty, S Bellusci. *MiR-154 controls alveologenesis in lung development via TGF- β signaling*. **Pneumologie** 2018, 72(3), DOI:10.1055/s-0037-1615335

2) A Moiseenko, E El Agha, V Kheirollahi, **CM Chao**, S De Langhe, S Bellusci. *Cellular and molecular characterization of the mesenchymal niche for bronchiolar epithelial progenitor cells after naphthalene injury in mice*. **Pneumologie** 2018, 72(3), DOI:10.1055/s-0037-1615333

3) **CM Chao**, G Carraro, Z Rako, J Kolck, RE Morty, RJ Rottier, S Bellusci. *MiR-154 controls branching morphogenesis and alveologenesis in lung development involving Tgf- β signaling*. **European Respiratory Journal** 2017, DOI:10.1183/1393003.congress-2017.OA3229, ERS International Congress 2017 abstracts

4) T Shahzad, **CM Chao**, S Bellusci, H Ehrhardt. *Role of trail in the pathogenesis of bronchopulmonary dysplasia (BPD)*. **European Respiratory Journal** 2017, DOI:10.1183/1393003.congress-2017.OA3231, ERS International Congress 2017 abstracts

5) G Barreto, A Mehta, J Cordero, S Dobersch, AJ Romero-Olmedo, R Savai, J Bodner, **CM Chao**, L Fink, E Guzman-Diaz, I Singh, G Dobrev, UR Rapp, S Günther, O Ilinskaya, S Bellusci, RH Dammann, T Braun, W Seeger, S Gattenlöhner, A Tresch, A Günther. *Non-invasive lung cancer diagnosis by detection of GATA6 and NKX2-1 isoforms in exhaled breath condensate*. **European Respiratory Journal** 2017, DOI:10.1183/1393003.congress-2017.PA2033, ERS International Congress 2017 abstracts

6) N Ahmadvand, **CM Chao**, S Bellusci. *Fgfr2b signaling in Alveolar Epithelial type II cells following pneumonectomy in mice*. **European Respiratory Journal** 2017, DOI:10.1183/1393003.congress-2017.PA2085, ERS International Congress 2017 abstracts

7) **CM Chao**, F Yahya, A Moiseenko, A Shrestha, N Ahmadvand, J Quantius, J Wilhelm, E El-Agha, KP Zimmer, S Bellusci. *Fgf10 deficiency leads to disturbed formation of alveolar epithelial cell type II (AEC II) which causes lethality in a mouse model of bronchopulmonary dysplasia*. **Molecular and Cellular Pediatrics** 2017, 4 (Suppl. 1):A3

8) **CM Chao**, D Al Alam, S Schermuly, H Ehrhardt, KP Zimmer and S Bellusci.

*Impact of embryonic Fgf10 expression deficiency on embryonic mouse lung development and repair following hyperoxia injury. **Molecular and Cellular Pediatrics** 2015 2(Suppl 1):A14*

9) F Yahya*, **CM Chao***, S Bellusci. *Constitutive Fgf10 deficiency leads to impaired embryonic lung development. **Pneumologie** 2015; 69 - A32, DOI: 10.1055/s-0035-1556624, * equally contributed*

10) A Moiseenko, E El Agha, **CM Chao**, A Shrestha, S Dilai, V Kheirollahi, S De Langhe, S Bellusci. *Origin of the smooth muscle cells during murine lung development. **Pneumologie** 2015; 69(07) – A34, DOI:10.1055/s-0035-1556626*

11) **CM Chao**, D Al Alam, R Schermuly, H Ehrhardt, KP Zimmer and S Bellusci. *Therapeutic effects of Fibroblast growth factor 10 (Fgf10) after hyperoxic lung injury in mice. **Arch Dis Child** 2014 Oct; 99(Suppl 2)*

12) **CM Chao**, D Al Alam, R Schermuly, H Ehrhardt, KP Zimmer and S Bellusci. *Evidence that Fgf10 offers therapeutic opportunities after hyperoxic lung injury in mice. **Molecular and Cellular Pediatrics** 2014 1(Suppl 1):A27*

13) B MacKenzie, D Al Alam, I Henneke, E El Agha, J Quantius, **CM Chao**, J Wilhelm, M Königshoff, S Herold, W Klepetko, A Günther, W Seeger, S Bellusci. ***Endogenous Fgfr2b ligands are dispensable for fibrosis formation and resolution in bleomycin-injured mice. **Pneumologie** 2014; 68 - A33, DOI: 10.1055/s-0034-1376802***

14) F Rochais, R Sturny, **CM Chao**, K Mesbah, M Bennett, TJ Mohun, S Bellusci,

RG. Kelly. *FGF10 regulates regional proliferation in the fetal heart through a FOXO3/p27kip1 pathway and promotes cell cycle reentry of adult cardiomyocytes.*

Cardiovasc Res (2014) 103 (suppl 1): S58. doi: 10.1093/cvr/cvu091.6

15) **CM Chao**, D Al Alam, R Schermuly, H Ehrhardt, KP Zimmer and S Bellusci.

Therapeutic effects of fibroblast growth factor 10 (FGF 10) after hyperoxic lung injury in mice. **Pneumologie** 2014; 68 - A26, DOI: 10.1055/s-0034-1376795

16) **CM Chao**, D Al Alam, C Tiozzo, KP Zimmer, S Bellusci. *Fgf10 induziert de novo Alveogenese im Mausmodell der BPD.* **Monatsschrift Kinderheilkunde** 2013/S2: 93, DOI: 10.1007/s00112-013-2984-6. DGKJ 2013/ 49. Arbeitstagung Pädiatrische Forschung Düsseldorf

17) **CM Chao**, C Tiozzo, D Al Alam, KP Zimmer, S Bellusci. *Fgf-10-Expression schützt vor Hyperoxie-induzierter Lungenschädigung in einem Mausmodell der Bronchopulmonalen Dysplasie.* **Monatsschrift Kinderheilkunde** Juni 2013, Band 161:1-93, Suppl.1:FV-52

18) **CM Chao**, C Tiozzo, D Al Alam, S Bellusci. *Fgf10 overexpression prevents lung injury in a BPD model.* **Pneumologie** 2012; 66 - A809, DOI: 10.1055/s-0032-1315554

19) G Carraro, J Rostkiovius, M Jones, K Goth, E El Agha, B MacKenzie, **CM Chao**, A Moiseenko, S Bellusci. *MicroRNA regulation of embryonic lung mesenchyme*

proliferation and differentiation. Pneumologie 2012; 66 - A407, DOI: 10.1055/s-0032-1315505

20) **CM Chao**, D Al Alam, C Tiozzo, R Virender, S Bellusci. *The role of Fgf10 for alveologenesis in bronchopulmonary dysplasia. Arch Dis Child* 2012 Oct; 97(Suppl 2):A51; DOI: 10.1136/archdischild-2012-302724.0176

21) **CM Chao**, J Su and S Ullrich. *Dexamethasone-induced inhibition of insulin secretion is accompanied by an increased expression of voltage-dependent K⁺ channels. Diabetologia* 2002,45: A165

22) M Peterhoff, A Sieg, **CM Chao**, M Brede, L Hein and S Ullrich. *Impaired Glucose-induced insulin secretion in α 2-adrenoceptor knock out mice. Pflügers Arch.* 2002, 443: S200

23) M Peterhoff, M Buchenau, A Sieg, M Brede, L Hein, **CM Chao**, J Su, J Bryan, L Aguilar-Bryan, S Ullrich. *Impaired insulin secretion in knock out mice islets due to alterations of inhibitory pathways. Diabetologia* 2002,45: A146

10. Appendix

Appendix A

Jones, M., Lingampally, A., Dilai, S., Shrestha, A., Stripp, A., Helmbacher, F., **Chao, CM.***, Bellusci, S.*:

“Characterization of Etv4GFP and Etv5RFP reporter lines in the context of fibroblast growth factor 10 signalling during mouse embryonic lung development.”

Front Genet. 2019 Mar 14;10:178 | doi: 10.3389/fgene.2019.00178

Appendix B

Jones, M.[#], Dilai, S.[#], Lingampalli, A., **Chao, CM.**, Danopoulos, S., Carraro, G., Mukhametshina, R., Wilhem, J., Baumgart-Vogt, E., Al Alam, D., Chen, C., Minoo, P., Zhang, J.S.*, Bellusci, S.*:

“A comprehensive analysis of fibroblast growth factor receptor 2b signalling on epithelial tip progenitor cells during early mouse lung branching morphogenesis.”

Front Genet. 2019 Jan 23;9:746 | doi:10.3389/fgene.2018.00746

Appendix C

El Agha E, Herold S, Al Alam D, Quantius J, MacKenzie B, Carraro G, Moiseenko A, **Chao CM**, Minoo P, Seeger W, Bellusci S.:

“Fgf10-positive cells represent a progenitor cell population during lung development and postnatally.”

Development. 2014 Jan;141(2):296-306 | doi:10.1242/dev.099747

Appendix D

Al Alam D, El Agha E, Sakurai R, Kheirollahi V, Moiseenko A, Danopoulos S, Shrestha A, Schmoldt C, Quantius J, Herold S, **Chao CM**, Tiozzo C, De Langhe S, Plikus MV, Thornton M, Grubbs B, Minoo P, Rehan VK, Bellusci S.:

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

Development. 2015 Dec 1;142(23):4139-50 | doi: 10.1242/dev.109173

Appendix E

Chao CM, Yahya F, Moiseenko A, Tiozzo C, Shrestha A, Ahmadvand N, El Agha E, Quantius J, Dilai S, Kheirollahi V, Jones M, Wilhem J, Carraro G, Ehrhardt H, Zimmer KP, Barreto G, Ahlbrecht K, Morty RE, Herold S, Abellar RG, Seeger W, Schermuly RT, Zhang JS, Minoo P and Bellusci S.:

“Fgf10 deficiency is causative for lethality in a mouse model of bronchopulmonary dysplasia.”

J Pathol. 2017 Jan;241(1):91-103 | doi:10.1002/path.4834

Appendix F

Chao CM, Moiseenko A, Kosanovic D, Rivetti S, El Agha E, Wilhelm J, Kampschulte M, Yahya F, Ehrhardt H, Zimmer KP, Barreto G, Rizvanov AA, Schermuly RT, Reiss I, Morty RE, Rottier RJ, Bellusci S and Zhang JS.:

“Impact of Fgf10 deficiency on pulmonary vasculature formation in a mouse model of bronchopulmonary dysplasia.”

Hum Mol Genet 2018 Dec 19 | doi: 10.1093/hmg/ddy439

Appendix G

Volckaert T*, Yuan TT*, **Chao CM**, Bell H, Sitaula A, Szimmetenings L, El Agha E, Chanda D, Majka S, Bellusci S, Thannickal VJ, Faessler R, de Langhe SP.:

“Fgf10-Hippo epithelial-mesenchymal crosstalk maintains and recruits lung basal stem cells.”

Dev Cell. 2017 Oct 9;43(1):48-59.e5 | doi: 10.1016/j.devcel.2017.09.003

Appendix H

Yuan, T.[#], Volckaert, T.[#], Redente, E.F., Yuan, J., **Chao, C.M.**, Wasnick, R., Yao, C., Majka, S., Stripp, B., Günther, A., Riches, D.W.H., Bellusci, S., Thannickal, V., De Langhe, S.P.:

“Fgf10-Fgfr2b signaling generates basal cells and drives alveolar epithelial regeneration by bronchial epithelial stem cells after lung injury.”

Stem Cell Reports 2019 Apr 16. pii: S2213-6711(19)30125-0 | doi:10.1016/j.stemcr. 2019. 04. 003.

Appendix I

Carraro G, Shrestha A, Rostkovius J, Contreras A, **Chao CM**, El Agha E, Mackenzie B, Dilai S, Guidolin D, Taketo MM, Günther A, Kumar ME, Seeger W, De Langhe S, Barreto G, Bellusci S.:

“MiR-142-3p balances proliferation and differentiation of mesenchymal cells during lung development.”

Development. 2014 Mar;141(6):1272-81 | doi:10.1242/dev.105908

Appendix J

Shrestha A, Carraro G, El Agha E, Mukhametshina R, **Chao CM**, Rizvanov A, Barreto G, Bellusci S.:

“Generation and Validation of miR-142 Knock Out Mice.”

PLOSone. 2015 Sep 1;10(9):e0136913 | doi: 10.1371/journal.pone.0136913

Appendix K

Shrestha, A., Carraro, G., Mari, B., Nottet, N., Vasquez-Armendariz, A.I., Herold, S., Cordero, J., Singh, I., Barreto, G., Wilhelm, J., Gunther, S., El Agha, E., Morty, R., Zhang, J.S., **Chao, C.M.** *, Bellusci, S.*:

“A critical role for miR-142 in alveolar epithelial lineage formation during lung development.”

Cell Mol Life Sci. 2019 Mar 18 | doi: 10.1007/s00018-019-03067-8

Appendix L

Chao CM, van den Bruck R, Lork S, Merkle J, Krampen L, Weil P, Aydin M, Bellusci S, Jenke AC, Postberg J.:

“Neonatal exposure to hyperoxia leads to persistent disturbances in pulmonary histone signatures associated with NOS3 and STAT3 in a mouse model.”

Clinical Epigenetics. 2018 Mar 20;10:37 | doi:10.1186/s13148-018-0469-0



Characterization of *Tg(Etv4-GFP)* and *Etv5^{RFP}* Reporter Lines in the Context of Fibroblast Growth Factor 10 Signaling During Mouse Embryonic Lung Development

OPEN ACCESS

Edited by:

Emmanuel S. Tzanakakis,
Tufts University, United States

Reviewed by:

Laszlo Farkas,
Virginia Commonwealth University,
United States
Jincheng Wu,
Novartis Institutes for BioMedical
Research, United States

*Correspondence:

Cho-Ming Chao
Cho-Ming.Chao@
paediat.med.uni-giessen.de
Saverio Bellusci
saverio.bellusci@
innere.med.uni-giessen.de

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Stem Cell Research,
a section of the journal
Frontiers in Genetics

Received: 28 September 2018

Accepted: 18 February 2019

Published: 14 March 2019

Citation:

Jones MR, Lingampally A, Dilai S,
Shrestha A, Stripp B, Helmbacher F,
Chen C, Chao C-M and Bellusci S
(2019) Characterization of
Tg(Etv4-GFP) and *Etv5^{RFP}* Reporter
Lines in the Context of Fibroblast
Growth Factor 10 Signaling During
Mouse Embryonic Lung Development.
Front. Genet. 10:178.
doi: 10.3389/fgene.2019.00178

Matthew R. Jones^{1,2†}, Arun Lingampally^{2†}, Salma Dilai², Amit Shrestha², Barry Stripp³,
Francoise Helmbacher⁴, Chengshui Chen¹, Cho-Ming Chao^{2,5,6*} and Saverio Bellusci^{1,2,6*}

¹ Department of Pulmonary and Critical Care Medicine, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China, ² Department of Internal Medicine II, Member of the German Lung Center, Cardio-Pulmonary Institute, University of Giessen Lung Center, Giessen, Germany, ³ Department of Medicine, Cedars-Sinai Medical Center, Lung and Regenerative Medicine Institutes, Los Angeles, CA, United States, ⁴ Aix Marseille University, CNRS, IBDM, UMR7288, Marseille, France, ⁵ Department of General Pediatrics and Neonatology, University Children's Hospital Gießen, Justus-Liebig-University, Gießen, Germany, ⁶ International Collaborative Center on Growth Factor Research, Life Science Institute, Wenzhou University-Wenzhou Medical University, Wenzhou, China

Members of the PEA3 transcription factors are emerging as bone fide targets for fibroblast growth factor (FGF) signaling. Among them, ETV4 and ETV5 appear to mediate FGF10 signaling during early embryonic lung development. In this paper, recently obtained *Tg(Etv4-GFP)* and *Etv5^{CreERT2-RFP}* fluorescent reporter lines were generally characterized during early embryonic development and in the context of FGF10 signaling, in particular. We found that both *Tg(Etv4-GFP)* and *Etv5^{CreERT2-RFP}* were primarily expressed in the epithelium of the lung during embryonic development. However, the expression of *Etv5^{CreERT2-RFP}* was much higher than that of *Tg(Etv4-GFP)*, and continued to increase during development, whereas *Tg(Etv4-GFP)* decreased. The expression patterns of the surrogate fluorescent protein GFP and RFP for ETV4 and ETV5, respectively, agreed with known regions of FGF10 signaling in various developing organs, including the lung, where ETV4-GFP was seen primarily in the distal epithelium and to a lesser extent in the surrounding mesenchyme. As expected, ETV5-RFP was restricted to the lung epithelium, showing a decreasing expression pattern from distal buds to proximal conducting airways. FGF10 inhibition experiments confirmed that both *Etv4* and *Etv5* are downstream of FGF10 signaling. Finally, we also validated that both fluorescent reporters responded to FGF10 inhibition *in vitro*. In conclusion, these two reporter lines appear to be promising tools to monitor FGF10/FGFR2b signaling in early lung development. These tools will have to be further validated at later stages and in other organs of interest.

Keywords: ETV4, ETV5, FGF10, lung development, branching morphogenesis

INTRODUCTION

PEA3 transcription factors are a subfamily of the E26 transformation-specific (ETS) transcription factor family consisting of three members: ETV1 (also known as Er81), ETV4 (also known as PEA3), and ETV5 (also known as ERM) (reviewed by Sharrocks et al., 1997). Evidence from multiple studies has demonstrated that ETV4 and ETV5 are primary mediators of fibroblast growth factor (FGF) signaling via fibroblast growth factor receptor 2b (FGFR2b), and play overlapping roles in the patterning, morphogenesis, differentiation, and homeostasis of multiple organs and structures. For example, ETV4 and ETV5 have been shown to repress *Shh* expression in mouse limb buds, thus promoting bud outgrowth and proper anterior-posterior patterning (Mao et al., 2009; Zhang et al., 2009); studies in the mouse lung have shown that ETV4- and ETV5-mediated induction of *Shh* appear to regulate branching morphogenesis (Herriges et al., 2015); furthermore, ETV5 was shown to maintain alveolar type 2 (AT2) cell identity during mouse lung homeostasis and repair after injury, and has been implicated in lung tumorigenesis (Zhang et al., 2017); during development of the lacrimal gland in mice, it was demonstrated that PEA3 transcription factors control epithelial cell fate determination (Garg et al., 2018); in the kidney as well, research has shown that ETV4 and ETV5 play a wide range of functions within multiple signaling pathways, including FGF signaling, to regulate Wolffian duct and ureteric bud morphogenesis (Kuure et al., 2010); finally, a study in zebrafish has found that knocking down *Etv4* and *Etv5* resulted in embryonic abnormalities similar to a loss of FGF signaling, including cardiac, and left/right patterning defects (Znosko et al., 2010).

Lung organogenesis is a complex process involving a number of signaling pathways, and is divided into distinct stages comprising embryonic (E) and postnatal (P) development. In the mouse, these stages include the pseudoglandular stage (E9.5–16.5), the canalicular stage (E16.5–17.5), the saccular stage (E17.5–P5), and the alveolar stage (P5–P30) (reviewed in Chao et al., 2015). During each of the embryonic and postnatal stages, FGF signaling plays critical roles. For instance, during the pseudoglandular stage of lung development, the majority of the lung architecture is established via branching morphogenesis, and also most of the epithelial and mesenchymal cell types are formed (reviewed in El Agha and Bellusci, 2014). Disruption of FGFR2b signaling during this stage leads to morphological and differentiation defects (Bellusci et al., 1997b), while either *Fgf10*- or *Fgfr2b*-null embryos display complete lung agenesis (Sekine et al., 1999; De Moerloose et al., 2000).

Another critical signaling pathway during pseudoglandular lung development is the sonic hedgehog (SHH) pathway. SHH is a negative regulator of *Fgf10* expression in the distal mesenchyme, and serves to limit the action of FGF10/FGFR2b signaling (Bellusci et al., 1997a). Recent studies propose that FGF10 acts on SHH via ETV4 and ETV5, creating an FGF10/ETV4/ETV5/SHH axis required for orchestrating proper branching morphogenesis (Herriges et al., 2015). It is unclear, however, how FGF10 acts on ETV4 and ETV5; whether it acts directly to regulate the expression of these transcription

factors, for example, or whether these are regulated indirectly via another pathway.

To aid the study of the role of ETV4 and ETV5 during organogenesis, two transgenic reporter mice have recently been developed, *Tg(Etv4-GFP)* (Lamballe et al., 2011) and *Etv5*^{CreERT2-RFP} (also referred to as *Etv5*^{RFP}). These lines report, via green and red fluorescent markers, the expression of *Etv4* and *Etv5*, respectively. Furthermore, *Etv5*^{CreERT2-RFP} can be used to drive inducible genetic recombination in *Etv5* positive cells, upon administration of tamoxifen. These lines can, therefore, potentially be used as invaluable research tools to study the role of ETV4 and ETV5 during the development of multiple organs, especially in the context of FGF signaling.

In this paper, we characterize these two reporter mouse lines during early embryogenesis (up to E16.5). We report the areas and organs of expression of ETV4-GFP and ETV5-RFP in the mouse embryo. We then focus on the expression of *Etv4* and *Etv5* in isolated embryonic lung mesenchyme and epithelium as well as the dynamic expression of the ETV4-GFP and ETV5-RFP protein during pseudoglandular development. Finally, various FGF10 inhibition experiments were carried out *in vitro* to determine the impact on the expression of ETV4-GFP and ETV5-RFP activity.

MATERIALS AND METHODS

Animal Husbandry and Experimental Embryos

All animals were housed in a specific-pathogen-free (SPF) facility in accordance with local, state, and national laws. Timed pregnancies were set-up to obtain experimental embryos for analysis at the desired embryonic stage (from E9.5 to E18.5). Noon on the day of the vaginal plug was considered E0.5.

To obtain experimental and littermate control embryos, mice heterozygous for *Etv5*^{CreERT2-RFP} (B6.Cg-*Etv5*<*tm1*(*cre*/ERT2)*Brst*>), or hemizygous for *Tg(Etv4-GFP)* (B6-*Tg(Etv4/EGFP)4Fhel*) (Lamballe et al., 2011), were crossed with wild type mice. Embryos were harvested at the desired embryonic stage. Briefly, pregnant females were sacrificed with an overdose of pentobarbital sodium (dosage: 0.4 mg pentobarbital / g mouse weight), embryos removed, and washed in PBS for ~2 min. Organs were dissected under a stereoscope and positioned for imaging.

DNA was extracted from tails and limbs following basic lab procedures and prepared for PCR-based genotyping. The *Tg(Etv4-GFP)* gene was detected using the following primer sequences: Forward—5'-GGA ATC TTG GGC CTT GAG AAC AGC-3'; reverse—5'-CGC TGA ACT TGT GGC CGT TTA CG-3'. The cycling protocol was as follows: denaturation at 94°C for 3 min; 40 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min; finish with a final extension at 72°C for 5 min. The *Etv5*^{CreERT2-RFP} gene as well as the wild type *Etv5*, was detected using the following primer sequences: *Etv5*^{CreERT2-RFP} forward—5'-TCG ATG CAA CGA GTG ATG AG-3'; *Etv5*^{CreERT2-RFP} reverse—5'-TTC GGC TAT ACG TAA CAG GGT-3'; *Etv5* forward—5'-AAA GAG

GAA CGC GGT CTG AG-3'; *Etv5* reverse—5'-CCA GCT GAG TCT CGT GTG AT-3'. The cycling protocol was as follows: denaturation at 95°C for 3 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 80 s. Product bands were detected by capillary gel electrophoresis.

FACS-based Isolation of Epithelial and Mesenchymal Cells

Lungs from embryonic mice were dissected at desired time points (E14.5, E16.5, and E18.5), and transferred to ice-cold Hank's Balanced Salt Solution (HBSS). Lungs were finely chopped with a sterile razor blade on a glass plate. The tissue was then added to a falcon tube and digested in 0.5% collagenase at 37°C for 45 min, with constant mixing. Single cell suspensions were made by successively flushing the samples through 18, 20, and 24 g grade needles and then filtering the samples through 70 and 40 μ m nylon strainers (BD Biosciences). The cell suspensions were diluted with 5 ml HBSS and centrifuged at 12,000 rpm for 5 min, and the supernatant was discarded. The pellet was resuspended in 10 μ l blocking buffer and the following antibodies were added: 488-CD31 (1:50); FITC-CD45 (1:50); and Apc Cy7 EpCam (Epithelial cell adhesion molecule) (1:50) (Biolegend), for 20 min at 4°C. The samples were washed 2x with 100 μ l FACS buffer and centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was discarded. The pellet was resuspended in 100 μ l FACS buffer.

Cell sorting and isolation was performed using the FACSariaTM III (BD Biosciences) cell sorter. Alveolar epithelial cells were identified as CD45^{-ve}/CD31^{-ve}/Epcam^{+ve} and mesenchymal cells as CD45^{-ve}/CD31^{-ve}/Epcam^{-ve}. Cells were sorted through a flow chamber with a 100- μ m nozzle tip under 25 psi sheath fluid pressure. Isolated cells were used for RNA isolation. As a main criterion for gating, we used the settings allowing to capture 98% of the cells in the isotype control and then we applied these gating conditions to the stained cells.

RT-qPCR

Either FACS-isolated cells, or embryonic lungs were lysed in Qiazol lysis reagent, and total RNA isolated using a miRNeasy mini RNA extraction kit according to the manufacturer's instructions (Qiagen). Please note that from cell dissociation to RNA isolation, the process takes 3–4 h. Our results indicate that there is very little variability between samples from the same time point and same group (epithelium or mesenchyme).

Up to 1 μ g RNA was reverse-transcribed using the QuantiTect reverse transcription kit (Qiagen). cDNA was used to specifically amplify the desired DNA sequence by quantitative PCR (qPCR). Primers were designed to amplify specific mature mRNAs using NCBI's primer-BLAST option (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (last accessed, 01-08-2018). Primers were further validated by PCR-based gel electrophoresis. The following primer sequences were used: *Etv4* (FWD-5'-CAGACTTCGCCTAC GACTCA-3'; REV-5'-GCCATAACCCATCACTCCAT-3'); *Etv5* (FWD-5'-GTGGCCGCTCAGGAGTA-3'; REV-5'-GTGCTTC CTTCCAAAGTCTCCG CT-3'); *RFP* (FWD-5'-GCGTGATG AACTTCGAGGAC-3'; REV-5'-TTCACCTTG TAGATC

AGCGTG-3'); *Hprt* (FWD-5'-CCTAAGATGAGCGCAAGTT GAA-3'; REV-5'-CCACAGGACTAGAACACCTGCTAA-3').

qPCR reaction mixtures were set up using the PowerUp SYBR Green Master Mix kit following the manufacturer's instructions (Thermo Fisher). Samples were run with three technical replicates on a LightCycler 480II (Roche) using the following protocol: UDG activation at 50°C for 2 min; DNA polymerase activation at 95°C for 2 min; and 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and extension at 72°C for 1 min. To validate amplification specificity, a dissociation step was also included for each sample. Threshold cycles (Ct) were calculated and used for relative expression analyses, using mouse *Hprt* as the reference gene.

Δ Ct and $\Delta\Delta$ Ct values were calculated according to the following formulas:

$$\Delta Ct = Ct_{\text{Reference}} - Ct_{\text{gene of interest}}$$

Note, this equation accounts for the fact that Ct is proportional to the $-\log$ of gene expression. Δ Ct is therefore positively related to the expression of the gene of interest.

$$\text{Expression relative to } Hprt = 2^{\Delta Ct}$$

$$\Delta\Delta Ct_{\text{Experimental-control}} = \text{Mean}\Delta Ct_{\text{Experimental}} - \text{Mean}\Delta Ct_{\text{Control}}$$

Unpaired two-tailed Student's *t*-tests were performed on the Δ Ct values, which can be assumed to be normally distributed. Number of "n" form of graphical representation, and significance level is indicated either in the figures or in the figure legends.

Lung Explant Culture for *in vitro* Experiments

Embryonic lungs used for *in vitro* experiments were obtained either from genetically modified embryos generated as described above, or from C57BL/6 wild-type embryos.

Embryonic lungs were dissected and cultured on 13 mm Whitman Track-Etch polycarbonate membranes, with 8.0 μ m pores (Merck) positioned atop DMEM culture medium in a 24-well culture dish [medium contained: Dulbecco's Modified Eagle Medium (1x DMEM), supplemented with D-Glucose, L-Glutamine, HEPES, Pyruvate, and Phenol red (Gibco), 10% fetal bovine serum (FBS), 1% penicillin (10,000 units/ml)-streptomycin (10 mg/ml)]. Lungs were incubated at 5% CO₂ and 37°C for ~45 min to allow them to settle. At the desired time, recombinant FGF7 (50 ng/ml), FGF10 (250 ng/ml), soluble FGFR2b (5 μ g/ml) (R and D Systems), or anti-FGF10 blocking antibody (20 μ g/ml) (Santa Cruz Biotechnology) was added to the experimental lungs (as described in Sakaue et al., 2002), while the vehicle was added to control samples. Lungs were incubated at 5% CO₂ and 37°C for the duration of the experiment.

In vivo Model to Inhibit FGFR2b Signaling

In vivo studies were conducted using an inducible dominant negative mouse model: *Rosa26^{rtTA/+}*; *Tg(tetO-Fgfr2b)/+* (*B6-Cg-Gt(ROSA)26Sor^{tm1.1(rtTA,EGFP)Nagy}* *Tg(tetO-Fgfr2b/lgh)1.3Jaw/sbel*). Doxycycline was administered via

intraperitoneal injection to timed-pregnant females, as previously described (Danopoulos et al., 2013).

Imaging Acquisition and Measurements

Brightfield images of lungs from *in vivo* and *in vitro* experiments were captured either on a Leica MZ 125 stereoscopic dissecting microscope using a Spot Insight 2.0 Mp Color Mosaic camera and Spot 4.5.9 imaging software, or were obtained from live imaging experiments using a Leica DM6000B inverted microscope, DFC 305FX camera, and Leica Application Suite Advanced Fluorescence imaging software. Fluorescence intensities were quantified using FIJI software (version 2.0.0-rc-68/1.52 g).

Branching was quantified by counting and averaging the distal tips of the left lobe of control and experimental samples at 0 and 24 h. These groups were analyzed using a two-factorial quasi-Poisson model, with the dispersion parameter estimated as 0.317. Differences were considered statistically significant at $p < 0.05$.

Gene Expression Patterns

To assess the expression patterns of genes in early stage embryonic lungs (E14.5), the online database genepaint.org was used (last accessed 01-08-2018). Each of the genes significantly regulated in our *in vivo* studies was entered in genepaint. The whole embryo section displaying the clearest gene expression in the lung, along with a magnification of the lung itself, was chosen for the figures.

RESULTS AND DISCUSSION

Tg(Etv4-GFP) Expression During Early Embryonic Development

The Tg(Etv4-GFP) mouse line was created by Lamballe et al. (2011). These researchers used a bacterial artificial chromosome containing the *Etv4* gene fused with a green fluorescent protein gene (GFP) in frame with exon 9, to generate the Tg(Etv4-GFP) transgenic mouse line (Figure 1A). In the process, exons 10–12 of the *Etv4* construct were deleted. Thus, the randomly inserted Tg(Etv4-GFP) transgene is non-functional, and does not interfere with endogenous *Etv4* activity.

To characterize Tg(Etv4-GFP) expression in various organs during development, embryos were generated at different stages (E9.5, E11.5, E12.5, and E13.5). Tg(Etv4-GFP) was crossed with wild type mice to generate controls (Etv4^{+/+}) and transgenic embryos (Tg(Etv4-GFP)/+) at different developmental stages. Figure 1B shows the PCR-based strategy designed to detect the band corresponding to the presence of the transgene (see sequence of the primers in materials and methods). Figure 1C shows embryos negative for GFP (Figures 1Ca,g,n,u) and positive for GFP (Figures 1Cb,r,h,v) at different developmental stages. At E9.5, GFP was detected in the nasal placode, the mandibullary and maxillary processes, and at the mid-brain/hindbrain junction (Figures 1Cc), the anterior limb bud and the mammary line (Figures 1Ce), the otic placode (Figures 1Cd), and the tail bud (Figures 1Cf). Similar expression sites were found at E11.5 (Figures 1Ch–l). Close examination of the lung indicated that GFP was expressed specifically at the tips in the epithelium and mesenchyme (Figures 1Cm). This

expression overlaps with the previously reported endogenous *Etv4* expression in the E12.5 lung (Yin et al., 2011). At E12.5, the external expression of GFP did not appear as specific. However, upon dissection of internal organs, GFP was enriched in the posterior (glandular) stomach (Figures 1Co), the developing kidneys (Figures 1Cq), the epithelium of the cecum (Figures 1Cs), and the lung epithelium and mesenchyme (Figures 1Ct, see also insert in figure 1t). At E13.5, GFP was found in similar places (Figures 1Cw,y,z). In addition, GFP was located at the tip of the forming digits (Figures 1Cx).

Etv5^{CreERT2-RFP/+} Expression During Early Embryonic Development

The Etv5^{CreERT2-RFP/+} mouse line was recently generated by Barry Stripp (Cedars-Sinai Medical Center, Los Angeles, USA). In this line, a knock-in of *CreERT2/RFP* in the 3' untranslated region of the *Etv5* gene was carried in embryonic stem cells (Figure 1D). Recombinant clones were used to generate Etv5^{CreERT2-RFP/+} knock-in mice. Figure 1E shows the PCR based strategy to detect the presence of the Etv5^{RFP} knock in allele in heterozygous or homozygous embryos (see sequence of the primers in materials and methods). We performed a similar characterization on this line as we did on the Tg(Etv4-GFP) mouse line.

Etv5^{CreERT2-RFP/+} mice were crossed with wild type mice to generate controls (Etv5^{+/+}) and heterozygous embryos (Etv5^{CreERT2-RFP/+}) at different developmental stages. During the E9.5 and E11.5 stages, RFP expression was general throughout the embryo (Figures 1Fa,g). At E9.5, expression was seen in the heart (Figures 1Fb), otic placode (Figures 1Fd), and nasal placode (Figures 1Fc). Expression in the tail was seen at E9.5 and E11.5 (Figures 1Ff,k), and in limb buds, limbs and the tips of forming digits in all stages (Figures 1Ff,h,q,w). Expression was seen in whisker regions (Figures 1Fn,t). After dissection, RFP was found to be enriched in the anterior stomach (Figures 1Fp), kidneys (Figures 1Fr,x), and epithelium of the cecum (Figures 1Fj,v). The expression of RFP in the lung at E11.5 did not appear enriched in the epithelium (Figures 1Fl). However, in later stages, RFP expression did appear enriched in the epithelium (Figures 1Fo,s,u), not only distally, but throughout the epithelium, with the expression disappearing in the main bronchi and trachea (e.g., Figures 1Fo,s, see also Figures 4, 6).

Expression of Etv4 and Etv5 During Embryonic Lung Development

To assess the relative expression of *Etv4* and *Etv5* during embryonic lung development, epithelial and mesenchymal cells from embryonic wild type mouse lungs were separated using FACS at different stages (E14.5, E16.5, and E18.5). Please note that while our protocol allows excluding the CD31-positive endothelium and CD45-positive hematopoietic cells, the isolated mesenchyme (negative for the surface marker Epcam, an epithelial cell adhesion molecule used to isolate epithelial cells) was a mixture of nerve cells, smooth muscle cells, mesothelial cells and resident mesenchymal cells. RNA

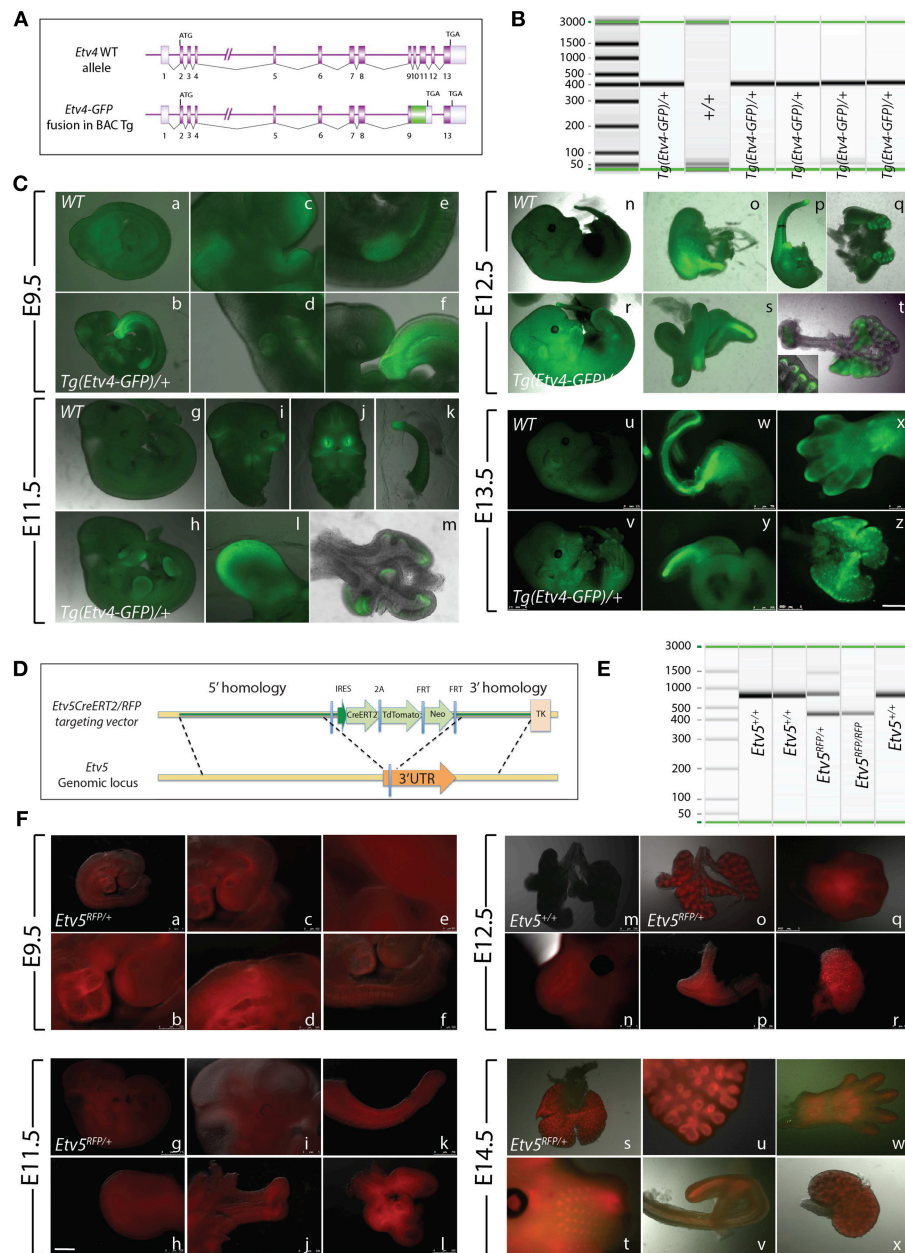


FIGURE 1 | Early embryonic expression of ETV4-GFP and ETV5-RFP **(A)** *Tg(Etv4-GFP)* genetic construct. **(B)** Genotype detecting the *Tg(Etv4-GFP)* transgene at ~400 bp. **(C)** ETV4-GFP expression pattern in various embryonic organs at E9.5 (a–f), E11.5 (g–m), E12.5 (n–t), and E13.5 (u–z). See text for details. Note the increased expression of ETV4-GFP in the distal tips of the lung (see inset in “t,” and “z”). Scale bar: (a,b,g,h,n,r,u,v) 2.5 mm; (c–f,i,l,o–q,s,y) 500 μ m; (w,x) 750 μ m; (t,m,z) 1,000 μ m. **(D)** *Etv5^{RFP}* genetic construct. **(E)** Genotype detecting the *Etv5^{RFP}* transgene at ~450 bp and the wild type *Etv5* at ~827 bp. **(F)** ETV5-RFP expression pattern in various embryonic organs at E9.5 (a–f), E11.5 (g–l), E12.5 (o–r), and E14.5 (s–x). Please note that the *Etv5^{+/+}* lungs do not show any autofluorescence (**Figure 1Fm**). See text for details. Note the differential expression level of ETV5-RFP in the distal lung epithelium vs. the proximal conducting airways (see “o” and “s”). Scale bar: (a,i,n,t) 1 mm; (b) 250 μ m; (c,d,f,h,j,l,m,o,q–s,u,w,x) 500 μ m; (e) 100 μ m; (g) 2.5 mm; (k,p,v) 750 μ m.

was extracted from the Epcam-positive (epithelial) and Epcam-negative (mesenchymal) cells and qPCR analysis was performed (**Figure 2A**). *Etv4* expression was seen in both the mesenchyme and epithelium during embryonic development. At E14.5, *Etv4* was more highly expressed in the epithelium than in the mesenchyme. However, at later stages (E16.5 and E18.5), *Etv4*

expression decreased in the epithelium while remaining relatively constant in the mesenchyme (**Figure 2B**).

Etv5 was likewise expressed in both the mesenchymal and epithelial compartments throughout embryonic development. However, its expression in the epithelium was much higher than in the mesenchyme (around 10 times higher at E14.5,

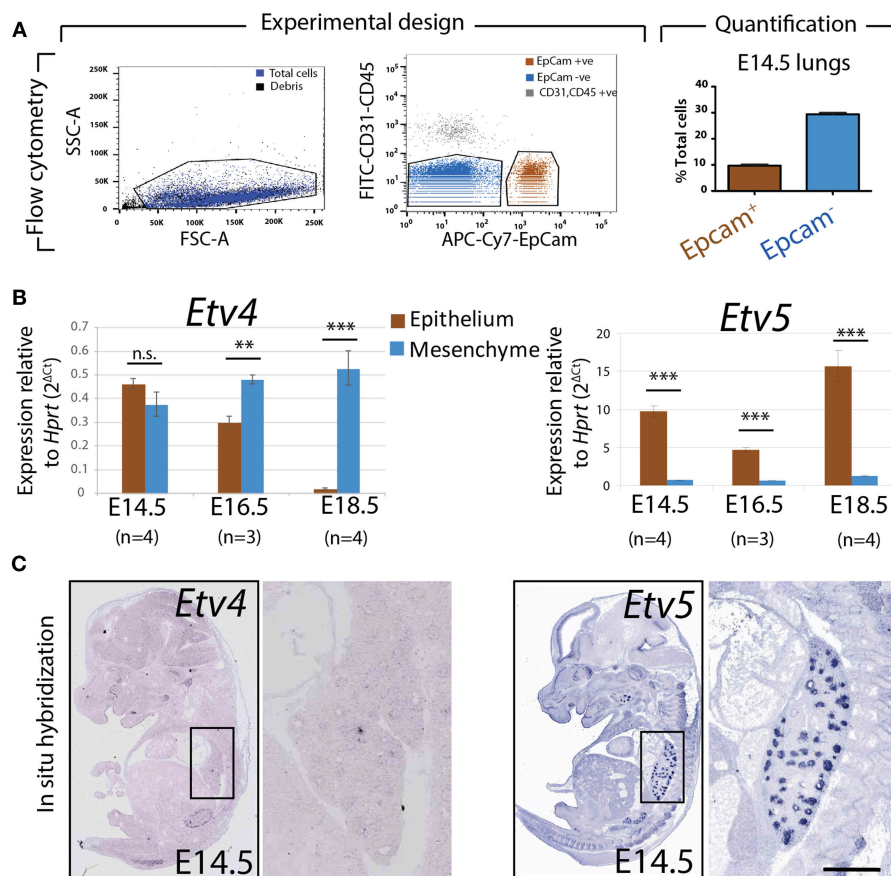


FIGURE 2 | Expression of *Etv4* and *Etv5* in isolated embryonic lung epithelium and mesenchyme **(A)** Experimental design for the FACS-based isolation of embryonic lung epithelial and mesenchymal cells at E14.5 ($n = 4$; 44.88 ± 17.54 ng/ μ l RNA from epithelial cells, 64.70 ± 18.10 ng/ μ l RNA from mesenchymal cells), E16.5 ($n = 3$; 16.38 ± 5.69 ng/ μ l RNA from epithelial cells, 75.18 ± 13.79 ng/ μ l RNA from mesenchymal cells), and E18.5 ($n = 4$; 28.77 ± 3.06 ng/ μ l RNA from epithelial cells, 44.89 ± 15.30 ng/ μ l RNA from mesenchymal cells). Approximately 10 and 30% of the cells in E14.5 lungs were epithelial and mesenchymal, respectively. **(B)** RT-qPCR for *Etv4* and *Etv5* in isolated epithelial and mesenchymal cells. Note the different scales of the y-axis between *Etv4* and *Etv5* graphs. (Data are presented as geometric mean $\pm$ SE; n.s. = not significant, $**p < 0.01$, $***p < 0.001$). **(C)** *In situ* hybridization from the genepaint database showing the expression of *Etv4* and *Etv5* in E14.5 embryos.

and 15 times higher at E18.5). *Etv5* epithelial expression decreased at E16.5 before greatly increasing at E18.5, compared to E14.5 (**Figure 2B**).

The expression of *Etv4* and *Etv5* was also validated using the publicly available genepaint.org data base. While *Etv4* expression was diffusely seen in the lung epithelium and surrounding mesenchyme, *Etv5* showed very strong expression restricted to the epithelium (compare insets in **Figure 2C**).

Dynamic GFP Expression Reports FGF10 Signaling

To monitor the expression of Tg(*Etv4*-GFP) in the epithelial buds of pseudoglandular stage lungs in the context of FGF10 signaling, E12.5 Tg(*Etv4*-GFP) lungs were cultured and live imaged for 24 h, after which FGF10 signaling was blocked using an anti-FGF10 antibody for an additional 24 h (**Figure 3A**). During the first 24 h, ETV4-GFP was dynamically expressed,

showing greater expression at the distal tips of growing buds, in likely regions of active FGF10 signaling (see **Figures 3Ba–c** and **Supplementary Movie 1**). During the FGF10 inhibition, ETV4-GFP expression was greatly reduced. Still images from multiple time points during the live imaging from three independent lungs were used to quantify these global changes in fluorescence intensity (**Figure 3C**). We also confirmed that the loss of GFP expression of the E12.5 lungs after 24 h in culture was neither due to bleaching, nor to a normal decrease in ETV4-GFP expression. **Supplementary Figure 1** shows that the expression of ETV4-GFP in E12.5 transgenic lungs was maintained in culture for at least 38 h. Furthermore, still images of individual buds were used to quantify the dynamic expression of ETV4-GFP in three regions of the bud during a branching event (**Figures 3D,E**). Intensity was measured during new branch formation at the tip (regions 4 and 6), stalk (regions 1–3; 7–9) and cleft (region 5). Tip ETV4-GFP fluorescence intensity initially increased before stabilizing (**Figures 3Ea**), whereas stalk

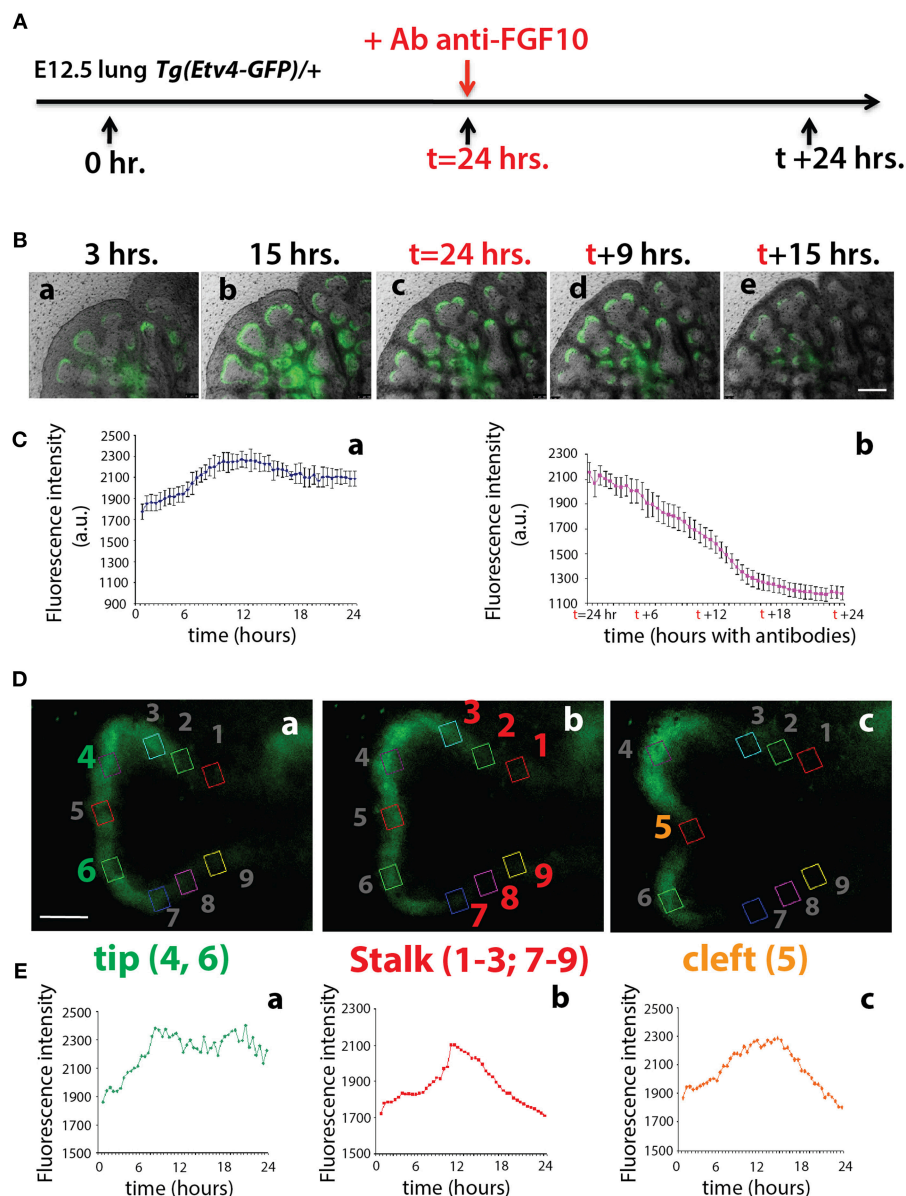


FIGURE 3 | ETV4-GFP is dynamically expressed in regions of active FGF10 signaling during early lung development **(A)** Experimental design: E12.5 *Tg(Etv4-GFP)* lungs were isolated, cultured and live imaged for 48 h. After 24 h anti-FGF10 antibody was added to inhibit FGF10 activity. **(B)** Still images from different time points during the live imaging experiment. Note how ETV4-GFP expression increases before leveling off within the first 24 h (a–c), and, once the antibody is added, the expression decreases to almost zero by the end of the experiment (c–e). Scale bar: 125 μ m. **(C)** Global ETV4-GFP fluorescence intensity measured at various time points before (a) and after (b) adding the FGF10 blocking antibody. ($n = 3$; data are presented as average fluorescence intensity in arbitrary units (a.u.) $\pm$ standard deviation). **(D)** Example images of a branching tip at three successive time points (a–c), highlighting three regions of dynamic ETV4-GFP expression: the tip (4 and 6), the stalk (1–3, and 7–9), and the cleft (5). See text for details. Scale bar: 30 μ m. **(E)** Representative plot of ETV4-GFP expression in three independent regions [(a) tip, (b) stalk, and (c) cleft] of a single bud over a period of 24 h ($n = 1$; a.u. = arbitrary units).

and cleft fluorescence intensity increased before decreasing to initial levels (**Figures 3Eb,c**). The expression patterns of the stalk and cleft reflected the initial single bud branching into two buds.

These results suggest that the *Tg(Etv4-GFP)* mouse line can be used as a valid tool to report FGF10 signaling in the distal tips

of lungs during pseudoglandular development. This conclusion is supported by the previously described dynamic expression pattern of *Sprouty2* during the branching process (Mailleux et al., 2001). *Sprouty2* is a well-accepted target of FGF10 signaling in the lung, and displays a remarkably similar expression pattern to what we found for *Tg(Etv4-GFP)*.

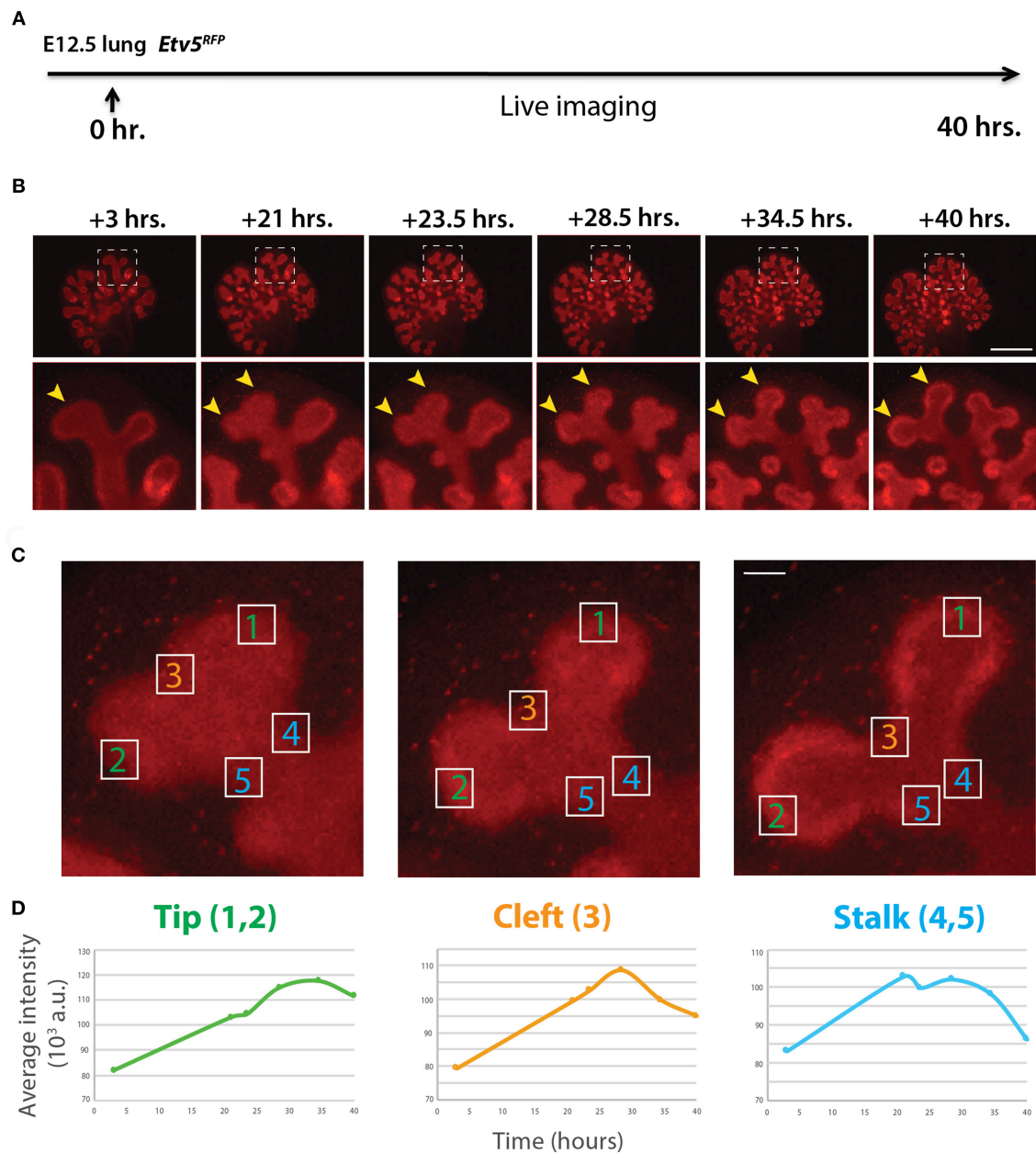


FIGURE 4 | Expression pattern of ETV5-RFP in distal lung epithelium during early development **(A)** Experimental design: E12.5 *Etv5^{RFP}* lung explants were cultured for 40 h and live imaged. **(B)** Still images showing representative ETV5-RFP expression both globally and in the distal tips (boxes). Arrows indicate increased expression in the tips over time. Scale bar: (Top row) 500 μ m; (Bottom row) 125 μ m. **(C)** Example images of a branching tip at three successive time points (a–c), highlighting three regions of dynamic ETV5-RFP expression: the tip (1 and 2), the cleft (3), and the stalk (4 and 5). See text for details. Scale bar: 37 μ m. **(D)** Representative plot of ETV5-RFP expression in three independent regions [(a) tip, (b) stalk and (c) cleft] of a single bud over a period of 40 h ($n = 1$; a.u. = arbitrary units).

Quantification of Dynamic ETV5-RFP Fluorescence

Similar to the analysis of ETV4-GFP expression in early lung development, the expression of ETV5-RFP in branching epithelial buds was assessed. E12.5 *Etv5^{CreERT2-RFP/+}* lungs were cultured and live imaged for 40 h (**Figure 4A**). During this time ETV5-RFP was expressed throughout the epithelial

tree at the exception of the primary bronchi and trachea. This was in contrast with the expression of ETV4-GFP, which was only at the tip. Interestingly, the more proximal regions of ETV5-RFP expression were not localized to known areas of FGF10 signaling. Close-up examination of ETV5-RFP indicated higher expression in distal tips compared to more proximal regions (see arrows in **Figure 4B** and

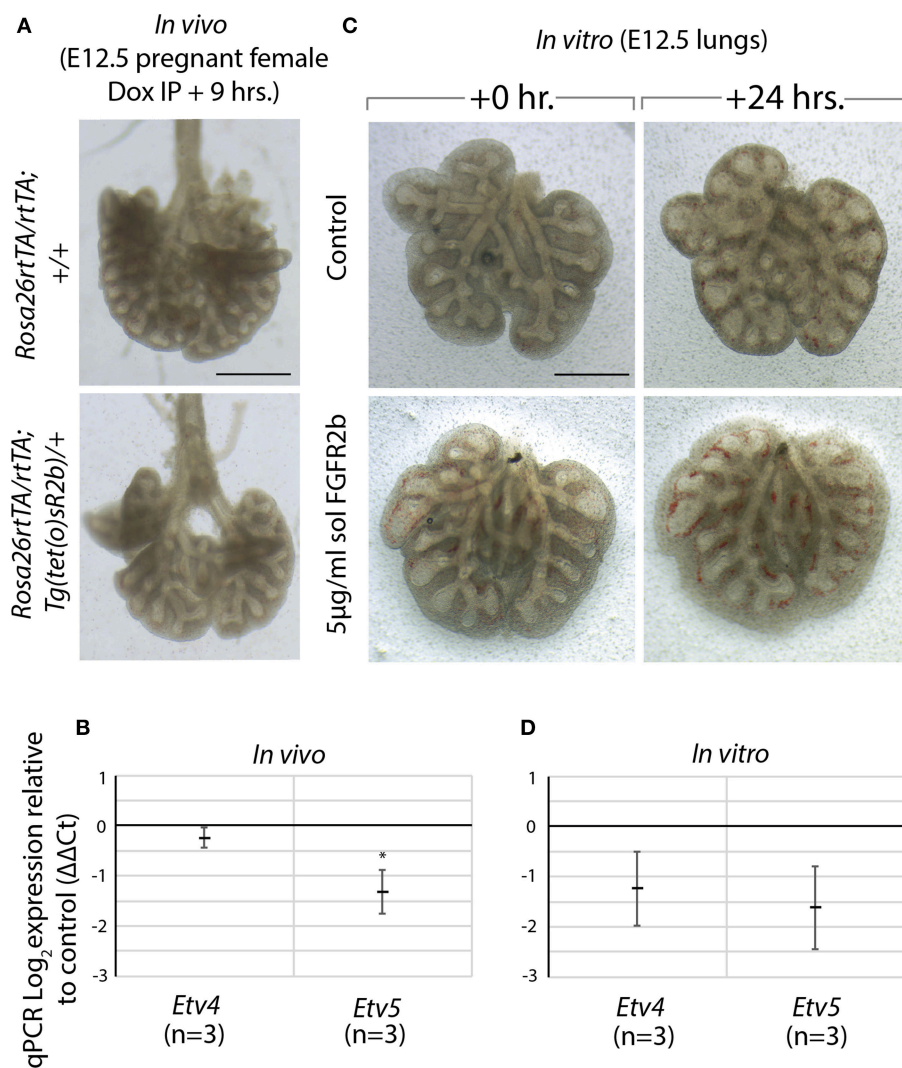


FIGURE 5 | Model to inhibit FGF10 *in vivo* and *in vitro* demonstrates *Etv4* and *Etv5* are downstream of FGF10/FGFR2b signaling **(A)** *In vivo* model to inhibit FGF10/FGFR2b signaling: pregnant females carrying experimental (*Rosa26rtTA/rtTA*; *Tg(tet(o)sR2b)/+*) and littermate control (*Rosa26rtTA/rtTA*; *Tg*^{+/+}) embryos were injected with doxycycline. Embryonic lungs were isolated 9 h later. Scale bar: 500 μ m. **(B)** Corresponding RT-qPCR analysis showing *Etv4* and *Etv5* expression in experimental vs. control lungs. **(C)** *In vitro* model to inhibit FGF10/FGFR2b signaling: E12.5 lung explants were cultured for 24 h with (experimental) or without (control) 5 μ g/ml recombinant (soluble) FGFR2b. Scale bar: 500 μ m. **(D)** Corresponding RT-qPCR analysis showing *Etv4* and *Etv5* expression in experimental vs. control lungs. (Data are presented as mean $\pm$ SEM; * p < 0.05).

Supplementary Movie 2). Finally, we quantified the fluorescence intensity of bud regions during a branching event using still images at multiple time points (Figures 4Ca–c). Fluorescence intensity at the tip (regions 1 and 2) increased before leveling off around 30 h, and then decreased slightly (Figures 4Da), whereas intensity at the cleft and stalk regions (regions 3 and 4–5, respectively) initially increased before decreasing to original levels (Figures 4Db,c). This temporal expression pattern was similar to what was found for *Tg(Etv4-GFP)* lungs, and is likely a consequence of the initial bud branching into two buds.

Model to Inhibit FGF10 Confirms *Etv4* and *Etv5* Are Downstream of FGF10/FGFR2b Signaling

We made use of a model to inhibit all FGFR2b ligands *in vivo* via inducible expression of a dominant negative form of the FGFR2b receptor, called soluble FGFR2b (*Rosa26rtTA/rtTA*; *Tg(tet(o)sR2b)/+*) (Parsa et al., 2008, 2010). Upon administration of doxycycline, soluble FGFR2b is produced and secreted from cells, functionally inhibiting all FGFR2b ligands from properly signaling. We have previously shown that at E12.5, FGF10 is the predominant FGF ligand signaling in the lung (Bellusci et al.,

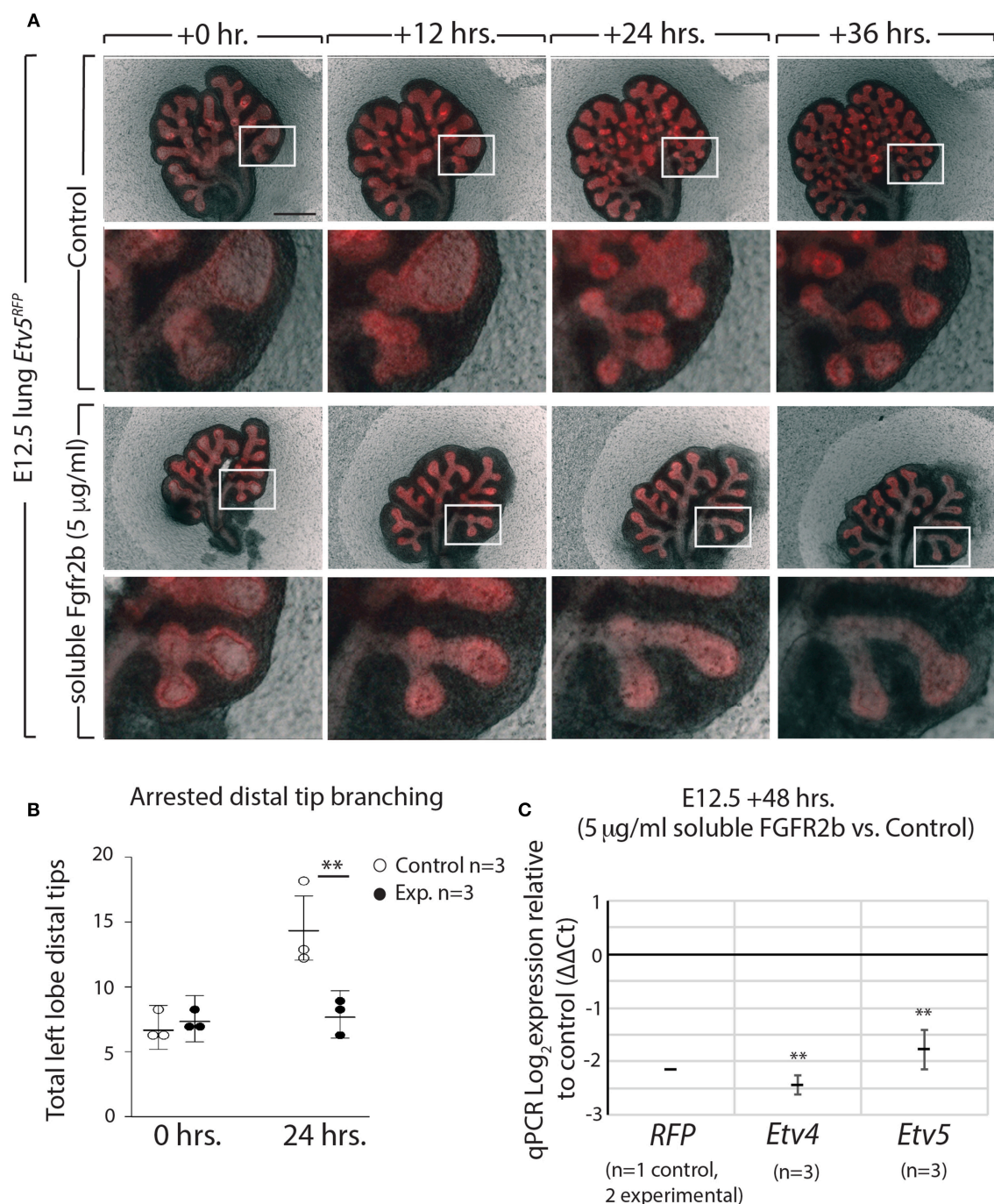


FIGURE 6 | ETv5-RFP reports FGF10/FGFR2b signaling *in vitro* (A) E12.5 *Etv5*^{RFP} lungs were harvested and cultured with (experimental) or without (control) 5 µg/ml recombinant (soluble) FGFR2b for 48 h. Scale bar: (Top rows) 500 µm; (Boxes) 125 µm. (B) Left lobe distal tips show arrested branching in experimental lungs after 24 h. FGF10/FGFR2b inhibition, compared to control lungs. While both control lungs and experimental lungs had a similar number of average tips at 0 h, only control lungs showed an increase in branching over time (Data are presented as mean with 95% CI; ***p* < 0.01). (C) RT-qPCR analysis of the lungs showing downregulation of *RFP*, *Etv4*, and *Etv5*, in experimental lungs, demonstrating the usefulness of the *Etv5*^{RFP} line to report FGF10/FGFR2b signaling. Note, we only had one control *Etv5*^{RFP} lung and two experimental *Etv5*^{RFP} lungs, thus statistical analysis was not possible (Data are presented as mean ± SEM; ***p* < 0.01).

1997b). Therefore, induction of soluble FGFR2b at this time point inhibits FGF10 signaling. As **Figure 5A** shows, after only 9 h of FGF10 inhibition, experimental lungs were smaller, displayed

simplified branching, and had fewer distal buds. Furthermore, the expressions of *Etv4* and *Etv5*, as evaluated by RT-qPCR, were reduced (**Figure 5B**).

A similar approach to inhibiting FGFR2b signaling was conducted *in vitro*. In this experiment, wild type E12.5 lungs were harvested and cultured with (experimental) or without (control) recombinant FGFR2b added to the culture medium for 24 h (Figure 5C). A similar phenotype to that seen *in vivo* was produced in experimental lungs, while the downregulation of *Etv4* and *Etv5* mRNA was even more pronounced (Figure 5D).

Taken together, these results suggest that FGF10 signaling can be inhibited both *in vitro* and *in vivo*, and that *Etv4* and *Etv5* are regulated by FGF10.

ETV5-RFP Expression Reports FGF10 Signaling *in vitro*

Using our *in vitro* approach to inhibit FGF10/FGFR2b signaling in pseudoglandular stage lungs, we investigated whether the *Etv5*^{CreERT2-RFP} line could be used as a tool to report FGF10/FGFR2b signaling. E12.5 *Etv5*^{CreERT2-RFP} lungs were cultured and live imaged for 48 h with (experimental) or without (control) recombinant soluble FGFR2b (Figure 6A). As can be seen in the still images, the soluble FGFR2b treated lungs showed arrest in branching over time, compared to the control (Figure 6B). This phenotype is a hallmark of inhibited FGF10 signaling. Furthermore, demonstrating the usefulness of this line to report FGF10/FGFR2b signaling, the fluorescence intensity of the ETV5-RFP decreased over time in experimental lungs, while that of the controls remained constant. This finding is supported by RT-qPCR results after 48 h, which show a decrease in the expression of *RFP*, *Etv4*, and *Etv5* in experimental lungs compared to controls (Figure 6C).

These results not only confirm that soluble FGFR2b can be used to inhibit FGF10 signaling in *Etv5*^{CreERT2-RFP} lung explants, but they also suggest that the *Etv5*^{CreERT2-RFP} line responds to FGF10/FGFR2b signaling in the developing lung.

CONCLUSION

In conclusion, the Tg(*Etv4*-GFP) and *Etv5*^{RFP} reporter mouse lines appear to be promising tools to monitor FGF10/FGFR2b signaling in early lung development. These tools will have to be further validated at later stages and in other organs of interest.

ETHICS STATEMENT

Animal experiments, harvesting organs and tissues from wild type and mutant mice following euthanasia using pentobarbital was approved at Justus Liebig University Giessen by the federal authorities for animal research of the Regierungspraesidium

REFERENCES

Bellusci, S., Furuta, Y., Rush, M. G., Henderson, R., Winnier, G., and Hogan, B. L. (1997a). Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. *Development* 124, 53–63.

Giessen, Hessen, Germany (Approved Protocol GI 20/10 Nr. G 84/2016).

AUTHOR CONTRIBUTIONS

SB, CC, C-MC, BS, and FH concept and design. MJ, AL, SD, and AS acquisition of data. MJ, SB, C-MC, and SD analysis and interpretation. MJ and SB drafting and editing of the manuscript. All authors read and approved the final manuscript.

FUNDING

SB was supported by grants from the Deutsche Forschungsgemeinschaft [DFG; BE4443/1-1, BE4443/4-1, BE4443/6-1, KFO309 P7 and SFB1213-projects A02 and A04, EXC2026 (Project ID 390649896)], Landes-Offensive zur Entwicklung Wissenschaftlich-Ökonomischer Exzellenz (LOEWE), UKGM, Universities of Giessen and Marburg Lung Center (UGMLC), DZL, and COST (BM1201). C-MC also acknowledges the support of the University Hospital Giessen and Marburg (UKGM) and the German Center for Lung Research (DZL). CC acknowledges the support of The National Nature Science Foundation of China (81570075, 81770074, 31471269, 81600062), Zhejiang Provincial Natural Science Foundation (LZ15H010001, LY18H010006), Zhejiang Provincial Science Technology Department Foundation (2015103253, 2018264229), the National Key Research and Development Program of China (2016YFC1304000), Wenzhou public welfare science and technology project (Y20180125), Scientific research incubation project of the First Affiliated Hospital of Wenzhou Medical University (FHY2015037).

ACKNOWLEDGMENTS

We acknowledge the precious help provided by Kerstin Goth and Jana Rostkovius in managing the mouse colonies and for the genotyping of the mice. We thank Dr. Jochen Wilhelm for the statistical interpretation of the generated data.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2019.00178/full#supplementary-material>

Supplementary Movie 1 | ETV4-GFP in the E12.5 developing lung for 24 h.

Supplementary Movie 2 | ETV5-RFP in the E12.5 developing lung for 24 h.

Supplementary Figure 1 | ETV4-GFP expression is stable for at least 38 h *in vitro* To monitor the expression pattern of ETV4-GFP in E12.5 lungs *in vitro*, lungs were cultured and live imaged in control medium (a–d), with additional recombinant FGF7 (e–h), or additional recombinant FGF10 (i–l) for 38 h. Scale bar: 125 μm.

Bellusci, S., Grindley, J., Emoto, H., Itoh, N., and Hogan, B. L. (1997b). Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. *Development* 124, 4867–4878.

Chao, C. M., El Agha, E., Tiozzo, C., Minoo, P., and Bellusci, S. (2015). A breath of fresh air on the mesenchyme: impact of impaired mesenchymal

- development on the pathogenesis of bronchopulmonary dysplasia. *Front. Med.* 2:27. doi: 10.3389/fmed.2015.00027
- Danopoulos, S., Parsa, S., Al Alam, D., Tabatabai, R., Baptista, S., Tiozzo, C., et al. (2013). Transient Inhibition of FGFR2b-ligands signaling leads to irreversible loss of cellular beta-catenin organization and signaling in AER during mouse limb development. *PLoS ONE* 8:e76248. doi: 10.1371/journal.pone.0076248
- De Moerlooze, L., Spencer-Dene, B., Revest, J. M., Hajihosseini, M., Rosewell, I., and Dickson, C. (2000). An important role for the IIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. *Development* 127, 483–492. doi: 10.1042/cs099005P
- El Agha, E., and Bellusci, S. (2014). Walking along the Fibroblast growth factor 10 route: a key pathway to understand the control and regulation of epithelial and mesenchymal cell-lineage formation during lung development and repair after injury. *Scientifica* 2014:538379. doi: 10.1155/2014/538379
- Garg, A., Hannan, A., Wang, Q., Collins, T., Teng, S., Bansal, M., et al. (2018). FGF-induced Pea3 transcription factors program the genetic landscape for cell fate determination. *PLoS Genet.* 14:e1007660. doi: 10.1371/journal.pgen.1007660
- Herriges, J. C., Verheyden, J. M., Zhang, Z., Sui, P., Zhang, Y., Anderson, M. J., et al. (2015). FGF-regulated ETV transcription factors control FGF-SHH feedback loop in lung branching. *Dev. Cell* 35, 322–332. doi: 10.1016/j.devcel.2015.10.006
- Kuure, S., Chi, X., Lu, B., and Costantini, F. (2010). The transcription factors Etv4 and Etv5 mediate formation of the ureteric bud tip domain during kidney development. *Development* 137, 1975–1979. doi: 10.1242/dev.051656
- Lamballe, F., Genestine, M., Caruso, N., Arce, V., Richelme, S., Helmbacher, F., et al. (2011). Pool-specific regulation of motor neuron survival by neurotrophic support. *J. Neurosci.* 31, 11144–11158. doi: 10.1523/JNEUROSCI.2198-11.2011
- Mailleux, A. A., Tefft, D., Ndiaye, D., Itoh, N., Thierry, J. P., Warburton, D., et al. (2001). Evidence that SPROUTY2 functions as an inhibitor of mouse embryonic lung growth and morphogenesis. *Mech. Dev.* 102, 81–94. doi: 10.1016/S0925-4773(01)00286-6
- Mao, J., McGlinn, E., Huang, P., Tabin, C. J., and McMahon, A. P. (2009). Fgf-dependent Etv4/5 activity is required for posterior restriction of Sonic Hedgehog and promoting outgrowth of the vertebrate limb. *Dev. Cell* 16, 600–606. doi: 10.1016/j.devcel.2009.02.005
- Parsa, S., Kuremoto, K., Seidel, K., Tabatabai, R., Mackenzie, B., Yamaza, T., et al. (2010). Signaling by FGFR2b controls the regenerative capacity of adult mouse incisors. *Development* 137, 3743–3752. doi: 10.1242/dev.051672
- Parsa, S., Ramasamy, S. K., De Langhe, S., Gupte, V. V., Haigh, J. J., Medina, D., et al. (2008). Terminal end bud maintenance in mammary gland is dependent upon FGFR2b signaling. *Dev. Biol.* 317, 121–131. doi: 10.1016/j.ydbio.2008.02.014
- Sakaue, H., Konishi, M., Ogawa, W., Asaki, T., Mori, T., Yamasaki, M., et al. (2002). Requirement of fibroblast growth factor 10 in development of white adipose tissue. *Genes Dev.* 16, 908–912. doi: 10.1101/gad.983202
- Sekine, K., Ohuchi, H., Fujiwara, M., Yamasaki, M., Yoshizawa, T., Sato, T., et al. (1999). Fgf10 is essential for limb and lung formation. *Nat. Genet.* 21, 138–141. doi: 10.1038/5096
- Sharrocks, A. D., Brown, A. L., Ling, Y., and Yates, P. R. (1997). The ETS-domain transcription factor family. *Int. J. Biochem. Cell Biol.* 29, 1371–1387. doi: 10.1016/S1357-2725(97)00086-1
- Yin, Y., Wang, F., and Ornitz, D. M. (2011). Mesothelial- and epithelial-derived FGF9 have distinct functions in the regulation of lung development. *Development* 138, 3169–3177. doi: 10.1242/dev.065110
- Zhang, Z., Newton, K., Kummerfeld, S. K., Webster, J., Kirkpatrick, D. S., Phu, L., et al. (2017). Transcription factor Etv5 is essential for the maintenance of alveolar type II cells. *Proc. Natl. Acad. Sci. U.S.A.* 114, 3903–3908. doi: 10.1073/pnas.1621177114
- Zhang, Z., Verheyden, J. M., Hassell, J. A., and Sun, X. (2009). FGF-regulated ETV genes are essential for repressing Shh expression in mouse limb buds. *Dev. Cell* 16, 607–613. doi: 10.1016/j.devcel.2009.02.008
- Znosko, W. A., Yu, S., Thomas, K., Molina, G. A., Li, C., Tsang, W., et al. (2010). Overlapping functions of Pea3 ETS transcription factors in FGF signaling during zebrafish development. *Dev. Biol.* 342, 11–25. doi: 10.1016/j.ydbio.2010.03.011

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Jones, Lingampally, Dilai, Shrestha, Stripp, Helmbacher, Chen, Chao and Bellusci. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



A Comprehensive Analysis of Fibroblast Growth Factor Receptor 2b Signaling on Epithelial Tip Progenitor Cells During Early Mouse Lung Branching Morphogenesis

Matthew R. Jones^{1,2†}, Salma Dilai^{2†}, Arun Lingampally², Cho-Ming Chao^{1,2}, Soula Danopoulos³, Gianni Carraro⁴, Regina Mukhametshina⁵, Jochen Wilhelm², Eveline Baumgart-Vogt², Denise Al Alam³, Chengshui Chen¹, Parviz Minoo⁶, Jin San Zhang^{1,7,8*} and Saverio Bellusci^{1,2,3,7,8*}

OPEN ACCESS

Edited by:

Maria Caterina Mione,
University of Trento, Italy

Reviewed by:

Poongodi Geetha-Loganathan,
SUNY Oswego, United States
Liping Xiao,
UCONN Health, United States

*Correspondence:

Jin San Zhang
zhang.jinsan@mayo.edu
Saverio Bellusci
Saverio.Bellusci@
innere.med.uni-giessen.de

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Stem Cell Research,
a section of the journal
Frontiers in Genetics

Received: 26 October 2018

Accepted: 27 December 2018

Published: 23 January 2019

Citation:

Jones MR, Dilai S, Lingampally A, Chao C-M, Danopoulos S, Carraro G, Mukhametshina R, Wilhelm J, Baumgart-Vogt E, Al Alam D, Chen C, Minoo P, Zhang JS and Bellusci S (2019) A Comprehensive Analysis of Fibroblast Growth Factor Receptor 2b Signaling on Epithelial Tip Progenitor Cells During Early Mouse Lung Branching Morphogenesis. *Front. Genet.* 9:746. doi: 10.3389/fgene.2018.00746

¹ Department of Pulmonary and Critical Care Medicine, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China, ² Department of Internal Medicine II, Member of the German Lung Center, Excellence Cluster Cardio-Pulmonary Systems, University of Giessen Lung Center, Giessen, Germany, ³ Developmental Biology and Regenerative Medicine Program, Saban Research Institute of Children's Hospital Los Angeles and University of Southern California, Los Angeles, CA, United States, ⁴ Department of Medicine, Cedars-Sinai Medical Center, Lung and Regenerative Medicine Institutes, Los Angeles, CA, United States, ⁵ Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russia, ⁶ Division of Newborn Medicine, Department of Pediatrics, Children's Hospital Los Angeles, University of Southern California, Los Angeles, CA, United States, ⁷ Institute of Life Sciences, Wenzhou University, Zhejiang, China, ⁸ International Collaborative Research Center on Growth Factors, Wenzhou Medical University, Zhejiang, China

This study demonstrates that FGF10/FGFR2b signaling on distal epithelial progenitor cells, via β -catenin/EP300, controls, through a comprehensive set of developmental genes, morphogenesis, and differentiation. Fibroblast growth factor (FGF) 10 signaling through FGF receptor 2b (FGFR2b) is mandatory during early lung development as the deletion of either the ligand or the receptor leads to lung agenesis. However, this drastic phenotype previously hampered characterization of the primary biological activities, immediate downstream targets and mechanisms of action. Through the use of a dominant negative transgenic mouse model (*Rosa26rtTA; tet(o)sFgfr2b*), we conditionally inhibited FGF10 signaling *in vivo* in E12.5 embryonic lungs via doxycycline IP injection to pregnant females, and *in vitro* by culturing control and experimental lungs with doxycycline. The impact on branching morphogenesis 9 h after doxycycline administration was analyzed by morphometry, fluorescence and electron microscopy. Gene arrays at 6 and 9 h following doxycycline administration were carried out. The relationship between FGF10 and β -catenin signaling was also analyzed through *in vitro* experiments using IQ1, a pharmacological inhibitor of β -catenin/EP300 transcriptional activity. Loss of FGF10 signaling did not impact proliferation or survival, but affected both adherens junctions (up-regulation of E-cadherin), and basement membrane organization (increased laminin). Gene arrays identified multiple direct targets of FGF10, including main transcription factors. Immunofluorescence showed a down-regulation of the distal epithelial marker SOX9 and mis-expression distally of the proximal marker SOX2. Staining for the transcriptionally-active form of β -catenin showed a reduction in experimental vs.

control lungs. *In vitro* experiments using IQ1 phenocopied the impacts of blocking FGF10. This study demonstrates that FGF10/FGFR2b signaling on distal epithelial progenitor cells via β -catenin/EP300 controls, through a comprehensive set of developmental genes, cell adhesion, and differentiation.

Keywords: FGF10, FGFR2b, lung, branching morphogenesis, differentiation, β -catenin

INTRODUCTION

In mice, the first morphological evidence of lung development is seen at embryonic day (E) 9.5 with the budding of the ventral foregut endoderm, forming the tracheal primordium ventrally and the esophagus dorsally. Concomitantly, distal to the tracheal primordium, two primary lung buds form, initiating the early stages of pseudoglandular development (E9.5–E12.5) [for reviews on early lung development, see (Warburton et al., 2008, 2010; El Agha and Bellusci, 2014)]. During this early stage, the lung epithelium undergoes branching morphogenesis, a semi-stereotypical, and reiterative budding process whereby a tree-like structure, the scaffold of the future conducting airway network, is formed. At the tip of each bud reside multipotent epithelial progenitor cells, which are positive for the transcription factors SOX9 and ID2. These cells either self-renew, if they remain distally, or give rise to bronchial progenitors when they exit the tip domain, subsequently acquiring SOX2 expression (Rawlins, 2008).

Branching morphogenesis and epithelial differentiation depend on poorly understood cross-talk among a number of signaling pathways, involving fibroblast growth factors (FGF), sonic hedgehog (SHH), bone morphogenic proteins (BMP), and wingless/integrase 1 (WNT) ligands (El Agha and Bellusci, 2014). For example, fibroblast growth factor 10 (FGF10), signaling via its epithelial receptor FGFR2b, is sufficient to induce branching morphogenesis of isolated lung endoderm grown in Matrigel (Bellusci et al., 1997b). Additionally, demonstrating that FGF10 signaling is necessary to control branching morphogenesis, both *Fgf10*- and *Fgfr2b*-null embryos display lung agenesis (Sekine et al., 1999; De Moerloose et al., 2000), while *Fgf10* hypomorphic lungs display decreased ramifications (Ramasamy et al., 2007). Less is known about the regulation of distal tip multipotent epithelial stem cell maintenance and differentiation. Interestingly, *Fgf10* gain-of-function experiments prevent the differentiation of epithelial tip cells toward the bronchial progenitor lineage (Volckaert et al., 2013).

Almost twenty years after the discovery of FGF10 as a key growth factor regulating branching morphogenesis, the primary targets and biological activities controlled by FGF10 are still unclear (El Agha and Bellusci, 2014). Addressing this issue has been difficult, since loss of *Fgf10* leads to lung agenesis, therefore leaving little tissue to study; and while conditional deletions are possible, the lapse of time separating either constitutive or inducible Cre activity (in the case of a CreERT2 system) from complete gene inactivation is usually 24 to 48 h, it is difficult to distinguish between primary and secondary effects (Abler et al., 2009). Furthermore, genetic deletion of *Fgf10*

does not necessarily mean simultaneous loss of corresponding functional protein. The stability of the protein depends, for example, on the degradation rate of FGF10 present in the extracellular matrix and bound to heparin sulfate proteoglycans (Makarenkova et al., 2009; Patel et al., 2017), and it might take hours or days before a complete loss of function is achieved.

Genetically modified mouse strains, based on the reverse tetracycline transactivator (rtTA) system, do exist to conditionally inhibit FGF ligand activity at the protein level, and employ a dominant negative soluble form of FGFR2b. Soluble FGFR2b is a hybrid protein, where the extracellular part of the receptor, responsible for binding FGF ligands, is fused to the heavy chain of mouse immunoglobulin (Celli et al., 1998). This hybrid protein, once secreted, sequesters all FGFR2b ligands in the extracellular matrix.

Little research has explicitly used soluble FGFR2b to study the role of FGF signaling on early lung development, while the papers that do exist only touch on the question tangentially. For example, Hokuto et al. (2003) induced soluble FGFR2b at various pre- and postnatal stages to elicit the role played by FGF signaling in alveologenesis. While the authors reported a clear phenotype in early lungs (for example, branching defects) associated with FGF inhibition, detailed analyses of these defects were missing. Therefore, in this paper, we focused our study at E12.5, a stage where FGF10 is the only FGFR2b ligand significantly expressed. We performed both *in vitro* and *in vivo* experiments to analyze the impact of blocking FGF10 activity at the protein level. We used the developing lung as a model system to decipher the primary role of FGF10 in branching morphogenesis. Through the use of a previously validated dominant negative transgenic mouse model (*Rosa26rtTA; tet(o)sFgfr2b*; Parsa et al., 2008, 2010; Volckaert et al., 2011, 2017) we conditionally inhibited FGF10 signaling *in vivo* in E12.5 embryonic lungs via doxycycline IP injection to pregnant females, and *in vitro* by culturing lungs with doxycycline. The impact on branching morphogenesis 9 h after doxycycline administration was analyzed by morphometry, fluorescence, and electron microscopy. Gene arrays at 6 and 9 h following doxycycline administration were performed. The relationship between FGF10 and β -catenin signaling was also analyzed using IQ1, a pharmacological inhibitor of β -catenin/EP300 transcriptional activity.

This study demonstrates that FGF10/FGFR2b signaling on distal epithelial progenitor cells via β -catenin/EP300 controls, through a comprehensive set of developmental genes, cell adhesion, and differentiation. Altogether, our results clarify

the role of FGF10 on tip epithelial progenitor cells during development. Our transcriptomic approach has also provided a valuable dataset for future mechanistic studies aiming to characterize the role of newly found players in FGF10 signaling. Such knowledge will be instrumental to better understanding the role of FGF10 signaling at later stages of lung development, as well as during the repair process after injury.

RESULTS

Expression of *Fgf* Genes Encoding the Main FGFR2b Ligands During Early Lung Development and Validation of the Transgenic Approach to Inactivate FGFR2b Ligands

First, at different stages during embryonic lung development, we monitored by qPCR the expression of *Fgf* genes encoding ligands of FGFR2b, of which only *Fgf1*, 7, and 10 were detected ($n = 3$; **Figure 1A**). At E12.5, *Fgf10* was the predominantly expressed *Fgf* gene, while the expressions of *Fgf1* and 7 progressively increased during development, as previously described (Bellusci et al., 1997b). Next, we validated the double transgenic approach to block the activity of all FGFR2b ligands via the inducible expression of soluble FGFR2b using the *Rosa26^{rtTA/rtTA}; tet(o)sFgfr2b/+* transgenic mouse line (**Figure 1B**). The ubiquitous expression of soluble FGFR2b is achieved upon exposure to doxycycline (Dox), delivered via food, water, or intraperitoneal (IP) injection. Efficient inhibition of FGF10 activity occurs shortly after the soluble receptor is produced (usually within minutes after exposure to Dox; Danopoulos et al., 2013). **Figure S1A** shows the *in vivo* validation of this approach on lung development. Our results indicate severely impaired branching of the lung, with long, non-ramified epithelial tubes reminiscent of the primary bronchi. As expected, the earliest time points of treatment were the ones leading to the more severe phenotype. Dox-food exposure from E9.5 onwards led to complete lung agenesis (data not shown), similar to the genetic loss of *Fgf10* or *Fgfr2b* (Sekine et al., 1999; De Moerloose et al., 2000). **Figure S1B** shows the *in vitro* validation of our transgenic approach. While control lungs underwent significant branching over time (**Figure S1Ba,c,e,g**), experimental lungs failed to branch, but instead formed long tubular extensions (**Figure S1Bb,d,f,h**) similar to that observed *in vivo*.

Therefore, our results indicate that *Fgf10* is the predominantly expressed FGFR2b ligand at E12.5 and that we have a validated transgenic system allowing the inducible blockade at the protein level of FGFR2b ligands. Due to our choice of E12.5 to run our experiments, we therefore conclude that inhibiting FGFR2b ligands at this stage is functionally equivalent to inhibiting FGF10 activity. In addition, supporting our choice to focus this study on FGF10, *Fgf1* and *Fgf7* knock out mice are viable and do not display any respiratory defects (Guo et al., 1996; Miller et al., 2000).

Impact of FGF10 Inhibition on Branching Morphogenesis

We performed *in vitro* live imaging experiments for up to 24 h on control and experimental E12.5 lungs cultured with Dox ($n = 3$). This enabled us to characterize the changes occurring in the branching of the epithelium. We focused on the left lobe over this 24-h period (**Figure 1C**). While the number of distal buds in control lungs increased (from 12.0 ± 1.2 to 17 ± 1.5), no new buds were observed in experimental lungs (**Figure 1Da**). We also measured the total epithelial surface vs. the total surface of the left lobes (**Figure 1Db**). Our results indicate a phase of retraction of the epithelial surface in experimental lungs ($-12.0\% \pm 4.0$ at 3 h, $-15.0\% \pm 4.2$ at 6 h, $-16.6\% \pm 5.7$ at 9 h, and $-9.8\% \pm 5.7$ at 24 h). At the same time-points, the epithelial surface ratio increased in the control lungs ($+4.0\% \pm 0.7$ at 3 h, $+13.0\% \pm 3.3$ at 6 h, $+12.7\% \pm 0.9$ at 9 h, and $+24.0\% \pm 3.5$ at 24 h). Next, we measured the distance between the epithelium at the tip of the buds and the adjacent mesothelium (see white arrows **Figures 1C, Dc**). We observed a progressive increase in the space between the tip epithelium and the mesothelium in experimental lungs over time ($+18.0\% \pm 12.4$ at 3 h, $+24.0\% \pm 15.7$ at 6 h, $29.5\% \pm 16.0$ at 9 h, and $31.0\% \pm 17.0$ at 24 h), while this distance decreased in control lungs ($-8.7\% \pm 3.7$ at 3 h, $-10.5\% \pm 4.5$ at 6 h, $-16\% \pm 3.9$ at 9 h, and $-36.6\% \pm 1.2$ at 24 h). Finally, we quantified the lengths of the different epithelial domain branches over time (**Figure 1Dd–i**). These domain branches were named according to the previously described nomenclature (Metzger et al., 2008b). In both control and experimental lungs, L1, L2, L3, L4, D1, and D2 were clearly visible. Note that the experimental lung shown in **Figure 1Cf** was slightly delayed in terms of branching, compared to the lung shown in **Figure 1Ca**, as is often the case between lungs within a given litter. Consequently, in the control lung, L1 and L2 were already ramified, while only L1 was in the experimental lung. In both control and experimental lungs, L3, L4, D1, and D2 were not ramified. Our results indicate that the temporal increase in length of D1 and D2 was less in experimental lungs, compared to controls (**Figure 1Dh,i**). Furthermore, when comparing branches that were already ramified (such as L1 in control and experimental), there was no difference between experimental and control lungs during the time period considered (**Figure 1Dd**). However, caution should be exercised when comparing ramified branches with non-ramified branches (such as L2 in the control and experimental lungs shown), as there tended to be an increase in the rate of lengthening of the initially non-ramified branch, compared to the initially ramified branch. This could explain why the average L2 branch length in the experimental lungs showed a greater increase over time compared to the control branch (**Figure 1De**).

In conclusion, our detailed analysis reveals that subtle branching defects were already apparent 3 h after exposure to Dox. The major impact of inhibiting FGF10 activity was on the epithelium, where a complete arrest in budding, and a transient retraction of the epithelium (which correlated with an increase in the distance between the mesothelium and the distal tip epithelium), was observed.

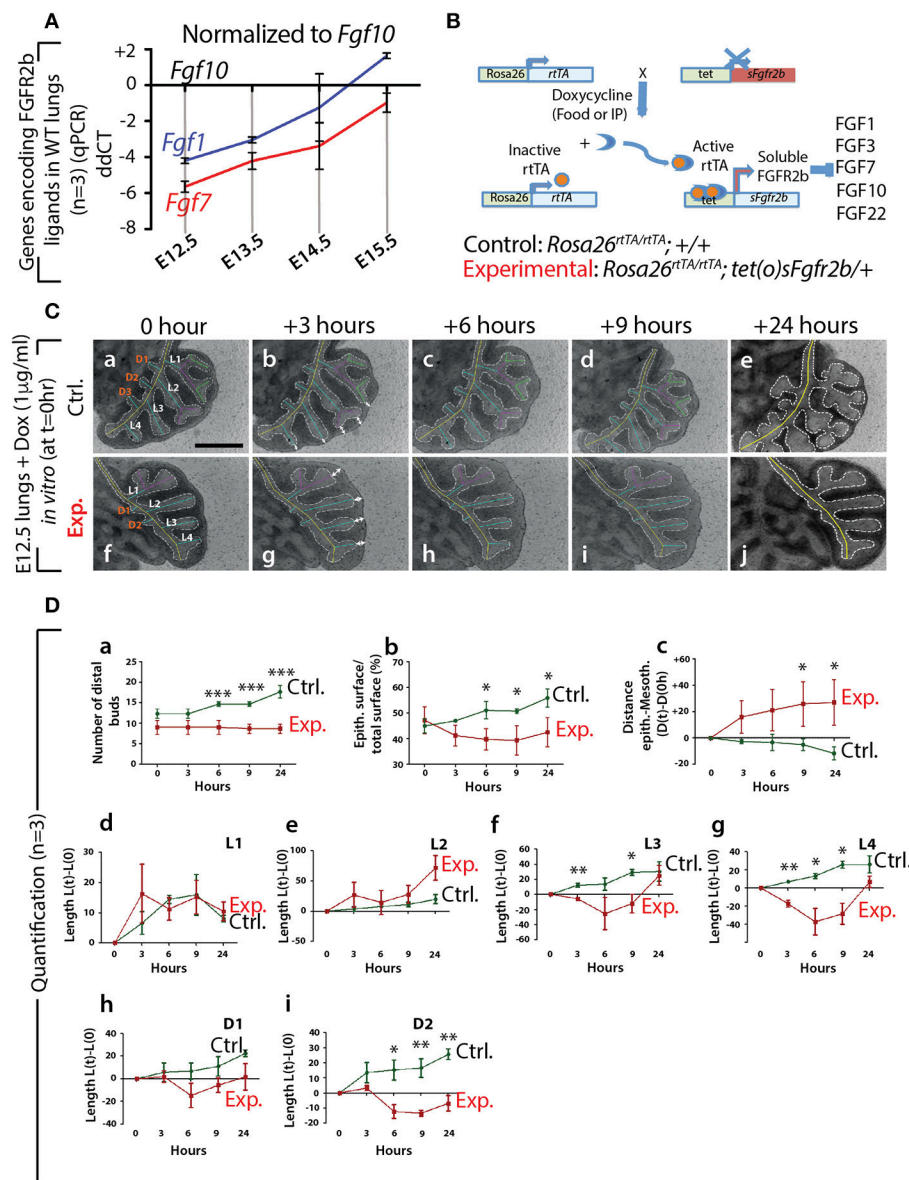


FIGURE 1 | Expression of genes encoding the main FGFR2b ligands during early lung development and impact of FGFR2b ligand inactivation on branching morphogenesis **(A)** qPCR for *Fgf1*, 7, and 10 in mouse embryonic lungs at E12.5, E13.5, E14.5, and E15.5. *Fgf10* is the main ligand expressed at E12.5 ($n = 3$). **(B)** *Rosa26^{rtTA/rtTA}; tet(o)sFgfr2b/+* double transgenic system inducing, upon doxycycline exposure (via food, water, or IP), the expression of a soluble form of FGFR2b acting as a dominant negative receptor. **(C)** Impact of FGFR2b ligand inactivation on the branching process. Branches (a and f) are labeled according to established domain branching nomenclature (L, lateral branch; D, dorsal branch). Arrows (b and g) indicate the distance between distal tip epithelium and the adjacent mesothelium. Scale bar: 400 μm. **(D)** Quantification of the branching defects. (Data are presented as mean ± SEM; significance determined by unpaired two-tailed Student's *t*-test; $n = 3$; * p -value < 0.05, ** p -value < 0.01, *** p -value < 0.001).

Inhibition of FGF10 Activity Leads to Reduction of Epithelial Bud Lumen Area Associated With Cell Rearrangements

Next, we analyzed the branching defects at the cellular level using 3D-reconstructions of serial confocal images of distal epithelial buds in control and experimental lungs. These lungs were isolated 9 h following a Dox-IP to pregnant females carrying E12.5 embryos, and were whole-mount stained with

CDH1 (E-cadherin) antibody. **Figure 2A** shows a longitudinal section, cross section, and 3D projection of control (a, c, e) and experimental (b, d, f) buds (see corresponding movies in **Supplementary Materials**). Quantification of the relative lumen area at different positions within the bud shows a clear reduction in this ratio in experimental buds, compared to controls ($n = 3$; **Figure 2B**). In addition, the average epithelial thickness was larger in experimental buds, compared to controls (**Figure 2B**).

This increased thickness was likely a consequence of epithelial cells piling atop one another, and failing to form an ordered monolayer, as seen in control buds. Altogether, inhibition of FGF10 activity led to the collapse of the lumen within the bud, and to increased epithelial thickness, which we think are the consequences of cell rearrangements within the epithelial layer. This conclusion is supported by the fact that extensive analysis of cell proliferation and cell death in the epithelium and mesenchyme, at this time point, did not indicate any difference between control and experimental lungs ($n = 3$; **Figure 3**).

We also analyzed the appearance of epithelial tip cells in control and experimental lungs by transmission electron microscopy (TEM; **Figure 2C**). Our results reveal numerous interesting impacts of blocking FGF10 signaling in experimental vs. control epithelial tip cells, including altered Golgi morphology, decreased microvilli size and number, and opened tight junctions (see **Figure S2**). In terms of impacts on cell rearrangement, in experimental lungs, the thickness of the basement membrane was consistently greater than that of control lungs (see dashed line in **Figure 2Cb,d**). This increase in basement membrane thickness was confirmed by immunofluorescence for laminin (LAMA1; **Figure 2De–h**). Second, epithelial cell-cell adhesion was affected in experimental lungs, compared to controls. This is evidenced by the many large gaps between adjacent epithelial cells in experimental lungs (see the asterisks in **Figure 2Cc,d**), whereas adjacent epithelial cells in control samples formed tight associations with few gaps. Impacts on adhesion are further demonstrated by looking at the adherens junctions, where a darker staining was observed in experimental lungs, compared to controls (**Figure 2Ce–h**). This darker staining suggests an accumulation of adherens junction associated protein, most likely CDH1. Indeed, immunofluorescent staining revealed drastically increased CDH1 expression in the distal epithelium of experimental lungs vs. controls (**Figure 2Da–d**).

Our results demonstrate that inhibition of FGF10 signaling leads to impaired distal bud morphology, including collapsed bud lumens and thicker epithelial layers. It is likely that this phenotype is primarily a result of epithelial disorganization caused by adhesion and rearrangement defects.

Identification of Early FGF10 Target Genes by Gene Array

Based on the previous results, we selected E12.5 as the ideal time point to determine the specific transcriptomic targets of FGF10 *in vivo* using our transgenic system. Since a single Dox-IP is sufficient to quickly induce soluble FGFR2b expression at E12.5 (Danopoulos et al., 2013), we administered single Dox-IPs to pregnant females and isolated embryos 6 or 9 h later (**Figure 4A**). Compared to control lungs (**Figure 4Ba,b**), the experimental lungs showed branching simplification and an increased distance between the distal epithelium and the mesothelium at 6 h (**Figure 4Bc,d**), a phenotype which was more pronounced at 9 h (**Figure 4Be,f**). RNA isolated from single experimental lungs at 6 and at 9 h was used for whole transcriptome analysis by gene array. As the difference in terms of size and branching between

control lungs at 6 and 9 h was minimal, we chose two controls at 6 h and one control at 9 h for the gene array. **Figure 4C** shows the corresponding volcano plots, demonstrating significant sets of genes being either up- or down-regulated between experimental and control lungs at these two time points. **Figure 4D** shows the heatmap of the top 100 regulated genes (selected according to p -value; $n = 3$) between experimental and control lungs at 6 h, as well as the corresponding expression levels for those genes at 9 h. **Figure 4F** shows the heatmap of the top 100 regulated genes (selected according to p -value; $n = 3$) between experimental and control lungs at 9 h, as well as the corresponding expression levels for those genes at 6 h. At both the 6 and 9 h reference points, four gene clusters can be identified based on similar expression patterns (**Figures 4E,G**). Of great interest are the genes contained in the Early 4 and Late 4 clusters. These genes showed early down-regulation upon attenuation of FGF10 signaling, and their expression continued to decrease over time. These genes, therefore, are likely primary targets of FGF10. The heatmaps for the other gene clusters, as well as their regions of expression in the embryonic lung using the genepaint database, are shown in supplementary data (**Figures S3–S10**).

Figure 4H displays the list of genes found in the Early 4 and Late 4 clusters (representing the “FGF10 gene signature”), as well as Late 3 and Late 1 (presented as a validation for the array). Interestingly, Early 4 (6 h) contains only 14 genes while Late 4 (9 h) contains 43 genes (10 of which overlap with the Early 4 genes), suggesting progressive transcriptional-level changes with time. As expected, Early 4 contains genes linked to multipotent epithelial cell differentiation, such as *Sftpc* and *Id2*. The transcription factor *Etv5*, an accepted bona fide target of FGF signaling, was also among the first genes to be down-regulated. The Early 4 cluster also contains genes linked to cell adhesion (e.g., *Tspan8* and *Lin7a*), signaling and transcriptional regulation (e.g., *Gprc5a* and *Sp5*), neuronal processes (e.g., *Gprn3*, *Enc1*, and *Sema4f*), apoptosis and cell cycle (e.g., *Bid* and *Rai14*), and transport (e.g., *Arrdc1* and *Slco2a1*).

The Late 4 cluster displays supplementary genes linked to epithelial differentiation, such as *Sftpa1*, *Sftpb*, and *Hopx*. *Etv5* was also found among these top-regulated genes. *Etv4*, a related transcription factor working redundantly with *Etv5* (Herriges et al., 2015), was also down-regulated, albeit at a lower level (data not show). Furthermore, the Late 4 cluster includes many additional genes regulating the biological processes described for the Early 4 cluster (e.g., cell adhesion genes *Lama3*, *Lamc2*, and *Ctnnd2*; signaling and transcriptional regulation genes *Kiss1*, *Akap5*, *Cytl1*, *Apcdd1*, and *Shh*; and apoptosis and cell cycle genes *Bex4*, *Dhx33*, *Klf16*, and *Aen*). **Figure 4J** shows KEGG pathway analyses for the 6 and 9 h time points, highlighting the cellular and signaling processes significantly regulated in experimental vs. control lungs. Significant changes in cytokine-cytokine receptor interactions, calcium signaling, WNT signaling, and Hedgehog signaling were observed at 6 h. In addition to these changes, at 9 h, we also observed significant alterations in the ECM-receptor interaction and cell adhesion molecules, reflecting the morphological alterations observed in **Figure 2**.

In order to gain more insight into the genes identified in each of the four groups, we made use of the online expression-profiling

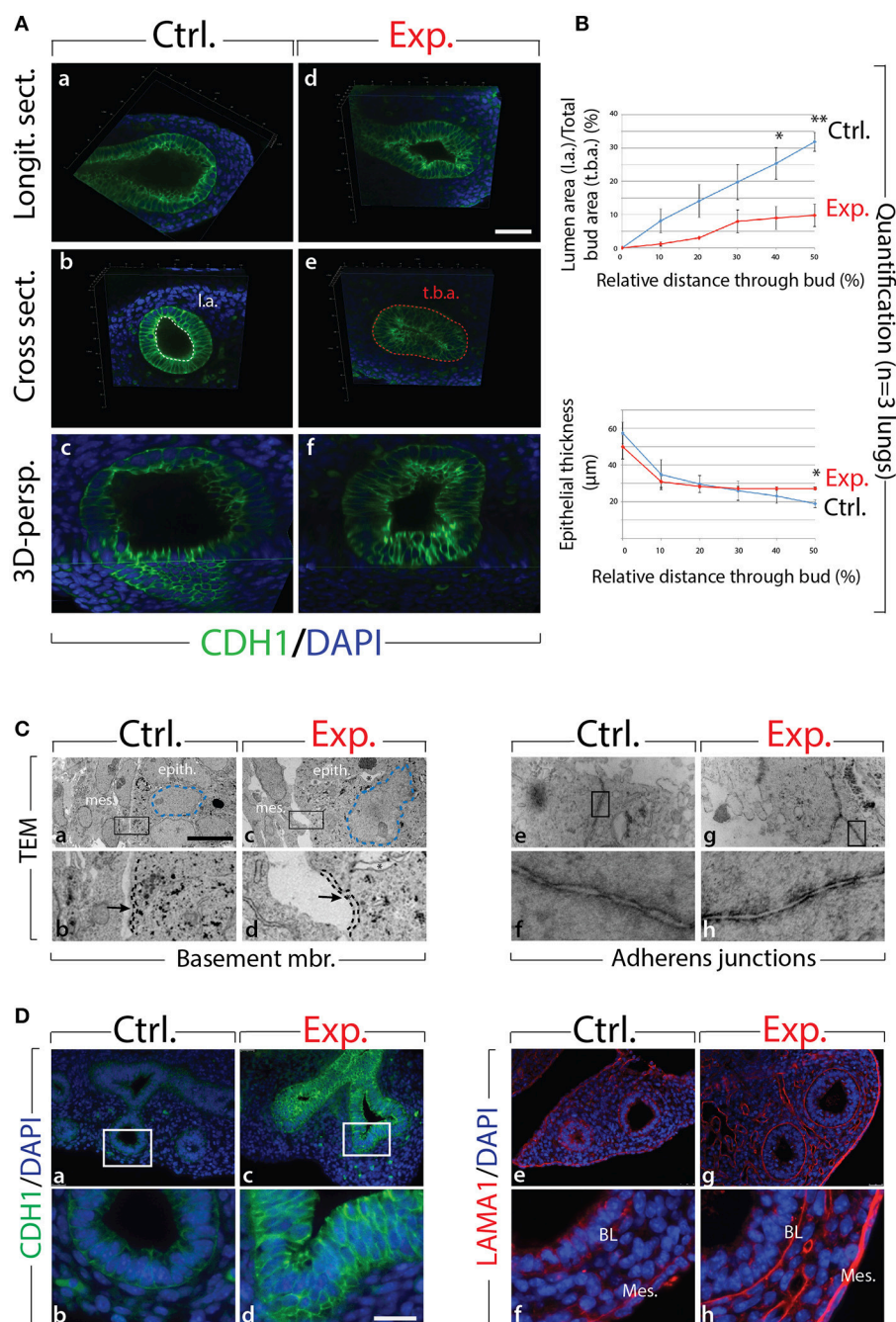


FIGURE 2 | Inhibition of FGF10 activity for 9 h in E12.5 lungs leads to collapse of the epithelial bud associated with cell rearrangements and altered cell-cell adhesion **(A)** whole-mount confocal images of distal lung buds. Control buds show open lumens and an ordered epithelial monolayer (a–c), while experimental lung buds show collapsed lumens associated with multi-layered epithelium (d–f). Please note that due to our fluorescence acquisition requirements, the intensity of the signal cannot be compared between control and experimental samples. l.a. = lumen area; t.b.a. = total bud area. Scale bar: (a,b,d,e) 40 μm; (c,f) 16 μm. **(B)** Quantification of relative lumen area (l.a./t.b.a.) and epithelial thickness. (Data are presented as mean ± SEM; significance determined by unpaired two-tailed Student's *t*-test; *n* = 3; **p*-value < 0.05, ***p*-value < 0.01). **(C)** TEM of distal lung buds highlighting the thickened basement membrane (black dashed line and arrows; a–d) and the darker staining around the adherens junctions (arrows; e–h) in experimental vs. control lungs. Note also the enlarged and irregularly shaped nuclei (blue dashed line; a,c) and the gaps between adjacent epithelial cells (asterisk; c,d) in experimental vs. control lungs. mes. = mesenchyme; epith. = epithelium. Magnification: (a,c) 7,750x; (e,g) 27800x; (b,d) 38,750x; (f,h) 139,000x. **(D)** Immunofluorescent staining for E-cadherin (CDH1) and laminin (LAMA1). E-cadherin shows increased expression in experimental lungs (c,d) compared to controls (a,b). Laminin deposition is increased in the basal lamina (BL) and mesothelium (Meso.) in experimental (g,h) vs. control lungs (e,f). Scale bar: (a,c) 75 μm; (b,d) 19 μm.

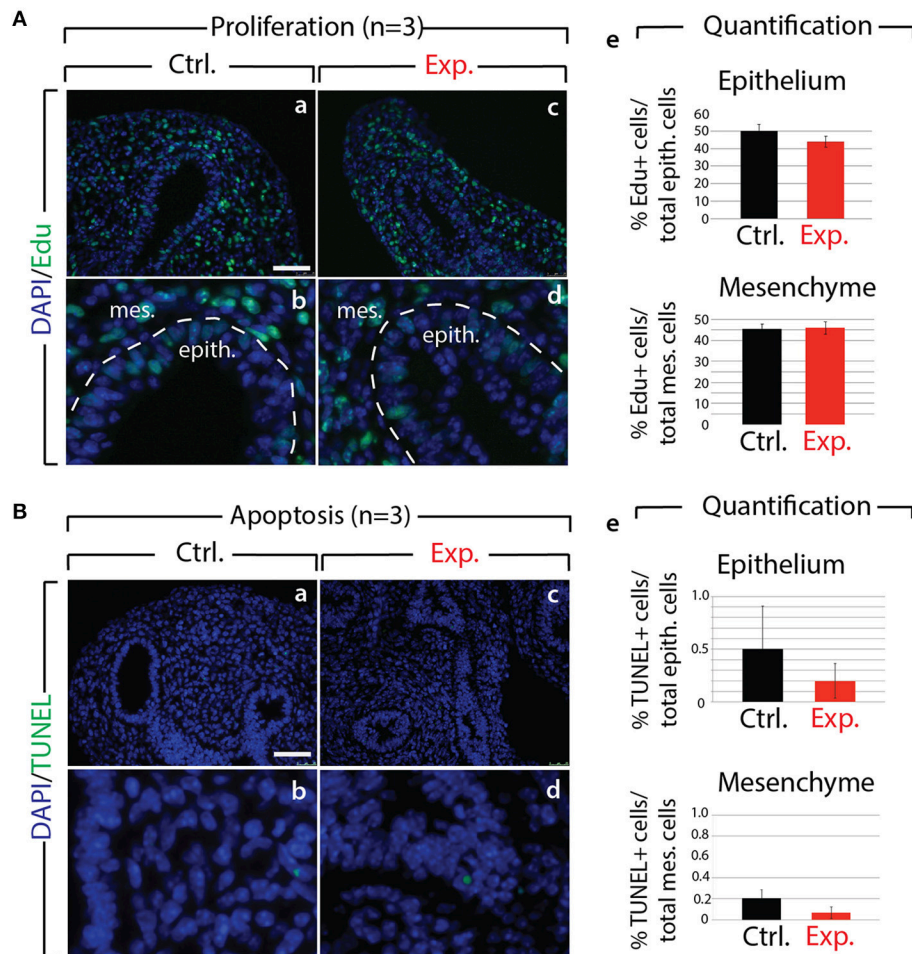


FIGURE 3 | Analysis of proliferation and cell death in control and experimental lungs **(A)** Proliferation analysis following Edu injection to pregnant females in control (a,b) and experimental (c,d) E12.5 lungs. Quantification of Edu signal indicates no major difference in the number of proliferating cells in control vs. experimental lungs (e). ($n = 3$) Scale bar: (a,c) 50 μm ; (b,d) 17 μm . **(B)** Apoptosis analysis by TUNEL showing no major difference (e) in the number of apoptotic cells in control (a,b) vs. experimental (c,d) lungs. ($n = 3$) Scale bar: (a,c) 50 μm ; (b,d) 17 μm .

database “Genepaint.org” to identify the expression domain of the genes found in the different groups (**Figures S3–S10**). In addition, we carried out a gene array between isolated distal tip epithelial and mesenchymal cells of E12.5 wild type lungs ($n = 3$). Our results allowed us to determine the genes differentially expressed between the two compartments (see **Figure S11A** for details). With this data, we reanalysed the genes in the Early 4, Late 4, Late 3, and Late 1 clusters according to their relative expression in the epithelium or mesenchyme. All the genes present in the Early 4 group were more highly expressed in the epithelium, supporting the idea that at this early time point, and for this group, our global transcriptomic approach was able to identify mainly epithelial specific changes.

We then compared the differential expression of the Early 4 and Late 4 genes (FGF10 signature genes), determined from the epithelium vs. mesenchyme gene array, with their expression after FGF10 inhibition (**Figures S11C,D**). Genes which are

expressed at high or medial levels in the epithelium, and which are highly or moderately regulated following inhibition of FGF10 activity, should be prioritized for further investigation. Promising candidates include *Lin7a*, *Sp5*, *Tspan8*, *Cytl1*, *Pthlh*, *Sftpa1*, *Sftpc*, and *Bspry* (**Figure S11G**).

A similar analysis was performed for the Late 3 and Late 1 groups (**Figures S11E,F**), which likely contain late acting, or secondary FGF10 targets. Particularly interesting from the Late 3 group is *Wnt7b*, as we have previously demonstrated that an FGF10/WNT7b loop regulates repair in conducting airways following naphthalene injury (Volckaert et al., 2011, 2017). Equally interesting in this group is the down-regulation of genes expressed preferentially in the mesenchyme and involved in Hedgehog signaling (*Foxl1*, *Foxf1*, *Gli1*, and *Ptch2*), which suggests our array captures one of the best-known epithelial-mesenchymal interactions: the FGF10-SHH interaction (reviewed in Warburton et al., 2008, 2010).

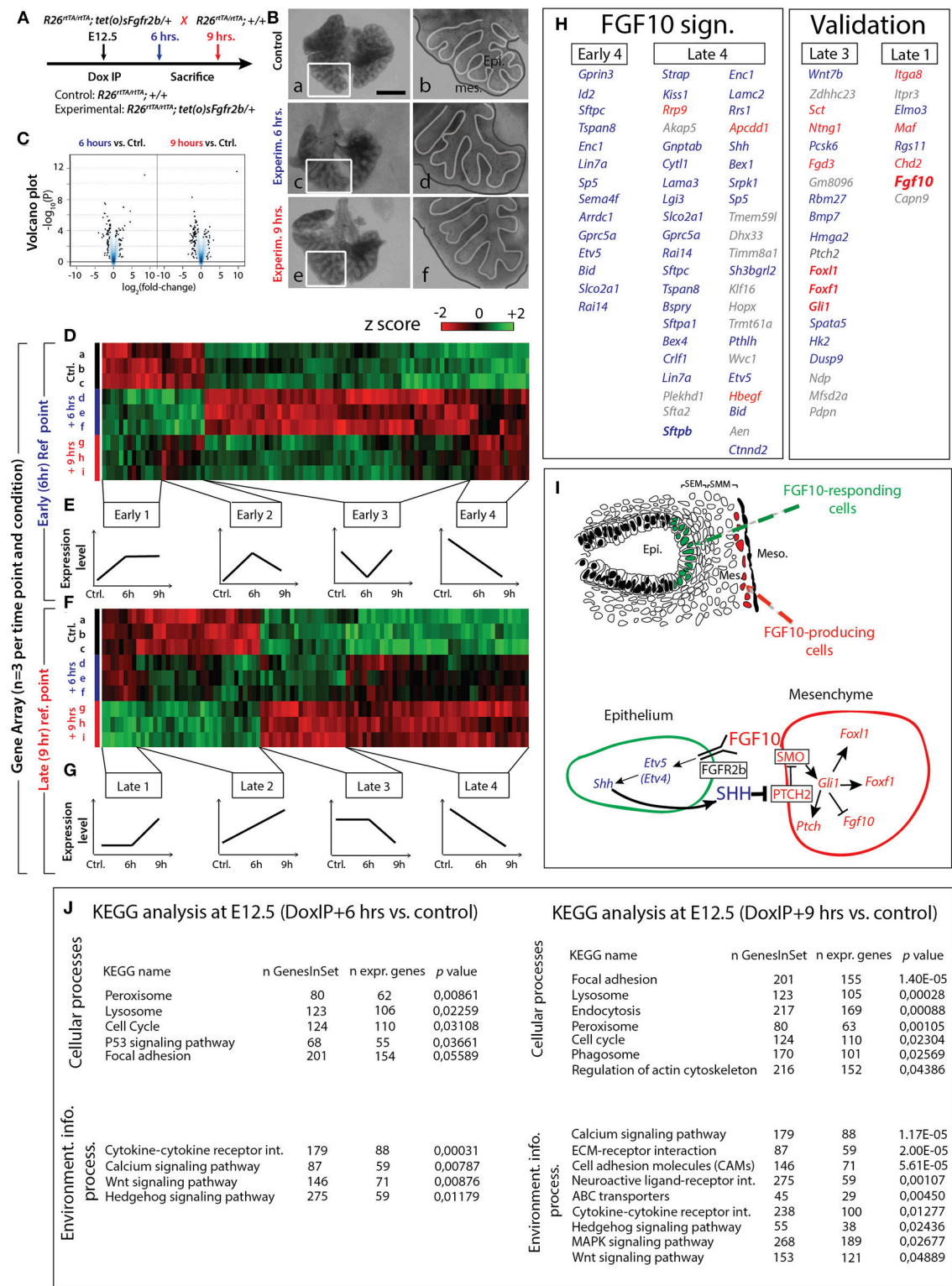


FIGURE 4 | Identification of early FGF10 target genes by a gene array approach **(A)** E12.5 littermate control and experimental lungs were collected 6 or 9 h after a single Dox-IP injection to pregnant females. **(B)** FGFR2b signaling attenuation leads to visible branching defects at Dox-IP+9 h (increased mesothelial-epithelial distance and branching inhibition). Meso.=Mesothelium; Epi.=Epithelium. Scale bar: (a,c,e) 500 μ m; (b,d,f) 175 μ m. **(C)** Volcano plots showing genes which are either down- or up-regulated at 6 or 9 h Dox-IP compared to the time-matched control lungs. **(D)** Heatmap of the top 100 genes differentially expressed between
(Continued)

FIGURE 4 | experimental and control lungs at 6 hrs (based on *p*-values). **(E)** Four classes of genes, grouped according to expression pattern (Early 1–4). **(F)** Heatmap of the top 100 genes differentially expressed between experimental and control lungs at 9 h (based on *p*-values). **(G)** Four classes of genes, grouped according to expression pattern (Late 1–4). **(H)** Genes identified in the Early 4 and Late 4 clusters are direct targets of FGF10 signaling and comprise the “FGF10 gene signature.” Many genes in the Late 3 and Late 1 clusters are involved in the FGF10/SHH regulatory feedback loop (see **I**), validating the gene array approach (note the presence of *Fgf10* in the Late 1 cluster). Genes enriched in the epithelium or mesenchyme are color coded in blue or red, respectively. **(I)** Reconstitution of the FGF10-FGFR2b-ETV4/5-SHH-FGF10 signaling axis from the gene array. **(J)** KEGG results for 6 and 9 h Dox-IP vs. control. **[(D,F)** *n* = 3; see *Materials and Methods* for details on the statistical analysis of gene arrays].

Shh is an epithelial gene encoding a secreted growth factor, and was down-regulated in our array concomitantly with *Etv4* and *Etv5*, beginning 6 h after FGF10 inhibition (Late 4). The combined decrease of *Etv4* and *Etv5* was likely causative for the loss of *Shh* (Herriges et al., 2015; see Discussion). Following the decrease in *Shh* expression, the mesenchymal-specific Hedgehog signaling genes *Foxl1*, *Foxf1*, *Gli1*, and *Ptch2* all showed a delayed down-regulation (only between 6 and 9 h). Furthermore, as SHH is known to regulate *Fgf10* transcription in the mesenchyme (Bellusci et al., 1997a; Lebeche et al., 1999), we also determined if *Fgf10* expression was affected by *Shh* down-regulation. We found that the Late 1 cluster, containing genes up-regulated between experimental and control at 9 h, but not at 6 h, contained *Fgf10*. Taken together, this evidence functionally validates our gene array, and leads to the model proposed in **Figure 4I**.

Identification of Lung-Specific Transcription Factors Controlled by FGF10

Next, we evaluated the expression of the major transcription factors expressed in the lung at E12.5. A previous report indicated that out of 1100 transcription factors analyzed (covering 90% of all transcription factors encoded in the mouse genome), only 62 exhibited localized expression in the epithelium and/or mesenchyme of the developing lung (Herriges et al., 2012). Of these 62 genes, from our gene array, 17 were either induced or repressed with significance in experimental vs. control lungs at 6 or 9 h (*n* = 3; **Figure 5A**). From these 17, 11 were exclusively or predominantly expressed in the epithelium (**Figure 5B** shows the expression of some of these genes in the developing lung by *in situ* hybridization). Among the repressed genes in the epithelium, we found early regulation of genes such as *Etv4* and *Etv5* (belonging to the TF1 cluster), delayed regulation of *Sox9* (belonging to the TF2 cluster), as well as transiently regulated genes such as *Grhl2*, *Nkx2-1*, and *Id2* (from the TF3 cluster). These “repressed” genes are likely direct targets of FGF10, required to elicit the different facets of FGF10 activity. Among the induced genes in the epithelium, we found the early regulation of *Sox2* (forming the TF4 cluster), the transiently regulated genes *Nkx1-2* and *Pitx2* (from the TF5 cluster), and the late regulation of *Lmo1* and *Elf5* (from cluster TF6). These “induced” genes are likely repressed by FGF10 during early lung development.

FGF10/FGFR2b Signaling Is Required to Maintain the Differentiation Status of the Epithelial Multipotent Progenitors

Next, we examined the impact of attenuated FGFR2b signaling on the differentiation of the multipotent epithelial progenitor

cells. **Figure 5C** confirms the reduced expression of SOX9 distally in experimental (d–f) vs. control (a–c) lungs at 9 h post Dox-IP. In control lungs, SOX2 expression in the proximal epithelium established a clear boundary between proximal and distal regions (**Figure 5Cg–i**). However, in experimental lungs, SOX2 expression in the proximal epithelium expanded more distally, showing a salt and pepper expression pattern with increased expression in the mesenchyme around the conducting airway (**Figure 5Cj–l**).

Until recently, the close examination of epithelial tip cell differentiation was limited, as only a few signature genes denoting differentiation status were known to be expressed in those cells. However, this limitation has been overcome after a paradigm-shifting paper published by Treutlein et al. (2014), which used single cell transcriptomic approaches to characterize the genetic signatures of lung distal epithelial cells at E14.5, at E16.5, and at E18.5. The authors proposed a list of specific markers for alveolar epithelial cell type I (AT1) and type II (AT2) cells, and showed that a common progenitor cell, called a bipotent progenitor, expressed markers of both cell types. Our gene array data shown in **Figure 4** indicate that some of these markers of differentiation (*Sftpc*, *Etv5*, *Sftpa1*, *Sftpb*, *Hopx*, *Pdpn*) are actually regulated by FGF10 in multipotent epithelial progenitors. In order to probe more extensively the status of these differentiation markers in the epithelial tip cells, we assessed the AT1 and AT2 signatures from our gene array (experimental vs. control) at the 9 h time point. **Figure 5D** shows a clear global reduction in the markers characteristic of the AT2 signature, with minimal change in the AT1 signature. Gene set analysis confirms a very significant difference in the AT2 signature in experimental vs. control lungs (*n* = 3; *p* = 0.000004). No significant change was observed in the AT1 signature. We therefore conclude that upon inhibition of FGF10, the tip epithelial cells, which include the progenitors for the bipotent cells, lose expression of the markers characteristic of the AT2 signature, and that globally the expression of these genes is under the control of FGF10.

Connecting FGF10 and β -Catenin Signaling

Our KEGG analysis indicated that WNT signaling was also significantly regulated by FGF10 inhibition (**Figure 4J**), in particular, those genes involved in the canonical WNT pathway (*n* = 3; **Figure 6B**). Of note, *Wnt7b*, which codes for a growth factor secreted by the epithelium and acting on the mesenchyme, appears highly regulated by FGF10 (**Figure 6B**; see also **Figure S11E**). Downstream targets of WNT7b, such as the transcription factors *Lef1* and *Tcf7*, were also transcriptionally down-regulated. To confirm the down-regulation of WNT signaling in experimental lungs at the protein level, we examined

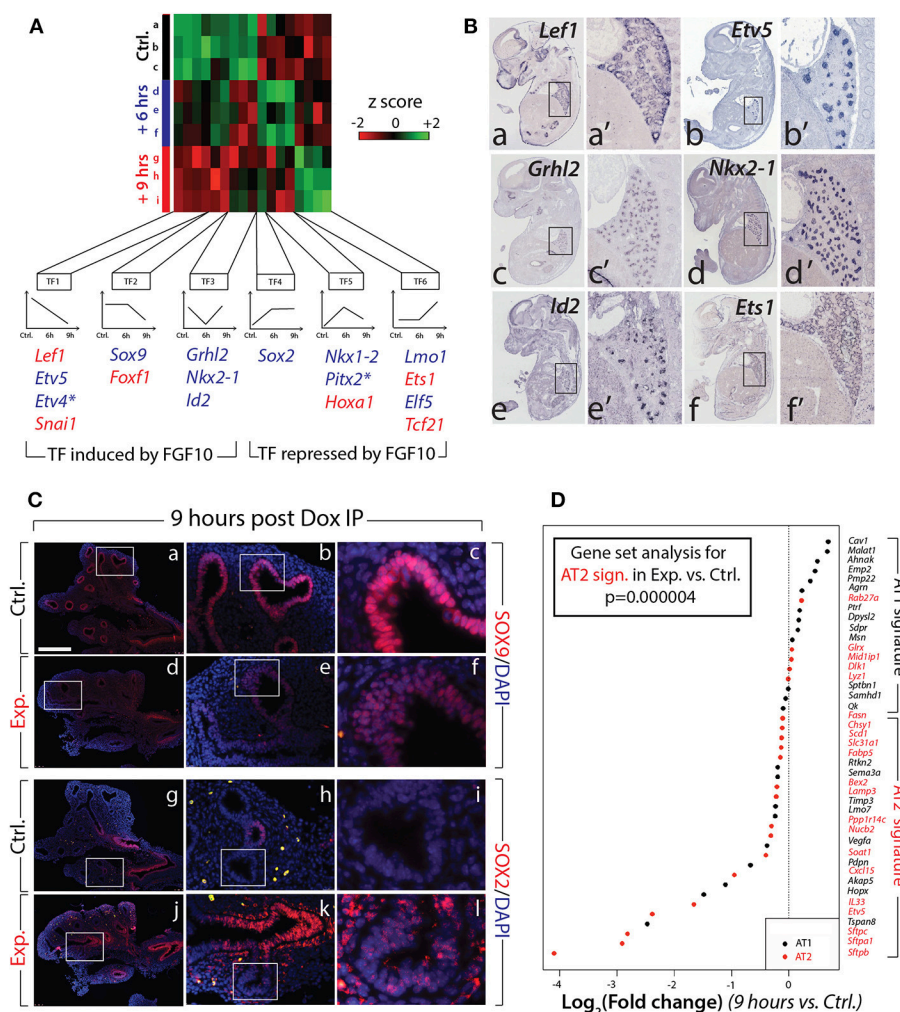


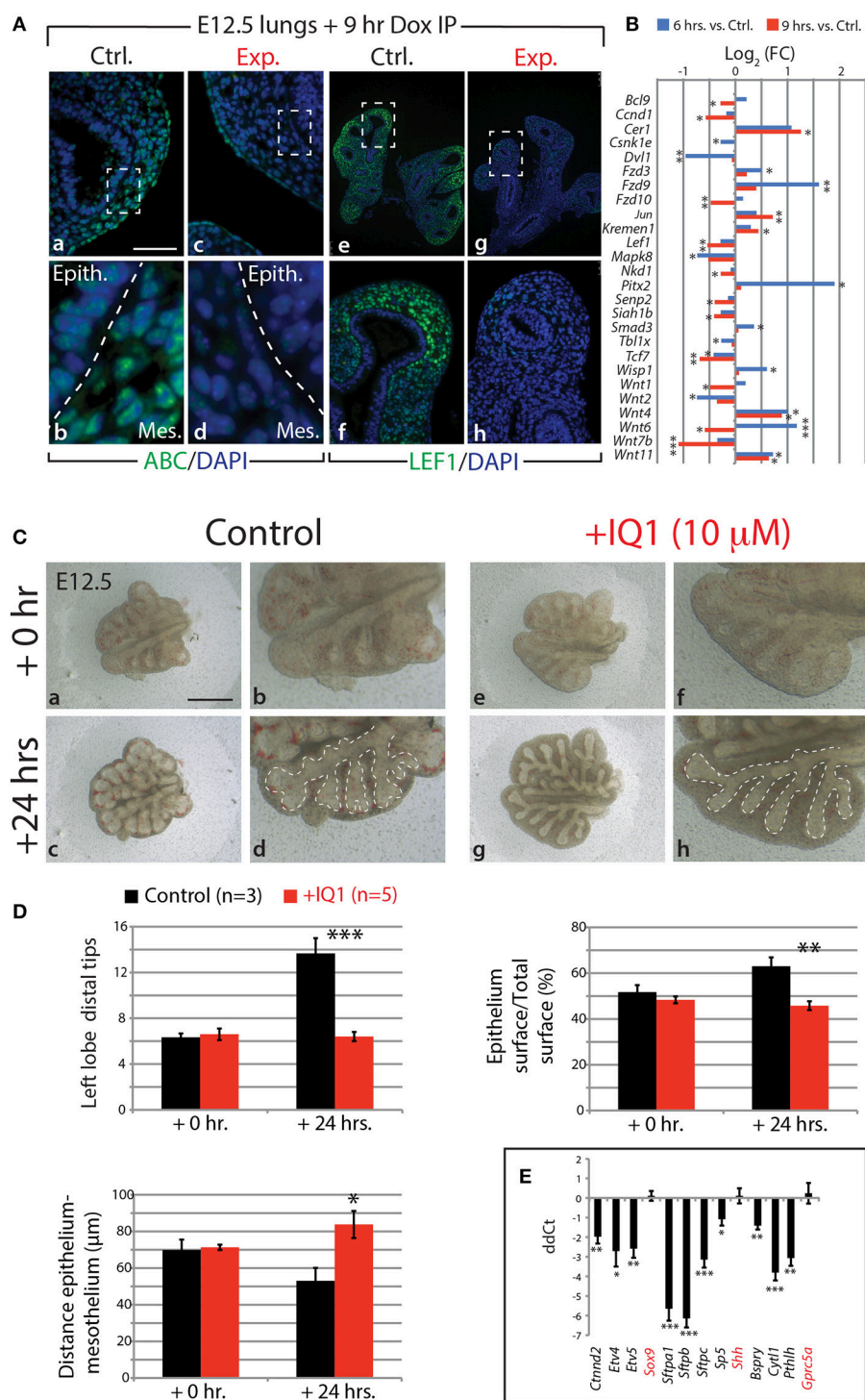
FIGURE 5 | Identification of the transcription factors regulated by FGF10 and impacts on epithelial differentiation **(A)** Heatmap of highly regulated transcription factors between control, Dox-IP+6h and Dox-IP+9h conditions. Six classes of transcription factors (TF1-6) were identified based on their expression profiles. Genes in blue and in red are preferentially expressed in the epithelium and mesenchyme, respectively (asterisks denote genes found in both compartments). **(B)** *In situ* hybridization on E14.5 lungs for *Lef1* (a,a'), *Etv5* (b,b'), *Grhl2* (c,c'), *Nkx2-1* (d,d'), *Id2* (e,e'), and *Ets1* (f,f'). **(C)** Validation by IF of changes in SOX9 (a-f) and SOX2 (g-l) expression in experimental vs. control lungs. Scale bar: (a,d,g,j) 300 μ m; (b,e,h,k) 100 μ m; (c,f,i,l) 33 μ m. **(D)** Expression of AT1 and AT2 markers in 9 h vs. control lungs. Note the significant global reduction in the expression of the AT2 markers. ($n = 3$; see *Materials and Methods* for details on the statistical analysis of gene sets).

by immunofluorescence the levels of the transcriptionally active form of CTNNB1 (β -catenin) and the transcription factor LEF1 (**Figure 6A**). A loss of expression of activated β -catenin was observed in both the epithelium and mesenchyme of experimental lungs (**Figure 6Aa–d**). In addition, LEF1 was also lost in the mesenchyme, correlating with the decrease in *Wnt7b* expression (**Figure 6Ae–h**).

Given that WNT/ β -catenin signaling is vital for proper branching morphogenesis and cellular differentiation (for a review see De Langhe and Reynolds, 2008) and that we observed a drastic reduction in activated β -catenin in our experimental lungs, we investigated the degree to which the effect of inhibiting FGF10 was mediated through WNT/ β -catenin signaling. We made use of a pharmacological inhibitor

of β -catenin transcriptional activity, called IQ1. This inhibitor decreases the interaction between β -catenin and one of its transcriptional coactivators, EP300, and has been shown to disrupt branching morphogenesis, and proximalize the lung epithelium, during the pseudoglandular stage of lung development (Sasaki and Kahn, 2014).

Figures 6C–E shows the results of inhibiting, *in vitro*, the β -catenin/EP300 interaction for 24 h in E12.5 lung explants. While control lung explants displayed continued branching over this time (**Figure 6Ca–d**), the experimental lungs failed to branch, forming instead long epithelial tubes (**Figure 6Ce–h**). The failure of experimental lungs to branch, as well as the relative area of the epithelial surface and the distance between the distal epithelium and mesothelium, is quantified in **Figure 6D** ($n = 3$ for control



and $n = 5$ for experimental). These phenotypic impacts are very similar to those seen by blocking FGF10 *in vitro* (see for comparison **Figure 1Dk–m**).

We also compared, by qPCR, the expression levels of a number of genes found in the “FGF10 gene signature” between experimental and control lung explants ($n = 3$ for control and $n = 5$ for experimental; **Figure 6E**). Ten of the 13 genes assessed showed drastic reductions in expression in IQ1 treated lung explants, similar to the down-regulation seen in our *in vivo* inhibition of FGF10. Interestingly, the transcription factor *Sox9*, as well as *Shh*, showed no change in expression between control and experimental lungs at this time point, indicating, perhaps, compensatory mechanisms.

In the attempt to produce results more comparable to the 9 h *in vivo* inhibition of FGF10, we also conducted a 9 h inhibition of the β -catenin/EP300 interaction (**Figure 7**). While the branching defects, after this time point, were less pronounced than those seen in either the 24 h IQ1 treatment, or the 9 h *in vivo* FGF10 inhibition (**Figure 7Ae–h**), noticeable effects on the relative epithelial surface and the distance between distal epithelium and mesothelium could be observed ($n = 6$; **Figure 7B**). We also conducted a gene array on control vs. IQ1 treated lung explants after 9 h of inhibition ($n = 2$ for control and $n = 3$ for experimental). A gene set analysis using the “FGF10 gene signature” revealed that this set of genes was significantly down-regulated in IQ1 treated lung explants ($p < 0.001$; **Figure 7C**). Finally, immunofluorescent stains for SOX9 and SOX2 protein revealed a decrease in SOX9 expression (**Figure 7Dd–f**), and a concomitant advancement of SOX2 expression distally (**Figure 7Dj–l**), reminiscent of the proximalization of lung epithelium after 9 h of FGF10 inhibition (see **Figure 5C**).

In summary, our data suggest that a large proportion of the regulation by FGF10 signaling of epithelial branching morphogenesis and differentiation is mediated specifically through β -catenin/EP300 transcriptional activity.

Discussion

In this paper, we report the impacts on the epithelial tip cells of E12.5 lungs by blocking FGFR2b ligands, primarily FGF10. Both *in vivo* and *in vitro* experiments inhibiting FGF10 resulted in arrested epithelial branching and collapsed distal bud lumens associated with abnormal cellular adhesion. Gene arrays at 6 and 9 h inhibition revealed the transcriptomic regulation of FGFR2b signaling. From these arrays, we identified an FGF10 gene signature primarily composed of genes enriched in the epithelium and positively regulated by FGF10. We also highlighted a set of lung-specific transcription factors significantly regulated by FGF10. Our data on SOX9 and SOX2 expression, as well as gene-set analyses on differentiation markers of AT2 cells, which are found to be expressed in multipotent epithelial progenitor cells, demonstrated the proximalization of the tip epithelium and a loss of distal differentiation markers 9 h after FGF10 inhibition. Finally, the effects of blocking FGF10 signaling on β -catenin activity were assessed. *In vitro* experiments using IQ1, a pharmacological

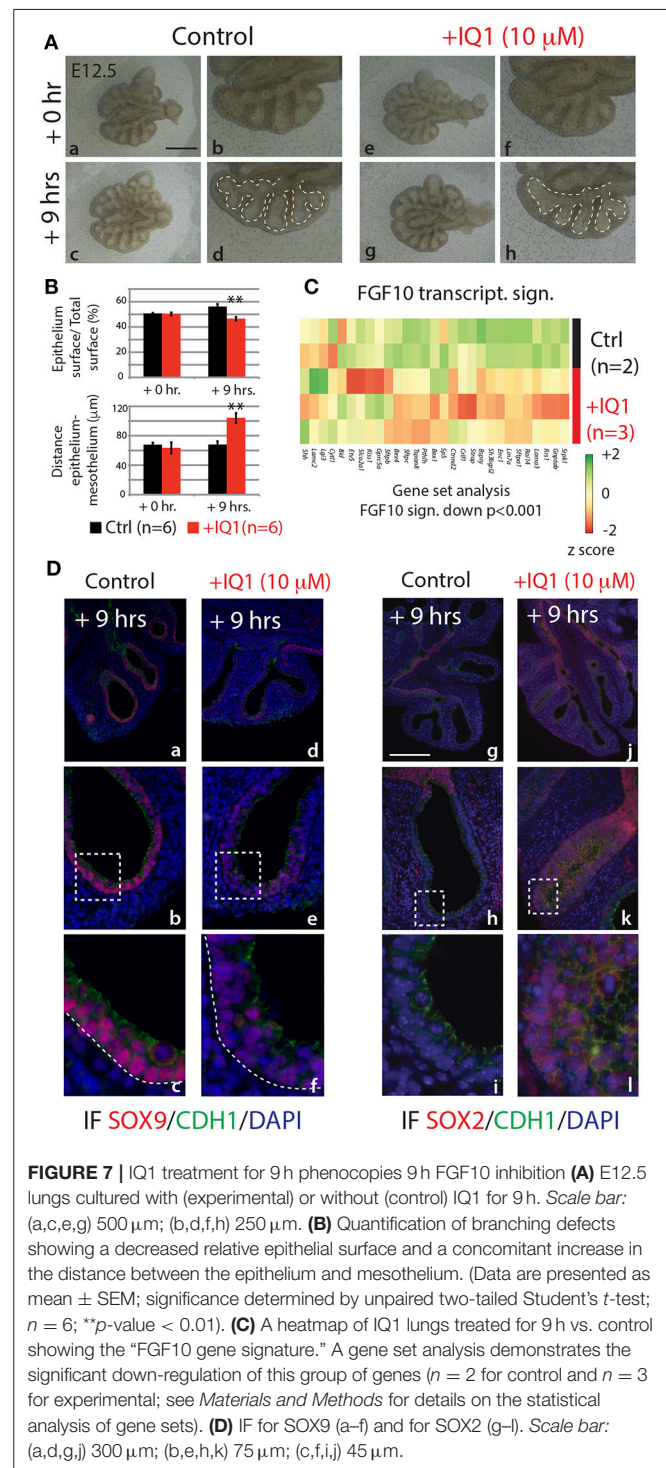


FIGURE 7 | IQ1 treatment for 9 h phenocopies 9 h FGF10 inhibition **(A)** E12.5 lungs cultured with (experimental) or without (control) IQ1 for 9 h. Scale bar: (a,c,e,g) 500 μm; (b,d,f,h) 250 μm. **(B)** Quantification of branching defects showing a decreased relative epithelial surface and a concomitant increase in the distance between the epithelium and mesothelium. (Data are presented as mean $\pm$ SEM; significance determined by unpaired two-tailed Student's *t*-test; $n = 6$; $**p$ -value < 0.01 .) **(C)** A heatmap of IQ1 lungs treated for 9 h vs. control showing the “FGF10 gene signature.” A gene set analysis demonstrates the significant down-regulation of this group of genes ($n = 2$ for control and $n = 3$ for experimental; see *Materials and Methods* for details on the statistical analysis of gene sets). **(D)** IF for SOX9 (a–f) and for SOX2 (g–l). Scale bar: (a,d,g,i) 300 μm; (b,e,h,k) 75 μm; (c,f,i,j) 45 μm.

inhibitor of the β -catenin/EP300 interaction, were able to phenocopy a large proportion of the impacts found by FGF10 inhibition, suggesting that FGF10 signaling on E12.5 distal tip progenitors is significantly mediated via β -catenin/EP300. These findings are summarized in a model of FGF10 signaling during pseudoglandular lung development provided in **Figure 8**.

Validation, and Limitations, of *in vivo* Inhibition of FGF10 Signaling

One of the major limitations of our *in vivo* model to inhibit FGF10 signaling during pseudoglandular lung development (E12.5) is that the production of soluble FGFR2b is global, potentially inducing secondary effects. Furthermore, soluble FGFR2b is secreted into the mesenchyme, potentially inhibiting mesenchyme-specific FGF signaling. Indeed, we have recently reported that FGF10 can directly act on the rat lung mesenchyme during the late canalicular/early saccular stage (E19) to control the differentiation of lipofibroblast progenitors (Al Alam et al., 2015). Although we could not detect any significant increase in P-ERK on primary culture of lung mesenchyme isolated at the mid-pseudoglandular stage (E14.5; De Langhe et al., 2006), we cannot exclude that FGF10 is active on discrete mesenchymal subpopulations throughout the pseudoglandular stage.

To control for the potential secondary effects of our model, we validated the location of FGF10's primary targets by a gene array comparing expression of genes in the epithelium vs. mesenchyme of E12.5 wild type lungs (Figure S11), and also by the online expression-profiling database "genepaint.org" (Figures S3–S10). We are confident that our global *in vivo* approach does indeed detect the impacts of FGF10 signaling on epithelial-specific targets.

Furthermore, we assessed the well-established FGF10-SHH regulatory feedback loop during lung development as a means of validating our array (Figures 4H,I). Our array detects the down-regulation of *Shh* within 6 h of FGF10 inhibition, and also the delayed impacts on the downstream targets of SHH at 9 h, including the up-regulation of *Fgf10*. Additionally, our array supports the recently reported data showing that the inactivation of FGF-regulated *Etv4* and *Etv5* in the multipotent epithelial progenitor cells during lung development leads to the loss of *Shh* expression (Herriges et al., 2015). We therefore propose that FGF10 acts via FGFR2b, positively regulating the expression of ETV4/ETV5, and, subsequently, the SHH pathway.

FGF10 Primary Targets

From our gene array data, we identified an "FGF10 gene signature." These genes, primarily enriched in the epithelium, show decreased expression shortly after FGF10 inhibition, and continue to decrease during inhibition; therefore, these genes likely represent primary targets of FGF10, and are potential key mediators of FGF10/FGFR2b signaling.

We also found that FGF10 regulates many previously identified lung specific transcription factors (Herriges et al., 2012). Some of these transcription factors are established mediators of FGF10 signaling (e.g., *Etv4*, *Etv5*, *Sox9*), whereas little is known of the others in the context of FGF10 signaling. Knock-out and over-expression studies on many of these transcription factors show impacts very similar to the effects seen in our study. For example, Metzger et al. (2007, 2008a) found that *Elf5* (group TF6, Figure 5) is regulated by FGF10 and FGF7, and that over-expression of *Elf5* leads to branching defects and delayed AT2 differentiation; Quaggin et al. (1999) reported that *Tcf21* (group TF6) knock-out mice display reduced branching,

smaller lungs, and a proximalization of lung epithelium at E14.5; finally, Varma et al. (2012) studied the transcription factor *Grhl2* (group TF 3, Figure 5) during lung development, and found that GRHL2 controls cell adhesion and migration, forms a positive feedback loop with NKX2-1 during branching morphogenesis, and is associated with proper AT2 differentiation.

We propose that the comprehensive set of target genes and transcription factors identified in our study is a valuable resource for future investigations on early lung branching morphogenesis and differentiation.

FGF10's Regulation of Tip Cell Differentiation and Morphology via SOX9

Sustained SOX9 expression in the tip epithelium of the developing lung has been associated with epithelial stem cell self-renewal. The current model predicts that individual tip cells, under the influence of FGF10, are prone to remain in the tip domain; as these cells divide, some of the daughter cells acquire bronchial progenitor characteristics associated with the exit from the tip domain.

The transcription factor SOX9 has been extensively studied during early lung development (Perl et al., 2005; Chang et al., 2013; Rockich et al., 2013). For example, Chang et al. (2013) found that knocking out *Sox9* before E12 leads to branching defects, an increase between the distal epithelium and mesothelium, and smaller lungs. Furthermore, it was found that FGFR2b signaling regulates *Sox9*, and that SOX9 suppresses the initiation of alveolar differentiation. Rockich et al. (2013) found similar findings, and also assessed the impacts on cell adhesion in *Sox9* loss-of-function E14.5 lungs. Using TEM, the authors found multiple cellular defects similar to what we found in our *in vivo* dominant negative model, including: irregularly shaped epithelial cells, gaps with protruding pseudopodia between adjacent cells, partial to complete loss of microvilli, and a disjointed basement membrane filled with gaps.

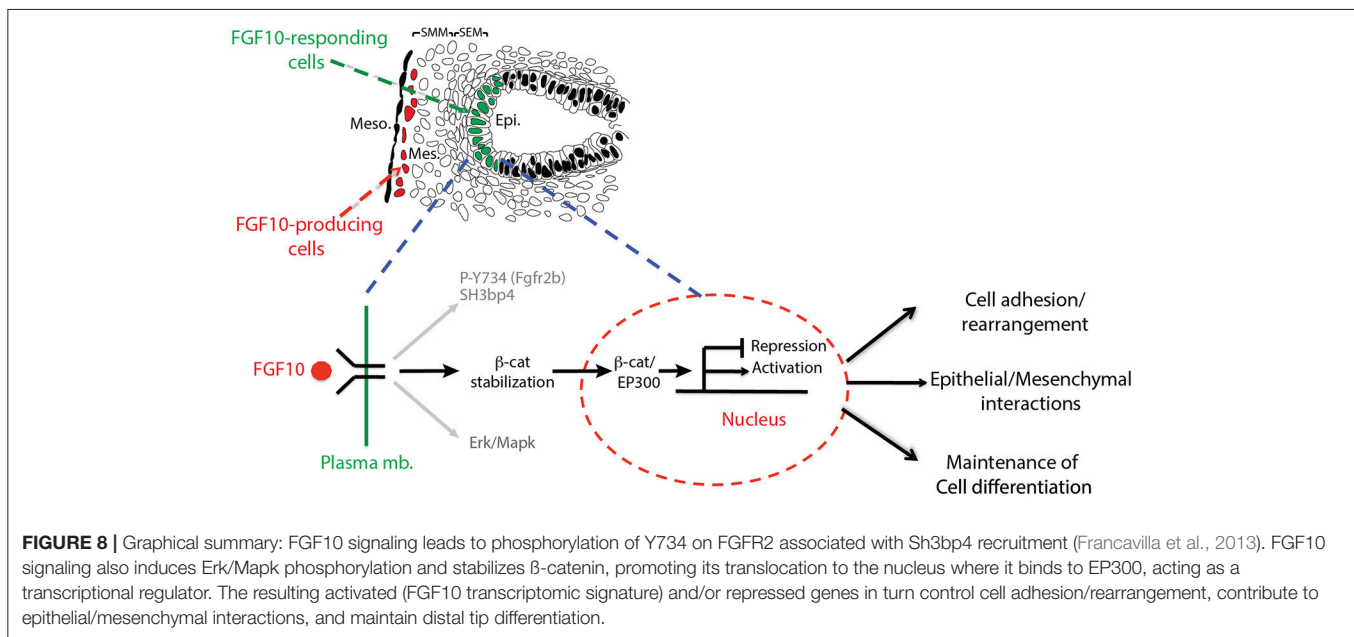
As SOX9 is lost in the distal epithelial cells of experimental lungs, the expression of SOX2 in these cells increases, further suggesting these cells are losing their multipotency, and are adopting a proximal fate. This idea is supported by the evidence, at this stage, of a loss of the AT2 signature in the multipotent progenitors upon FGF10 inhibition.

Taken together, our data suggest that the loss of SOX9, downstream of FGF10 signaling, affects the morphogenesis and multipotent potential of distal epithelial cells.

FGF10 Regulation of β -Catenin Levels

The importance of β -catenin signaling during pseudoglandular branching morphogenesis has been extensively studied (reviewed in De Langhe and Reynolds, 2008). In our dominant negative *in vivo* model, transcriptionally active β -catenin was greatly reduced in experimental lungs after 9 h inhibition (Figure 6). Since *Ctnnb1* gene expression was unaltered in our gene array, the drastic decrease in activated β -catenin was likely a result of post-translational modifications, such as altered phosphorylation and/or reduced cytoplasmic pools of available β -catenin.

An intriguing possibility concerning available cytoplasmic β -catenin is the relationship between β -catenin and CTNND2,



which regulate E-cadherin stability at the adherens junction complex of epithelial cells. While β -catenin stabilizes E-cadherin to promote cell adhesion, CTNND2 leads to E-cadherin destabilization, the release of β -catenin to the cytoplasm, and cell motility (Lu et al., 1999; Kim et al., 2012). Indeed, high levels of stable E-cadherin have been shown to inhibit budding and branching morphogenesis in isolated embryonic lung epithelium (Liu et al., 2008). Once β -catenin is released from E-cadherin, it contributes to the pool of transcriptionally active β -catenin (Kam and Quaranta, 2009). With reduced CTNND2, levels of E-cadherin remain relatively high, and β -catenin becomes sequestered at the adherens junction, being removed from the transcriptionally active pool. Thus, E-cadherin destabilization by CTNND2 regulates proper branching morphogenesis and the cytoplasmic availability of β -catenin. Interestingly, loss of CTNND2 in humans is associated with the “Cri du chat” syndrome (Medina et al., 2000), wherein affected babies may develop breathing problems at birth and require respiratory treatments. Additionally, a high rate of lung infection is also common during the first years of life. In the future, it will be important to delineate the impact of loss of CTNND2 on the formation and maturation of the lung epithelium in the context of homeostasis, as well as repair after injury.

Our data support the importance of the proposed CTNND2/CDH1/CTNNB1 regulatory axis. Contained in the FGF10 gene signature is the drastic reduction of *Ctnnd2* (group Late 4, **Figure 4H**), while E-cadherin is greatly increased in experimental lungs (**Figure 2D**), which could explain to an appreciable degree not only the morphological defects, but also the lack of activated β -catenin seen in these samples.

FGF10 Regulation via β -Catenin/EP300

In the nucleus, β -catenin associates with a number of transcriptional co-activators to regulate gene expression via the

TCF/LEF family of DNA-bound transcription factors (reviewed in Hoppler and Kavanagh, 2007). Two of these co-activators, CBP and EP300, are critical for balancing cellular differentiation and branching morphogenesis (Miyabayashi et al., 2007; Sasaki and Kahn, 2014). Through the use of a pharmacological inhibitor of the β -catenin/EP300 interaction, IQ1, Sasaki and Kahn (2014) report numerous impacts on pseudoglandular stage lungs, including arrested branching and an increase between the distal epithelium and adjacent mesothelium, similar to what we found in our *in vivo* inhibition of FGF10 (compare **Figures 1C, 6C**). Furthermore, Sasaki and Kahn (2014) show that inhibiting β -catenin/EP300 leads to the proximalization of lung epithelium via a reduction in distal marker expression (*Bmp4* and *Fgf10*), and an increase in the proximal markers *Sox2* and *Scgb1a1*. In our 24h IQ1 inhibition experiment, we likewise found a number of distal marker genes down regulated, including *Etv4*, *Etv5*, *Sftpa1*, *Sftpb*, and *Sftpc* (see **Figure 6E**). Interestingly, *Sox9* showed no change in expression between control and experimental lungs at this time point. In our 9h IQ1 inhibition experiment, we were able to detect significant overall down regulation of the FGF10 gene signature, although some of the individual genes showed little regulation at this time point (see **Figure 7C**). This suggests that not all the genes regulated in our *in vivo* inhibition of FGF10 are similarly regulated by inhibiting β -catenin/EP300. Furthermore, staining for SOX9 and SOX2 supports the idea that β -catenin/EP300 sustains the multipotency of distal epithelial cells (see **Figure 7D**). This data is in line with a recent report showing that β -catenin deletion in the SOX9-positive cells leads to ectopic expression of SOX2 (Ostrin et al., 2018).

In summary, the *in vitro* inhibition of β -catenin/EP300 produces branching defects and the proximalization of epithelial cells as early as 9h. Phenotypically, these impacts greatly overlap with the impacts of inhibiting FGF10 signaling. Many of the

direct targets of FGF10 signaling are similarly regulated by β -catenin/EP300, suggesting that FGF10 regulation is significantly mediated through β -catenin/EP300.

In conclusion, we have carried out a comprehensive analysis of FGF10/FGFR2b signaling on epithelial tip progenitor cells during E12.5 mouse lung development. This analysis revealed a new “FGF10 transcriptomic signature” which will be instrumental in designing new mechanistic studies concerning the role of FGF10 in alveolar epithelium formation during development, as well as maintenance during homeostasis and repair after injury.

MATERIALS AND METHODS

Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to, and will be fulfilled by, the corresponding author, Dr. Saverio Bellusci (saverio.bellusci@innere.med.uni-giessen.de).

Experimental Model and Subject Details

Ethical Statement and Husbandry

Animal experiments were performed at Children's Hospital Los Angeles under the research protocols (31–08 and 31–11) approved by the Animal Research Committee 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 Children's Hospital Los Angeles is AAALAC A3276-01. Harvesting organs and tissues from wild type and mutant mice following euthanasia using pentobarbital was approved at Justus Liebig University Giessen by the federal authorities for animal research of the Regierungspräsidium Giessen, Hessen, Germany (Approved Protocol GI 20/10 Nr. G 84/2016).

All mice used to generate experimental and control embryos were housed in a specific pathogen free (SPF) environment with free access to food and water. Up to five females were housed together, while males were housed singly. Females between 9 and 12 weeks of age were used to generate embryos.

In vivo Mouse Model to Inhibit FGFR2b Ligands

In vivo studies were conducted using an inducible dominant negative mouse model: *Rosa26^{rtTA/+}; tet(o)sFgfr2b/+* (B6-Cg-Gt(ROSA)26Sor^{tm1.1(rtTA,EGFP)Nagy} Tg(tetO-Fgfr2b/Igh)1.3Jaw/sbel). This mouse model employs a reverse tetracycline transactivator (rtTA) under the transcriptional control of the ubiquitous *Rosa26* locus. Upon administration of doxycycline, the rtTA is able to bind to the tetracycline operator (tetO), inducing the transcription of a soluble dominant negative form of *Fgfr2b* (*sFgfr2b*). These mice were generated by crossing *Rosa26^{rtTA/+}; (Gt(ROSA)26Sor^{tm1.1(rtTA,EGFP)Nagy})* with *tet(o)sFgfr2b/+* mice (Tg(tetO-Fgfr2b/Igh)1.3Jaw, obtained from Dr. Jeffrey Whitsett, Cincinnati, USA). Experimental (*Rosa26^{rtTA/rtTA}; tet(o)sFgfr2b/+*) and littermate control (*Rosa26^{rtTA/rtTA}; +/+*) embryos were generated by crossing *Rosa26^{rtTA/rtTA}; tet(o)sFgfr2b/+* and *Rosa26^{rtTA/rtTA}; +/+* animals.

Timed-pregnant females were used to conduct *in vivo* experiments. Doxycycline was administered either through food (625 mg doxycycline/kg food pellets), or via an intraperitoneal injection (i.p.; Dosage: 0.0015 mg doxycycline/g mouse weight) at the desired embryonic time point (E), where E0.5 was assumed to be noon on the day a vaginal copulation plug was found. At a determined time post-doxycycline i.p., a lethal dose of pentobarbital sodium was administered to animals via i.p. (Dosage: 0.4 mg pentobarbital/g mouse weight). After breathing ceased and a lack of pupil response to light was observed, cervical dislocation was performed to ensure death. Embryos were then harvested and washed in PBS with gentle shaking for ~2 min. Embryonic lungs were then dissected and prepared for subsequent analyses.

In vitro Lung Culture

Embryonic lungs used for *in vitro* experiments were obtained either from genetically modified embryos generated as described above, or from C57BL/6 wild-type embryos.

Embryonic lungs were dissected and cultured on 13 mm Whatman Track-Etch polycarbonate membranes, with 8.0 μ m pores (Merck, Darmstadt, Germany) positioned atop DMEM culture medium in a 24-well culture dish [medium contained: Dulbecco's Modified Eagle Medium (1x DMEM), supplemented with D-Glucose, L-Glutamine, HEPES, Pyruvate, and Phenol red (Gibco, Paisley, UK), 10% fetal bovine serum (FBS), 1% penicillin (10,000 units/ml)-streptomycin (10 mg/ml)]. Lungs were incubated at 5% CO₂ and 37°C for ~45 min to allow them to settle. At the beginning of the experiment ($t = 0$ h.), doxycycline dissolved in PBS (1 μ g/ml) or IQ1 dissolved in DMSO (10 μ M; Selleckchem, Munich, Germany) was added to the experimental lungs, while the vehicle (either PBS or DMSO) was added to control samples. Lungs were incubated at 5% CO₂ and 37°C for the duration of the experiment.

Method Details

Still and Live Image Acquisition

Brightfield images of lungs from *in vivo* and *in vitro* experiments were captured either on a Leica MZ 125 stereoscopic dissecting microscope using a Spot Insight 2.0 Mp Color Mosaic camera and Spot 4.5.9 imaging software, or were obtained from live imaging experiments using a Leica DM6000B inverted microscope, DFC 305FX camera, and Leica Application Suite Advanced Fluorescence imaging software.

Separation of Mesenchyme and Epithelium to Assess Relative Gene Expression in the Distal Tips Via Microarray

To assess the expression of genes in the epithelial and mesenchymal compartments of distal E12.5 lung tips, C57BL/6 wild type embryos were used. Embryonic lungs were isolated in culture medium, and the distal epithelial buds, along with the surrounding mesenchyme and mesothelium, were carefully dissected with fine-tipped pincers. The dissected tips were then immediately transferred to 500 μ l undiluted dispase (Corning, Amsterdam, The Netherlands) where they were incubated for 20–30 min on ice. The dispase-digested samples were then

transferred to pure FBS, and incubated for 15 min on ice, thus blocking the enzymatic activity of dispase. Using tungsten microdissection needles, the epithelium was gently separated from the surrounding mesenchyme. Separated tissues were then prepared for total RNA extraction and microarray analysis.

Immunofluorescence

Freshly dissected E12.5 lungs were washed in sterile PBS (2 × 5 min), fixed in 4% PFA for 20 min on ice, and then washed again (3 × 5 min). Lungs were dehydrated by successive washes in a graded ethanol series (30, 50, 70, 100, 100%) for 5 min each, and stored in 100% ethanol at −20°C.

To embed the lungs in paraffin, they were first washed in Xylol (2 × 5 min, or until clear), incubated for 1 h at 60°C in a 1:1 Xylol/paraffin mixture, washed in pure paraffin (3 × 20 min) at 60°C, and then stored in pure paraffin overnight at 60°C. Lungs were then embedded in paraffin blocks and sectioned to a thickness of 4 or 5 µm. Sections were placed in a 40°C water bath for ~30 min, and then placed on glass slides and incubated at 37°C overnight.

Before antibody staining, sections were first washed with gentle shaking in Xylol (3 × 10 min), and then in serial dilutions of ethanol (100, 70, 50, and 30%) for 3 min each, and finally in distilled water for 5 min. For each stain an antigen retrieval step was performed, which involved incubating the slides in 75–90°C citrate-based antigen unmasking solution (pH 6.0; Vector Laboratories, Peterborough, UK) for 15 min and then cooling on ice for ~30 min. Sections were then washed with PBST (1x PBS + 0.1% TWEEN20; 3 × 5 min). Blocking solution (1x PBS + 3% BSA + 0.4% TritonX) was then added atop each section for 1 h at room temperature. Primary antibodies were added to incubation buffer (1x PBS + 1.5% BSA + 0.2% TritonX) and samples were incubated overnight at 4°C (anti-SOX2, anti-Phospho-βCatenin (Ser552), and anti-LEF1 were added at 1:100 dilution; anti-CDH1, anti-LAMA1, and anti-SOX9 were added at 1:200 dilution; see **Table S1** for antibody details). Following primary antibody incubation, samples were washed in PBST (3 × 5 min) and secondary antibodies were added (all at a 1:500 dilution) for 1 h at room temperature, in the dark. Samples were washed in PBST (3 × 10 min) and PBS for 5 min, with gentle shaking. Finally, ProLong Gold antifade reagent with DAPI (Invitrogen, Schwerte, Germany) was added to each section and covered with a glass coverslip.

Sections were imaged on a Leica DM 5500B upright fluorescent microscope system, with a DFC 360FX camera, and Leica Application Suite Advanced Fluorescence imaging software. Signal intensity was optimized to either a control or experimental sample for an experiment, and the acquisition and intensity values were similarly applied to each sample in that experiment, thus ensuring valid comparisons.

Whole Mount Immunofluorescence and Confocal Microscopy

To assess the morphology of intact distal lung buds in control and experimental embryos, whole mount immunofluorescence followed by confocal imaging was performed.

E12.5 lungs were dissected and fixed in 4% PFA for 20 min on ice. Samples were washed in PBS + 1% TritonX (3 × 10 min),

and incubated in blocking buffer (1x PBS + 1% TritonX + 10% FBS) for 1.5 h at room temperature, followed by two washes in blocking buffer. Samples were then incubated for 2 h with FITC-conjugated anti-CDH1 (Dilution: 1:200) diluted in 1/4 blocking buffer and PBS, at 4°C in the dark. Lungs were then washed in PBS (3 × 10 min), and transferred to custom made imaging dishes (composed of a 35,0/10 mm glass bottom cell culture dish (Greiner Bio-One, Frickenhausen, Germany) and a 10,0/1 mm rubber washer fixed to the middle of the dish with a suitable adhesive, thus creating an ideal well to mount and image the sample). ProLong Gold antifade reagent with DAPI was added to each well and covered with a glass coverslip.

Z-stacks of distal lung buds were obtained on a Leica TCS SP5 confocal microscopy system using Leica Application Suite X software. For each bud, the first optical section of the z-stack was acquired at the basal edge of the epithelium. Z-stack images were taken at 0.5 µm increments through the bud, until imaging was no longer possible due to complete loss of signal intensity. Compensation of intensity loss through the bud was obtained using the linear compensation by AOTF option. 3-D reconstructions and movies were created using Leica Application Suite X software.

Transmission Electron Microscopy

To identify the effects of FGFR2b ligand inhibition on the ultrastructure of distal epithelial and mesenchymal cells, control and experimental E12.5 lungs were prepared for transmission electron microscopy.

The chest cavities of freshly harvested E12.5 embryos were gently opened by incising from the lower abdomen, through the sternum, to just under the chin using a pair of fine dissection scissors. An incision was made along the diaphragm from the midline to the spine. The embryos were then immediately placed, ventral side up, in an immersion fixative solution containing 4% PFA + 2% sucrose + 0.05% calcium chloride + 1x PIPES buffer (0.1M, pH 7.4; Sigma-Aldrich, Taufkirchen, Germany) in a 50 ml Falcon tube, such that each sample was immersed in 5X the volume of fixative in its own tube. Tubes were placed on ice and gently shaken for 2 h, after which the fixative was removed and replaced by 4% PFA + 0.05% glutaraldehyde (GA; Sigma-Aldrich, Taufkirchen, Germany), 2% sucrose, 0.05% calcium chloride + 1X PIPES buffer (0.1 M, pH 7.4). Samples remained in this fixative overnight at 4°C.

The next morning, the samples were processed for routine transmission electron microscopy. Briefly, the fixed lungs were dissected and placed into molten agar, which was allowed to harden before the samples were cut longitudinally in half. Each half was fixed for 30 min in 1.5% GA fixative containing 2% sucrose + 0.05% calcium chloride + 1X PIPES buffer (0.1 M, pH 7.4). The fixative solution was then removed and samples were washed with 1X PIPES (3 × 5 min). Samples were then incubated for 1 h at room temperature in reduced osmium fixation solution containing 0.15% sodium hexacyanoferrate(II) and 2% reduced osmium, then washed very briefly with distilled water, and dehydrated via washes in a graded ethanol series (70, 80, 90, 100%), 3 times 10 min each step. Samples were then embedded by immersion in propylene oxide (3 × 5 min), in 1:1 propylene oxide:Agar 100 epoxy resin (1 × 30 min) following

the manufacturer's instructions to produce blocks of medium hardness (Agar Scientific, Essex, UK), and finally in pure Agar 100 epoxy resin in a desiccation chamber at room temperature overnight.

The Agar 100 resin-penetrated lungs were then flat embedded into fresh Agar 100 resin and polymerized at 60°C for at least 2 days, or until complete polymerization was achieved. Ultrathin sections were then prepared and micrographs were obtained using a Zeiss LEO 906 transmission electron microscope equipped with a TRS slow-scan 2K CCD camera and ImageSP software.

DNA Isolation and PCR

DNA was isolated from the tails and hind limbs of E12.5 embryos. Gene-specific primers were used to detect the presence of *Rosa26rtTA* (wild type and transgene specific forward primer 5'- GAG TTC TCT GCT GCC TCC TG; wild type specific reverse primer 5'- CGA GGC GGA TAC AAG CAA TA; transgene specific reverse primer 5'- AAG ACC GCG AAG AGT TTG TC; expected product size of ~200 bp for the transgene and 322 bp for the wild type) and *tet(o)sFgfr2b* (transgene specific forward primer 5'- GAA GGA GAT CAC GGC TTC C; transgene specific reverse primer 5'- AGA CAG ATG ATA CTT CTG GGA CTG T; expected product size of 110 bp). The PCR reaction mix was calculated for 20 µl per reaction, and included 10 µl 2xTaq PCR Master Mix (Qiagen, Hilden, Germany), primers at a final concentration of 0.2 µM, RNase-free water, and up to 1 µg of genomic DNA. PCRs were performed in a C1000 Thermocycler (Bio-Rad). The cycling protocol to amplify *Rosa26rtTA* was as follows: initial denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, and extension at 72°C for 1.5 min; final extension at 72°C for 5 min; and hold at 4°C. The protocol to amplify *tet(o)sFgfr2b* was as follows: initial denaturation at 95°C for 2 min; 35 cycles of denaturation at 95°C for 5 seconds, annealing at 58°C for 5 s, and extension at 72°C for 20 s; final extension at 72°C for 2 min; and hold at 4°C. Capillary gel electrophoresis was performed using a QIAxcel Advanced capillary electrophoresis system (Qiagen).

RNA Isolation and RT-qPCR

Whole embryonic lungs or separated epithelium and mesenchyme used for total RNA isolation were first put in 700 µl QIAzol Lysis Reagent (Qiagen, Hilden, Germany). For tissue disruption and homogenization, the samples were transferred to gentleMACS M Tubes and homogenized in a gentleMACS Dissociator (Miltenyi Biotec) for 1 min. Total RNA was then isolated using the miRNeasy Mini Kit (Qiagen, Hilden, Germany), and eluted in 30 µl RNase-free water. RNA amount and purity was assessed with a NanoDrop 2000c (Thermo Scientific). Up to 1 µg of total RNA for each sample was then reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany).

Primers were designed to amplify specific mature mRNAs using NCBI's primer-BLAST option (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>; last accessed, 01-08-2018). Primers were further validated by PCR-based gel electrophoresis (see Table S2 for primer sequences). qPCR reaction mixtures were

set up using the PowerUp SYBR Green Master Mix (Thermo Fisher, Schwerte, Germany), with a final volume of 20 µl for each reaction. Reaction mixtures included the following components: 10 µl of 2X PowerUp SYBR Green Master Mix, between 300 and 800 nM of each primer, between 1 and 10 ng cDNA, and nuclease-free water. Samples were run with two or three technical replicates on a LightCycler 480II (Celli et al.) using the following protocol: UDG activation at 50°C for 2 min; DNA polymerase activation at 95°C for 2 min; and 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and extension at 72°C for 1 min. To validate amplification specificity, a dissociation step was also included for each sample. Threshold cycles (Ct) were calculated and used for relative expression analyses, using mouse *Hprt* as the reference gene.

Microarray Analysis

Differential gene expression was investigated using microarray analysis. Depending on the amount of RNA isolated per sample in an experiment, one of two possible microarray protocols was used. For RNA concentrations >50 ng/µl, the T7-protocol was followed. In this protocol, purified total RNA was amplified and Cy3-labeled using the LIRAK kit (Agilent Technologies, Waldbronn, Germany) following the kit instructions. Per reaction, 200 ng of total RNA was used. The Cy3-labeled aRNA was hybridized overnight to 8 × 60 K 60mer oligonucleotide spotted microarray slides (Agilent Technologies, design ID 028005).

For experiments where samples yielded <50 ng/µl of RNA, the SPIA-protocol was utilized. In this protocol, purified total RNA was amplified using the Ovation PicoSL WTA System V2 kit (NuGEN Technologies, Leek, The Netherlands). Per sample, 2 µg amplified cDNA was Cy-labeled using the SureTag DNA labeling kit (Agilent Technologies). The Cy3-labeled aRNA was hybridized overnight to 8 × 60 K 60mer oligonucleotide spotted microarray slides (Agilent Technologies, design ID 074809).

For each protocol, hybridization, and subsequent washing and drying of the slides were performed following the Agilent hybridization protocol. The dried slides were scanned at 2 µm/pixel resolution using the InnoScan is900 (Innopsys). Image analysis was performed with Mapix 6.5.0 software, and calculated values for all spots were saved as GenePix results files. Stored data were evaluated using the R software (version 3.3.2) and the limma package (version 3.30.13) from BioConductor. Gene annotation was supplemented by NCBI gene IDs via biomaRt (last accessed 08-03-2018).

Assessing Proliferation and Apoptosis

Proliferation in E12.5 lungs was assessed using the Click-iT EdU Imaging Kit (Invitrogen, Schwerte, Germany). 5-ethynyl-2'-deoxyuridine (EdU), a nucleoside analog of thymidine incorporated into DNA during DNA synthesis, was injected (i.p.) 2 h before pregnant females were sacrificed (Dosage: 0.005 mg EdU / g mouse weight). Embryonic lungs were harvested, paraffin embedded, sectioned and placed on glass slides. Sections were then deparaffinised and stained for EdU according to the manufacturer's protocol.

Apoptosis was assessed using the TdT-mediated dUTP Nick-End Labeling (TUNEL) assay. The assay was performed using the DeadEnd Fluorometric TUNEL System (Promega, Mannheim, Germany). E12.5 lungs were harvested, paraffin embedded, sectioned and placed on glass slides. The assay was performed according to the manufacturer's protocol.

Gene Expression Patterns

To assess the expression patterns of genes in early stage embryonic lungs (E14.5), the online database genepaint.org was used (last accessed 01–08–2018). Each of the genes significantly regulated in our *in vivo* studies was entered in [genepaint](http://genepaint.org). The whole embryo section displaying the clearest gene expression in the lung, along with a magnification of the lung itself, was chosen for the figures.

Quantification and Statistical Analysis

Morphometric Quantifications

Using still images of lungs, mesothelium and airways were traced in Adobe Illustrator CS6 (version 16.0.4) to create skeletal outlines. These outlines were exported and lengths and areas were quantified either using MetaMorph (version 1.5.0) or FIJI (version 2.0.0-rc-68/1.52g) software.

Significance was determined by unpaired two-tailed Student's *t*-tests. All data are presented as mean $\pm$ SEM. Values of $p < 0.05$ were considered significant. The number of independent samples (*n*) can be found in the figures.

Relative Gene Expression From qPCR Data

ΔCt and $\Delta\Delta Ct$ values were calculated according to the following formulas:

$$\Delta Ct = Ct_{\text{Reference}} - Ct_{\text{gene of interest}} \quad (1)$$

Note, this equation accounts for the fact that Ct is proportional to the $-\log$ of gene expression. ΔCt is therefore positively related to the expression of the gene of interest.

$$\Delta\Delta Ct_{\text{Experimental-control}} = \text{Mean}\Delta Ct_{\text{Experimental}} - \text{Mean}\Delta Ct_{\text{Control}} \quad (2)$$

Unpaired two-tailed Student's *t*-tests were performed on the ΔCt values, which can be assumed to be normally distributed. Number of "*n*" and significance level is indicated either in the figures or in the figure legends.

Microarray Analyses

Mean spot signals were background corrected with an offset of 1 using the NormExp procedure on the negative control spots. The logarithms of the background-corrected values were quantile-normalized. The normalized values were then averaged for replicate spots per array. From different probes addressing the same NCBI gene ID, the probe showing the maximum average signal intensity over the samples was used in subsequent analyses. Genes were ranked for differential expression using an unpaired two-tailed Student's *t*-test on a moderated *t*-statistic, and heatmaps were generated displaying genes according to descending *p*-values. Gene set tests were done on the ranks of the *t*-values, using the function "geneSetTest" in the *limma* package from BioConductor. The number of independent samples (*n*) can

be found in the figures. Gene sets were either user defined or, for pathway analyses, according to the KEGG database (last accessed 08–03–2018). The data from the microarray experiments have been deposited in the NCBI's gene expression omnibus (GEO accession GSE124157).

AUTHOR CONTRIBUTIONS

SB, MJ, JZ, PM, and CC: concept and design; MJ, SaD, AL, SoD, JW, GC, and RM: acquisition of data; SB, MJ, JW, EB-V, and DA: analysis and interpretation; MJ, SaD, SB, and JZ: drafting and editing of the manuscript. All authors read and approved the final manuscript.

FUNDING

SB was supported by grants from the Deutsche Forschungsgemeinschaft (DFG; BE4443/1-1, BE4443/4-1, BE4443/6-1, KFO309 P7 and SFB1213-projects A02 and A04), Landes-Offensive zur Entwicklung Wissenschaftlich-ökonomischer Exzellenz, the Universitätsklinikum Giessen Marburg, the University of Giessen Marburg Lung Center, the German Center for Lung Research (DZL) and COST (BM1201). SB was supported by the Deutsche Forschungsgemeinschaft (SFB1221, C05; SFB-TR84, B2; EXC147), the Universitätsklinikum Giessen & Marburg (UKGM, FOKOOPV), and the German Center for Lung Research (DZL). JZ was supported by the National Natural Science Foundation of China (81472601).

ACKNOWLEDGMENTS

We would like to thank Kerstin Goth and Jana Rostkovius for managing and genotyping the mice used for experiments. We also thank Dr. Elie El Agha for the critical reading of this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2018.00746/full#supplementary-material>

Figure S1 | Experimental validation of the *Rosa26^{rtTA/rtTA}; tet(o)sFgfr2b/+* double transgenic mice **(A)** Pregnant females carrying control (*Rosa26^{rtTA/rtTA}; +/+*) and experimental (*Rosa26^{rtTA/rtTA}; tet(o)sFgfr2b/+*) embryos were fed with Dox food starting either at E10.5, E11.5, E13.5, or E14.5 and sacrificed at E18.5. The lungs were dissected and shown in c–f. *Scale bar*: (b–f) 500 μ m. **(B)** Pregnant females carrying control (a,c,e,g and a',c',e',g') and experimental (b,d,f,g and b',d',f',h') embryos were sacrificed at E12.5. The lungs were dissected and cultured for 72 h in the presence of Dox added to the culture medium. *Scale bar*: (a–h) 500 μ m; (a'–h') 125 μ m.

Figure S2 | Transmission electron microscopy: Compared to controls, experimental lungs (DoxIP + 9 h) show reduced numbers and stunted microvilli (see black arrows, a–d), opened tight junctions (see black arrows in e and g; white asterisks in f and h), and flattened Golgi with increased staining (see black arrows, i–l). *epith.* = epithelium. *Magnification*: (a,c,e,g,i,k) 27,800 $\times$; (b,d,f,h,j,l) 139,000 $\times$.

Figure S3 | Genes and Expression pattern found in the Early 1 cluster **(A)** Graphical representation of changes in the level of gene expression over time. **(B)** Heat map. **(C)** Corresponding *in situ* hybridization results at E14.5 from [genepaint](http://genepaint.org).

Figure S4 | Genes and Expression pattern found in the Early 2 cluster (A) Graphical representation of changes in the level of gene expression over time. (B) Heat map. (C) Corresponding *in situ* hybridization results at E14.5 from genepaint.

Figure S5 | Genes and Expression pattern found in the Early 3 cluster (A) Graphical representation of changes in the level of gene expression over time. (B) Heat map. (C) Corresponding *in situ* hybridization results at E14.5 from genepaint.

Figure S6 | Genes and Expression pattern found in the Early 4 cluster (A) Graphical representation of changes in the level of gene expression over time. (B) Heat map. (C) Corresponding *in situ* hybridization results at E14.5 from genepaint.

Figure S7 | Genes and Expression pattern found in the Late 1 cluster (A) Graphical representation of changes in the level of gene expression over time. (B) Heat map. (C) Corresponding *in situ* hybridization results at E14.5 from genepaint.

Figure S8 | Genes and Expression pattern found in the Late 2 cluster (A) Graphical representation of changes in the level of gene expression over time. (B) Heat map. (C) Corresponding *in situ* hybridization results at E14.5 from genepaint.

Figure S9 | Genes and Expression pattern found in the Late 3 cluster (A) Graphical representation of changes in the level of gene expression over time. (B) Heat map. (C) Corresponding *in situ* hybridization results at E14.5 from genepaint.

Figure S10 | Genes and Expression pattern found in the Late 4 cluster (A) Graphical representation of changes in the level of gene expression over time. (B) Heat map. (C) Corresponding *in situ* hybridization results at E14.5 from genepaint.

Figure S11 | Relative level of expression of the genes of interest in epithelium and mesenchyme of WT E12.5 lungs and regulation of gene expression upon FGF10 inhibition (A) Determination of genes differentially expressed in the distal epithelium vs. mesenchyme of E12.5 wild type lungs by gene array ($n = 3$; see *Materials and Methods* for details on the statistical analysis of gene arrays). (B) Impact of *in vivo* FGF10 inhibition on lung branching at 6 and 9 h using our double transgenic system. Corresponding gene arrays allowed the identification of genes belonging to Early 4, Late 4, Late 3, and Late 1 groups. (C) Analysis of the genes found in the Early 4 cluster. The first LogFC (identified with “A” after the gene) represents the differential expression of this gene in the epithelium vs. mesenchyme of WT E12.5 lungs. The second LogFC (identified with “B” after the gene) represents the level of regulation upon FGF10 inhibition. Blue indicates genes enriched in the epithelium, and red indicates genes enriched in the mesenchyme. Genes in black were not found in our gene array in (A). Note that all the genes in Early 4 are blue, and therefore enriched in the epithelium. Some of these genes were differentially expressed in the epithelium at a high level (Log₂FC more than 2; *Id2*, *Sftpc*, *Tspan8*, *Lin7a*, *Sp5*, *Sema4f*, *Arrdc1*, *Gprc5a*, and *Slco2a1*), a medial level (Log₂FC between 1 and 2; *Etv5* and *Rai14*), or a lower level (Log₂FC between 0 and 1; *Gprn3*, *Enc1*, and *Bid*). We found that some of these genes were highly regulated upon FGF10 inhibition (Log₂FC < -2; *Gprn3*), moderately regulated (Log₂FC between -1 and -2; *Id2*, *Sftpc*, *Tspan8*, *Lin7a*, *Sp5*, *Sema4f*, *Gprc5a*, *Etv5*, *Slco2a1*), or weakly regulated (Log₂FC between 0 and -1; *Enc1*, *Arrdc1*, *Bid*, *Rai14*). It is likely that genes which are expressed at high or medial levels in

the epithelium, and which are highly or moderately regulated following inhibition of FGF10 activity, are involved in mediating FGF10 activity. One exception is *Gprn3*, which is only weakly enriched in the epithelium, but which nonetheless is highly regulated by FGF10. (D) Analysis of the genes found in the Late 4 cluster. Note again that most of the genes are blue, confirming the enrichment in the Late 4 cluster for epithelial genes. Out of 43 genes, 29 were preferentially expressed in the epithelium, two were preferentially expressed in the mesenchyme, while information on the 11 other genes was not available in the array. With the exception of *Gprn3*, *Id2*, *Arrdc1*, and *Sema4f*, all the other genes found in the Early 4 cluster are also found in the Late 4 group. Some of these genes were expressed at a high level in the epithelium (Log₂FC more than 2; *Cytl1*, *Lama3*, *Slco2a1*, *Gprc5a*, *Sftpc*, *Tspan8*, *Bspry*, *Sftpa1*, *Bex4*, *Crif1*, *Lin7a*, *Shh*, *Bex1*, *Sp5*, *Ctnnd2*, and *Pthlh*), at a medial level (Log₂FC between 1 and 2; *Kiss1*, *Lgi3*, *Rai14*, *Sftpb*, *Sh3bgrl2*, and *Etv5*), or at a lower level (Log₂FC between 0 and 1; *Strap*, *Gnptab*, *Enc1*, *Lamc2*, *Rrs1*, *Srpkl*, and *Bid*). We also classified the genes in the Late 4 cluster according to their regulation following FGF10 inactivation: highly regulated (Log₂FC < -2; *Cytl1*, *Sftpc*, *Bspry*, *Sftpa1*, *Lin7a*, *Sftpb*, *Sp5*, *Pthlh*, and *Etv5*), moderately regulated (Log₂FC between -1 and -2; *Strap*, *Kiss1*, *Lgi3*, *Slco2a1*, *Gprc5a*, *Bex4*, *Enc1*, *Lamc2*, *Sh3bgrl2*, and *Ctnnd2*), or weakly regulated (Log₂FC between 0 and -1; *Rrp9*, *Gnptab*, *Lama3*, *Rai14*, *Crif1*, *Rrs1*, *Shh*, *Bex1*, *Srpkl*, and *Bid*). As with the Early 4 analysis, it would be logical to prioritize genes for future studies based both on their expression in the epithelium and their level of regulation by FGF10. *Cytl1*, *Sftpc*, *Bspry*, *Sftpa1*, *Lin7a*, *Sp5*, and *Pthlh* fit these prioritization criteria. (E) Analysis of the genes found in the Late 3 cluster. Note the presence of genes belonging to the Hedgehog signaling pathway. This group contains 20 genes, eight of which were preferentially expressed in the epithelium, five preferentially expressed in the mesenchyme, and six other genes not identified in the epithelium vs. mesenchyme array. Of the genes contained in the Late 3 cluster, some were expressed at a high level in the epithelium (Log₂FC more than 2; *Wnt7b*, and *Pcsk6*), at a medial level (Log₂FC between 1 and 2; *Bmp7*, and *Dusp9*), or at a lower level (Log₂FC between 0 and 1; *Rbm27*, *Hmga2*, *Spata5*, and *Hk2*). We also classified the genes according to their level of regulation following FGF10 inactivation: highly regulated (Log₂FC < -2; no genes), moderately regulated (Log₂FC between -1 and -2; *Wnt7b*, *Pcsk6*, *Fgd3*, *Hmga2*, *Gli1*, *Hk2*, and *Dusp9*), or weakly regulated (Log₂FC between 0 and -1; *Sct*, *Ntng1*, *Rbm27*, *Bmp7*, *Foxf1*, and *Spata5*). (F) Analysis of the genes found in Late 1 cluster. Note the presence of *Fgf10* in this cluster. (G) Graphical representation of the FGF10 gene signature showing those genes both enriched in the epithelium (X-axis) and down-regulated after 9 hrs FGF10 inhibition (Y-axis).

Table S1 | Primary antibodies.

Table S2 | Primer sequences for qRT-PCR.

Movie S1 | Longitudinal section of control bud.

Movie S2 | Longitudinal section of experimental bud (9 h Dox-IP).

Movie S3 | Cross section of control bud.

Movie S4 | Cross section of experimental bud (9 h Dox-IP).

REFERENCES

- Abler, L. L., Mansour, S. L., and Sun, X. (2009). Conditional gene inactivation reveals roles for Fgf10 and Fgfr2 in establishing a normal pattern of epithelial branching in the mouse lung. *Dev. Dyn.* 238, 1999–2013. doi: 10.1002/dvdy.22032
- Al Alam, D., El Agha, E., Sakurai, R., Kheirollahi, V., Moiseenko, A., Danopoulos, S., et al. (2015). Evidence for the involvement of fibroblast growth factor 10 in lipofibroblast formation during embryonic lung development. *Development* 142, 4139–4150. doi: 10.1242/dev.109173
- Bellusci, S., Furuta, Y., Rush, M. G., Henderson, R., Winnier, G., and Hogan, B. L. (1997a). Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. *Development* 124, 53–63.
- Bellusci, S., Grindley, J., Emoto, H., Itoh, N., and Hogan, B. L. (1997b). Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. *Development* 124, 4867–4878.
- Celli, G., LaRochelle, W. J., Mackem, S., Sharp, R., and Merlino, G. (1998). Soluble dominant-negative receptor uncovers essential roles for fibroblast growth factors in multi-organ induction and patterning. *EMBO J.* 17, 1642–1655. doi: 10.1093/emboj/17.6.1642
- Chang, D. R., Martinez Alanis, D., Miller, R. K., Ji, H., Akiyama, H., McCrea, P. D., et al. (2013). Lung epithelial branching program antagonizes alveolar differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 110, 18042–18051. doi: 10.1073/pnas.1311760110
- Danopoulos, S., Parsa, S., Al Alam, D., Tabatabai, R., Baptista, S., Tiozzo, C., et al. (2013). Transient Inhibition of FGFR2b-ligands signaling leads to irreversible loss of cellular beta-catenin organization and signaling in AER during mouse limb development. *PLoS ONE* 8:e76248. doi: 10.1371/journal.pone.0076248
- De Langhe, S. P., Carraro, G., Warburton, D., Hajihosseini, M. K., and Bellusci, S. (2006). Levels of mesenchymal FGFR2 signaling modulate smooth muscle progenitor cell commitment in the lung. *Dev. Biol.* 299, 52–62. doi: 10.1016/j.ydbio.2006.07.001
- De Langhe, S. P., and Reynolds, S. D. (2008). Wnt signaling in lung organogenesis. *Organogenesis* 4, 100–108. doi: 10.4161/org.4.2.5856
- De Moerloose, L., Spencer-Dene, B., Revest, J. M., Hajihosseini, M., Rosewell, I., and Dickson, C. (2000). An important role for the IIIb isoform of fibroblast

- growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. *Development* 127, 483–492.
- El Agha, E., and Bellusci, S. (2014). Walking along the fibroblast growth factor 10 route: a key pathway to understand the control and regulation of epithelial and mesenchymal cell-lineage formation during lung development and repair after injury. *Scientifica* 2014:538379. doi: 10.1155/2014/538379
- Francavilla, C., Rigbolt, K. T., Emdal K. B., Carraro, G., Vernet, E., Bekker-Jensen, D. B., et al. (2013). Functional proteomics defines the molecular switch underlying FGF receptor trafficking and cellular outputs. *Mol. Cell.* 51, 707–722. doi: 10.1016/j.molcel.2013.08.002
- Guo, L., Degenstein, L., and Fuchs, E. (1996). Keratinocyte growth factor is required for hair development but not for wound healing. *Genes Dev.* 10, 165–175. doi: 10.1101/gad.10.2.165
- Herriges, J. C., Verheyden, J. M., Zhang, Z., Sui, P., Zhang, Y., Anderson, M. J., et al. (2015). FGF-regulated ETV transcription factors control FGF-SHH feedback loop in lung branching. *Dev. Cell* 35, 322–332. doi: 10.1016/j.devcel.2015.10.006
- Herriges, J. C., Yi, L., Hines, E. A., Harvey, J. F., Xu, G., Gray, P. A., et al. (2012). Genome-scale study of transcription factor expression in the branching mouse lung. *Dev. Dyn.* 241, 1432–1453. doi: 10.1002/dvdy.23823
- Hokuto, I., Perl, A. K., and Whitsett, J. A. (2003). Prenatal, but not postnatal, inhibition of fibroblast growth factor receptor signaling causes emphysema. *J. Biol. Chem.* 278, 415–421. doi: 10.1074/jbc.M208328200
- Hoppler, S., and Kavanagh, C. L. (2007). Wnt signalling: variety at the core. *J. Cell. Sci.* 120, 385–393. doi: 10.1242/jcs.03363
- Kam, Y., and Quaranta, V. (2009). Cadherin-bound beta-catenin feeds into the Wnt pathway upon adherens junctions dissociation: evidence for an intersection between beta-catenin pools. *PLoS ONE* 4:e4580. doi: 10.1371/journal.pone.0004580
- Kim, H., He, Y., Yang, I., Zeng, Y., Kim, Y., Seo, Y.-W., et al. (2012). δ -Catenin promotes E-cadherin processing and activates β -catenin-mediated signaling: implications on human prostate cancer progression. *Biochim. Biophys. Acta Mol. Basis Dis.* 1822, 509–521. doi: 10.1016/j.bbdis.2011.12.015
- Lebeche, D., Malpel, S., and Cardoso, W. V. (1999). Fibroblast growth factor interactions in the developing lung. *Mech. Dev.* 86, 125–136. doi: 10.1016/S0925-4773(99)00124-0
- Liu, Y., Martinez, L., Ebine, K., and Abe, M. K. (2008). Role for mitogen-activated protein kinase p38 alpha in lung epithelial branching morphogenesis. *Dev. Biol.* 314, 224–235. doi: 10.1016/j.ydbio.2007.12.003
- Lu, Q., Paredes, M., Medina, M., Zhou, J., Cavallo, R., Peifer, M., et al. (1999). delta-catenin, an adhesive junction-associated protein which promotes cell scattering. *J. Cell. Biol.* 144, 519–532. doi: 10.1083/jcb.144.3.519
- Makarenkova, H. P., Hoffman, M. P., Beenken, A., Eliseenkova, A. V., Meech, R., Tsau, C., et al. (2009). Differential interactions of FGFs with heparan sulfate control gradient formation and branching morphogenesis. *Sci. Signal.* 2:ra55. doi: 10.1126/scisignal.2000304
- Medina, M., Marinescu, R. C., Overhauser, J., and Kosik, K. S. (2000). Hemizygosity of delta-catenin (CTNND2) is associated with severe mental retardation in cri-du-chat syndrome. *Genomics* 63, 157–164. doi: 10.1006/geno.1999.6090
- Metzger, D. E., Stahlman, M. T., and Shannon, J. M. (2008a). Misexpression of ELf5 disrupts lung branching and inhibits epithelial differentiation. *Dev. Biol.* 320, 149–160. doi: 10.1016/j.ydbio.2008.04.038
- Metzger, D. E., Xu, Y., and Shannon, J. M. (2007). Elf5 is an epithelium-specific, fibroblast growth factor-sensitive transcription factor in the embryonic lung. *Dev. Dyn.* 236, 1175–1192. doi: 10.1002/dvdy.21133
- Metzger, R. J., Klein, O. D., Martin, G. R., and Krasnow, M. A. (2008b). The branching programme of mouse lung development. *Nature* 453, 745–750. doi: 10.1038/nature07005
- Miller, D. L., Ortega, S., Bashayan, O., Basch, R., and Basilico, C. (2000). Compensation by fibroblast growth factor 1 (FGF1) does not account for the mild phenotypic defects observed in FGF2 null mice. *Mol. Cell. Biol.* 20, 2260–2268. doi: 10.1128/MCB.20.6.2260-2268.2000
- Miyabayashi, T., Teo, J. L., Yamamoto, M., McMillan, M., Nguyen, C., and Kahn, M. (2007). Wnt/beta-catenin/CBP signaling maintains long-term murine embryonic stem cell pluripotency. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5668–5673. doi: 10.1073/pnas.0701331104
- Ostrin, E. J., Little, D. R., Gerner-Mauro, K. N., Sumner, E. A., Rios-Corzo, R., Ambrosio, E., et al. (2018). Beta-Catenin maintains lung epithelial progenitors after lung specification. *Development* 145:5. doi: 10.1242/dev.160788
- Parsa, S., Kuremoto, K., Seidel, K., Tabatabai, R., Mackenzie, B., Yamaza, T., et al. (2010). Signaling by FGFR2b controls the regenerative capacity of adult mouse incisors. *Development* 137, 3743–3752. doi: 10.1242/dev.051672
- Parsa, S., Ramasamy, S. K., De Langhe, S., Gupte, V. V., Haigh, J. J., Medina, D., et al. (2008). Terminal end bud maintenance in mammary gland is dependent upon FGFR2b signaling. *Dev. Biol.* 317, 121–131. doi: 10.1016/j.ydbio.2008.02.014
- Patel, V. N., Pineda, D. L., and Hoffman, M. P. (2017). The function of heparan sulfate during branching morphogenesis. *Matrix Biol.* 57–58, 311–323. doi: 10.1016/j.matbio.2016.09.004
- Perl, A. K., Kist, R., Shan, Z., Scherer, G., and Whitsett, J. A. (2005). Normal lung development and function after Sox9 inactivation in the respiratory epithelium. *Genesis* 41, 23–32. doi: 10.1002/gene.20093
- Quaggin, S. E., Schwartz, L., Cui, S., Igarashi, P., Deimling, J., Post, M., et al. (1999). The basic-helix-loop-helix protein pod1 is critically important for kidney and lung organogenesis. *Development* 126, 5771–5783.
- Ramasamy, S. K., Mailleux, A. A., Gupte, V. V., Mata, F., Sala, F. G., Veltmaat, J. M., et al. (2007). Fgf10 dosage is critical for the amplification of epithelial cell progenitors and for the formation of multiple mesenchymal lineages during lung development. *Dev. Biol.* 307, 237–247. doi: 10.1016/j.ydbio.2007.04.033
- Rawlins, E. L. (2008). Lung epithelial progenitor cells: lessons from development. *Proc. Am. Thorac. Soc.* 5, 675–681. doi: 10.1513/pats.200801-006AW
- Rockich, B. E., Hrycaj, S. M., Shih, H. P., Nagy, M. S., Ferguson, M. A., Kopp, J. L., et al. (2013). Sox9 plays multiple roles in the lung epithelium during branching morphogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 110, E4456–E4464. doi: 10.1073/pnas.1311847110
- Sasaki, T., and Kahn, M. (2014). Inhibition of beta-catenin/p300 interaction proximalizes mouse embryonic lung epithelium. *Transl. Respir. Med.* 2:8. doi: 10.1186/s40247-014-0008-1
- Sekine, K., Ohuchi, H., Fujiwara, M., Yamasaki, M., Yoshizawa, T., Sato, T., et al. (1999). Fgf10 is essential for limb and lung formation. *Nat. Genet.* 21, 138–141. doi: 10.1038/5096
- Treutlein, B., Brownfield, D. G., Wu, A. R., Neff, N. F., Mantalas, G. L., Espinoza, F. H., et al. (2014). Reconstructing lineage hierarchies of the distal lung epithelium using single-cell RNA-seq. *Nature* 509, 371–375. doi: 10.1038/nature13173
- Varma, S., Cao, Y., Tagne, J. B., Lakshminarayanan, M., Li, J., Friedman, T. B., et al. (2012). The transcription factors Grainyhead-like 2 and NK2-homeobox 1 form a regulatory loop that coordinates lung epithelial cell morphogenesis and differentiation. *J. Biol. Chem.* 287, 37282–37295. doi: 10.1074/jbc.M112.408401
- Volckaert, T., Campbell, A., Dill, E., Li, C., Minoo, P., and De Langhe, S. (2013). Localized Fgf10 expression is not required for lung branching morphogenesis but prevents differentiation of epithelial progenitors. *Development* 140, 3731–3742. doi: 10.1242/dev.096560
- Volckaert, T., Dill, E., Campbell, A., Tiozzo, C., Majka, S., Bellusci, S., et al. (2011). Parabrachial smooth muscle constitutes an airway epithelial stem cell niche in the mouse lung after injury. *J. Clin. Invest.* 121, 4409–4419. doi: 10.1172/JCI58097
- Volckaert, T., Yuan, T., Chao, C. M., Bell, H., Sitaula, A., Szimmetenings, L., et al. (2017). Fgf10-hippo epithelial-mesenchymal crosstalk maintains and recruits lung basal stem cells. *Dev. Cell* 43, 48–59 e45. doi: 10.1016/j.devcel.2017.09.003
- Warburton, D., El-Hashash, A., Carraro, G., Tiozzo, C., Sala, F., Rogers, O., et al. (2010). Lung organogenesis, organogenesis in development. *Curr Top Dev Biol.* 90, 73–158. doi: 10.1016/S0070-2153(10)90003-3
- Warburton, D., Perin, L., Defilippo, R., Bellusci, S., Shi, W., and Driscoll, B. (2008). Stem/progenitor cells in lung development, injury repair, and regeneration. *Proc. Am. Thorac. Soc.* 5, 703–706. doi: 10.1513/pats.200801-012AW

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Jones, Dilai, Lingampally, Chao, Danopoulos, Carraro, Mukhametshina, Wilhelm, Baumgart-Vogt, Al Alam, Chen, Minoo, Zhang and Bellusci. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

RESEARCH ARTICLE

STEM CELLS AND REGENERATION

Fgf10-positive cells represent a progenitor cell population during lung development and postnatally

Elie El Agha^{1,2}, Susanne Herold^{1,2}, Denise Al Alam³, Jennifer Quantius², BreAnne MacKenzie^{1,2}, Gianni Carraro^{1,2}, Alena Moiseenko^{1,2}, Cho-Ming Chao^{1,2}, Parviz Minoo⁴, Werner Seeger^{1,2} and Saverio Bellusci^{1,2,3,*}

ABSTRACT

The lung mesenchyme consists of a widely heterogeneous population of cells that play crucial roles during development and homeostasis after birth. These cells belong to myogenic, adipogenic, chondrogenic, neuronal and other lineages. Yet, no clear hierarchy for these lineages has been established. We have previously generated a novel *Fgf10*^{Cre} knock-in mouse line that allows lineage tracing of *Fgf10*-positive cells during development and postnatally. Using these mice, we hereby demonstrate the presence of two waves of *Fgf10* expression during embryonic lung development: the first wave, comprising *Fgf10*-positive cells residing in the submesothelial mesenchyme at early pseudoglandular stage (as well as their descendants); and the second wave, comprising *Fgf10*-positive cells from late pseudoglandular stage (as well as their descendants). Our lineage-tracing data reveal that the first wave contributes to the formation of parabronchial and vascular smooth muscle cells as well as lipofibroblasts at later developmental stages, whereas the second wave does not give rise to smooth muscle cells but to lipofibroblasts as well as an Nkx2.1⁺ E-Cad⁺ Epcam⁺ Pro-Spc⁺ lineage that requires further in-depth analysis. During alveologenesis, *Fgf10*-positive cells give rise to lipofibroblasts rather than alveolar myofibroblasts, and during adult life, a subpopulation of *Fgf10*-expressing cells represents a pool of resident mesenchymal stromal (stem) cells (MSCs) (Cd45⁺ Cd31⁺ Sca-1⁺). Taken together, we show for the first time that *Fgf10*-expressing cells represent a pool of mesenchymal progenitors in the embryonic and postnatal lung. Our findings suggest that *Fgf10*-positive cells could be useful for developing stem cell-based therapies for treating interstitial lung diseases.

KEY WORDS: Lung mesenchyme, Lineage tracing, *Fgf10*-positive cells, Resident MSCs, Mouse

INTRODUCTION

The adult mouse lung consists of at least 40–60 different types of cells that are organized in a highly sophisticated 3D structure inside the thoracic cavity (Crapo et al., 1982; McQualter and Bertoncello, 2012). These cell types can be broadly classified into epithelial cells

(populating the airways) and mesenchymal cells (populating the surrounding extracellular matrix). Along the proximodistal axis, the mouse lung is rich with stem/progenitor cells that are capable of self-renewal, multipotent differentiation and pollutant resistance.

Whereas the lineage tree of the lung epithelium has been thoroughly studied during embryonic development and postnatally (Giangreco et al., 2002; Hong et al., 2004; Kim et al., 2005; Rawlins et al., 2009; Rock et al., 2009; Stripp et al., 1995), the lineage tree of the postnatal lung mesenchyme is poorly understood. This part of the lung consists of a wide array of cell types such as smooth muscle cells, endothelial cells, chondrocytes, nerve cells, lipofibroblasts, myofibroblasts, lymphatic cells and others.

Most of our knowledge regarding the lineage tree of the lung mesenchyme has been achieved by studying embryonic development. For example, our research group has previously used a fibroblast growth factor 10 (*Fgf10*)-*lacZ* transgenic line (originally reported as *Mlc1v-nLacZ-24*) to demonstrate that *Fgf10*-expressing cells are progenitors for parabronchial smooth muscle cells (PBSMCs) in the distal lung during early embryonic development (Mailleux et al., 2005). Shan et al., on the other hand, identified a population of mesenchymal cells, initially located near the lung hilum, as progenitors for airway smooth muscle cells (Shan et al., 2008). Using Wilms tumor 1 homolog (*Wt1*)-*Cre* transgenic line, Que et al. have suggested that the mesothelium is a source of vascular – but not airway – smooth muscle cells (Que et al., 2008). On the contrary, Greif et al. have shown that vascular smooth muscle cells (VSMCs) arise from *Pdgfrb*-positive mesenchymal cells rather than *Wt1*-positive mesothelial cells (Greif et al., 2012). More recently, Dixit et al. utilized *Wt1*^{Cre-ERT2/+} mice to demonstrate that *Wt1*-positive cells are indeed progenitors for airway and VSMCs as well as desmin⁺ fibroblasts (Dixit et al., 2013). Fetal liver kinase 1 (*Flk-1*; *Kdr* – Mouse Genome Informatics), another term for vascular endothelial growth factor receptor 2 (*Vegfr2*), has been identified as the earliest marker for endothelial progenitors (angioblasts) that respond to Vegfa and give rise to the capillary plexus surrounding the airways (Del Moral et al., 2006; Kappel et al., 1999; Yamaguchi et al., 1993). Using a prospero homeobox 1 (*Prox1*)-*Cre-ERT2* knock-in line, Srinivasan et al. have shown that lymphatic cells arise from *Prox1*-positive progenitor cells (Srinivasan et al., 2007). Finally, Langsdorf et al. used receptor tyrosine kinase (*Ret*)-*EGFP* knock-in line to transiently trace neuronal progenitors during embryonic development and show that nerve cells originate from the neural crest (Langsdorf et al., 2011).

One of the early markers of the lung mesenchyme is *Fgf10*. During early lung development, *Fgf10* is expressed in the distal (submesothelial) mesenchyme and it acts on the opposite epithelium expressing *Fgfr2b* to maintain the epithelial cells in a progenitor-like state and induce branching and migration (Bellusci et al., 1997; Lü et al., 2005; Park et al., 1998). Gain of function of *Fgf10* during

¹Excellence Cluster Cardio-Pulmonary System (ECCPS), D-35392 Giessen, Hessen, Germany. ²Member of the German Center for Lung Research, Department of Internal Medicine II, Universities of Giessen and Marburg Lung Center (UGMLC), D-35390 Giessen, Hessen, Germany. ³Developmental Biology and Regenerative Medicine Program, Saban Research Institute of Childrens Hospital Los Angeles and University of Southern California, Los Angeles, CA 90027, USA. ⁴Department of Pediatrics, Division of Newborn Medicine, University of Southern California, Childrens Hospital Los Angeles, Los Angeles, CA 90027, USA.

*Author for correspondence (saverio.bellusci@innere.med.uni-giessen.de)

Received 3 June 2013; Accepted 15 October 2013

development leads to epithelial progenitor state arrest and distalization of the lung (Nyeng et al., 2008; Volckaert et al., 2013), whereas loss of function of *Fgf10* results in branching simplification and decreased numbers of epithelial progenitors. Although the primary target of *Fgf10* is the epithelium, severe mesenchymal abnormalities are also observed when *Fgf10* signaling is attenuated (Ramasamy et al., 2007).

We have previously generated a novel *Fgf10*^{Cre-ERT2/+} (or *Fgf10*^{iCre/+}) knock-in mouse line that allows specific labeling of *Fgf10*-expressing cells, which can be lineage-traced temporally and spatially (El Agha et al., 2012). Using this line, combined with *tomato*^{lox} Cre-reporter line, we performed extensive lineage tracing of *Fgf10*-positive cells during lung development and postnatal homeostasis. We used a combination of time-lapse imaging, immunofluorescence, fluorescence-activated cell sorting (FACS) analysis and sorting, analysis of cytospin-prepared cells and reverse transcription polymerase chain reaction (RT-PCR) on isolated cells to characterize the progeny of *Fgf10*-expressing cells labeled at distinct time points throughout lung development and postnatally. Our data indicate that *Fgf10*-positive cells represent a pool of progenitors for multiple lineages in the embryonic and postnatal lung mesenchyme.

RESULTS

Fgf10^{iCre} knock-in line is not leaky and does not show signs of ectopic expression

We have previously shown that the *Fgf10*^{iCre/+}; *tomato*^{lox/+} lineage-tracing tool allows permanent labeling of *Fgf10*-positive cells (as well as their descendants) and thus opening the way to study their fate in a time-controlled manner (El Agha et al., 2012). To further validate this line, *Fgf10*^{iCre/+}; *tomato*^{lox/lox} mice were crossed with the previously established *Fgf10-lacZ* reporter line (Kelly et al., 2001; Mailleux et al., 2005) and pregnant mice received a single intraperitoneal (IP) injection of tamoxifen at embryonic day (E) 11.5 and embryonic lungs were harvested at E13.5 (supplementary material Fig. S1). We reasoned that lineage-labeled cells from E11.5 are likely to retain *Fgf10* expression at E13.5. Double immunostaining for red fluorescent protein (RFP; reporting for *Fgf10*-expressing cells at E11.5 as well as their descendants) and *lacZ* (reporting for total *Fgf10*-expressing cells at E13.5) showed that all lineage-labeled cells were indeed *lacZ* positive, indicating the absence of ectopic expression from the *Fgf10*^{iCre} locus and demonstrating that *Fgf10*-expressing cells are specifically targeted with this tool. Quantification revealed that 65.44±4.27% (*n*=3) of total *lacZ*-positive cells were also positive for the lineage label (RFP), indicating that this model allows the targeting of a subset of *Fgf10*-expressing cells. Like any other lineage-tracing tool, the level of recombination greatly depends on the dose of tamoxifen used. For this study, a tolerable dose of 0.1 mg/g of body weight was used.

Throughout the study, *Fgf10*^{+/+}; *tomato*^{lox/+} littermates from tamoxifen-injected mice and/or *Fgf10*^{iCre/+}; *tomato*^{lox/+} offspring from corn-oil-injected mice were used as controls and no recombination was observed in these offspring at the level of whole-mount fluorescence imaging, fluorescence microscopy of lung sections and FACS analysis, confirming the absence of leakiness in the *Fgf10*^{iCre/+}; *tomato*^{lox/+} tool.

Fgf10-positive cells are amplified and migrate during early lung development *ex vivo*

In order to study the behavior of *Fgf10*-positive cells during the pseudoglandular stage of lung development, pregnant mice carrying *Fgf10*^{iCre/+}; *tomato*^{lox/+} embryos received a single IP injection of tamoxifen at E11.5 and embryos were harvested at E12.5. Lungs were cultured in an air-liquid interphase and brightfield/fluorescence time-

lapse imaging was carried out for 72 hours (Fig. 1; supplementary material Movie 1; *n*=3). Lineage-labeled cells were originally detected as small populations in the distal mesenchyme and were mostly abundant in the accessory lobe (Fig. 1B-B"). During the first 24 hours of culture, tomato-positive cells amplified exponentially and populated other regions of the growing lung (Fig. 1D-D",J). At the end of the three-day culture, lineage-labeled cells were highly abundant and dispersed throughout the lung mesenchyme (Fig. 1E-E"). After *ex vivo* culture, lungs were fixed, processed and stained for α Sma (Acta2 – Mouse Genome Informatics) (Fig. 1F-I). An average of 81±0.2 (*n*=3) tomato-positive cells was detected per section (Fig. 1G,G',K) and a subpopulation of *Fgf10*-positive cells (5.67±0.57 cells; *n*=3) appeared in the PBSMC layer (Fig. 1I,I',K).

Fgf10-positive cells are progenitors for parabronchial and VSMCs *in vivo*

To study the commitment of *Fgf10*-positive cells to the smooth muscle lineage *in vivo*, pregnant mice carrying *Fgf10*^{iCre/+}; *tomato*^{lox/+} embryos received a single IP injection of tamoxifen at E10.5 and embryonic lungs were harvested at E13.5, E15.5 and E18.5 (Fig. 2A-C). Because tomato-positive cells were mostly abundant in the accessory lobe, this lobe was used for immunostaining. α Sma staining of *Fgf10*^{iCre/+}; *tomato*^{lox/+} lungs revealed a subpopulation of tomato-positive cells in the PBSMC layer at all three developmental stages (Fig. 2G,G',K,K',O,O'). Among total tomato-positive cells, tomato-positive PBSMCs were significantly abundant at E13.5 (14.03±1.31% of total tomato-positive cells; *n*=3) (Fig. 2A) and E15.5 (18.47±3.75%; *n*=3) (Fig. 2B) but to a less extent at E18.5 (7.51±0.72%; *n*=3) (Fig. 2C). Another population of tomato-positive cells was identified in the VSMC compartment at E15.5 (3.44±0.96%; *n*=3) (Fig. 2B; supplementary material Fig. S2) and E18.5 (4.88±0.83%; *n*=3) (Fig. 2C; supplementary material Fig. S2). A third population of lineage-labeled cells was observed in the vicinity of parabronchial and VSMCs at all three stages. Initially, we thought that these cells could have a neuronal nature, as *Fgf10* is known to be involved in neurogenesis (Haan et al., 2013; Hajihosseini et al., 2008). However, β -III Tubulin (tubulin, beta 3 class III – Mouse Genome Informatics) immunostaining did not show any overlap with the lineage label (data not shown).

Fgf10-positive cells from early pseudoglandular stage are progenitors for smooth muscle cells and lipofibroblasts *in vivo*

The genetic model used in this study revealed that only a minor proportion of *Fgf10*-positive cells commits to the smooth muscle lineage (Fig. 2). *Fgf10* is known to be expressed by adipocyte precursors and *Fgf10*-null neonates suffer from impaired development of the white adipose tissue (Sakaue et al., 2002; Yamasaki et al., 1999). Thus, we decided to investigate whether *Fgf10*-positive cells give rise to an adipose-related cell lineage in the lung, the lipofibroblasts. *Fgf10*-positive cells were labeled during early pseudoglandular stage (E11.5) and lungs were harvested at E12.5, E15.5 and E18.5 (Fig. 3A-C). FACS analysis showed a drastic increase in the total number of RFP⁺ cells from E12.5 to E15.5 and then E18.5 (Fig. 3D). Our unpublished data show that lipofibroblasts start to emerge in the lung at E15.5; thus, α Sma and Adrp (Plin2 – Mouse Genome Informatics) immunostaining was performed at E18.5. For convenience, the left lung lobe was used to carry out immunofluorescence staining and quantification (Fig. 3F). An average of 450±10.12 (*n*=3) tomato-positive cells was counted per sample and a minor proportion of RFP-positive cells stained for

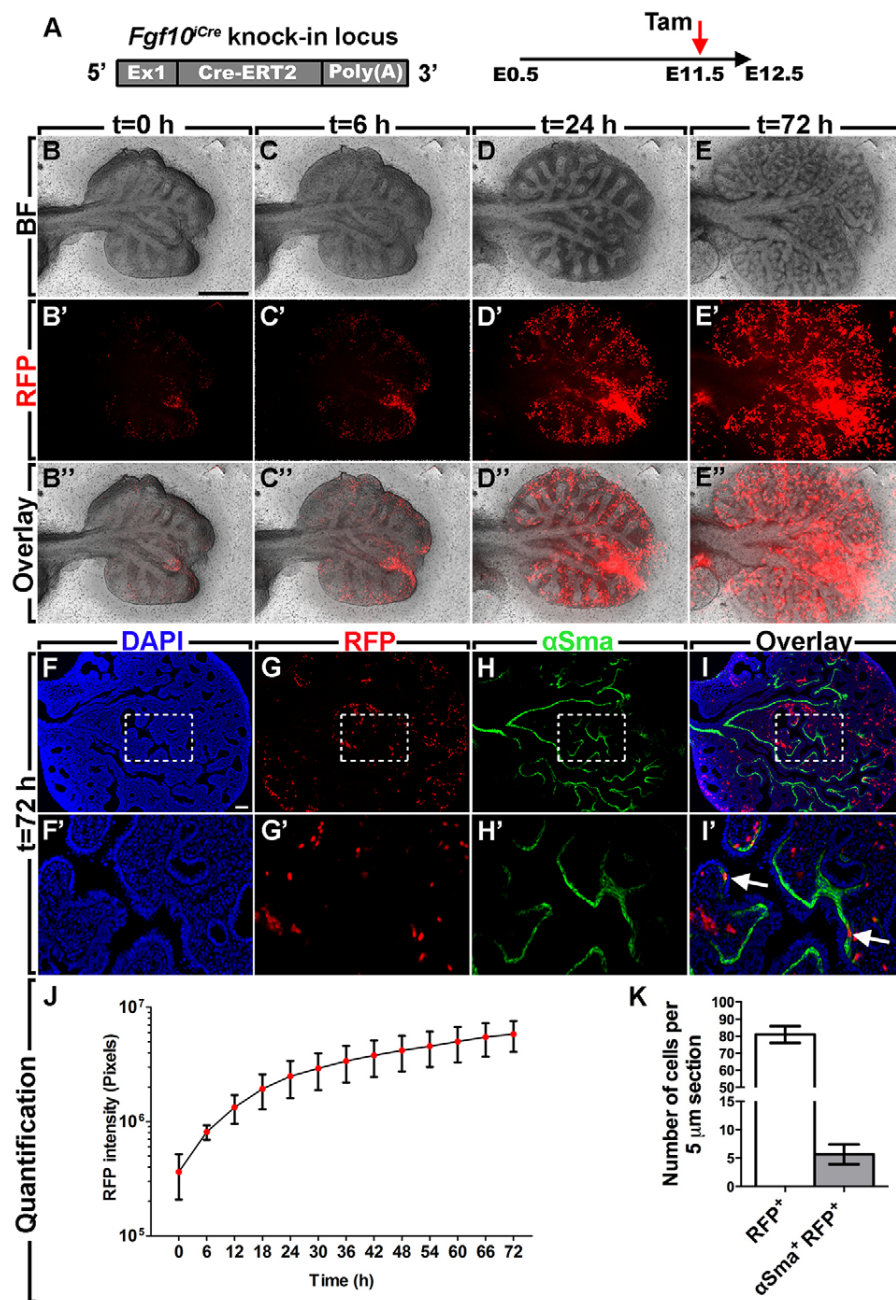


Fig. 1. Time-lapse imaging of E12.5 lungs from *Fgf10*^{Cre/+}; *tomato*^{lox/+} embryos.

(A) Schematic of the *Fgf10* locus in *Fgf10*^{Cre} knock-in mice. Recombination was induced *in vivo* by a single IP injection of tamoxifen at E11.5. (B-E) Brightfield imaging of an E12.5 *Fgf10*^{Cre/+}; *tomato*^{lox/+} lung undergoing branching morphogenesis. (B'-E') Whole-mount fluorescence imaging showing the progressive amplification and migration of lineage-labeled cells. Note the high tomato expression in the accessory lobe at t=0 hour. (B''-E'') Overlay of brightfield and fluorescent images. (F-I) Immunofluorescent detection of αSma (green) in the lung after 72 hours of culture. The areas in white boxes are magnified in F'-I'. A subpopulation of lineage-labeled cells lies in the PBSMC layer (white arrows in I'). (J) Quantification of the RFP signal over time. (K) Quantification of lineage-labeled cells after αSma immunostaining. *n*=3. Data are shown as average values ± s.e.m. Scale bars: 500 μm (B-E); 75 μm (F-I). BF, brightfield.

αSma around the epithelium ($5.68 \pm 0.39\%$; *n*=3) (Fig. 3E,G) and within vascular walls ($4.31 \pm 0.23\%$; *n*=3) (Fig. 3E,H). Similarly to *Fgf10*-positive cells labeled at E10.5, a population of αSma⁺ RFP⁺ cells was identified in the vicinity of PBSMCs ($10.17 \pm 0.88\%$; *n*=3) (Fig. 3E,I) and VSMCs ($3.92 \pm 0.72\%$; *n*=3) (Fig. 3E). These cells did not express the neuronal marker *β-III tubulin* (supplementary material Fig. S3). However, a significant population of RFP-positive cells stained for Adrp ($29.96 \pm 5.17\%$; *n*=3) (Fig. 3E,J) and the remaining population of RFP-positive cells ($45.97 \pm 4.2\%$; *n*=3) that did not stain for αSma or Adrp was characterized by its big size and the presence of filopodia (Fig. 3E,K). The lungs were also stained for other markers, such as Cd45 (Ptpcr – Mouse Genome Informatics) and Cd31 (Pecam1 – Mouse Genome Informatics), but no overlap was observed between these markers and the RFP signal (supplementary material Fig. S3).

Fgf10*-positive cells from late pseudoglandular stage are progenitors for lipofibroblasts *in vivo

Fgf10-positive cells from E11.5 showed to be precursor cells for PBSMCs, VSMCs and lipofibroblasts at E18.5 (Fig. 3). However, the commitment of these early progenitors to the smooth muscle lineage showed a decline beyond E15.5 (Fig. 2). To investigate this observation further and to test whether lipofibroblast progenitors are restricted to early developmental stages, *Fgf10*-positive cells were labeled at E15.5 and embryonic lungs were harvested at E16.5 and E18.5 (Fig. 4). RFP expression was low at E16.5 (Fig. 4A) and was more pronounced at E18.5 (Fig. 4B). The left lung lobe was used to carry out immunofluorescence staining and quantification (Fig. 4D). An average of 499 ± 71.04 (*n*=3) tomato-positive cells was counted per sample at E18.5 and αSma staining showed minimal overlap with the RFP signal in the parabronchial ($0.26 \pm 0.03\%$; *n*=3) and

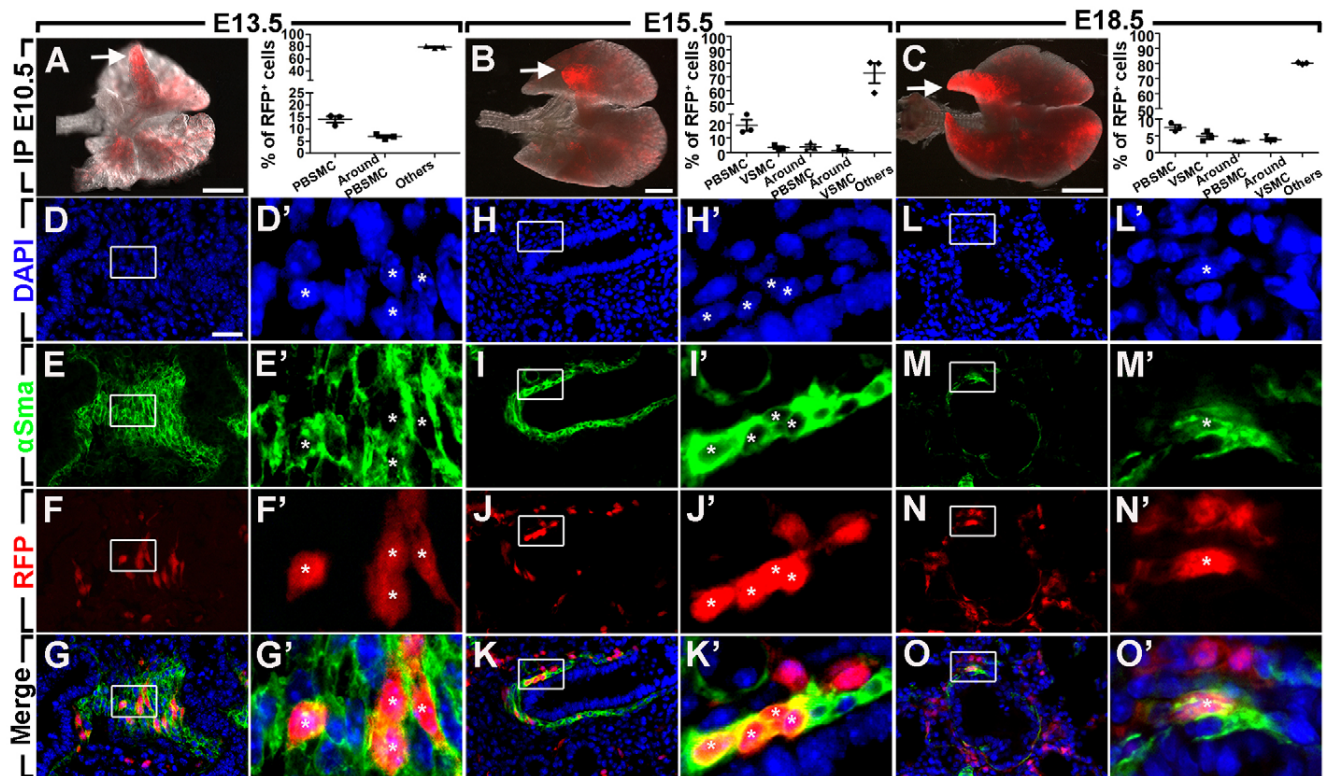


Fig. 2. Contribution of *Fgf10*-positive cells labeled at E10.5 to the parabronchial smooth muscle lineage *in vivo*. (A–C) Overlay of brightfield and whole-mount fluorescent images of the lung at E13.5, E15.5 and E18.5. Note the strong RFP signal in the accessory lobe at all three stages (white arrows). The right panels in A–C show the quantification of multiple RFP⁺ populations based on αSma staining for each stage. (D–O) Single-channel and merged images of αSma staining (green) of the accessory lobes of the lungs from the upper panel showing RFP⁺ cells (red) in the PBSMC layer at all three stages (asterisks). The areas in the white boxes are magnified in D'–O'. *n*=3. Data are shown as average values ± s.e.m. Scale bars: 500 μm (A); 1000 μm (B); 750 μm (C); 25 μm (D–O).

vascular ($0.75 \pm 0.34\%$; *n*=3) compartments (Fig. 4E). Significant numbers of αSma⁺ RFP⁺ cells around PBSMCs ($10.33 \pm 1.65\%$; *n*=3) (Fig. 4E,F) and VSMCs ($4.99 \pm 0.32\%$; *n*=3) (Fig. 4E,G) were detected and Adrp⁺ RFP⁺ cells ($39.98 \pm 8.28\%$; *n*=3) (Fig. 4E,H,I). Similarly to cells labeled at E11.5, αSma⁺ Adrp⁺ β-III Tub⁺ Cd45⁺ Cd31⁺ cells ($43.69 \pm 8.32\%$; *n*=3) were also observed (Fig. 4E,J,K; supplementary material Fig. S3).

***Fgf10* expression identifies lipofibroblast rather than alveolar myofibroblast progenitors during alveologenesis**

The alveolar stage of lung development is known for the prevalence of alveolar myofibroblasts. These cells, in addition to lipofibroblasts, are believed to play a crucial role in secondary septa formation and alveolar maturation during postnatal alveolarization (O'Hare and Sheridan, 1970; Rehan and Torday, 2012; Rubin et al., 2004; Torday and Rehan, 2007; Vaccaro and Brody, 1978; Yamada et al., 2005). To determine whether *Fgf10*-positive cells contribute to these lineages postnatally, pregnant mice carrying *Fgf10*^{Cre/+}; *tomato*^{lox/+} embryos were left to develop to term and then fed tamoxifen-containing pellets starting on postnatal day 2 (P2). At P14, pups were sacrificed, lungs were harvested, and the left lung lobe was used for immunostaining (Fig. 5). No tomato signal was detected in lungs from *Fgf10*^{+/+}; *tomato*^{lox/+} pups (Fig. 5A,L) whereas the signal was abundant in their *Fgf10*^{Cre/+}; *tomato*^{lox/+} counterparts (Fig. 5B), especially near the main bronchus (Fig. 5C). An average

of 141 ± 40 (*n*=3) tomato-positive cells was counted per *Fgf10*^{Cre/+}; *tomato*^{lox/+} sample and αSma immunostaining revealed a subpopulation of lineage-labeled cells expressing αSma ($19.70 \pm 1.88\%$; *n*=3) (Fig. 5D–H,M). These cells were located in the lung parenchyma and not at the tips of secondary septa. On the other hand, Adrp immunostaining showed that a major proportion of *Fgf10*-expressing cells gives rise to lipofibroblasts ($67.74 \pm 1.27\%$; *n*=3) (Fig. 5I–K,M).

***Fgf10*-positive cells from various developmental stages exhibit various molecular signatures and are retained postnatally**

Fgf10-expressing cells were labeled at E11.5 or E15.5 and lungs were harvested at E18.5. Lungs were dissociated into single-cell suspensions and stained for Cd45, Cd31, Epcam, Pdgfra, Kit and Sca-1 (Ly6a – Mouse Genome Informatics). A FACSaria III cell sorter was used to analyze 50,000–100,000 cells per sample per time point. No tomato signal was detected in lungs from *Fgf10*^{+/+}; *tomato*^{lox/+} embryos (Fig. 6A) or from *Fgf10*^{Cre/+}; *tomato*^{lox/+} embryos exposed to corn oil instead of tamoxifen (data not shown). Whereas lineage-labeled cells from E11.5 accounted for ~0.5% of the total lung, those from E15.5 accounted for ~1.5% (Fig. 6B,C) and most of these cells were Cd45⁺ Cd31⁺ (data not shown). Almost all lineage-labeled cells from both time points expressed low levels of *Sca-1* (*Sca-1*^{low}) at E18.5 (Fig. 6E,G) and a subpopulation of *Fgf10*-positive cells from E11.5 and E15.5 also expressed the

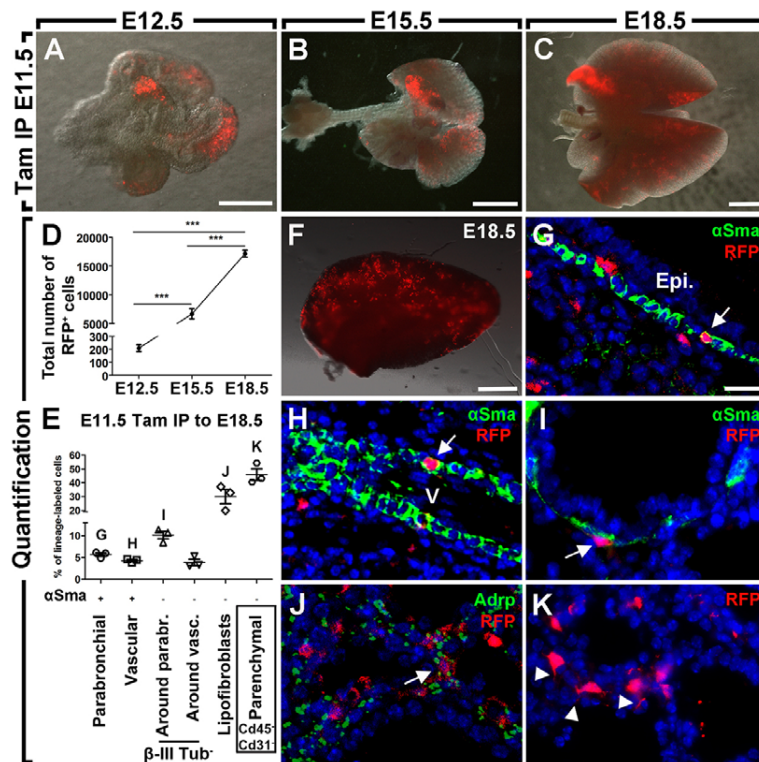


Fig. 3. In vivo lineage tracing of *Fgf10*-positive cells labeled at E11.5. (A-C) Overlay of brightfield and whole-mount fluorescent images of the lung at E12.5, E15.5 and E18.5. (D) The total number of RFP⁺ cells in the lungs shown in A-C increases drastically over time, as determined by FACS analysis. (E) Quantification of RFP-positive populations. (F) Overlay of brightfield and whole-mount fluorescent images of the left lung lobe at E18.5. (G-I) α Sma staining showing RFP-positive cells in the PBSMC layer (G), vascular walls (H) and around PBSMCs (I). (J) Immunofluorescence staining showing an Adrp⁺ RFP⁺ cell. (K) Big RFP-positive cells with filopodia (arrowheads) are present in the lung parenchyma. $n=3$. Data are shown as average values $\pm$ s.e.m. *** $P<0.001$ (one-way ANOVA). Scale bars: 250 μ m (A); 1000 μ m (B,C); 500 μ m (F); 12.5 μ m (G-K). Epi, epithelium; v, vessel.

mesenchymal marker *Pdgfra* at E18.5 (~9.9% and ~17.5%, respectively) (Fig. 6F,H). Interestingly, almost no tomato-positive cells from E11.5 and ~18.1% from E15.5 stained for the pan-epithelial cell marker *Epcam* at E18.5 (Fig. 6E,G). *Kit* expression was very low at this stage (E18.5) but staining revealed ~7.3% and ~1.4% overlap with the tomato signal in E11.5 and E15.5 time

points, respectively (Fig. 6F,H). RT-PCR and cytospin analysis of sorted *Fgf10*-positive cells (labeled at E15.5) showed high expression of *Adrp* (Fig. 6D), confirming the immunofluorescence data obtained from processed lung tissues (Figs 3 and 4). Histological analysis of E15.5 *Fgf10*^{Cre/+}; *tomato*^{lox/+} lungs (labeled at E11.5) showed a subpopulation of RFP⁺ *Pdgfra*⁺ cells (Fig. 6I,J).

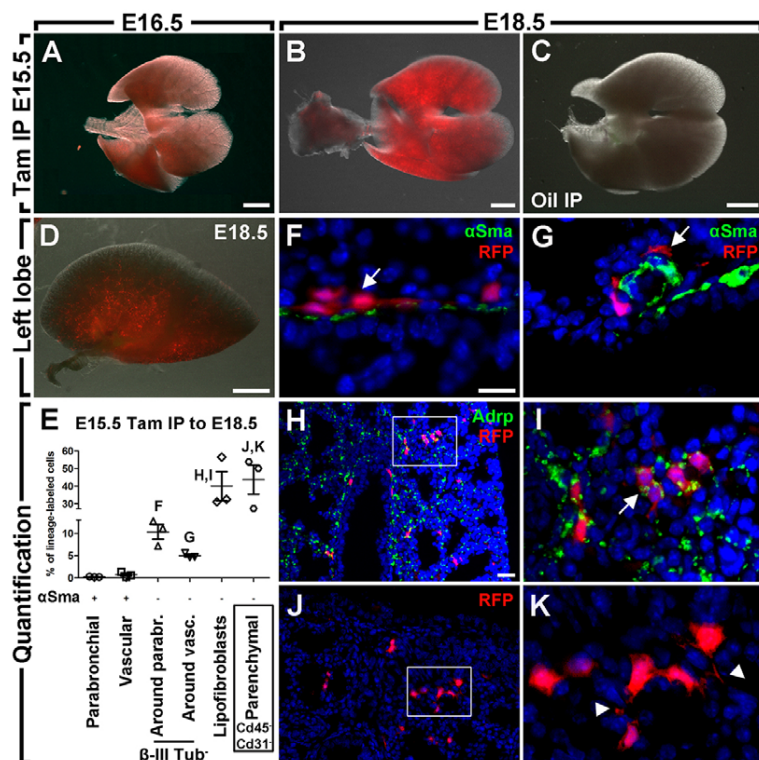


Fig. 4. In vivo lineage tracing of *Fgf10*-positive cells labeled at E15.5. (A,B) Overlay of brightfield and whole-mount fluorescent images of the lung at E16.5 and E18.5. The RFP signal is more intense at E18.5 than at E16.5. (C) Overlay of brightfield and whole-mount fluorescent images of an E18.5 *Fgf10*^{Cre/+}; *tomato*^{lox/+} lung exposed to corn oil instead of tamoxifen. (D) Overlay of brightfield and whole-mount fluorescent images of the left lung lobe at E18.5. (E) Quantification of RFP-positive populations. (F,G) α Sma staining showing α Sma⁺ RFP⁺ cells around PBSMCs and VSMCs. (H,I) Adrp immunostaining showing Adrp⁺ RFP⁺ cells. The area in the white box is magnified in I. (J,K) Big RFP-positive cells with filopodia (arrowheads) are present in the lung parenchyma. The area in the white box is magnified in K. $n=3$. Data are shown as average values $\pm$ s.e.m. Scale bars: 750 μ m (A,D); 1000 μ m (B,C); 12.5 μ m (F,G,I,K); 25 μ m (H,J).

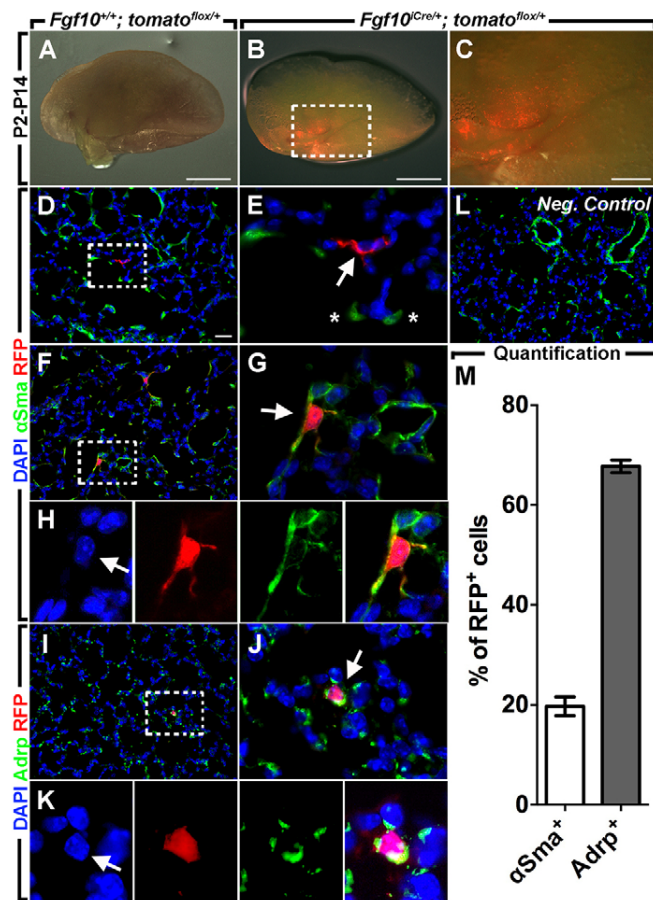


Fig. 5. *Fgf10*-positive cells preferentially give rise to lipofibroblasts rather than alveolar myofibroblasts during alveologenesis. Nursing female mice were fed tamoxifen-containing pellets from P2 to P14. (A) Overlay of brightfield and whole-mount fluorescent images of the left lung lobe from an *Fgf10*^{Cre/+}; *tomato*^{flox/+} pup. (B,C) Representative image of the left lung lobe from *Fgf10*^{Cre/+}; *tomato*^{flox/+} pups. Note the strong tomato signal around the mainstem bronchus (C; higher magnification of the boxed area in B). (D,E) Immunostaining for αSma showing an αSma⁺ RFP⁺ cell. The area in the dashed box is magnified in E. The asterisks mark alveolar myofibroblasts in the secondary septa. (F,G) Immunostaining for αSma showing an αSma⁺ RFP⁺ cell (white arrow). The area in the dashed box is magnified in G. (H) Single-channel fluorescent images of the αSma⁺ RFP⁺ cell shown in G. (I,J) Immunostaining for Adrp showing an Adrp⁺ RFP⁺ cell. The area in the dashed box is magnified in J. (K) Single-channel fluorescent images of the Adrp⁺ RFP⁺ cell shown in J. (L) Immunostaining for αSma of an *Fgf10*^{Cre/+}; *tomato*^{flox/+} lung. (M) Quantification of lineage-labeled cells according to αSma and Adrp expression. *n*=3. Data are shown as average values ± s.e.m. Scale bars: 2.5 mm (A,B); 750 μm (C); 25 μm (D-L).

Fgf10-expressing cells, labeled postnatally, represented ~0.1% of the total lung (a total of one million cells was analyzed) (Fig. 6L). ~15.7% and ~22.8% of total RFP-positive cells stained for Epcam and Sca-1, respectively (Fig. 6M). A subpopulation (accounting for ~10.5% of total RFP-positive cells) exhibited the molecular signature of resident MSCs (Cd45⁺ Cd31⁺ Sca-1⁺) (Fig. 6N).

In a separate experiment, pregnant mice carrying *Fgf10*^{Cre/+}; *tomato*^{flox/+} embryos received a single IP injection of tamoxifen or corn oil at E11.5 and were left to develop to term. The pups from these mice were sacrificed at P30 and lungs were harvested for FACS analysis. Lungs from corn-oil-exposed pups revealed no tomato expression (Fig. 6S), whereas lineage-labeled cells from

tamoxifen-exposed lungs appeared in the distal mesenchyme, especially at the tip of the accessory lobe (Fig. 6T,U). These cells accounted for ~0.4% of total lung suspensions (Fig. 6O) and similarly to the cells labeled at E11.5 and harvested at E18.5, minimal overlap between tomato and Epcam was detected (Fig. 6P). Nevertheless, ~11.1% of total lineage-labeled cells expressed *Sca-1*, half of which representing a subset of resident MSCs (Fig. 6P,Q). LipidTOXTM staining revealed that most tomato-positive cells (~83.7%) contained neutral lipids at P30 (Fig. 6R).

In order to further characterize Epcam⁺ RFP⁺ cells, *Fgf10*-expressing cells were labeled at E15.5 and RFP⁺ cells were sorted at E18.5 and stained for Pro-Spc (Sftpc – Mouse Genome Informatics) and E-Cadherin (Cdh1 – Mouse Genome Informatics) (Fig. 7A,B). Interestingly, a subpopulation of RFP⁺ cells was Pro-Spc⁺ but E-Cad⁺ (Fig. 7A',B'). A similar observation was made for lung sections where RFP⁺ cells could not be detected in the lung epithelium (E-Cad⁺) (Fig. 7D,D',F) and 20.23±0.89% (*n*=3) of total RFP⁺ cells was Pro-Spc⁺ (Fig. 7C,C',F). Finally, RT-PCR on sorted cells (labeled at P2 and collected at P30) showed that Epcam⁺ RFP⁺ cells were positive for *Spc*, but negative for *Nkx2.1* and *E-Cad*, whereas Epcam⁺ RFP⁺ cells were positive for *Nkx2.1*, *E-Cad* and *Spc*. Both populations showed lack of *Fgf10* expression (Fig. 7E).

DISCUSSION

The embryonic lung mesenchyme consists of cells that belong to distinct lineages, including myogenic, adipogenic, chondrogenic, neuronal and other lineages. Most of these cell types arise from endogenous (or resident) progenitor populations whereas few (e.g. nerve cells) arise from exogenous sources (the neural crest). Cell-fate determination probably arises from a complex network of autocrine and paracrine signals leading to the proliferation and differentiation of lineage-restricted as well as multipotent progenitor cell populations.

Fgf10 is an early marker for the submesothelial mesenchyme (Bellusci et al., 1997) and its expression levels have shown to be crucial for the maintenance of both epithelial and mesenchymal progenitors (Ramasamy et al., 2007). The domain of *Fgf10* expression in the embryonic lung has been thoroughly studied. Northern blotting and quantitative PCR (qPCR) data show that *Fgf10* transcripts progressively accumulate in the embryonic lung between E11.5 and E18.5 (Bellusci et al., 1997; El Agha et al., 2012). *In situ* hybridization for *Fgf10* and X-Gal staining of *Fgf10-lacZ* lungs show that *Fgf10* expression is restricted to the tips of the distal mesenchyme until E14.5, after which the expression becomes dispersed throughout the mesenchyme (El Agha et al., 2012; Mailleux et al., 2005; Ramasamy et al., 2007; Tiozzo et al., 2012). Our lineage-tracing data reveal two waves of *Fgf10* expression, each of which is characterized by a distinct pattern in terms of cell localization as well as differentiation potential (Fig. 8A). The first wave, consisting of *Fgf10*-positive cells labeled at E11.5 as well as their descendants, yields cells residing mostly in the distal mesenchyme at E18.5 (especially observed in the accessory lobe) and contributes to myogenic (PBMSCs and VSMCs) as well as adipogenic (lipofibroblasts) lineages. By contrast, the second wave, consisting of *Fgf10*-positive cells labeled at E15.5 as well as their descendants, yields cells that are dispersed throughout the mesenchyme at E18.5 and gives rise to adipogenic, but not myogenic, lineages.

Lineage tracing of *Fgf10*-positive cells from E10.5 showed that these cells contribute to the smooth muscle cell lineage when examined at E13.5, E15.5 and E18.5; however, the percentage of

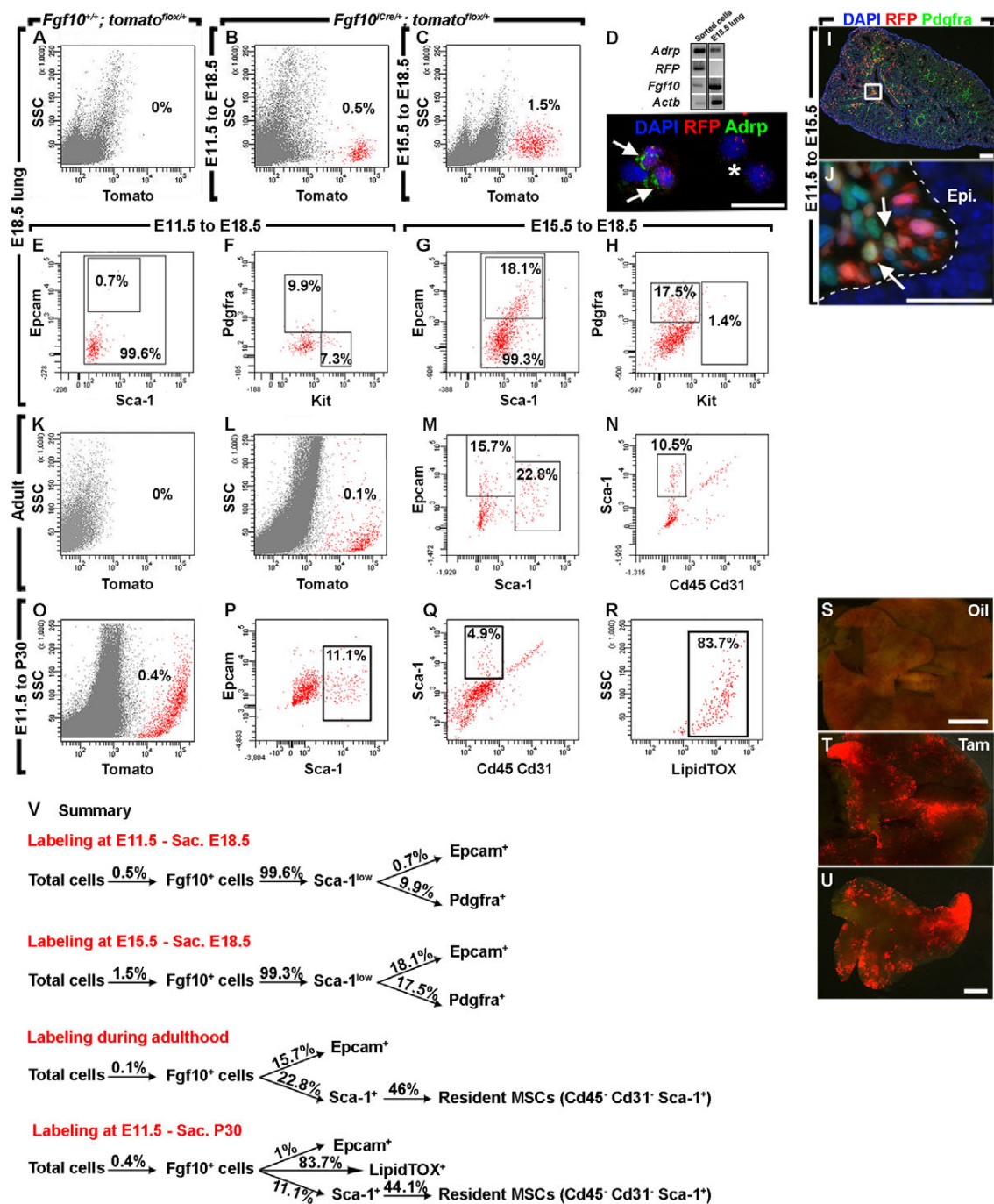


Fig. 6. Flow cytometry analysis of the *Fgf10*-expressing lineage during embryonic development and postnatally. (A) E18.5 lungs from *Fgf10*^{Cre/+}; *tomato*^{lox/+} embryos show no tomato signal. (B,C) Tomato-positive cells account for ~0.5% and ~1.5% of the total E18.5 lung suspension when labeled at E11.5 and E15.5, respectively. (D) RT-PCR and immunostaining of sorted *Fgf10*-positive cells (labeled at E15.5) show high expression of *Adrp* (white arrows). Note the presence of *Adrp*⁺ cells (asterisk). (E,F) Almost all *Fgf10*-expressing cells labeled at E11.5 express low levels of *Sca-1* (*Sca-1*^{low}) (E) and ~9.9% of these cells co-express *Pdgfra* (F). (G) Almost all *Fgf10*-expressing cells labeled at E15.5 express low levels of *Sca-1* (*Sca-1*^{low}) and ~18.1% of these cells co-express *Epcam*. (H) ~17.5% of lineage-labeled cells express *Pdgfra*. (I,J) Section of an E15.5 *Fgf10*^{Cre/+}; *tomato*^{lox/+} lung (labeled at E11.5) showing *Pdgfra* expression (green). The area in the white box is magnified in J. White arrows indicate RFP⁺ *Pdgfra*⁺ cells and the dashed line marks the epithelial-mesenchymal boundary. (K) Lungs from *Fgf10*^{Cre/+}; *tomato*^{lox/+} adult mice show no tomato signal when exposed to tamoxifen. (L) Tomato-positive cells account for ~0.1% of the total adult lung. (M) ~15.7% and ~22.8% of lineage-labeled cells express *Epcam* and *Sca-1*, respectively. (N) A subpopulation of *Fgf10*-expressing cells (~10.5%) shows the molecular signature of resident MSCs. (O) Tomato-positive cells from E11.5 are retained at P30, and account for ~0.4% of the total lung. (P) ~11.1% total RFP⁺ cells express *Sca-1* whereas minimal overlap with *Epcam* expression can be detected. (Q,R) ~4.9% of total tomato-positive cells are resident MSCs (Q) and the majority (~83.7%) of total labeled cells contains neutral lipids (R). (S-U) Whole-mount fluorescence images of lungs from P30 mice that were exposed to corn oil or tamoxifen at E11.5. Note the absence of tomato expression in corn-oil-exposed lungs. The accessory lobe of the tamoxifen-exposed lung shown in T is shown in U. Note the strong tomato signal at the tip of this lobe. (V) Summary of the flow cytometry analysis of the *Fgf10*-expressing lineage during embryonic lung development and postnatally. $n \geq 2$. Scale bars: 25 μ m (D); 100 μ m (I); 12.5 μ m (J); 5 mm (S,T); 2.5 mm (U). Epi, epithelium.

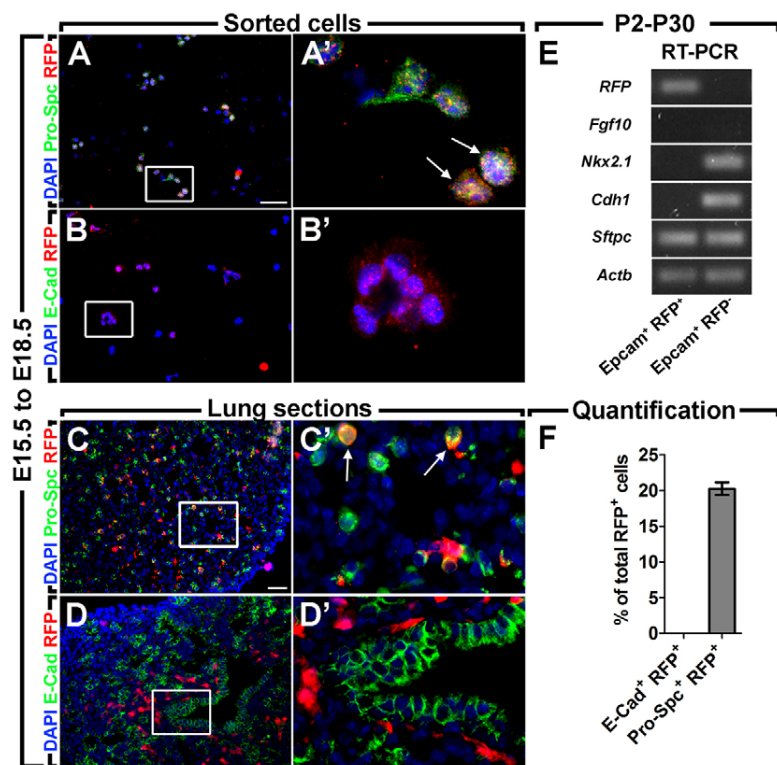


Fig. 7. Epcam⁺ lineage-labeled cells do not represent an epithelial lineage in the lung. (A–B') Immunofluorescence staining of sorted *Fgf10*-positive cells (labeled at E15.5 and harvested at E18.5) showing E-Cad⁺ Pro-Spc⁺ RFP⁺ cells. The areas in the white boxes are magnified in A', B'. The white arrows indicate Pro-Spc⁺ RFP⁺ cells. (C, C') Immunofluorescence staining of lung cryosections (exposed to tamoxifen at E15.5 and harvested at E18.5) showing Pro-Spc⁺ RFP⁺ cells in the lung mesenchyme. (D, D') Immunofluorescence staining of lung sections showing E-Cad⁺ RFP⁺ cells. The areas in the white boxes in C, D are magnified in C', D'. The white arrows indicate Pro-Spc⁺ RFP⁺ cells. (E) RT-PCR on sorted cells (labeled at P2 and harvested at P30) showing that Epcam⁺ RFP⁺ cells co-express *Spc*, but not *Nkx2.1* or *E-Cad* whereas Epcam⁺ RFP[−] cells co-express *Nkx2.1*, *E-Cad* and *Spc*, but not *Fgf10*. Note the absence of *Fgf10* expression also in Epcam⁺ RFP⁺ cells. (F) Quantification of E-Cad⁺ RFP⁺ and Pro-Spc⁺ RFP⁺ cells from the staining shown in C, D. *n*=3. Scale bars: 50 μ m (A, B); 25 μ m (C, D).

α Sma⁺ RFP⁺ cells relative to total RFP⁺ cells showed a decline between E15.5 and E18.5. As the overall number of *Fgf10*-positive cells (labeled at E11.5) showed a drastic increase from E12.5 to E15.5 and then E18.5 as demonstrated by FACS analysis (also demonstrated by time-lapse imaging of E12.5 lung undergoing branching morphogenesis), we conclude that the drop in the number of α Sma⁺ RFP⁺ cells relative to total RFP⁺ cells at E18.5 is likely to be due to the expansion of the α Sma[−] RFP⁺ population at the expense of the α Sma⁺ RFP⁺ population. In other words, the limited α Sma⁺ RFP⁺ fraction that forms between E10.5 and E15.5 gets diluted in the α Sma[−] RFP⁺ population, which continues to expand beyond E15.5. This is supported by our observation that α Sma⁺ cells rarely proliferate when examined at E13.5, E15.5 and E18.5 (supplementary material Fig. S4). The proliferative capacity of *Fgf10*-positive cells was also demonstrated by time-lapse imaging of E18.5 lung explants that were induced with tamoxifen at E11.5 (supplementary material Movie 2) or E15.5 (data not shown). Moreover, primary cultures of E18.5 lung mesenchyme (induced with tamoxifen at E11.5 or E15.5) showed that ~40% of RFP⁺ cells were undergoing mitosis during the 24 hours of culture (supplementary material Movie 3).

It was recently reported that a multipotent cardiopulmonary progenitor cell population (Wnt2⁺ Gli1⁺ Isl1⁺), originating from the second heart field (SHF), invades the lung, giving rise to airway and VSMCs in addition to other lineages (Peng et al., 2013). Our lineage-tracing system does not label any SHF cells as lineage-labeled cells could not be detected in the heart at any given time point, probably owing to the deletion of intronic sequences containing transcription factor binding sites for Isl1, Nkx2.5, Tbx5 and others (El Agha et al., 2012). Yet the possibility that a subset of *Fgf10*-positive cells arises from *Isl1*-positive cells coming from the SHF cannot be excluded.

FACS analysis revealed that the first wave of *Fgf10* expression accounts for ~0.5% of the total lung at E18.5, whereas the second

wave accounts for ~1.5% of the total E18.5 lung. This indicates that during embryonic lung development, not all *Fgf10*-expressing cells derive from *Fgf10*-positive mother cells; rather, the *Fgf10*-expressing domain expands by *de novo* induction of *Fgf10* expression (Fig. 8B). However, clonal expansion of *Fgf10*-positive cells needs to be further examined using confetti or rainbow Cre-reporter mice in order to assess the degree of heterogeneity of these cells and to determine whether they possess unipotent or multipotent capabilities. Flow cytometry analysis also revealed that a subpopulation of early and late lineage-labeled cells acquires *Pdgfra* expression at later developmental stages. Surprisingly, a subpopulation of late *Fgf10*-positive progenitors (second wave) also acquires *Epcam* expression. Epcam⁺ RFP⁺ cells were further analyzed following FACS and they showed lack of *Nkx2.1* and *E-Cad* expression, indicating that these cells do not represent a bona fide epithelial cell population. Yet they express significant levels of Pro-Spc, as shown by immunofluorescence (of lung sections and sorted cells) and RT-PCR, and appear to have lost *Fgf10* expression between P2 and P30. To date, there is no evidence for mesenchymal-to-epithelial transition events during lung development, and our previous work with the *Dermo1-Cre; R26R* line suggests that mesenchymal cells do not integrate into the lung epithelium (De Langhe et al., 2008; Tiozzo et al., 2012). Similarly, we show here that the *Fgf10*-expressing lineage is purely mesenchymal by nature and labeled cells were never detected in the lung epithelium. Whether these cells could 'completely' differentiate into bona fide epithelial cells under specific conditions (acute injury, for example) remains to be established.

Alveolar myofibroblasts and lipofibroblasts are two cell lineages that are highly abundant during postnatal alveolarization. These cell types play important roles in the formation of functional alveoli in newborns. In this study, the contribution of *Fgf10*-positive cells to these two lineages was addressed. When labeled during the alveolar

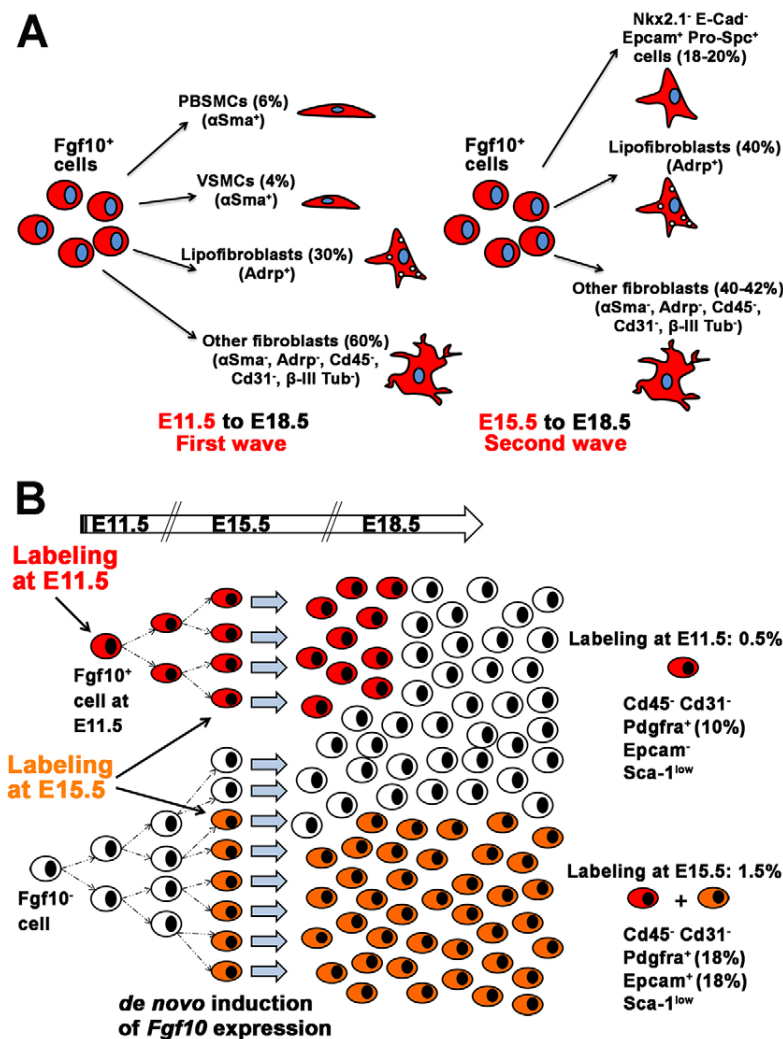


Fig. 8. The differentiation potential of *Fgf10*-positive cells varies throughout embryonic lung development.

(A) Model showing the differentiation capacity of *Fgf10*-positive cells labeled at E11.5 or E15.5 and analyzed at E18.5. (B) Model showing that the *Fgf10*-expressing domain expands by *de novo* induction of *Fgf10* expression.

stage of lung development, most *Fgf10*-expressing cells gave rise to lipofibroblasts that are believed to represent a stem cell niche that signals to adjacent type 2 alveolar epithelial cells in a paracrine fashion (Barkauskas et al., 2013). Furthermore, a minor population of *Fgf10*-expressing cells gave rise to myofibroblasts residing in the parenchyma rather than in secondary septae. Alveolar myofibroblasts are believed to derive from *Pdgfra*-positive cells as *Pdgfra*-null newborns suffer from arrest in alveologenesis because of the absence of these cells (Boström et al., 1996; Lindahl et al., 1997). More recently, McGowan et al. have shown that *Pdgfra* expression directly correlated with α Sma expression and inversely correlated with neutral lipid production (McGowan et al., 2008), indicating that *Pdgfra*-positive cells are progenitors for alveolar myofibroblasts rather than lipofibroblasts. Our FACS analysis shows that only ~10% of *Fgf10*-expressing cells co-express *Pdgfra* postnatally (data not shown), which could explain our observation that *Fgf10*-expressing cells preferentially give rise to lipofibroblasts rather than alveolar myofibroblasts (*Pdgfra*⁺). Lipofibroblasts originate from multiple progenitor pools and the *Fgf10*-expressing pool contributes to a subset of lipofibroblasts in the lung (D.A.A., E.E.A. and S.B., unpublished). Although it has been suggested that lipofibroblasts serve as a source of alveolar myofibroblasts during alveologenesis (Perl and Gale, 2009), our data suggest that at least *Fgf10*-expressing lipofibroblasts do not undergo this

transdifferentiation. However, as *Fgfr2b* ligands have shown to be required for the formation of alveolar myofibroblasts during homeostasis (Perl and Gale, 2009; Ramasamy et al., 2007) as well as regeneration after pneumonectomy (Chen et al., 2012), we speculate that paracrine signaling between *Fgf10*-positive cells and *Pdgfra*-positive progenitors is likely to be necessary for the formation of the alveolar myofibroblast lineage during alveologenesis.

Fgf10-positive cells were also examined during lung homeostasis in the adult mouse. Despite the low efficiency of the *Fgf10*^{Cre} line in labeling *Fgf10*-expressing cells postnatally (El Agha et al., 2012), FACS analysis of disaggregated adult lungs revealed that these cells constitute ~0.1% of the whole lung. A subpopulation of *Fgf10*-expressing cells (~10.5% of total *Fgf10*-expressing cells) exhibits the molecular signature of resident MSCs (Cd45⁻ Cd31⁻ Sca-1⁺) that were previously described by McQuilter et al. (McQuilter et al., 2009). In the latter study, the authors reported that the Cd45⁻ Cd31⁻ Sca-1⁺ fraction of the adult lung represents a mesenchymal population of resident progenitor cells that are also crucial for the growth of epithelial progenitors *in vitro*. Our data support the findings of McQuilter et al. and provide direct evidence that *Fgf10* expression identifies a subset of resident MSCs.

Finally, *Fgf10*-positive cells, labeled at E11.5, were retained after birth (P30), indicating that these cells have self-renewal

capabilities. Nevertheless, the majority of these cells stained for the neutral lipid dye, indicating their engagement into the lipofibroblast phenotype.

In summary, the reported genetic model of lineage tracing shows that *Fgf10*-expressing cells represent a population of mesenchymal progenitors in the lung *in vivo*. These cells give rise to airway and VSMCs as well as lipofibroblasts during embryonic development. Neonatally, these cells contribute to the formation of lipofibroblasts and during adult life, they represent a subset of resident MSCs that might play a crucial role in regeneration after lung injury. Our observation that some descendants of *Fgf10*-expressing cells express Pro-Spc shows how little is known about lineage hierarchy in the lung mesenchyme. More studies need to be performed in order to better characterize the various mesenchymal lineages in the lung, by combining lineage-tracing tools with responder lines allowing genetic manipulation of key signaling pathways (e.g. FGF, Wnt, Hedgehog), and eventually implementing the attained knowledge in novel therapeutic strategies for treating interstitial lung diseases.

MATERIALS AND METHODS

Mice and tamoxifen administration

Fgf10^{Cre} knock-in mice were generated as previously described (El Agha et al., 2012) and *tomato^{flax}* reporter mice (B6;129S6-*Gt(ROSA)26Sor^{tm9(CAG-tdTomato)Hze/J}*) were purchased from Jackson Lab (stock number 007905). *Fgf10-lacZ* hypomorph/reporter mice were described previously (Kelly et al., 2001; Mailloux et al., 2005; Ramasamy et al., 2007). Mice were housed in a specific pathogen-free environment and E0.5 was assigned to the day when a vaginal plug was detected. Harvesting organs and tissues from wild-type and transgenic mice was approved by Justus Liebig University Giessen (approval numbers 405_M and 437_M, respectively) and animal experiments were approved by the Regierungspräsidium Giessen (GI 20/10 Nr. 38/2011 and 117/2012) and the Animal Research Committee at Childrens Hospital Los Angeles (CHLA; Protocol number 31-11) in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The approval identification for CHLA is AAALAC A3276-01.

Tamoxifen solution was prepared as previously described (El Agha et al., 2012) and pregnant females carrying *Fgf10^{Cre/+}; tomato^{flax/+}* embryos received a single IP injection of 0.1 mg tamoxifen (Sigma, Schnelldorf, Germany) per gram of body weight. For continuous tamoxifen exposure, mice were fed tamoxifen-containing pellets (0.4 g of tamoxifen per kg of food) (Altromin, Lage, Germany).

Whole-mount fluorescence imaging and time-lapse microscopy

Lungs were harvested in PBS and examined using Leica M205 FA fluorescent stereoscope (Leica Microsystems, Wetzlar, Germany) equipped with Leica DFC360 FX camera. The endogenous tomato signal was detected using the RFP channel. For time-lapse microscopy, intact E12.5 lungs or E18.5 lung explants were harvested in Dulbecco's modified Eagle medium (DMEM) and cultured on Whatman Nuclepore membrane filters (GE Healthcare, Solingen, Germany) placed on 500 µl of DMEM with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Culture dishes with embryonic tissues were then transferred into the culture chamber (37°C; 5% CO₂) of Leica DM16000 B live imaging microscope (Leica Microsystems) connected to Leica DFC365 FX camera where imaging was performed. RFP signal intensity was measured using ImageJ software (NIH).

Tissue processing and immunostaining

Freshly isolated lungs were washed in PBS and then fixed in 4% paraformaldehyde (PFA) in PBS according to standard procedures. Then, tissues were embedded in paraffin and sectioned at 5 µm thickness. Slides were deparaffinized, blocked with 3% bovine serum albumin (BSA) and 0.4% Triton X-100 [in Tris-buffered saline (TBS)] at room temperature (RT) for 1 hour and then incubated with primary antibodies against αSma (Sigma; 1:100), Adipose differentiation-related protein (Adrp) (Abcam; 1:50), Pro-Spc (Seven Hills; 1:1000), E-Cadherin (BD; 1:200), β-III Tubulin (R&D; 1:50),

Cd45 or Cd31 (Biolegend; 1:100) at RT for 1 hour or at 4°C overnight. After incubation with primary antibodies, slides were washed three times in TBST (TBS buffer + 0.1% Tween 20) for 5 minutes, incubated with secondary antibodies at RT for 1 hour and then washed three times in TBST before being mounted with Prolong Gold Anti-fade Reagent with DAPI (4',6-diamidino-2-phenylindole; Invitrogen). Fluorescent images were acquired using Leica DM5500 B fluorescence microscope connected to Leica DFC360 FX camera and the endogenous tomato signal was detected using the RFP channel. Colocalization data were further confirmed by *z* stacks of multiple optical sections using Leica TCS SP5 X confocal microscope.

FACS analysis and sorting

Lungs were isolated, washed with Hanks' balanced salt solution (HBSS) and kept on ice. Then, they were cut into fine pieces using a sharp blade before being digested with 0.5% Collagenase Type IV in HBSS (Life Technologies, Invitrogen) at 37°C for 45 minutes. Single-cell suspensions were obtained by passing lung homogenates through 18, 21 and 24G syringes before being passed through 70 and 40 µm cell strainers (BD Biosciences, Heidelberg, Germany). Viable cell count was determined using Trypan Blue stain. Cells were then resuspended in cold staining solution (0.1% sodium azide/5% FCS/0.05% Triton X-100 in 1× PBS) containing antibodies (Biolegend, Fell, Germany) against Cd45 (FITC-conjugated; 1:100), Cd31 (FITC-conjugated; 1:100), Epcam (APC-Cy7-conjugated; 1:50), Sca-1 (Pacific Blue-conjugated; 1:100), Pdgfra (APC-conjugated; 1:100) and Kit (PE-Cy7-conjugated; 1:50) for 30 minutes on ice in the dark and were then washed with 0.1% sodium azide in PBS. LipidTOX staining was performed according to the manufacturer's instructions (Invitrogen). FACS measurements and sorting were carried out using a FACSAria III cell sorter (BD Biosciences) and the endogenous tomato signal was detected using the PE channel. Gates were set according to unstained controls.

Figure assembly

Quantitative data were assembled using GraphPad Prism software (GraphPad Software) and presented as average values ± s.e.m. Figures were assembled using Adobe Photoshop CS5.

Acknowledgements

We would like to thank Matthew Jones for his help in genotyping the mice used for these experiments, Jana Roskovius for contributing to the immunofluorescence protocol, Kerstin Goth for efficiently managing mouse colonies at the Justus Liebig University Giessen and Salma Dilai for her help with processing the movies. We also acknowledge Oleg Pak for his help with confocal microscopy. Finally, we would like to thank Jonathan Branch, Clarence Wigfall and Soula Danopoulos for their technical support at CHLA.

Competing interests

The authors declare no competing financial interests.

Author contributions

S.B., P.M. and W.S. conceived the study; E.E.A. and S.B. designed the experiments; S.B., S.H. and D.A.A. contributed to materials; E.E.A., D.A.A., J.Q., B.M., G.C., A.M. and C.-M.C. performed the experiments; E.E.A., S.H. and S.B. analyzed the data; E.E.A. and S.B. wrote the manuscript.

Funding

This work was funded by a German Research Foundation (DFG) grant [BE 4443/4-1 to S.B.]; a Landes-Offensive zur Entwicklung Wissenschaftlich-ökonomischer Exzellenz (LOEWE) grant (to S.B.); a Universities of Giessen and Marburg Lung Center (UGMLC) grant (to S.B. and S.H.); and by National Institutes of Health (NIH) grants [HL086322 to S.B.; and HL107307 to S.B. and P.M.]. P.M. was also supported by the Hastings Foundation. Deposited in PMC for release after 12 months.

Supplementary material

Supplementary material available online at
<http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.099747/-DC1>

References

Barkauskas, C. E., Cronic, M. J., Rackley, C. R., Bowie, E. J., Keene, D. R., Stripp, B. R., Randall, S. H., Noble, P. W. and Hogan, B. L. M. (2013). Type 2 alveolar cells are stem cells in adult lung. *J. Clin. Invest.* **123**, 3025-3036.

- Bellusci, S., Grindley, J., Emoto, H., Itoh, N. and Hogan, B. L. (1997). Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. *Development* **124**, 4867-4878.
- Boström, H., Willetts, K., Pekny, M., Levéen, P., Lindahl, P., Hedstrand, H., Pekna, M., Hellström, M., Gebre-Medhin, S., Schalling, M. et al. (1996). PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell* **85**, 863-873.
- Chen, L., Acciani, T., Le Cras, T., Lutzko, C. and Perl, A.-K. T. (2012). Dynamic regulation of platelet-derived growth factor receptor α expression in alveolar fibroblasts during realveolarization. *Am. J. Respir. Cell Mol. Biol.* **47**, 517-527.
- Crapo, J. D., Barry, B. E., Gehr, P., Bachofen, M. and Weibel, E. R. (1982). Cell number and cell characteristics of the normal human lung. *Am. Rev. Respir. Dis.* **126**, 332-337.
- De Langhe, S. P., Carraro, G., Tefft, D., Li, C., Xu, X., Chai, Y., Minoo, P., Hajhosseini, M. K., Drouin, J., Kaartinen, V., et al. (2008). Formation and differentiation of multiple mesenchymal lineages during lung development is regulated by beta-catenin signaling. *PLoS ONE* **3**, e1516.
- Del Moral, P. M., Sala, F. G., Tefft, D., Shi, W., Keshet, E., Bellusci, S. and Warburton, D. (2006). VEGF-A signaling through Flk-1 is a critical facilitator of early embryonic lung epithelial to endothelial crosstalk and branching morphogenesis. *Dev. Biol.* **290**, 177-188.
- Dixit, R., Ai, X. and Fine, A. (2013). Derivation of lung mesenchymal lineages from the fetal mesothelium requires hedgehog signaling for mesothelial cell entry. *Development* **140**, 4398-4406.
- El Agha, E., Al Alam, D., Carraro, G., MacKenzie, B., Goth, K., De Langhe, S. P., Vowsinkel, R., Hajhosseini, M. K., Rehan, V. K. and Bellusci, S. (2012). Characterization of a novel fibroblast growth factor 10 (Fgf10) knock-in mouse line to target mesenchymal progenitors during embryonic development. *PLoS ONE* **7**, e38452.
- Giangureco, A., Reynolds, S. D. and Stripp, B. R. (2002). Terminal bronchioles harbor a unique airway stem cell population that localizes to the bronchoalveolar duct junction. *Am. J. Pathol.* **161**, 173-182.
- Greif, D. M., Kumar, M., Lighthouse, J. K., Hum, J., An, A., Ding, L., Red-Horse, K., Espinoza, F. H., Olson, L., Offermanns, S. et al. (2012). Radial construction of an arterial wall. *Dev. Cell* **23**, 482-493.
- Haan, N., Goodman, T., Najdi-Samiei, A., Stratford, C. M., Rice, R., El Agha, E., Bellusci, S. and Hajhosseini, M. K. (2013). Fgf10-expressing tanycytes add new neurons to the appetite/energy-balance regulating centers of the postnatal and adult hypothalamus. *J. Neurosci.* **33**, 6170-6180.
- Hajhosseini, M. K., De Langhe, S., Lana-Elola, E., Morrison, H., Sparshott, N., Kelly, R., Sharpe, J., Rice, D. and Bellusci, S. (2008). Localization and fate of Fgf10-expressing cells in the adult mouse brain implicate Fgf10 in control of neurogenesis. *Mol. Cell. Neurosci.* **37**, 857-868.
- Hong, K. U., Reynolds, S. D., Watkins, S., Fuchs, E. and Stripp, B. R. (2004). Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium. *Am. J. Pathol.* **164**, 577-588.
- Kappel, A., Röncke, V., Damert, A., Flamme, I., Risau, W. and Breier, G. (1999). Identification of vascular endothelial growth factor (VEGF) receptor-2 (Flk-1) promoter/enhancer sequences sufficient for angioblast and endothelial cell-specific transcription in transgenic mice. *Blood* **93**, 4284-4292.
- Kelly, R. G., Brown, N. A. and Buckingham, M. E. (2001). The arterial pole of the mouse heart forms from Fgf10-expressing cells in pharyngeal mesoderm. *Dev. Cell* **1**, 435-440.
- Kim, C. F., Jackson, E. L., Woolfenden, A. E., Lawrence, S., Babar, I., Vogel, S., Crowley, D., Bronson, R. T. and Jacks, T. (2005). Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* **121**, 823-835.
- Langsdorf, A., Radzikas, K., Kroten, A., Jain, S. and Ai, X. (2011). Neural crest cell origin and signals for intrinsic neurogenesis in the mammalian respiratory tract. *Am. J. Respir. Cell Mol. Biol.* **44**, 293-301.
- Lindahl, P., Karlsson, L., Hellström, M., Gebre-Medhin, S., Willetts, K., Heath, J. K. and Betsholtz, C. (1997). Alveogenesis failure in PDGF-A-deficient mice is coupled to lack of distal spreading of alveolar smooth muscle cell progenitors during lung development. *Development* **124**, 3943-3953.
- Lü, J., Izvolosky, K. I., Qian, J. and Cardoso, W. V. (2005). Identification of FGF10 targets in the embryonic lung epithelium during bud morphogenesis. *J. Biol. Chem.* **280**, 4834-4841.
- Mailleux, A. A., Kelly, R., Veltmaat, J. M., De Langhe, S. P., Zaffran, S., Thiery, J. P. and Bellusci, S. (2005). Fgf10 expression identifies parabronchial smooth muscle cell progenitors and is required for their entry into the smooth muscle cell lineage. *Development* **132**, 2157-2166.
- McGowan, S. E., Grossmann, R. E., Kimani, P. W. and Holmes, A. J. (2008). Platelet-derived growth factor receptor- α -expressing cells localize to the alveolar entry ring and have characteristics of myofibroblasts during pulmonary alveolar septal formation. *Anat. Rec. (Hoboken)* **291**, 1649-1661.
- McQualter, J. L. and Bertoncello, I. (2012). Concise review: Deconstructing the lung to reveal its regenerative potential. *Stem Cells* **30**, 811-816.
- McQualter, J. L., Brouard, N., Williams, B., Baird, B. N., Sims-Lucas, S., Yuen, K., Nilsson, S. K., Simmons, P. J. and Bertoncello, I. (2009). Endogenous fibroblastic progenitor cells in the adult mouse lung are highly enriched in the sca-1 positive cell fraction. *Stem Cells* **27**, 623-633.
- Nyeng, P., Norgaard, G. A., Kobberup, S. and Jensen, J. (2008). FGF10 maintains distal lung bud epithelium and excessive signaling leads to progenitor state arrest, distalization, and goblet cell metaplasia. *BMC Dev. Biol.* **8**, 2.
- O'Hare, K. H. and Sheridan, M. N. (1970). Electron microscopic observations on the morphogenesis of the albino rat lung, with special reference to pulmonary epithelial cells. *Am. J. Anat.* **127**, 181-205.
- Park, W. Y., Miranda, B., Lebeche, D., Hashimoto, G. and Cardoso, W. V. (1998). FGF-10 is a chemotactic factor for distal epithelial buds during lung development. *Dev. Biol.* **201**, 125-134.
- Peng, T., Tian, Y., Boogerd, C. J., Lu, M. M., Kadzik, R. S., Stewart, K. M., Evans, S. M. and Morrissey, E. E. (2013). Coordination of heart and lung co-development by a multipotent cardiopulmonary progenitor. *Nature* **500**, 589-592.
- Perl, A.-K. T. and Gale, E. (2009). FGF signaling is required for myofibroblast differentiation during alveolar regeneration. *Am. J. Physiol.* **297**, L299-L308.
- Que, J., Wilm, B., Hasegawa, H., Wang, F., Bader, D. and Hogan, B. L. (2008). Mesothelium contributes to vascular smooth muscle and mesenchyme during lung development. *Proc. Natl. Acad. Sci. USA* **105**, 16626-16630.
- Ramasamy, S. K., Mailleux, A. A., Gupta, V. V., Mata, F., Sala, F. G., Veltmaat, J. M., Del Moral, P. M., De Langhe, S., Parsa, S., Kelly, L. K. et al. (2007). Fgf10 dosage is critical for the amplification of epithelial cell progenitors and for the formation of multiple mesenchymal lineages during lung development. *Dev. Biol.* **307**, 237-247.
- Rawlins, E. L., Clark, C. P., Xue, Y. and Hogan, B. L. M. (2009). The Id2+ distal tip lung epithelium contains individual multipotent embryonic progenitor cells. *Development* **136**, 3741-3745.
- Rehan, V. K. and Torday, J. S. (2012). PPAR γ signaling mediates the evolution, development, homeostasis, and repair of the lung. *PPAR Res.* **2012**, 289867.
- Rock, J. R., Onaitis, M. W., Rawlins, E. L., Lu, Y., Clark, C. P., Xue, Y., Randell, S. H. and Hogan, B. L. (2009). Basal cells as stem cells of the mouse trachea and human airway epithelium. *Proc. Natl. Acad. Sci. USA* **106**, 12771-12775.
- Rubin, L. P., Kovacs, C. S., De Paepe, M. E., Tsai, S.-W., Torday, J. S. and Kronenberg, H. M. (2004). Arrested pulmonary alveolar cytodifferentiation and defective surfactant synthesis in mice missing the gene for parathyroid hormone-related protein. *Dev. Dyn.* **230**, 278-289.
- Sakaue, H., Konishi, M., Ogawa, W., Asaki, T., Mori, T., Yamasaki, M., Takata, M., Ueno, H., Kato, S., Kasuga, M. et al. (2002). Requirement of fibroblast growth factor 10 in development of white adipose tissue. *Genes Dev.* **16**, 908-912.
- Shan, L., Subramaniam, M., Emanuel, R. L., Degan, S., Johnston, P., Tefft, D., Warburton, D. and Sunday, M. E. (2008). Centrifugal migration of mesenchymal cells in embryonic lung. *Dev. Dyn.* **237**, 750-757.
- Srinivasan, R. S., Dillard, M. E., Lagutin, O. V., Lin, F.-J., Tsai, S., Tsai, M.-J., Samokhvalov, I. M. and Oliver, G. (2007). Lineage tracing demonstrates the venous origin of the mammalian lymphatic vasculature. *Genes Dev.* **21**, 2422-2432.
- Stripp, B. R., Maxson, K., Mera, R. and Singh, G. (1995). Plasticity of airway cell proliferation and gene expression after acute naphthalene injury. *Am. J. Physiol.* **269**, L791-L799.
- Tiozzo, C., Carraro, G., Al Alam, D., Baptista, S., Danopoulos, S., Li, A., Lavarreda-Pearce, M., Li, C., De Langhe, S., Chan, B. et al. (2012). Mesodermal Pten inactivation leads to alveolar capillary dysplasia-like phenotype. *J. Clin. Invest.* **122**, 3862-3872.
- Torday, J. S. and Rehan, V. K. (2007). The evolutionary continuum from lung development to homeostasis and repair. *Am. J. Physiol.* **292**, L608-L611.
- Vaccaro, C. and Brody, J. S. (1978). Ultrastructure of developing alveoli. I. The role of the interstitial fibroblast. *Anat. Rec.* **192**, 467-479.
- Volckaert, T., Campbell, A., Dill, E., Li, C., Minoo, P. and De Langhe, S. (2013). Localized Fgf10 expression is not required for lung branching morphogenesis but prevents differentiation of epithelial progenitors. *Development* **140**, 3731-3742.
- Yamada, M., Kurihara, H., Kinoshita, K. and Sakai, T. (2005). Temporal expression of alpha-smooth muscle actin and drebrin in septal interstitial cells during alveolar maturation. *J. Histochem. Cytochem.* **53**, 735-744.
- Yamaguchi, T. P., Dumont, D. J., Conlon, R. A., Breitman, M. L. and Rossant, J. (1993). flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. *Development* **118**, 489-498.
- Yamasaki, M., Emoto, H., Konishi, M., Mikami, T., Ohuchi, H., Nakao, K. and Itoh, N. (1999). FGF-10 is a growth factor for preadipocytes in white adipose tissue. *Biochem. Biophys. Res. Commun.* **258**, 109-112.

RESEARCH ARTICLE

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

Denise Al Alam^{1,*}, Elie El Agha^{2,*}, Reiko Sakurai³, Vahid Kheirollahi², Alena Moiseenko², Soula Danopoulos¹, Amit Shrestha², Carole Schmoldt², Jennifer Quantius², Susanne Herold², Cho-Ming Chao², Caterina Tiozzo⁴, Stijn De Langhe⁵, Maksim V. Plikus⁶, Matthew Thornton⁷, Brendan Grubbs⁷, Parviz Minoo⁸, Virender K. Rehan^{3,†} and Saverio Bellusci^{1,2,9,‡}

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

¹Department of Surgery, Saban Research Institute of Children's Hospital Los Angeles, Los Angeles, CA 90027, USA. ²Excellence Cluster Cardio-Pulmonary System, Universities of Giessen and Marburg Lung Center, Member of the German Center for Lung Research, Department of Internal Medicine II, Klinikstrasse 36, Giessen, Hessen 35392, Germany. ³Department of Pediatrics, Los Angeles Biomedical Research Institute at Harbor-UCLA, Torrance, CA 90502, USA.

⁴Division of Neonatology, Department of Pediatrics, Columbia University, New York, NY 10027, USA. ⁵Department of Pediatrics, Division of Cell Biology, National Jewish Health, Denver, CO 80206, USA. ⁶Department of Developmental and Cell Biology, Sue and Bill Gross Stem Cell Research Center, Center for Complex Biological Systems, University of California Irvine, Irvine, CA 92697, USA. ⁷Maternal Fetal Medicine Division, University of Southern California, Los Angeles, CA 90033, USA. ⁸Division of Neonatal Medicine, University of Southern California, Los Angeles, CA 90033, USA. ⁹Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan 420008, Russia.

*These authors contributed equally to this work

†Authors for correspondence (vrehan@labiomed.org; saverio.bellusci@innere.med.uni-giessen.de)

Received 26 February 2014; Accepted 15 October 2015

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 retinoic 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 *Thy1*⁺ but not *Thy1*[−] 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 AECIIs (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 *Fgf10^{iCre}* 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 LIF formation was investigated, the results of which suggest that *Fgfr2b* compensates for the loss of *Fgfr1b*. The effect of recombinant FGF10 protein on mouse and human fetal lung mesenchymal cells was also tested. Taken together, our results demonstrate a novel role for mesenchymal *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 *Fgf10^{iCre/+};tomato^{lox/+}* 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 IF 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 Fgf10^{iCre/+};tomato^{lox}* (control) and *Fgf10^{iCre/lacZ};tomato^{lox}* (hypomorph) lungs (Fig. 3A). The *Fgf10^{iCre}* 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 *Fgf10^{iCre/lacZ};tomato^{lox}* embryos, thus validating phenotypically the reduced *Fgf10* expression (data not shown). The *Fgf10^{iCre/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 I,J). Quantification of the *Adrp* signal using MetaMorph software revealed a significant decrease in the number (Fig. 3M; *P*<0.05) and area (Fig. 3N; *P*<0.05) of *Adrp*

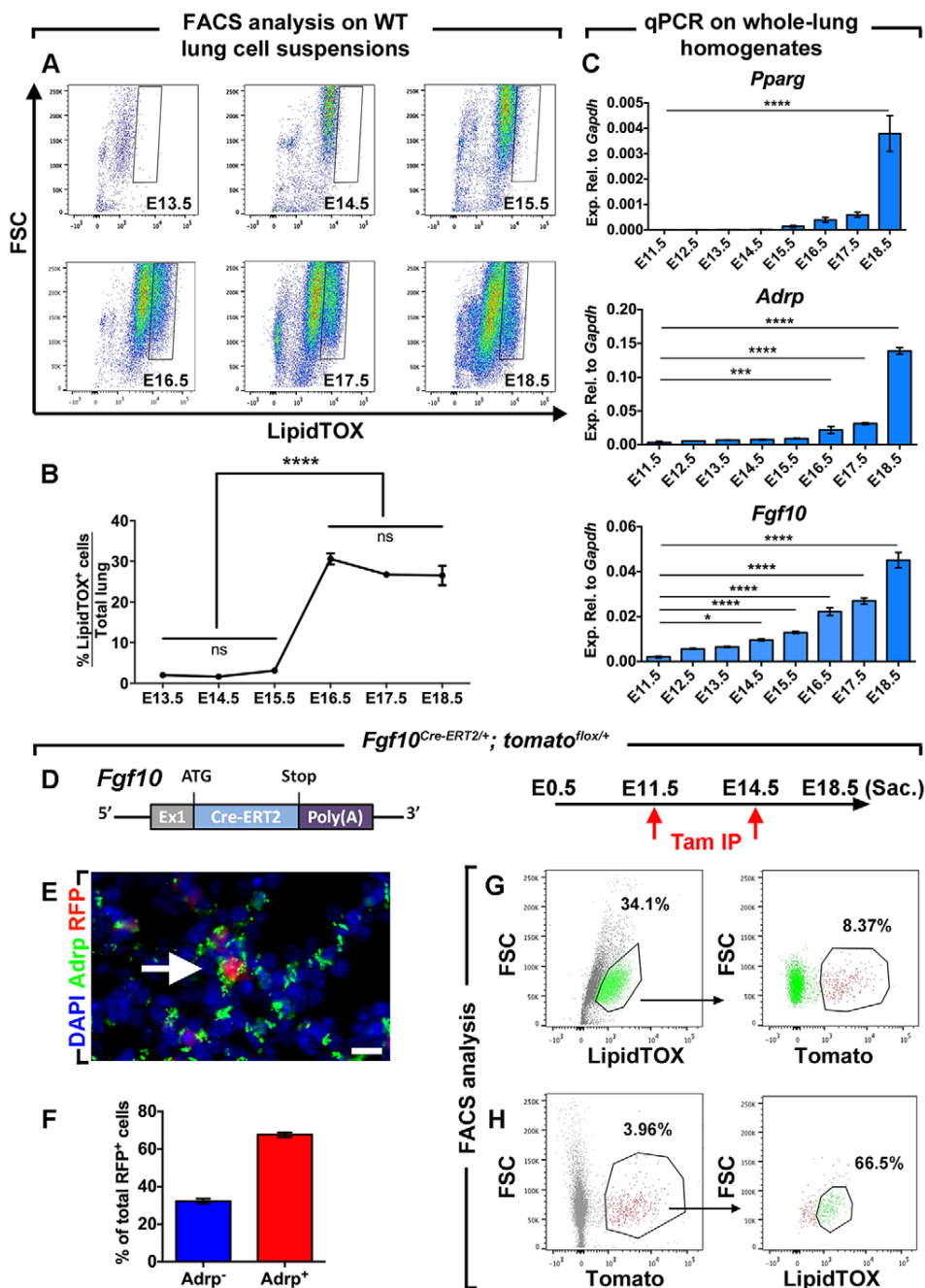


Fig. 1. Lipofibroblasts emerge in the mouse lung during the late pseudoglandular stage. (A) FACS analysis of LipidTOX-stained cell suspensions from embryonic CD1 lungs. Note the sudden increase in LipidTOX⁺ cells between E15.5 and E16.5. (B) Quantification of the FACS plots shown in A ($n=4$ per stage). (C) qPCR analysis showing the expression patterns of *Pparg*, *Adrp* and *Fgf10* during embryonic lung development ($n=3$ per stage). (D) Schematic representation of the *Fgf10*^{Cre} mouse construct and the time line of tamoxifen treatment and embryo harvest. (E) IF for Adrp (green). The endogenous Tomato signal was detected using the RFP channel (red). The arrow indicates an Adrp⁺ RFP⁺ cell. (F) Quantification of IF shown in E. (G) FACS analysis showing that RFP⁺ cells represent 8.37% of total LipidTOX⁺ cells. (H) FACS analysis showing that 66.5% of RFP⁺ cells are also LipidTOX⁺. Scale bar: 10 μ m. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$. Tam IP, intraperitoneal injection of tamoxifen; FSC, forward scatter; Sac., sacrifice.

immunoreactive spots in hypomorphic lungs ($n=3$) compared with littermate controls ($n=3$).

The *Tomato*^{flox} reporter allele was introduced in the background of *Fgf10*^{Cre/+} (control) and *Fgf10*^{Cre/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 hypomorph 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

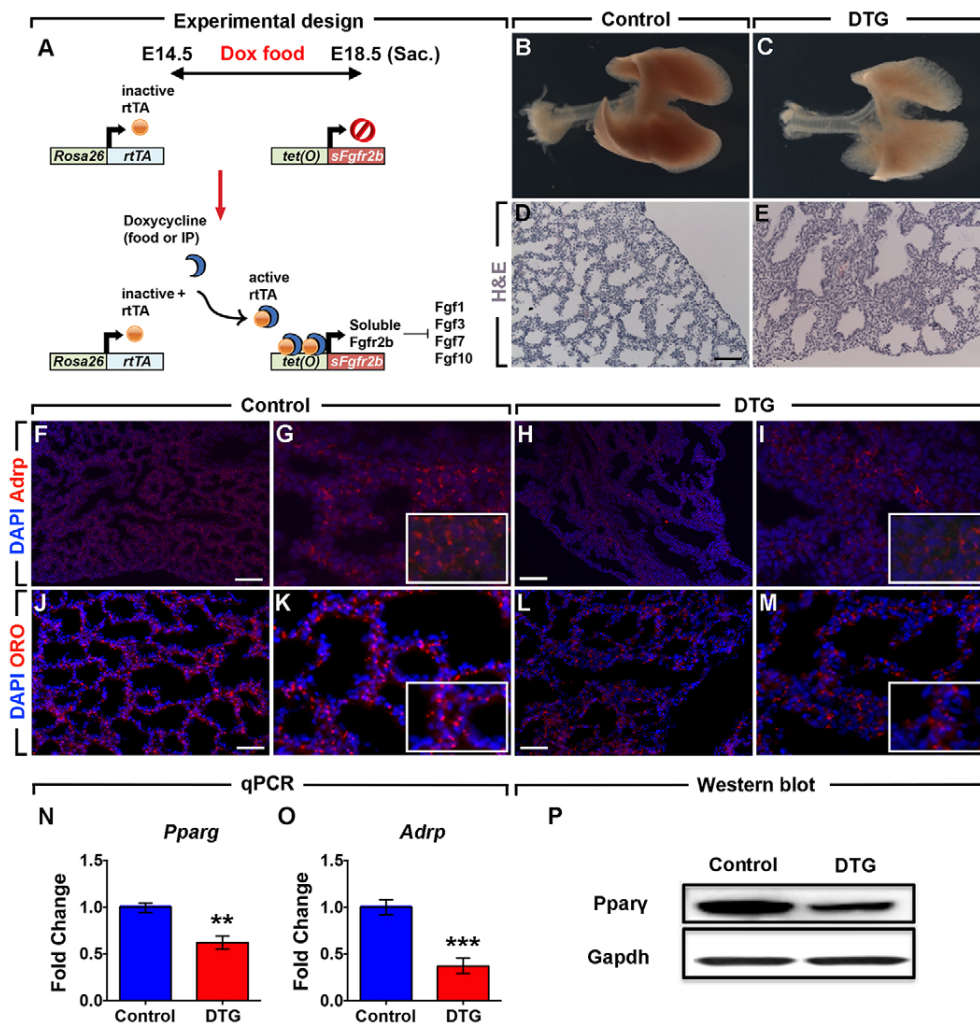


Fig. 2. Attenuation of *Fgfr2b* ligand activity leads to impaired LIF formation. (A) Inducible mouse model used to overexpress the soluble form of *Fgfr2b* receptor acting as a decoy receptor for *Fgfr2b* ligands between E14.5 and E18.5. (B, C) Whole-mount views of control (B) and DTG (C) lungs. (D, 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) ORO staining of frozen sections from control lungs. (K) High magnification of J. The inset in K shows a higher magnification. (L) ORO staining of frozen sections from DTG lungs. (M) High magnification of L. The inset in M shows a higher magnification. (N, O) 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 μ m in D, J, L; 100 μ m in F, H. $n=4$ per group. ** $P<0.01$, *** $P<0.001$. DTG, double transgenic.

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 *Pparg* 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. 5L, M) compared with controls (Fig. 5J, K). 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.

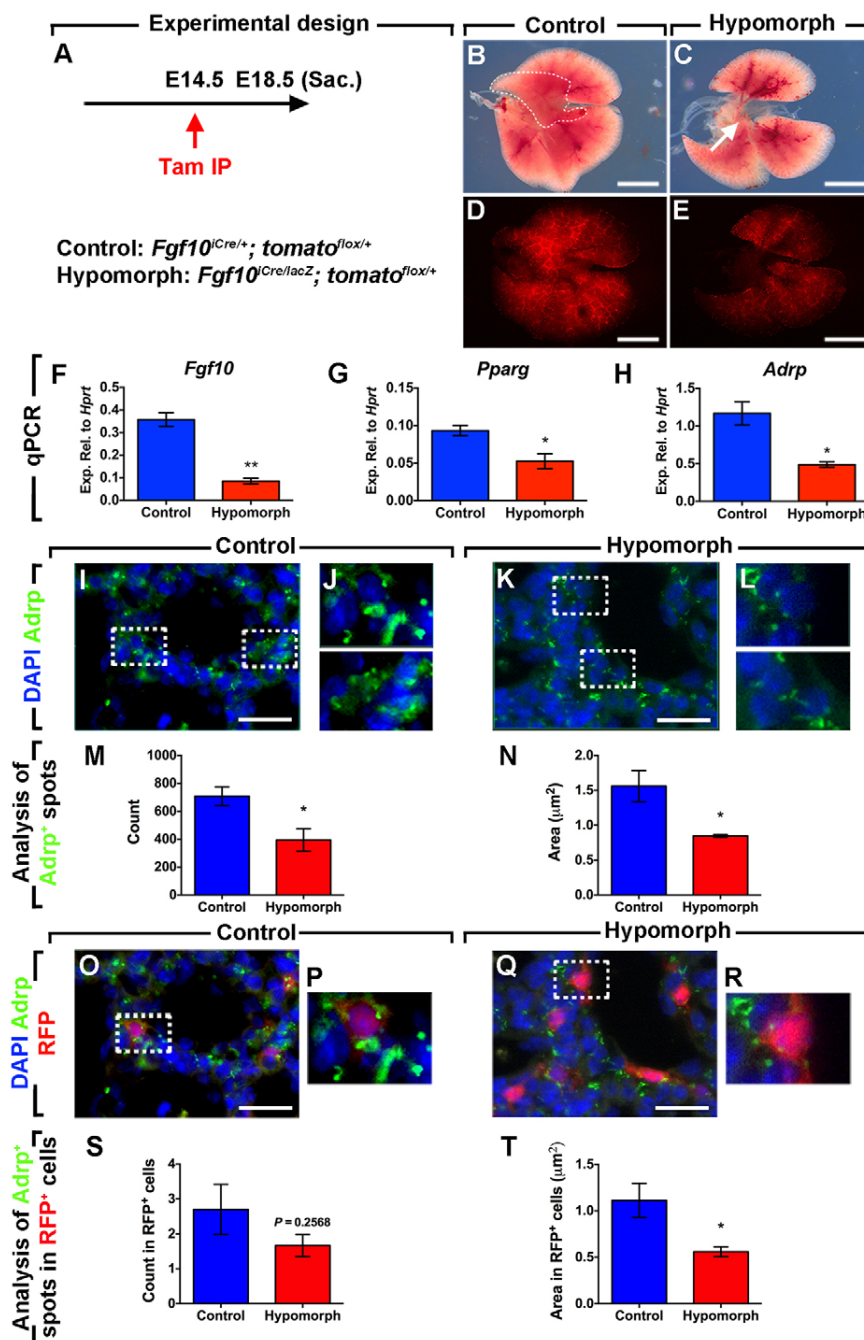


Fig. 3. *Fgf10* hypomorphs exhibit impaired LIF formation. (A) Experimental design showing the time line of tamoxifen treatment and tissue harvest. The control and hypomorph genotypes are shown. (B,C) Whole-mount views of control and hypomorph E18.5 lungs. Note the absence of the accessory lobe in the hypomorph (arrow in C). (D,E) Whole-mount fluorescent images of the lungs showing Tomato expression. (F-H) qPCR analysis showing decreased expression of *Fgf10*, *Pparg* and *Adrp* in the hypomorph lungs compared with control lungs. (I) IF for Adrp in control lungs. (J) High magnification of two fields from I. (K) IF for Adrp in hypomorph lungs. (L) High magnification of two fields from K. (M,N) Quantification of Adrp immunoreactivity shown in I-L using MetaMorph software. (O) IF for Adrp in control lungs showing Tomato expression. (P) High magnification of O. (Q) IF for Adrp in hypomorph lungs showing Tomato expression. (R) High magnification of Q. (S,T) Quantification of Adrp immunoreactivity in the lineage-labeled cells shown in O-R. Scale bars: 750 μ m in B-E; 25 μ m in I,K,O,Q. $n=3$ per group. * $P<0.05$, ** $P<0.01$.

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)/+*; *fgfr2b*^{+/+} (control) and *Tg(Tbx4-LMECreERT2)/+*; *fgfr2b*^{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 *Fgfr2b*^{lox} 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

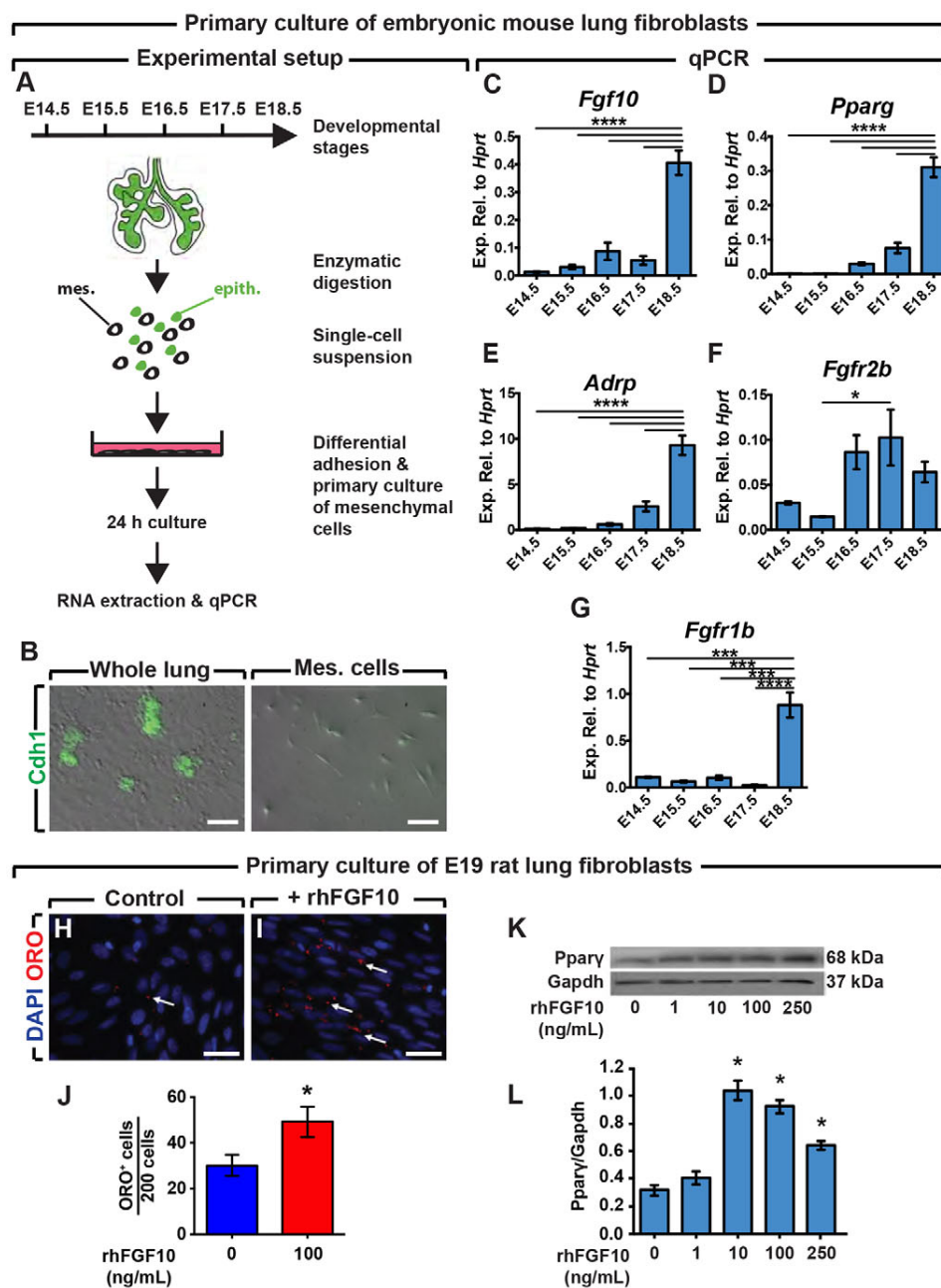


Fig. 4. Fgf10 acts directly on mesenchymal cells. (A) Time line for harvesting embryonic lungs and the subsequent experimental plan. (B) IF for Cdh1 showing minimal epithelial contamination in the primary culture of embryonic lung mesenchyme. (C–G) qPCR analysis for *Fgf10*, *Pparg*, *Adrp*, *Fgfr2b* and *Fgfr1b*. (H) ORO staining of control and rhFGF10-treated primary E19 rat lung fibroblasts. Note the increase in the number of ORO⁺ cells in rhFGF10-treated cells (white arrows). (I) Quantification of the ORO signal shown in H, I. (J) Western blot showing upregulation of Pparg upon treatment with increasing concentrations of rhFGF10 (from 1 to 250 ng/ml). (L) Densitometric analysis of the western blot data shown in J. Scale bars: 50 μ m in B; 25 μ m in H, I. $3 \leq n \leq 6$. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$. Mes, mesenchyme.

(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 ($3 \leq n \leq 11$ 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

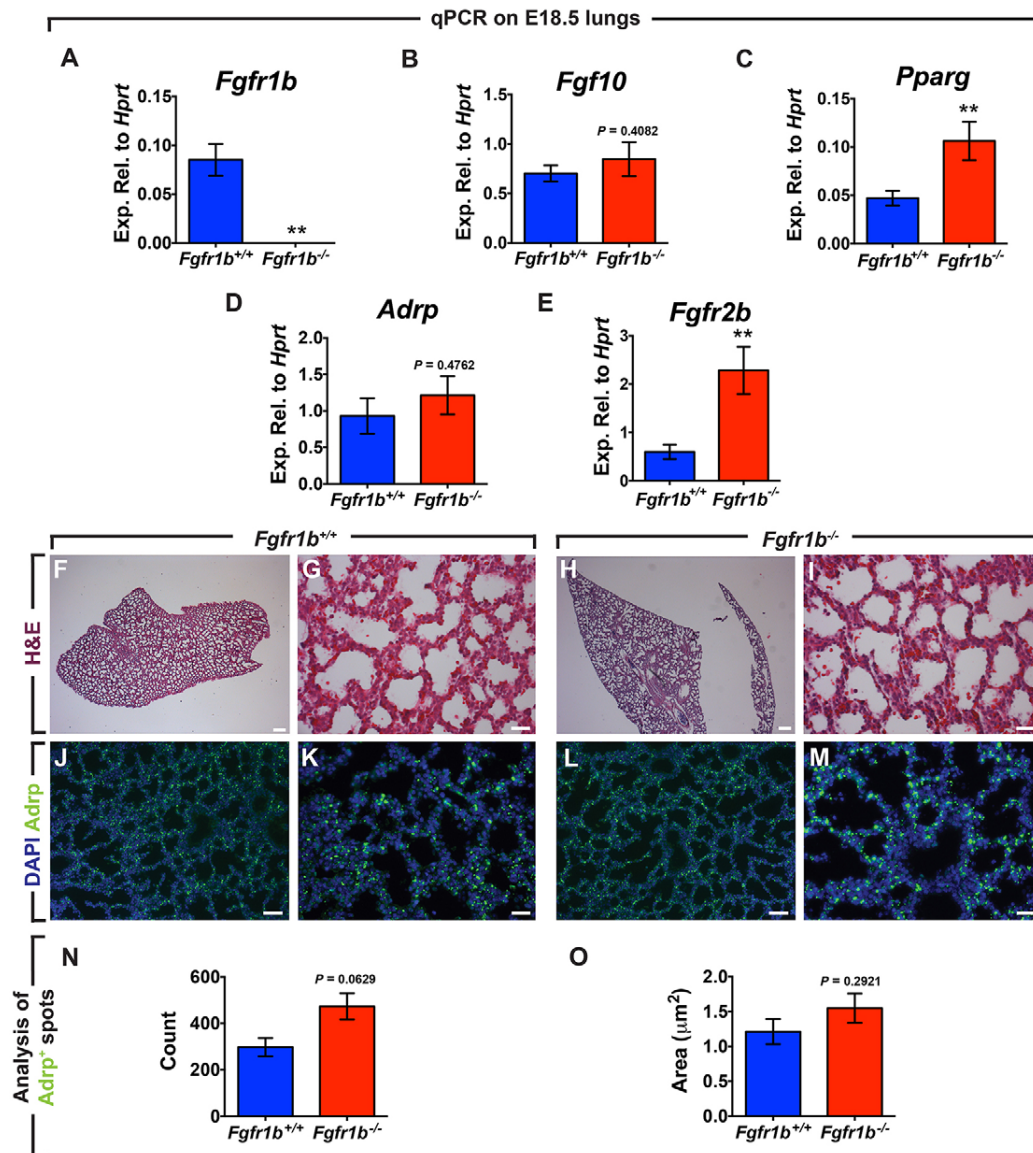


Fig. 5. *Fgfr1b* knockouts suggest compensation with *Fgfr2b*. (A–E) qPCR analysis of *Fgfr1b*, *Fgf10*, *Pparg*, *Adrp* and *Fgfr2b* in *Fgfr1b*^{-/-} lungs compared with littermate controls. (F–I) H&E staining showing no apparent histological differences between control (F,G) and *Fgfr1b* knockout lungs (H,I). (J–M) IF for *Adrp* showing a slight increase in immunoreactivity in *Fgfr1b* knockout lungs (L,M) compared with control lungs (J,K). (N,O) Quantification of *Adrp* immunoreactivity using MetaMorph software. Scale bars: 200 μm in F,H; 25 μm in G,I,K,M; 50 μm in J,L. (A–E) *Fgfr1b*^{+/+}, $n=7$; *Fgfr1b*^{-/-}, $n=4$. (N,O) *Fgfr1b*^{+/+}, $n=3$; *Fgfr1b*^{-/-}, $n=3$. ** $P<0.01$.

increasing concentrations of rhFGF10 also led to a dose-dependent increase in PPAR γ (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^{iCre}* 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

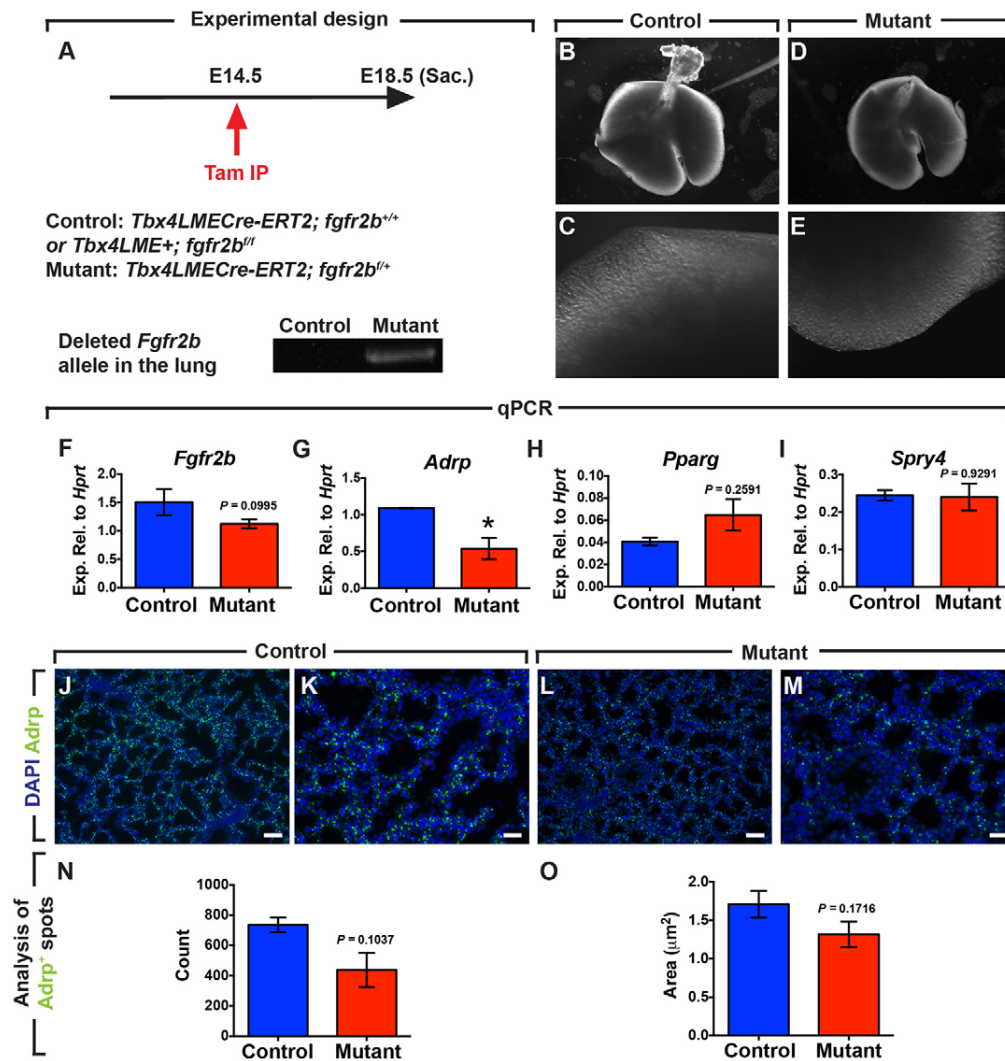


Fig. 6. Conditional deletion of *Fgfr2b* in the lung mesenchyme mildly impairs lipofibroblast formation. (A) Schematic representation of the time line of tamoxifen treatment and tissue harvest. Control and mutant genotypes are defined, and the detection of the deleted *Fgfr2b* allele is also shown. (B–E) Whole-mount view of control and mutant lungs showing no apparent phenotype. (F–I) qPCR analysis showing a trend towards decreased *Fgfr2b*, a statistically significant decrease in *Adrp* and unchanged *Pparg* and *Spry4* expression levels in mutant lungs compared with control lungs. (J–M) IF for Adrp showing a slight decrease in immunoreactivity in mutant lungs (L, M) compared with control lungs (J, K). (N, O) Quantification of Adrp immunoreactivity using MetaMorph software. Scale bars: 50 μm in J, L; 25 μm in K, M. $n=3$ for controls and $n=5$ for mutants. $*P<0.05$.

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 *Fgf10^{Cre}* 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 *Fgf10^{Cre/+};tomato^{lox/+}*

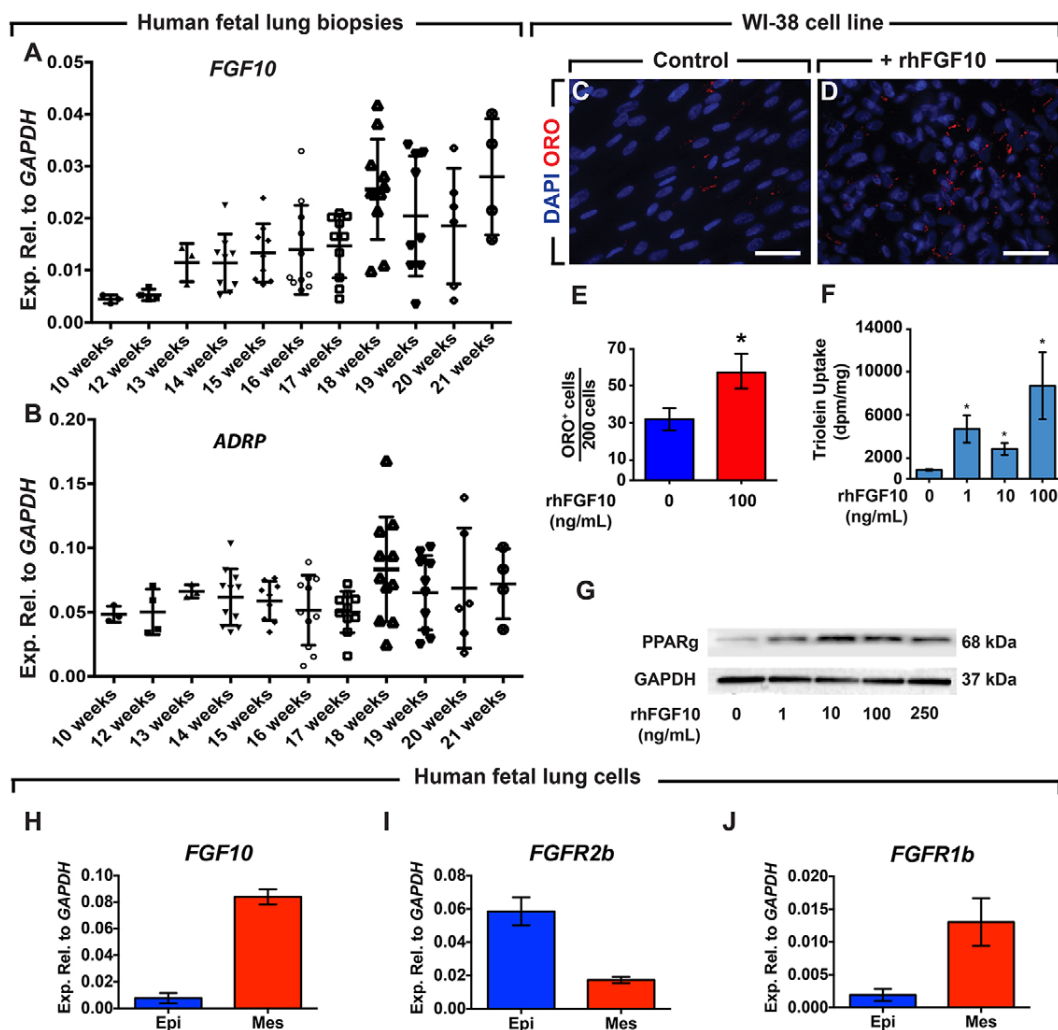


Fig. 7. FGF10 promotes lipofibroblast formation in human mesenchyme. (A,B) Expression levels of *FGF10* and *ADRP* in human fetal lungs. (C,D) ORO staining of untreated and rhFGF10-treated WI-38 cells. The increase in ORO⁺ cells is quantified in E. (F) Triolein uptake measured in protein extracts from WI-38 cells cultured for 24 h with serum-free media in the absence or the presence of rhFGF10 (1, 10 and 100 ng/ml). (G) Western blot for PPARγ and GAPDH in WI-38 cells cultured for 24 h with serum-free media in the absence or presence of rhFGF10. (H–J) qPCR for *FGF10*, *FGFR2b* and *FGFR1b* in epithelial (Epi) and mesenchymal (Mes) cells isolated from human lungs at 16 weeks gestational age. Scale bars: 25 μm. (A,B) 3 ≤ n ≤ 11; (E–G) n = 3; (H–J) n = 2 per group. *P < 0.05.

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*⁺ and *Fgf10*[−]) 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 phenotypic similarities between *Fgfr2b*-null pups (De Moerloose 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 *Sftpc* and *Scgb1a1* in the various mutants by qPCR (Fig. S3). *Sftpc* 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 *Sftpc*⁺ 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 understudied 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 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 *Rosa26^{rtTA/+};tet(O)sFgfr2b/+* double heterozygous mice were previously described (Parsa et al., 2008). To attenuate *Fgfr2b* 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 *Fgf10^{iCre}* mice crossed with *Tomato^{lox/lox}*

reporter mice as previously described (El Agha et al., 2012, 2014). Females carrying *Fgf10^{iCre};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, *Fgf10^{iCre/+};tomato^{lox/lox}* mice were crossed with *Fgf10-lacZ/+* mice (Kelly et al., 2001) to generate *Fgf10^{iCre/+};tomato^{lox/+}* (control) and *Fgf10^{iCre};tomato^{lox/+}* (hypomorph) embryos. The *Fgf10^{iCre}* 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* (Ramamany et al., 2007).

X-gal staining

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

FACS sorting

Lungs from E18.5 *Fgf10^{iCre};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 strained 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), Sftpc (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 40× 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 monocular camera attached to a Leica DM4000B microscope. Bright-field and differential interference contrast images were acquired on using 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 and incubated at 37°C for 10 min with an enzyme mix from the Miltenyi human tumor dissociation kit. The dissociation was then completed using

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 10⁷ 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 mesenchymal fractions 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 PCR (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]triolein (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 lipid 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.

Acknowledgements

We thank Salma Dilai for her help with MetaMorph software. We would also like to thank Drs Melissa L. Wilson (Department of Preventive Medicine, University of Southern California) and Rachel Steward (Department of Obstetrics and Gynecology, University of Southern California) for coordinating the efforts to obtain human fetal lungs.

Competing interests

The authors declare no competing or financial interests.

Author contributions

S.B., D.A.A., E.E.A., S.D.L., P.M. and V.K.R. developed the concepts or approach. D.A.A., E.E.A., R.S., V.K., A.M., S.D., A.S., C.S., J.Q., C.-M.C., S.D.L., M.T., B.G. and S.B. performed experiments or data analysis. S.B., D.A.A., E.E.A., S.H., C.T., S.D.L., M.V.P., P.M. and V.R. prepared or edited the manuscript prior to submission.

Funding

S.B., E.E.A., J.Q., C.S. and S.H. acknowledge the support from the German Lung Center for Lung Research, the Excellence Cluster Cardio-Pulmonary System, the Uniklinikum Giessen und Marburg and the Universities of Giessen and Marburg Lung Center. S.B. acknowledges the support from the Landes-Offensive zur Entwicklung wissenschaftlich-ökonomischer Exzellenz and Deutsche Forschungsgemeinschaft [projects BE 4443/1-1 and BE 443/4-1], the National Institutes of Health/National Heart, Lung, and Blood Institute [1R01HL086322-01A2], COST [BM1201] and the program of competitive growth of Kazan Federal

University. J.Q., C.S. and S.H. also acknowledge the Deutsche Forschungsgemeinschaft [project SFB1021 C05]. V.K.R. acknowledges the support of the National Institutes of Health [R01HD051857, R21HD071731 and R21HL107118]. D.A.A. acknowledges the support of American Lung Association [RTF09] and American Heart Association [11POST7440038]. P.M. is Hastings Professor of Pediatrics and acknowledges the support of National Institutes of Health/National Heart, Lung, and Blood Institute [HL107307]. S.D.L. acknowledges the support of National Institutes of Health/National Heart, Lung, and Blood Institute [R01HL092967], March of Dimes [1-FY15-352] and the Pulmonary Fibrosis Foundation [The Albert Rose established investigator award]. M.V.P. acknowledges support of the Edward Mallinckrodt Jr Foundation Research Grant and National Institutes of Health/National Institute of Arthritis and Musculoskeletal and Skin Diseases [R01AR067273]. Deposited in PMC for release after 12 months.

Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.109173/-DC1>

References

- Al Alam, D., Green, M., Tabatabai Irani, R., Parsa, S., Danopoulos, S., Sala, F. G., Branch, J., El Agha, E., Tiozzo, C., Voswinckel, R. et al. (2011). Contrasting expression of canonical Wnt signaling reporters TOPGAL, BATGAL and Axin2 (LacZ) during murine lung development and repair. *PLoS ONE* **6**, e23139.
- Asaki, T., Konishi, M., Miyake, A., Kato, S., Tomizawa, M. and Itoh, N. (2004). Roles of fibroblast growth factor 10 (Fgf10) in adipogenesis in vivo. *Mol. Cell. Endocrinol.* **218**, 119–128.
- Barkauskas, C. E., Crouse, M. J., Rackley, C. R., Bowie, E. J., Keene, D. R., Stripp, B. R., Randell, S. H., Noble, P. W. and Hogan, B. L. M. (2013). Type 2 alveolar cells are stem cells in adult lung. *J. Clin. Invest.* **123**, 3025–3036.
- Bellusci, S., Grindley, J., Emoto, H., Itoh, N. and Hogan, B. L. (1997). Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. *Development* **124**, 4867–4878.
- Berry, R. and Rodeheffer, M. S. (2013). Characterization of the adipocyte cellular lineage in vivo. *Nat. Cell Biol.* **15**, 302–308.
- De Langhe, S. P., Carraro, G., Warburton, D., Hajihosseini, M. K. and Bellusci, S. (2006). Levels of mesenchymal FGFR2 signaling modulate smooth muscle progenitor cell commitment in the lung. *Dev. Biol.* **299**, 52–62.
- De Moerloose, L., Spencer-Dene, B., Revest, J. M., Hajihosseini, M., Rosewell, I. and Dickson, C. (2000). An important role for the IIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. *Development* **127**, 483–492.
- El Agha, E., Al Alam, D., Carraro, G., MacKenzie, B., Goth, K., De Langhe, S. P., Voswinckel, R., Hajihosseini, M. K., Rehan, V. K. and Bellusci, S. (2012). Characterization of a novel fibroblast growth factor 10 (Fgf10) knock-in mouse line to target mesenchymal progenitors during embryonic development. *PLoS ONE* **7**, e38452.
- El Agha, E., Herold, S., Al Alam, D., Quantius, J., MacKenzie, B., Carraro, G., Moiseenko, A., Chao, C.-M., Minoo, P., Seeger, W. et al. (2014). Fgf10-positive cells represent a progenitor cell population during lung development and postnatally. *Development* **141**, 296–306.
- Ferguson, H. E., Thatcher, T. H., Olsen, K. C., Garcia-Bates, T. M., Bagloli, C. J., Kottmann, R. M., Strong, E. R., Phipps, R. P. and Sime, P. J. (2009). Peroxisome proliferator-activated receptor-γ ligands induce heme oxygenase-1 in lung fibroblasts by a PPARγ-independent, glutathione-dependent mechanism. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **297**, L912–L919.
- Guo, L., Degenstein, L. and Fuchs, E. (1996). Keratinocyte growth factor is required for hair development but not for wound healing. *Genes Dev.* **10**, 165–175.
- Jonker, J. W., Suh, J. M., Atkins, A. R., Ahmadian, M., Li, P., Whyte, J., He, M., Juguilon, H., Yin, Y.-Q., Phillips, C. T. et al. (2012). A PPARγ-FGF1 axis is required for adaptive adipose remodelling and metabolic homeostasis. *Nature* **485**, 391–394.
- Kaplan, N. B., Grant, M. M. and Brody, J. S. (1985). The lipid interstitial cell of the pulmonary alveolus. Age and species differences. *Am. Rev. Respir. Dis.* **132**, 1307–1312.
- Karadag, A., Sakurai, R., Wang, Y., Guo, P., Desai, M., Ross, M. G., Torday, J. S. and Rehan, V. K. (2009). Effect of maternal food restriction on fetal rat lung lipid differentiation program. *Pediatr. Pulmonol.* **44**, 635–644.
- Kelly, R. G., Brown, N. A. and Buckingham, M. E. (2001). The arterial pole of the mouse heart forms from Fgf10-expressing cells in pharyngeal mesoderm. *Dev. Cell* **1**, 435–440.
- Konishi, M., Asaki, T., Koike, N., Miwa, H., Miyake, A. and Itoh, N. (2006). Role of Fgf10 in cell proliferation in white adipose tissue. *Mol. Cell. Endocrinol.* **249**, 71–77.
- Lebeche, D., Malpel, S. and Cardoso, W. V. (1999). Fibroblast growth factor interactions in the developing lung. *Mech. Dev.* **86**, 125–136.
- Lee, J.-M., Kim, J.-Y., Cho, K.-W., Lee, M.-J., Cho, S.-W., Kwak, S., Cai, J. and Jung, H.-S. (2008). Wnt11/Fgfr1b cross-talk modulates the fate of cells in palate development. *Dev. Biol.* **314**, 341–350.

- Mailleux, A. A., Spencer-Dene, B., Dillon, C., Ndiaye, D., Savona-Baron, C., Itoh, N., Kato, S., Dickson, C., Thiery, J. P. and Bellusci, S. (2002). Role of FGF10/FGFR2b signaling during mammary gland development in the mouse embryo. *Development* **129**, 53–60.
- Mailleux, A. A., Kelly, R., Veltmaat, J. M., De Langhe, S. P., Zaffran, S., Thiery, J. P. and Bellusci, S. (2005). Fgf10 expression identifies parabronchial smooth muscle cell progenitors and is required for their entry into the smooth muscle cell lineage. *Development* **132**, 2157–2166.
- McGowan, S. E. and McCoy, D. M. (2015). Fibroblast growth factor signaling in myofibroblasts differs from lipofibroblasts during alveolar septation in mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **309**, L463–L474.
- McGowan, S. E. and Torday, J. S. (1997). The pulmonary lipofibroblast (lipid interstitial cell) and its contributions to alveolar development. *Annu. Rev. Physiol.* **59**, 43–62.
- McIntosh, J. C., Hagood, J. S., Richardson, T. L. and Simecka, J. W. (1994). Thy1 (+) and (–) lung fibrosis subpopulations in LEW and F344 rats. *Eur. Respir. J.* **7**, 2131–2138.
- O'Hare, K. H. and Sheridan, M. N. (1970). Electron microscopic observations on the morphogenesis of the albino rat lung, with special reference to pulmonary epithelial cells. *Am. J. Anat.* **127**, 181–205.
- Ohta, H. and Itoh, N. (2014). Roles of FGFs as adipokines in adipose tissue development, remodeling, and metabolism. *Front. Endocrinol.* **5**, 18.
- Ornitz, D. M., Xu, J., Colvin, J. S., McEwen, D. G., MacArthur, C. A., Coulier, F., Gao, G. and Goldfarb, M. (1996). Receptor specificity of the fibroblast growth factor family. *J. Biol. Chem.* **271**, 15292–15297.
- Parsa, S., Ramasamy, S. K., De Langhe, S., Gupte, V. V., Haigh, J. J., Medina, D. and Bellusci, S. (2008). Terminal end bud maintenance in mammary gland is dependent upon FGFR2b signaling. *Dev. Biol.* **317**, 121–131.
- Parsa, S., Kuremoto, K.-I., Seidel, K., Tabatabai, R., MacKenzie, B., Yamaza, T., Akiyama, K., Branch, J., Koh, C. J., Al Alam, D. et al. (2010). Signaling by FGFR2b controls the regenerative capacity of adult mouse incisors. *Development* **137**, 3743–3752.
- Penney, D. P., Keng, P. C., Derdak, S. and Phipps, R. P. (1992). Morphologic and functional characteristics of subpopulations of murine lung fibroblasts grown in vitro. *Anat. Rec.* **232**, 432–443.
- Phipps, R. P., Baecher, C., Frelinger, J. G., Penney, D. P., Keng, P. and Brown, D. (1990). Differential expression of interleukin 1 alpha by Thy-1+ and Thy-1– lung fibroblast subpopulations: enhancement of interleukin 1 alpha production by tumor necrosis factor-alpha. *Eur. J. Immunol.* **20**, 1723–1727.
- Qiao, J., Uzzo, R., Obara-Ishihara, T., Degenstein, L., Fuchs, E. and Herzlinger, D. (1999). FGF-7 modulates uretric bud growth and nephron number in the developing kidney. *Development* **126**, 547–554.
- Ramasamy, S. K., Mailleux, A. A., Gupte, V. V., Mata, F., Sala, F. G., Veltmaat, J. M., Del Moral, P. M., De Langhe, S., Parsa, S., Kelly, L. K. et al. (2007). Fgf10 dosage is critical for the amplification of epithelial cell progenitors and for the formation of multiple mesenchymal lineages during lung development. *Dev. Biol.* **307**, 237–247.
- Rehan, V. K. and Torday, J. (2003). Hyperoxia augments pulmonary lipofibroblast-to-myofibroblast transdifferentiation. *Cell Biochem. Biophys.* **38**, 239–250.
- Rehan, V. K., Sugano, S., Wang, Y., Santos, J., Romero, S., Dasgupta, C., Keane, M. P., Stahlman, M. T. and Torday, J. S. (2006). Evidence for the presence of lipofibroblasts in human lung. *Exp. Lung Res.* **32**, 379–393.
- Rehan, V. K., Sakurai, R., Wang, Y., Santos, J., Huynh, K. and Torday, J. S. (2007). Reversal of nicotine-induced alveolar lipofibroblast-to-myofibroblast transdifferentiation by stimulants of parathyroid hormone-related protein signaling. *Lung* **185**, 151–159.
- Rubin, L. P., Kovacs, C. S., De Paepe, M. E., Tsai, S.-W., Torday, J. S. and Kronenberg, H. M. (2004). Arrested pulmonary alveolar cytodifferentiation and defective surfactant synthesis in mice missing the gene for parathyroid hormone-related protein. *Dev. Dyn.* **230**, 278–289.
- Sakaue, H., Konishi, M., Ogawa, W., Asaki, T., Mori, T., Yamasaki, M., Takata, M., Ueno, H., Kato, S., Kasuga, M. et al. (2002). Requirement of fibroblast growth factor 10 in development of white adipose tissue. *Genes Dev.* **16**, 908–912.
- Schultz, C. J., Torres, E., Londos, C. and Torday, J. S. (2002). Role of adipocyte differentiation-related protein in surfactant phospholipid synthesis by type II cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **283**, L288–L296.
- Sekine, K., Ohuchi, H., Fujiwara, M., Yamasaki, M., Yoshizawa, T., Sato, T., Yagishita, N., Matsui, D., Koga, Y., Itoh, N. et al. (1999). Fgf10 is essential for limb and lung formation. *Nat. Genet.* **21**, 138–141.
- Simon, D. M. and Mariani, T. J. (2007). Role of PPARs and retinoid X receptors in the regulation of lung maturation and development. *PPAR Res.* **2007**, 91240.
- Torday, J. S., Torres, E. and Rehan, V. K. (2003). The role of fibroblast transdifferentiation in lung epithelial cell proliferation, differentiation, and repair in vitro. *Pediatr. Pathol. Mol. Med.* **22**, 189–207.
- Tordet, C., Marin, L. and Dameron, F. (1981). Pulmonary di-and-triacylglycerols during the perinatal development of the rat. *Experientia* **37**, 333–334.
- Tran, K.-V., Gealekman, O., Frontini, A., Zingaretti, M. C., Morroni, M., Giordano, A., Smorlesi, A., Perugini, J., De Matteis, R., Sbarbati, A. et al. (2012). The vascular endothelium of the adipose tissue gives rise to both white and brown fat cells. *Cell Metab.* **15**, 222–229.
- Varisco, B. M., Ambalavanan, N., Whitsett, J. A. and Hagood, J. S. (2012). Thy-1 signals through PPARgamma to promote lipofibroblast differentiation in the developing lung. *Am. J. Respir. Cell Mol. Biol.* **46**, 765–772.
- Volckaert, T., Dill, E., Campbell, A., Tiozzo, C., Majka, S., Bellusci, S. and De Langhe, S. P. (2011). Parabronchial smooth muscle constitutes an airway epithelial stem cell niche in the mouse lung after injury. *J. Clin. Invest.* **121**, 4409–4419.

Fgf10 deficiency is causative for lethality in a mouse model of bronchopulmonary dysplasia

Cho-Ming Chao,^{1,2} Faady Yahya,¹ Alena Moiseenko,¹ Caterina Tiozzo,³ Amit Shrestha,¹ Negah Ahmadvand,¹ Elie El Agha,¹ Jennifer Quantius,¹ Salma Dilai,¹ Vahid Kheirollahi,¹ Matthew Jones,¹ Jochen Wilhem,¹ Gianni Carraro,⁴ Harald Ehrhardt,² Klaus-Peter Zimmer,² Guillermo Barreto,⁵ Katrin Ahlbrecht,⁶ Rory E Morty,⁶ Susanne Herold,¹ Rosanna G Abellar,⁷ Werner Seeger,^{1,6} Ralph Schermuly,¹ Jin-San Zhang,⁸ Parviz Minoo⁹ and Saverio Bellusci^{1,8,10*}

¹ Universities of Giessen and Marburg Lung Center (UGMLC), Excellence Cluster Cardio-Pulmonary System (ECCPS), Member of the German Center for Lung Research (DZL), Department of Internal Medicine II, Aulweg 130, 35392, Giessen, Germany

² University Children's Hospital Gießen, Division of General Pediatrics and Neonatology, Justus-Liebig-University, Member of the German Lung Center (DZL), Gießen, Germany

³ Division of Neonatology, Department of Pediatrics, Columbia University, New York, NY, USA

⁴ Departments of Medicine and Biomedical Sciences, Lung and Regenerative Medicine Institutes, Cedars-Sinai Medical Center, Los Angeles, CA, 90048, USA

⁵ LOEWE Research Group, Lung Cancer Epigenetic, Max Planck Institute for Heart and Lung Research, Member of the German Lung Center (DZL), 61231, Bad Nauheim, Germany

⁶ Department of Lung Development and Remodeling, Max Planck Institute for Heart and Lung Research, Member of the German Lung Center (DZL), 61231, Bad Nauheim, Germany

⁷ Department of Pathology and Cell Biology, Columbia University, New York, NY 10032, USA

⁸ College of Life and Environmental Sciences, Wenzhou University, Wenzhou, Zhejiang 325027, PR China

⁹ Department of Pediatrics, Division of Newborn Medicine, University of Southern California, Children's Hospital Los Angeles, Los Angeles, CA 90027, USA

¹⁰ Developmental Biology and Regenerative Medicine Program, Saban Research Institute of Children's Hospital Los Angeles and University of Southern California, Los Angeles, CA, 90027, USA

*Correspondence to: S Bellusci, Universities of Giessen and Marburg Lung Center (UGMLC), Department of Internal Medicine II, Aulweg 130, 35392 Giessen, Germany. E-mail: Saverio.Bellusci@innere.med.uni-giessen.de

Abstract

Inflammation-induced FGF10 protein deficiency is associated with bronchopulmonary dysplasia (BPD), a chronic lung disease of prematurely born infants characterized by arrested alveolar development. So far, experimental evidence for a direct role of FGF10 in lung disease is lacking. Using the hyperoxia-induced neonatal lung injury as a mouse model of BPD, the impact of *Fgf10* deficiency in *Fgf10*^{+/-} versus *Fgf10*^{+/+} pups was investigated. In normoxia, no lethality of *Fgf10*^{+/-} or *Fgf10*^{+/+} pups was observed. By contrast, all *Fgf10*^{+/-} pups died within 8 days of hyperoxic injury, with lethality starting at day 5, whereas *Fgf10*^{+/+} pups were all alive. Lungs of pups from the two genotypes were collected on postnatal day 3 following normoxia or hyperoxia exposure for further analysis. In hyperoxia, *Fgf10*^{+/-} lungs exhibited increased hypoalveolarization. Analysis by FACS of the *Fgf10*^{+/-} versus control lungs in normoxia revealed a decreased ratio of alveolar epithelial type II (AECII) cells over total Epcam-positive cells. In addition, gene array analysis indicated reduced AECII and increased AECI transcriptome signatures in isolated AECII cells from *Fgf10*^{+/-} lungs. Such an imbalance in differentiation is also seen in hyperoxia and is associated with reduced mature surfactant protein B and C expression. Attenuation of the activity of Fgfr2b ligands postnatally in the context of hyperoxia also led to increased lethality with decreased surfactant expression. In summary, decreased *Fgf10* mRNA levels lead to congenital lung defects, which are compatible with postnatal survival, but which compromise the ability of the lungs to cope with sub-lethal hyperoxic injury. *Fgf10* deficiency affects quantitatively and qualitatively the formation of AECII cells. In addition, Fgfr2b ligands are also important for repair after hyperoxia exposure in neonates. Deficient AECII cells could be an additional complication for patients with BPD.

Copyright © 2016 Pathological Society of Great Britain and Ireland. Published by John Wiley & Sons, Ltd.

Keywords: fibroblast growth factor 10; bronchopulmonary dysplasia; AECII; differentiation; surfactant

Received 25 April 2016; Revised 31 August 2016; Accepted 12 October 2016

No conflicts of interest were declared.

Introduction

Fibroblast growth factor 10 (FGF10) protein deficiency has been reported in patients with bronchopulmonary dysplasia (BPD) [1]. BPD is a chronic lung disease

of prematurely born infants and remains a leading cause of morbidity and mortality. In humans, inflammation is known to increase risk for BPD [2,3]. Inflammatory mediators activated by bacterial-derived lipopolysaccharides (LPS) such as NF- κ B, Sp1, and

Sp3 were found to inhibit *Fgf10* transcription in mouse lung explants [4,5]. The inhibition of *Fgf10* expression is mediated by LPS receptors (Toll-like receptor 2 and 4) activation. In the context of the immature lung, the biotrauma and barotrauma induced by inflammation, mechanical ventilation, and oxygen toxicity are known to cause injury [6–9]. Due to advances in management and therapy, the survival of premature infants has increased. The histological characteristics of BPD have also changed. The ‘old’ BPD was characterized by emphysema, interstitial fibrosis, and airway squamous metaplasia. The ‘new’ BPD is thought to be a ‘developmental lung disease’, arising from arrested alveolar development resulting in hypoalveolization and dysmorphic microvasculature [10]. BPD treatment is a considerable burden on health care systems [11,12].

While the evidence confirming the key role of *Fgf10* in embryonic lung development is strong [13,14], comparatively less is known about the consequences of constitutive *Fgf10* insufficiency following lung injury. To demonstrate the effect of *Fgf10* deficiency for prenatal and postnatal lung development in normoxic conditions, we used a constitutive heterozygous *Fgf10*^{+/-} mouse line. Lung morphometry and gene array were performed to identify changes in the lung structure and global gene expression in *Fgf10*^{+/-} versus *Fgf10*^{+/+} wild-type (WT) littermate lungs at E18.5. Because oxygen toxicity is one of the major risk factors contributing to BPD, we used the mouse hyperoxia-induced BPD phenocopy model (85% oxygen from P0 to P8) to investigate the impact of *Fgf10* deficiency. Surprisingly, all *Fgf10*^{+/-} pups died within 8 days of hyperoxic injury. We therefore chose P3, a time point at which there was no observable lethality, to collect pups for further analysis consisting of lung morphometry, gene array, fluorescence-activated cell sorting (FACS), immunofluorescence (IF), reverse transcriptase-quantitative polymerase chain reactions (RT-qPCR), and western blotting. We also used a double transgenic system in mice [15–19] to attenuate all *Fgfr2b* ligands postnatally in the context of hyperoxic injury. Our detailed analysis may be critical in designing therapies to prevent lung injury in neonates at risk for BPD and in adult lung disorders characterized by FGF10 deficiency.

Materials and methods

Study approval

Animal studies

All experiments were performed in accordance with the National Institutes of Health Guidelines for the Use of Laboratory Animals. Animal experiments were approved by the Federal Authorities for Animal Research of the Regierungspraesidium Giessen, Hessen, Germany (protocols 105/2011).

Mice

C57BL/6 mice were crossed to generate WT pups. *Fgf10*^{+/-} mice were generated by crossing *Fgf10*^{flax/flax}

mice (*Fgf10*^{tm1.2Sms/J}; stock No 023729; The Jackson Laboratory, Bar Harbor, ME, USA) with *CMV-Cre* mice (*B6.C-Tg(CMV-cre)1Cgn/J*; stock No 006054; The Jackson Laboratory). The resulting *Fgf10*^{+/-} mice (*Fgf10*^{tm1.1Sms/J}) were crossed for at least five generations with C57BL/6 mice to remove the *CMV-Cre* allele and establish the *Fgf10*^{+/-} mice in the C57BL/6 background. *Fgf10*^{+/-} and *Fgf10*^{+/+} embryonic and postnatal mice were used (both males and females). The *Fgf10*^{LacZ/+} embryos were previously generated [20] by crossing the *Fgf10*^{LacZ/+} (*Fgf10*^{Tg(Myl3-lacZ)24Buck}) obtained from Dr Robert Kelly, Marseille, France and maintained on the C57BL/6 background for at least five generations) with the *Fgf10*^{+/-} mice previously described. The *Rosa26*^{rtTA/+}; *Tg(tet(o)sFgfr2b)/+* mice [*Gt(ROSA)26Sor*^{tm1.1(rTA,EGFP)Nagy} *Tg(tet0-sFgfr2b)1Jaw/CHC*] were generated by crossing *Rosa26*^{rtTA/+} + [*Gt(ROSA)26Sor*^{tm1.1(rTA,EGFP)Nagy}] with *Tg(tet(o)sFgfr2b)/+* mice [*Tg(tet0-sFgfr2b)1Jaw/CHC*], obtained from Dr Jeffrey Whitsett, Cincinnati, OH, USA. Mice were kept on the CD1 background for at least five generations. Both genders were used.

Hyperoxia injury (BPD mouse model)

Newborn pups were subjected to hyperoxia (HOX; 85% O₂) injury from P0 to P8 in a chamber (ProOx Model 110; BioSpherix, Ltd, Parish, NY, USA). To minimize oxygen toxicity and bias, nursing dams were rotated every 24 h between normoxia (NOX) and HOX. Pups and dams received food and water *ad libitum*.

Statistical analyses

Significance was determined by two-tailed Student's *t*-test using GraphPad Prism statistical analysis software (GraphPad, La Jolla, CA, USA). Chi-square analysis (software R, package ‘survival’) was used for the comparison of survival data of all hyperoxia-exposed litters. All data are presented as mean ± SEM. Values of *p* < 0.05 were considered significant.

Left lobe perfusion, alveolar morphometry, RNA extraction, RT-qPCR, isolation of primary AECII cells, microarray experiments, FACS, and western blot

Details may be found in the supplementary material online, Supplementary materials and methods.

The data from the microarray experiment have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE76302 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76302>).

Results

Fgf10 expression is reduced upon hyperoxia (HOX) exposure in neonatal wild-type (WT) lungs

The expression of *Fgf10* and associated *Fgf* genes belonging to the same *Fgf* subfamily (*Fgf1*, *Fgf3*, and

Fgf7) that encode Fgf ligands acting via *Fgfr2b* [21] was assessed by RT-qPCR in the lungs of C57BL/6 WT pups at different time points during normal alveologenesis (between P0 and P35). When normalized to their respective values for the first time point collected (P0), the results indicated a decrease in *Fgf10* expression over time, while the genes encoding other *Fgfr2b* ligands were maintained at stable levels (Figure 1A, a). When normalized to *Fgf10* expression at each of the considered time-points, *Fgf1* and *Fgf7* showed higher mRNA levels while *Fgf3* was generally present at lower level (Figure 1A, b). Comparison of the expression (normalized to the first time point collected, P2) of these ligands' mRNAs in HOX (85% O₂ from P0 onwards) revealed that *Fgf10* was the only one with significantly lower levels at P5 and P8 compared with NOX-exposed WT lungs (Figure 1A, c).

Fgf10^{+/-} newborn mice are indistinguishable from WT littermates in NOX, but display increased lethality following HOX injury

Considering the previously reported decreased FGF10 expression in human patients with BPD, we investigated whether *Fgf10* deficiency in mice could reproduce the clinical complications observed in BPD. We therefore crossed *Fgf10*^{+/-} and WT mice and collected embryos at E12.5 ($n = 3$ and $n = 4$ for WT and *Fgf10*^{+/-}, respectively) and E18.5 ($n = 3$ and $n = 7$ for WT and *Fgf10*^{+/-}, respectively) and assessed the levels of *Fgf10* mRNA in their lungs by RT-qPCR. The relative levels of *Fgf10* were 23.7% and 57.9% less at E12.5 ($p = 0.04$) and E18.5 ($p = 0.01$), respectively, in *Fgf10*^{+/-} versus WT lungs (data not shown). *Fgf10*^{+/-} ($n = 10$) and WT ($n = 12$) pups exposed to NOX between P0 and P8 (Figure 1B) were viable and without phenotypic differences (data not shown). Morphometric analysis of P3 lungs (Figure 1C, b, d versus a, c; $n = 4$ for each genotype) showed no significant differences between *Fgf10*^{+/-} and WT for the mean linear intercept (MLI), airspace or septal wall thickness (Figure 1D, a–c). To mimic the BPD phenotype, newborn pups were subjected to HOX for up to 8 days (Figure 1E) [22] (see also supplementary material online, Figure S1 for validation of the BPD model). Figure 1E–H displays the results for the pups from two litters combined together (14 pups). Survival analysis showed that all WT mice ($n = 8$) survived at day 8, while none of the *Fgf10*^{+/-} experimental mice ($n = 6$) were alive at this time point [chi-squared tests on the Cox proportional hazards models (group membership as only predictor): *Fgf10*^{+/-} versus WT: χ^2 : 11.6, $p < 0.001$ (1 degree of freedom, df)]. A 50% lethality was already observed at P5, suggesting that lung damage starts earlier (Figure 1F). We therefore analysed WT and *Fgf10*^{+/-} lungs at P3 during the course of HOX. Haematoxylin and eosin staining of *Fgf10*^{+/-} lungs ($n = 4$) revealed an abnormal phenotype compared with WT lungs ($n = 4$), with enlarged alveolar sacs and thinner interalveolar walls (Figure 1G, b, d versus a, c). Morphometric analysis demonstrated significantly

increased MLI and airspace (both $p = 0.001$), as well as decreased septal wall thickness ($p = 0.039$) (Figure 1H, a–c).

Fgf10^{+/-} lungs exhibit impaired morphometry at E18.5 associated with reduced Fgf signalling and epithelial marker expression as well as increased Tgfb signalling and ECM protein expression

We examined the presence of potential lung defects in E18.5 *Fgf10*^{+/-} embryos. We also used previously described *Fgf10*^{LacZ/-} hypomorphic mutants [20]. These two lines exhibit 50% and 20% *Fgf10* expression compared with WT lungs, respectively. Figure 2A, a–c shows the histology of the E18.5 WT ($n = 3$), *Fgf10*^{+/-} ($n = 7$), and *Fgf10*^{LacZ/-} hypomorphic ($n = 3$) lungs. Morphometric analyses (Figure 2B) indicated that *Fgf10*^{+/-} lungs exhibited increased MLI and airspace with no change in septal wall thickness compared with WT. Interestingly, *Fgf10*^{LacZ/-} hypomorphic lungs mostly showed increased septal wall thickness with moderately, but significantly, increased MLI. The reason for this difference is unclear. So far, our results have shown that at P3, there is no obvious difference between *Fgf10*^{+/-} and *Fgf10*^{+/+} in NOX. However, at E18.5, morphometric analyses revealed differences between the two genotypes. The cellular and molecular mechanisms involved in this apparent recovery from E18.5 to P3 are so far unknown. In order to further characterize the nature of the lung morphological differences between the three conditions, we isolated RNA from WT ($n = 3$), *Fgf10*^{+/-} ($n = 7$), and *Fgf10*^{LacZ/-} hypomorphic ($n = 3$) E18.5 lungs and carried out transcriptome analyses. Our approach using two distinct types of *Fgf10* mutants exhibiting different *Fgf10* levels allowed us to identify the top 70 mRNAs (selected based on p value) which were differentially regulated between *Fgf10*^{LacZ/-} hypomorph and WT lungs, and then to compare the expression of these genes between *Fgf10*^{+/-} and WT lungs. The heatmap in Figure 2C, a (see supplementary material online, Figure S2 for greater magnification) shows that these mRNAs were indeed also similarly decreased or increased in *Fgf10*^{+/-} versus WT lungs. The graph in Figure 2C, b summarizes the different cellular compartments and biological processes that are potentially affected based on the known function of the identified genes. Interestingly, the expected epithelial defects of *Fgf10* deficiency could, in part, be caused by the dysregulated expression of epithelium-specific genes, such as *lysophosphatidylcholine acyltransferase 1* (*Lpcat1*), *phosphatidylinositol-4-phosphate-5-kinase, type I, alpha* (*Pip5k1a*), *G-protein-coupled receptor 30* (*Gpr30*), *calpain 6* (*Capn6*), and *pleiotrophin* (*Ptn*). In particular, *Lpcat1* deficiency has been associated with neonatal death due to surfactant defects [23], while *Ptn* promotes the proliferation of alveolar epithelial type II (AECII) cells and prevents their differentiation into AECI [24]. In addition to those implicated in epithelial defects, we also identified changes in transcripts expressed in muscle, the immune system,

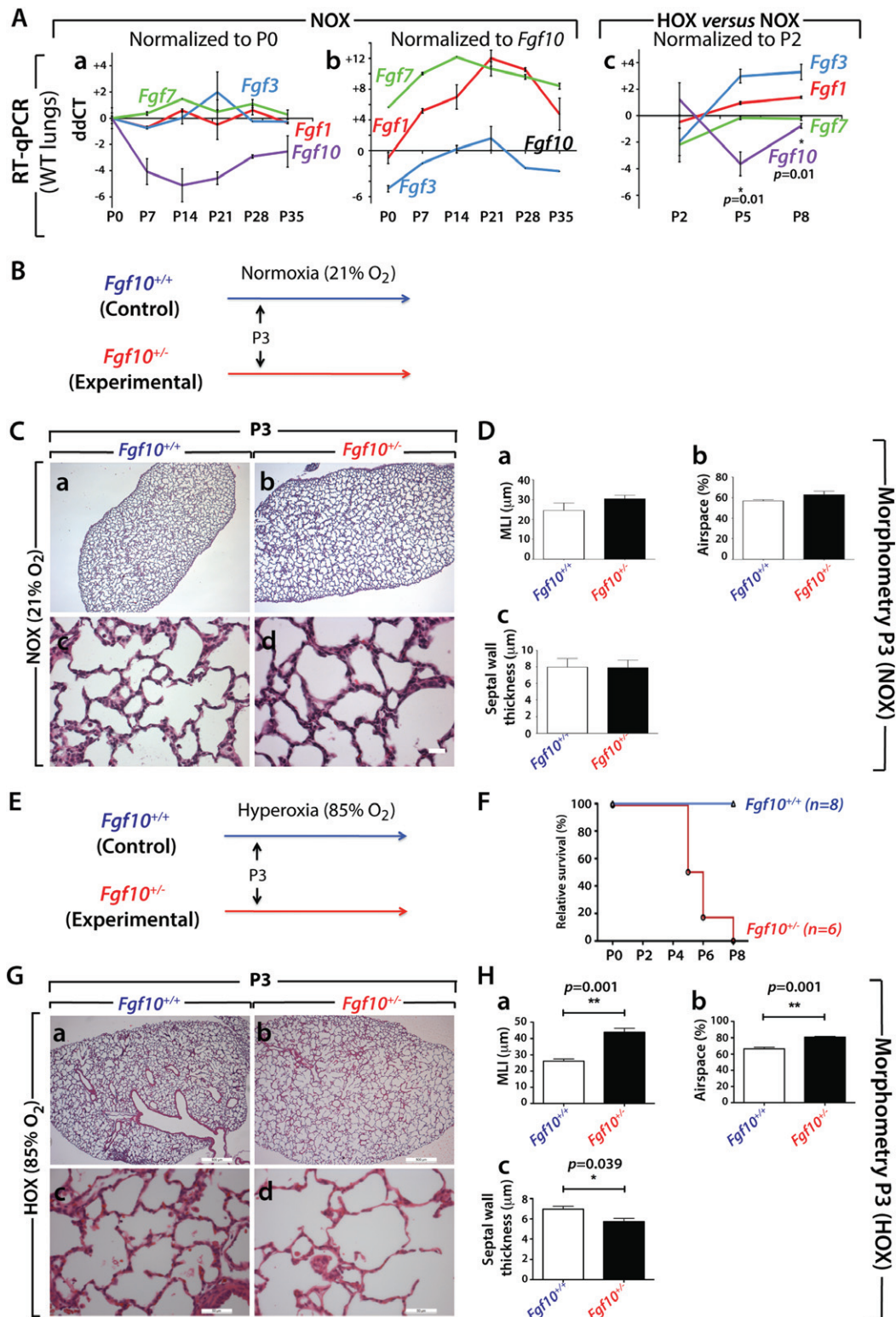


Figure 1. Congenital *Fgf10* insufficiency leads to neonatal death upon hyperoxia injury. (A) RT-qPCR for *Fgf1*, *Fgf3*, *Fgf7*, and *Fgf10* in lungs at various postnatal stages in normoxia (NOX). (a) Normalization to P0 for each of the ligands indicates that *Fgf10* expression decreases postnatally compared with P0 and compared with the other ligands. (b) Normalization to *Fgf10* expression at each time point indicates that *Fgf1* and *Fgf7* are expressed at higher levels than *Fgf10*. (c) Comparison of *Fgfr2b* ligands in hyperoxia (HOX) versus NOX indicates that *Fgf10* is the only *Fgfr2b* ligand mRNA to decrease significantly upon HOX injury. (B) Experimental set-up for NOX of *Fgf10*^{+/+} (control group) and *Fgf10*^{+/-} (experimental group) animals. (C) Haematoxylin and eosin (H&E) staining of NOX-exposed control (a, c) or experimental (b, d) lungs at P3. (D) Corresponding lung morphometric analysis. Note the absence of differences in MLI (a), airspace (b), and septal wall thickness (c). (E) Experimental set-up for HOX of *Fgf10*^{+/+} (control group) and *Fgf10*^{+/-} (experimental group) animals. (F) Survival curve for HOX-exposed animals showing that all of the experimental animals died within 8 days, whereas all of the control animals survived. (G) H&E staining of HOX-exposed control (a, c) or experimental (b, d) lungs at P3. (H) Corresponding lung morphometric analysis of HOX-exposed control and experimental animals. Note the increase in MLI (a) and airspace (b), and the decrease in septal wall thickness (c). Scale bars: (C, a, b) 500 μm; (C, c, d) 50 μm; (G, a, b) 500 μm; (G, c, d) 50 μm; *, significant *p* value; **, very significant *p* value.

nerves, and the extracellular matrix (ECM) (Figure 2C, c). These results highlight, as a consequence of either direct or indirect effects of *Fgf10* on the different cellular compartments, potential defects of *Fgf10*^{+/-} lungs. Next, we validated by RT-qPCR the changes in Fgf signalling and the associated epithelial markers between *Fgf10*^{+/-} and WT lungs at E18.5 (Figure 2D, a, b). We found that the mRNA levels of *Fgf10*, *Fgfr2b*, and *Bmp4*, the latter a downstream target of *Fgf10* in the epithelium, were decreased (all statistically significant). An overall trend of decreasing expression was also observed for other epithelial marker mRNAs. *Epcam* levels were significantly reduced, supporting the notion that alterations occur in the epithelial compartment, which is the main cellular target of Fgf10 signalling. As Fgf10 and Tgfb β 1 play opposite roles during lung development, we also investigated the expression of genes involved in Tgfb β signalling and the associated ECM (Figure 2D, c, d). We found significantly increased *Smad3*, *Smad7*, and *IL-1b* levels, but no changes for *Tgfb1* and *Tgfb3*. Additionally, mRNA levels for different types of collagen (*Col1a1*, *Col1a2*, *Col3a1*, and *Col5a2*) showed trends to increase. The increase in Tgfb β signalling was confirmed by IF using phospho-Smad3 antibodies (see supplementary material, Figure S3).

Characterization of the response to oxygen injury in AECII cells isolated from the lungs of WT and *Fgf10*^{+/-} mice

In order to identify the significant genes/pathways differentially affected in AECII cells by HOX versus NOX between *Fgf10*^{+/-} and WT, we isolated AECII cells from *Fgf10*^{+/-} and WT P3 lungs in NOX and HOX conditions and compared their transcriptomes (see supplementary material online, Figure S4). In particular, we carried out an interaction 'HOX $\times$ genotype' analysis (Figure 3). The volcano plot identified a set of genes that are either up- or down-regulated (Figure 3A). Interestingly, the majority of differences between the genotypes occurred in the down-regulated genes.

The top 100 regulated genes according to their *p* values are represented in the corresponding heatmaps (Figure 3B, a, b; see supplementary material online, Figure S5 for greater magnification). We found a gene set (located in the lower part of the array) whose expression was not drastically changed in HOX between the two genotypes (Figure 3B, a), but was differentially regulated in NOX conditions (Figure 3B, b). Furthermore, another gene set down-regulated in HOX (located in the top part of the array) was up-regulated in NOX conditions. KEGG analysis revealed that the processes altered were almost all linked to the immune system and were down-regulated in mutant AECII cells (Figure 3C). These processes were systemic lupus erythematosus, *Staphylococcus aureus* infection, antigen presentation, graft versus host disease, and autoimmune thyroid disease, amongst others. In order to assess the differentiation status of the isolated AECII cells in *Fgf10*^{+/-} versus WT lungs, we analysed the expression

of the previously reported signatures for AECII and AECI [25] in the arrays generated from these cells in both NOX (Figure 3D) and HOX conditions (Figure 3E) (see supplementary material online, Figure S6 for greater magnification). In both conditions, we found a decrease in the AECII signature, with a corresponding increase in the AECI signature. Figure 3F, comparing differential expression between genotypes in HOX versus NOX, confirms the differential clustering of the signatures for AECI and AECII.

Fgf10^{+/-} lungs in the context of HOX exhibit decreased surfactant expression

Using western blotting (whole lung homogenates, *n* = 4 for WT and *n* = 3 for *Fgf10*^{+/-} lungs at P3; Figure 4A, a, b), we quantified the protein expression of Fgf10, Fgfr2 (using an antibody which does not discriminate between the Fgfr2b versus the Fgfr2c isoform), and mature Sftpc and Sftpb. We noted significantly reduced expression of all of these markers in *Fgf10*^{+/-} versus WT (Figure 4A, a–f). A decrease in Sftpb expression was also supported by immunofluorescence (Figure 4B, a, b).

Next, we investigated by FACS the prevalence of total epithelial cells (using Epcam as a general marker), AECI [podoplanin (Pdpn)-positive], AECII (Sftpc-positive), and epithelial stem/progenitor cells (EpiSPCs; Epcam^{high} CD24^{low}). In NOX, we observed a significant decrease in the Epcam-positive fraction of cells in *Fgf10*^{+/-} versus WT lungs (Figure 4C, a). We also noted an increased proportion of AECI cells (Figure 4C, b) and a decreased proportion of AECII cells (Figure 4C, c) in *Fgf10*^{+/-} versus WT lungs. This defect is in addition to the previously described impaired differentiation of the AECII cells in *Fgf10*^{+/-} versus WT P3 lungs (Figure 3F). Interestingly, the prevalence of EpiSPCs was not different between *Fgf10*^{+/-} and WT lungs (Figure 4C, d). Following hyperoxic injury, the ratio of epithelial cells (Epcam+/whole lung) in *Fgf10*^{+/-} versus WT was no longer significant (Figure 4C, e). However, the quantitative imbalance in the AECI ratio in *Fgf10*^{+/-} versus WT remained (Figure 4C, f), while the AECII ratio in *Fgf10*^{+/-} versus WT was no longer significant (Figure 4C, g). As for NOX, no difference was observed in the ratio of EpiSPCs in *Fgf10*^{+/-} versus WT (Figure 4C, h). Next, we assessed cell proliferation using IF for Ki67 (Figure 4D). In this analysis, we did not discriminate between epithelium and mesenchyme. In NOX, we observed a trend towards increased proliferation in *Fgf10*^{+/-} versus WT lungs (Figure 4D, a–c). In HOX, a significant increase in Ki67-positive cells was observed in *Fgf10*^{+/-} versus WT lungs (Figure 4D, d–f). To confirm this observation, we assessed by IF the ratio of Ki67+, Cdh1+ double-positive cells (over the total number of cells stained by DAPI) in NOX and HOX (see supplementary material online, Figure S7). No difference was seen in NOX, but in contrast, HOX triggered a significant increase in Ki67+, Cdh1+ double-positive cells (*p* = 0.05; supplementary material online, Figure S7A, b versus a). Supporting the FACS

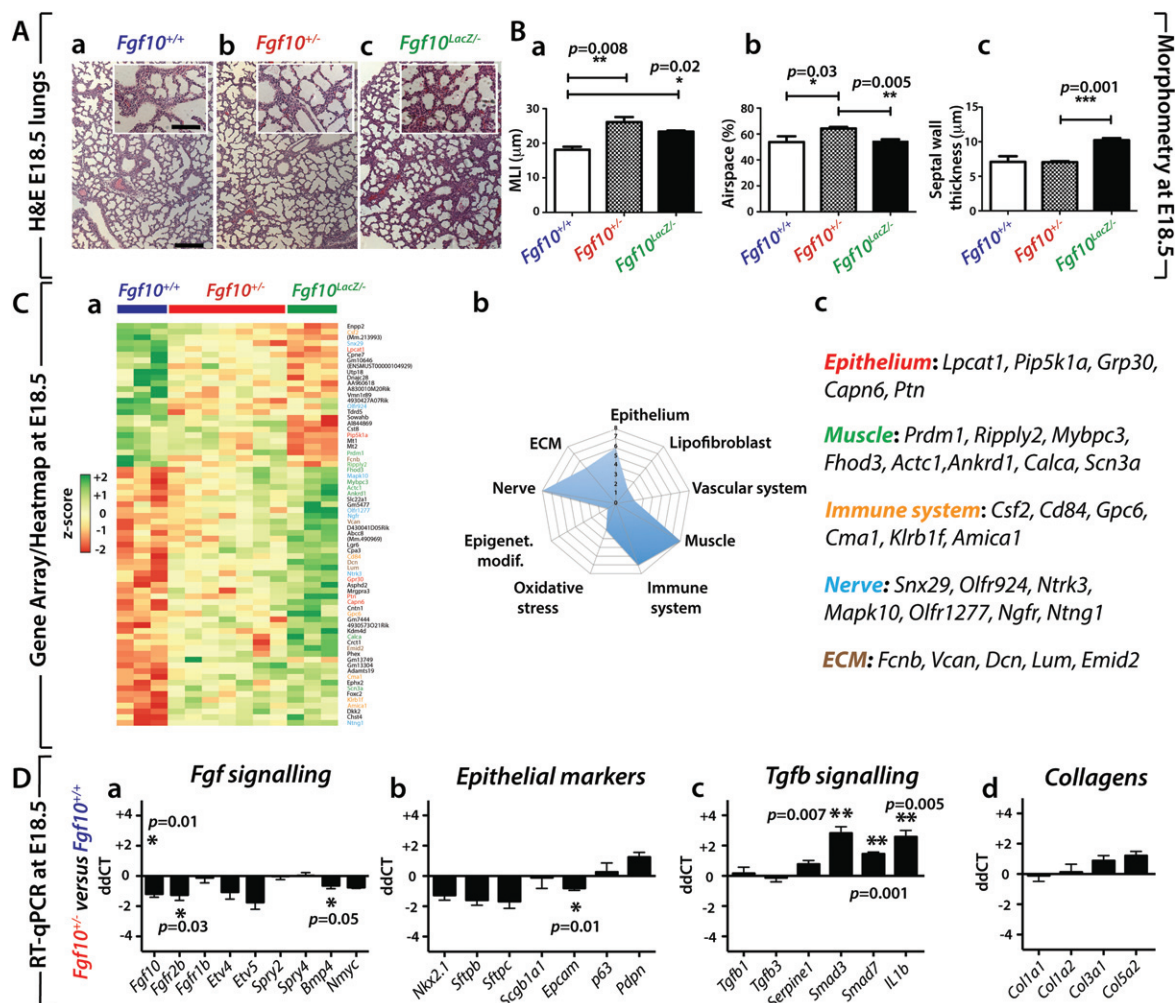


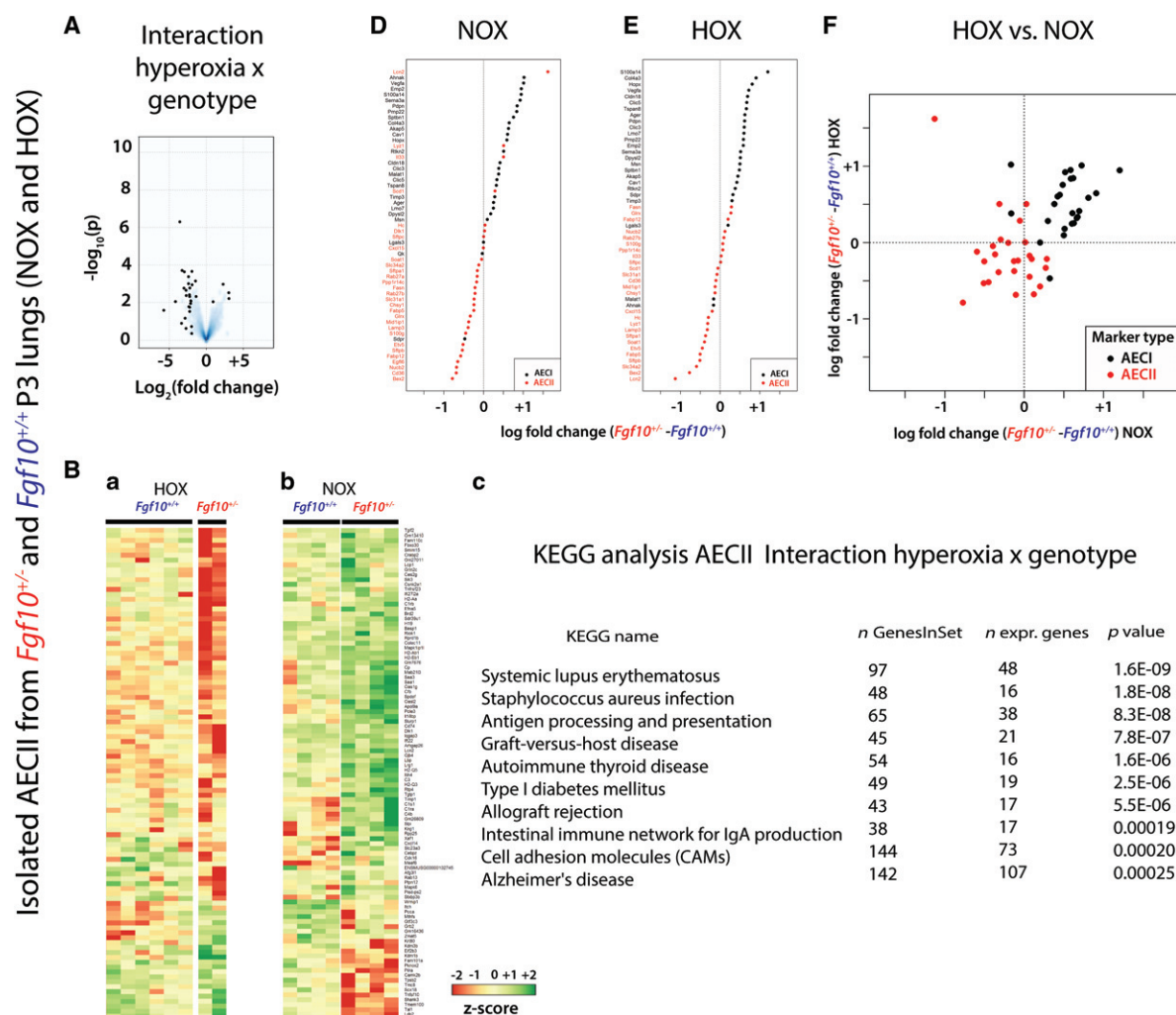
Figure 2. E18.5 *Fgf10*^{+/-} lungs display perturbed morphometry and impaired gene expression. (A) H&E staining of (a) *Fgf10*^{+/+}, (b) *Fgf10*^{+/-} (c), and *Fgf10*^{LacZ/-} lungs at E18.5. (B) Morphometry of the lungs shown in A. Note the increase in MLI (a) and airspace (b) and that there was no change in septal wall thickness (c) in *Fgf10*^{+/-} lungs compared with *Fgf10*^{+/+}. (C) (a) Gene array-based/heatmap analysis of the whole lung of E18.5 *Fgf10*^{+/+}, *Fgf10*^{+/-}, and *Fgf10*^{LacZ/-} lungs indicating that *Fgf10*-deficient lungs display an impaired pattern of gene expression compared with *Fgf10*^{+/+} lungs (see the supplementary material, Figure S2 for greater magnification). (b) Symbolic representation of the genes affected grouped by cell types or processes. (c) Some of the relevant genes pertinent for the epithelium, muscle, immune system, nerve, and ECM are listed. (D) Expression analysis by RT-qPCR of genes belonging to (a) FGF signalling, (b) epithelial markers, (c) Tgfb signalling, and (d) collagens between E18.5 *Fgf10*^{+/+} and *Fgf10*^{+/-} lungs. Note the decrease in *Fgf10* signalling mRNAs *Fgf2b* and *Bmp4*, the decrease in *Epcam*, and the increase in *Tgfb* signalling mRNAs *Smad3*, *Smad7*, and *IL1b*. Collagen mRNA levels were not significantly different at this stage. Scale bars: (A, a–c) 125 μm; (insets) 30 μm; *, significant *p* value; **, very significant *p* value.

data, which indicated reduced epithelial cell abundance (Epcam⁺ cells) in *Fgf10*^{+/-} versus WT lungs in NOX, we observed a reduction in the prevalence of Cdh1⁺ cells in NOX in *Fgf10*^{+/-} versus WT lungs (*p* = 0.004; supplementary material, Figure S7A, c). As observed for the FACS data, the difference in epithelial cell fraction between the two genotypes was no longer seen in HOX (supplementary material, Figure S7A, d). Investigating changes in the mesenchyme revealed significant increases, in NOX, for the fractions of CD45⁺, CD31⁺, and Epcam-negative over total cells in *Fgf10*^{+/-} versus WT lungs (supplementary material, Figure S7B, a). This difference in the fraction of mesenchymal cells between the two genotypes was no longer detected in HOX (supplementary material, Figure S7B, b). Measurement by FACS of the ratio of haematopoietic and endothelial cells (defined as CD45⁺, CD31⁺, Epcam-negative)

showed no difference in NOX and HOX between *Fgf10*^{+/-} and WT lungs at P3 (data not shown).

Postnatal attenuation of Fgfr2b ligands in NOX, during the saccular/alveolar stage of lung development, does not cause lung structural defects or lethality

As no congenital mutation in the *FGF10* gene in patients with BPD has been reported so far, it is likely that in BPD babies, the initial stages of lung development occur in the presence of normal levels of FGF10. To mimic this situation in a mouse model, we used a previously reported dominant negative soluble *Fgfr2b* expression approach [*Rosa26*^{rtTA/+}; *Tg(tet(O)sFgfr2b)*/+ mice] to scavenge, in a ubiquitous and doxycycline-inducible manner, all the Fgfr2b ligands, thereby turning off



15 pups) to NOX and HOX (P0–P8). In contrast to NOX, HOX induced lethality in 50% of the *Rosa26^{rtTA/+};Tg(tet(O)sFgf2b)/+* DTG experimental group beginning at P6 (Figure 5E, F; chi-squared tests on the Cox proportional hazards models (group membership as only predictor): *Rosa26^{rtTA/+};Tet(O)sFgf2b/+* versus single transgenic (STG) control: χ^2 : 6.2, $p=0.012$ (1 df)]. Compared with *Fgf10^{+/-}* neonatal mice, this lethality started 1 day later (see Figure 1F) and the lethality at P8 was less (100% versus 50% dead in *Fgf10^{+/-}* versus DTG, respectively). Morphometry at P3 (Figure 5G, H) showed increased MLI (+19.3%, $p=0.029$) in the DTG compared with the STG group control. This increase, compared with the *Fgf10^{+/-}* versus WT in HOX (Figure 1H, a) (+68.2%, $p=0.001$), supports our conclusion that in HOX, DTG lungs exhibit a less severe phenotype than do *Fgf10^{+/-}* lungs. The lungs of *Fgf10^{+/-}* mice at P3 in HOX had quantitative defects (increased AECl, decreased AEClI) as well

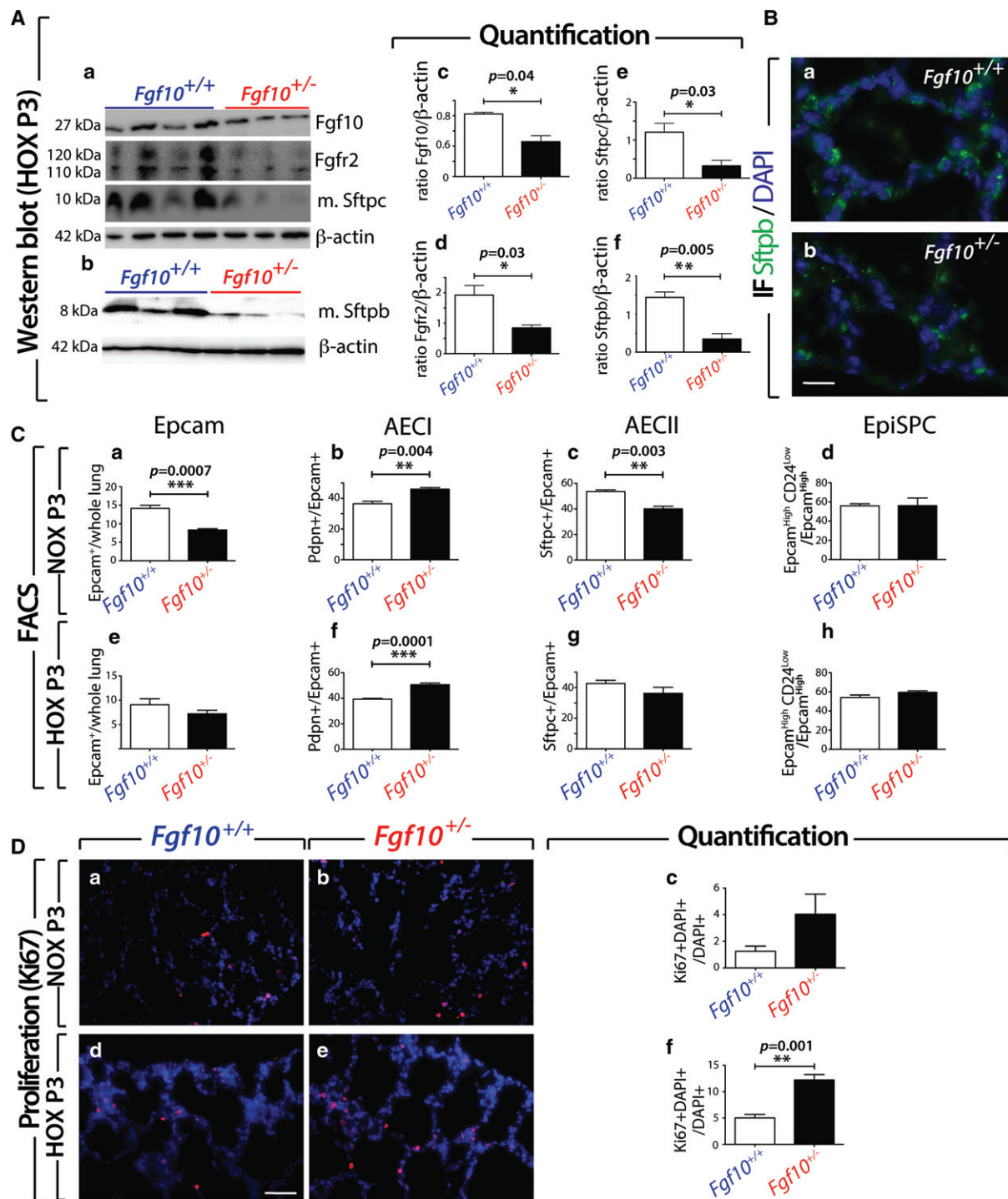


Figure 4. *Fgf10*^{+/-} lungs in the context of HOX exhibit less mature Sftpc and mature Sftpb. (A) Western blotting analysis (whole lung homogenates) for (a) Fgf10, Fgfr2, mature Sftpc, and β-actin, as well as (b) mature Sftpb and β-actin on *Fgf10*^{+/+} and *Fgf10*^{+/-} lungs exposed to HOX at P3. (c–f) Quantification of bands using ImageJ software (b–d). (B) Immunofluorescence for Sftpb in P3 HOX *Fgf10*^{+/+} and *Fgf10*^{+/-} lungs. (C) Fluorescence-activated cell sorting in NOX (a–d) and HOX (e–h) of *Fgf10*^{+/+} and *Fgf10*^{+/-} P3 lungs for (a, e) Epcam, (b, f) AECI, (c, g) AECII, and (d, h) epithelial stem progenitor cells (EpiSPC). (D) Ki67 labelling for proliferation in (a, b) NOX and (d, e) HOX for (a, d) *Fgf10*^{+/+} and (b, e) *Fgf10*^{+/-} P3 lungs. (c, f) The Ki67⁺ fraction of cells in (c) NOX and (f) HOX. Note the increase in Ki67⁺ cells in the *Fgf10*^{+/-} lungs exposed to HOX. Scale bars: (B, a, b) 20 μm; (D, a, b, d, e) 50 μm; *, significant *p* value; **, very significant *p* value.

as impaired AECII differentiation (reduced AECII signature, increased AECI signature) associated with reduced Sftpc expression, so we also carried out western blotting for Sftpc, as well as FACS analysis, to assess the total number of epithelial cells (using Epcam), AECI (Pdpn-positive), and AECII (Sftpc-positive) in DTG and

STG P3 lungs in NOX and HOX (Figure 6). In NOX, we did not find any significant differences between DTG and STG lungs (Figure 6A, B). By contrast, in HOX, we observed a reduction in Sftpc expression (Figure 6C), suggesting defective AECII cells. However, FACS analysis failed to show a quantitative difference between

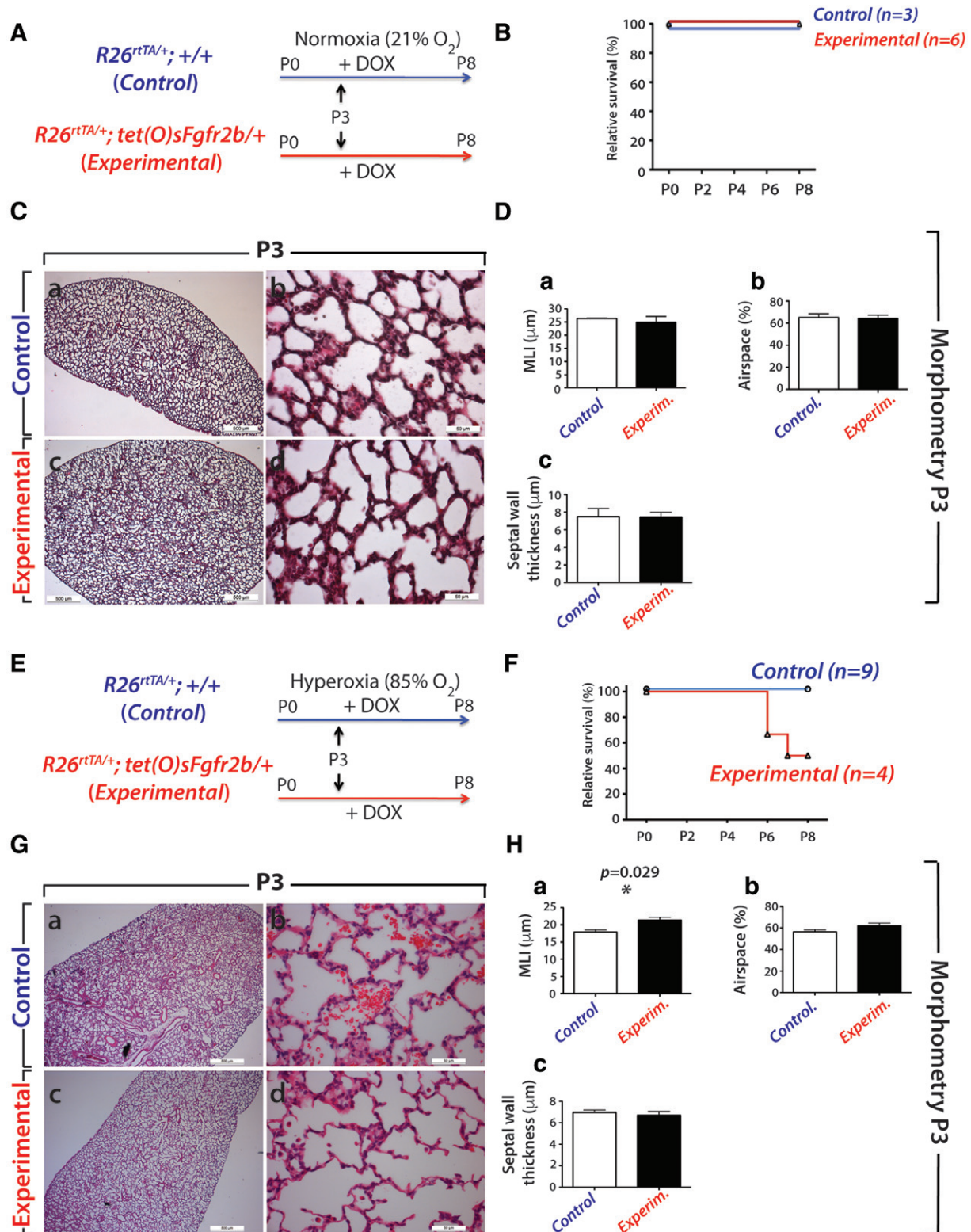


Figure 5. Attenuation of Fgfr2b ligands postnatally in the context of HOX-exposed pups leads to significant lethality. (A) Experimental set-up for NOX of $R26^{rtTA/+}; tet(O)sFgfr2b/+$ (DTG, experimental group) and $R26^{rtTA/+}; +/+$ (STG, control group) animals. Animals were fed with doxycycline-containing food to induce the dominant negative soluble Fgfr2b in the experimental group. (B) Survival curve for NOX-exposed animals indicating that all experimental and control animals survive in NOX. (C) H&E staining of NOX-exposed control (a, b) or experimental (c, d) lungs at P3. (D) Corresponding lung morphometric analysis. Note the absence of differences in MLI (a), airspace (b), and septal wall thickness (c). (E) Experimental set-up for HOX of $R26^{rtTA/+}; tet(O)sFgfr2b/+$ (experimental group) and $R26^{rtTA/+}; +/+$ (control group) animals. (F) Survival curve for HOX-exposed animals indicating that 50% of the experimental animals died within 8 days, while all the control animals survived. (G) H&E staining of HOX-exposed control (a, b) or experimental (c, d) lungs at P3. (H) Corresponding lung morphometric analysis of HOX-exposed control and experimental animals. Note the increase in MLI (a), while the airspace (b) and septal wall thickness (c) were not affected. Scale bars: (C, a, c; G, a, c) 500 μ m; (C, b, d; G, b, d) 50 μ m; *, significant p value.

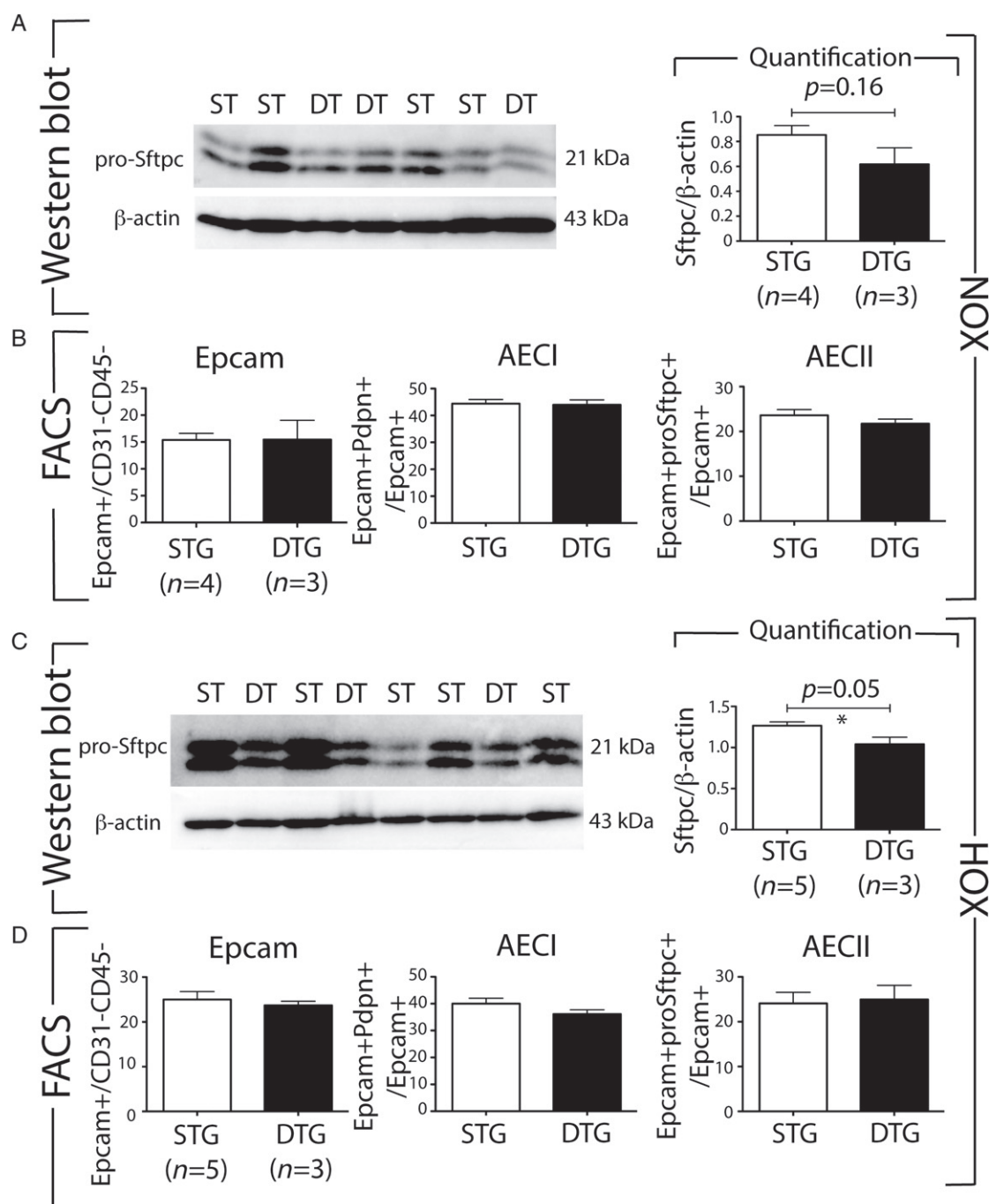


Figure 6. *Rosa26^{rtTA/+};Tg(tet(o)Sfgr2b)/+* (DTG) lungs in the context of HOX exhibit decreased Sftpc expression without quantitative change in the prevalence of Epcam, AECl, and AEClI cells. (A) Western blot analysis for pro-Sftpc and β-actin of STG and DTG P3 lungs exposed to NOX. Quantification of the western blot results shows no difference between STG and DTG mice. (B) Fluorescence-activated cell sorting of cells from STG and DTG P3 lungs in NOX for Epcam, AECl, and AEClI cells. No quantitative changes were observed in NOX. (C) Western blot analysis for pro-Sftpc and β-actin on STG and DTG P3 lungs exposed to HOX. Quantification of the western blot results shows a significant decrease in Sftpc expression in DTG versus STG mice. (D) Fluorescence-activated cell sorting of cells of STG and DTG P3 lungs in HOX for Epcam, AECl, and AEClI cells. No quantitative changes were observed in HOX; *, significant p value; **, very significant p value.

DTG and STG mice for Epcam, AECl or AEClI cell fractions (Figure 6D).

Discussion

Fgf10 maintains undifferentiated the Sox9/Id2-positive cells present in the distal lung epithelium [27,28]. These cells are multipotent epithelial

progenitor cells. Lineage tracing using *Id2-CreERT2* showed that they give rise to both bronchiolar and alveolar progenitors. Based on single cell transcriptome studies of the developing epithelium, the alveolar progenitors represent a population of 'bipotent progenitor cells'. These bipotent cells differentiate into either AECl or AEClI cells. However, what controls their differentiation remains unknown. A gene signature characteristic of each cell type has been recognized.

The bipotent progenitor cells exhibit both signatures [25]. Our results suggest that Fgf10 may play a key role in directing the differentiation of the bipotent progenitor cells towards the AECII lineage. This conclusion is based on the observation that while the epithelial compartment is decreased in *Fgf10*^{+/-} lungs, the ratio of AECII cells (to Epcam-positive cells) is also decreased, while that of AECI cells is increased. Interestingly, *Fgf10* hypomorphic lungs, exhibiting around 20% of the WT *Fgf10* mRNA level, also show a pronounced defect in AECII cells [20], supporting a role for Fgf10 in the ontogeny of the AECII lineage. The role, in this differentiation process, of the other Fgfr2b ligands abundantly expressed in the lung (Fgf1 and Fgf7) is unclear and will also need to be addressed. The observation that surfactant production, which is AECII's primary function, is compromised in *Fgf10*^{+/-} in HOX supports our conclusion that AECII cells from *Fgf10*^{+/-} lungs are deficient. Interestingly, the AECII cells of *Fgf10*^{+/-} lungs are still capable of producing enough surfactant to allow normal lung function in NOX. However, following HOX, these cells are prematurely lost due to built-in developmental (differentiation) defects that remain to be defined. The observed increase in epithelial proliferation and the FACS data from *Fgf10*^{+/-} versus WT lungs upon HOX injury appear to indicate an attempt to replenish the failing AECII pool. This newly formed AECII cell pool arising from either AECII stem cells [29] or other still unidentified sources is apparently not able to compensate for the loss of surfactant protein b and c production. Western blotting analysis at P3 clearly showed a significant decrease in Sftpc and Sftpb expression (Figure 4A, a, b). Decreased surfactant expression is likely a major contributor to the lethal phenotype upon injury. Impaired surfactant production following hyperoxia exposure in adult mice upon Fgfr2b ligand blocking has been reported previously [30]. In neonatal lung injury, the present results support the role of Fgfr2b ligands in the repair process of the AECII cells, and the associated surfactant expression.

Genome-wide association studies did not associate single-nucleotide polymorphisms in the *FGF10* gene with BPD [31]. Constitutive mutations in *FGF10* therefore appear to be rare, or non-existent, in BPD patients. However, the reduction in FGF10, which is linked to inflammation, may be associated with some of the clinical manifestations.

Our transcriptome results suggest that differences exist in the immune status of AECII cells in *Fgf10*^{+/-} versus WT lungs. The role of FGF signalling in controlling inflammation is still controversial. In primary cultures of human airway epithelial cells, FGF7 decreases transcript abundance for many interferon-induced genes, which may attenuate the response of epithelial cells to inflammatory mediators [32] (see ref 33 for a review of the role of Fgf7 in the lung). Short-term overexpression of Fgf7 *in vivo*, 3 days prior to acute lung injury, is protective. However, Fgf7 exposure for 7 days or longer increases lung inflammation and injury, suggesting differential cytoprotective

versus inflammatory effects of Fgf7, dependent on the duration of growth factor treatment [34]. To date, such negative inflammatory effects have not been reported for Fgf10. In the future, precise analysis of epithelial gene expression will be critical for a better understanding of the biological responses triggered by Fgf10 versus Fgf7. Fgf10 increases the recruitment of T-regulatory cells in the context of bleomycin injury [35] and mobilizes lung-resident mesenchymal stem cells and protects against acute lung injury [36]. Whether these effects are triggered by Fgf10-mediated epithelial signals or by Fgf10 acting on these inflammatory/stem cells remains to be elucidated.

Our results are also of interest regarding ALSG (aplasia of lacrimal and salivary glands) or LADD (lacrimo-auriculo-dento-digital syndrome) patients (exhibiting *FGF10* or *FGFR2b* mutations). While these patients initially appear to have normal lung function, they develop chronic obstructive pulmonary disease [37]. This is likely associated with deficient repair of the epithelium upon injury. It still remains to be investigated whether this is due to developmental defects in the epithelial progenitors and/or insufficient postnatal FGF10 levels that compromise epithelial maintenance. One aspect is clear; these patients will likely be more prone to lung disease than the normal population. At a minimum, prophylactic preventive measures should be considered for these 'at-risk' patients.

Acknowledgements

We would like to thank Kerstin Goth and Jana Roskovius for managing and genotyping the mice used for experiments. We also thank Professor Wellington Cardoso for critical reading of this manuscript. PM acknowledges the support from the Hastings Foundation. PM and SB acknowledge the NHLBI support (HL107307). SB was supported by grants from the Deutsche Forschungsgemeinschaft (BE4443/4-1 and BE4443/6-1), Landes-Offensive zur Entwicklung Wissenschaftlich-ökonomischer Exzellenz, the Universitätsklinikum Giessen Marburg, the University of Giessen Marburg Lung Center, the German Center for Lung Research (DZL), and COST (BM1201). SH was supported by the Deutsche Forschungsgemeinschaft (SFB1221, C05; SFB-TR84, B2; EXC147), the Universitätsklinikum Giessen & Marburg (UKGM, FOKOOPV), and the German Center for Lung Research (DZL). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contribution statement

The authors contributed in the following way: concept and design: CMC, SB, and PM; acquisition of data: CMC, CT, FY, AS, AM, EEA, VK, MJ, SD, JW, JQ, RGA, and NA; analysis and interpretation: JW, SB,

CMC, HE, KA, REM, SH, RS, GC, JSZ, and GB; drafting and editing of the manuscript: CMC, SB, WS, PM, KPZ, CT, EEA, and MJ. All authors read and approved the final manuscript.

References

- Benjamin JT, Smith RJ, Halloran BA, et al. FGF-10 is decreased in bronchopulmonary dysplasia and suppressed by Toll-like receptor activation. *Am J Physiol Lung Cell Mol Physiol* 2007; **292**: L550–L558.
- Watterberg KL, Demers LM, Scott SM, et al. Chorioamnionitis and early lung inflammation in infants in whom bronchopulmonary dysplasia develops. *Pediatrics* 1996; **97**: 210–215.
- Klinger G, Levy I, Sirota L, et al. Outcome of early-onset sepsis in a national cohort of very low birth weight infants. *Pediatrics* 2010; **125**: e736–e740.
- Benjamin JT, Carver BJ, Plosa EJ, et al. NF- κ B activation limits airway branching through inhibition of Sp1-mediated fibroblast growth factor-10 expression. *J Immunol* 2010; **185**: 4896–4903.
- Carver BJ, Plosa EJ, Stinnett AM, et al. Interactions between NF- κ B and SP3 connect inflammatory signaling with reduced FGF-10 expression. *J Biol Chem* 2013; **288**: 15318–15325.
- Allen J, Zwerdling R, Ehrenkranz R, et al. Statement on the care of the child with chronic lung disease of infancy and childhood. *Am J Respir Crit Care Med* 2003; **168**: 356–396.
- Baraldi E, Filippone M. Chronic lung disease after premature birth. *N Engl J Med* 2007; **357**: 1946–1955.
- Jain D, Bancalari E. Bronchopulmonary dysplasia: clinical perspective. *Birth Defects Res A Clin Mol Teratol* 2014; **100**: 134–144.
- Stoll BJ, Hansen NI, Bell EF, et al. Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. *Pediatrics* 2010; **126**: 443–456.
- Jobe AJ. The new BPD: an arrest of lung development. *Pediatr Res* 1999; **46**: 641–643.
- El Mazloum D, Moschino L, Bozzetto S, et al. Chronic lung disease of prematurity: long-term respiratory outcome. *Neonatology* 2014; **105**: 352–356.
- Jacob SV, Coates AL, Lands LC, et al. Long-term pulmonary sequelae of severe bronchopulmonary dysplasia. *J Pediatr* 1998; **133**: 193–200.
- Chao CM, El Agha E, Tiozzo C, et al. A breath of fresh air on the mesenchyme: impact of impaired mesenchymal development on the pathogenesis of bronchopulmonary dysplasia. *Front Med* 2015; **2**: 27.
- El Agha E, Bellusci S. Walking along the fibroblast growth factor 10 route: a key pathway to understand the control and regulation of epithelial and mesenchymal cell-lineage formation during lung development and repair after injury. *Scientifica* 2014; **2014**: 538379.
- Al Alam D, El Agha E, Sakurai R, et al. Evidence for the involvement of fibroblast growth factor 10 in lipofibroblast formation during embryonic lung development. *Development* 2015; **142**: 4139–4150.
- Danopoulos S, Parsa S, Al Alam D, et al. Transient inhibition of FGFR2b-ligands signaling leads to irreversible loss of cellular beta-catenin organization and signaling in AER during mouse limb development. *PLoS One* 2013; **8**: e76248.
- MacKenzie B, Henneke I, Hezel S, et al. Attenuating endogenous Fgfr2b ligands during bleomycin-induced lung fibrosis does not compromise murine lung repair. *Am J Physiol Lung Cell Mol Physiol* 2015; **308**: L1014–L1024.
- Parsa S, Kuremoto K, Seidel K, et al. Signaling by FGFR2b controls the regenerative capacity of adult mouse incisors. *Development* 2010; **137**: 3743–3752.
- Parsa S, Ramasamy SK, De Langhe S, et al. Terminal end bud maintenance in mammary gland is dependent upon FGFR2b signaling. *Dev Biol* 2008; **317**: 121–131.
- Ramasamy SK, Mailleux AA, Gupte VV, et al. Fgf10 dosage is critical for the amplification of epithelial cell progenitors and for the formation of multiple mesenchymal lineages during lung development. *Dev Biol* 2007; **307**: 237–247.
- Itoh N, Ornitz DM. Functional evolutionary history of the mouse *Fgf* gene family. *Dev Dyn* 2008; **237**: 18–27.
- Berger J, Bhandari V. Animal models of bronchopulmonary dysplasia. The term mouse models. *Am J Physiol Lung Cell Mol Physiol* 2014; **307**: L936–L947.
- Bridges JP, Ikegami M, Brilli LL, et al. LPCAT1 regulates surfactant phospholipid synthesis and is required for transitioning to air breathing in mice. *J Clin Invest* 2010; **120**: 1736–1748.
- Weng T, Gao L, Bhaskaran M, et al. Pleiotrophin regulates lung epithelial cell proliferation and differentiation during fetal lung development via beta-catenin and Dlk1. *J Biol Chem* 2009; **284**: 28021–28032.
- Treutlein B, Brownfield DG, Wu AR, et al. Reconstructing lineage hierarchies of the distal lung epithelium using single-cell RNA-seq. *Nature* 2014; **509**: 371–375.
- Volckaert T, Dill E, Campbell A, et al. Parabronchial smooth muscle constitutes an airway epithelial stem cell niche in the mouse lung after injury. *J Clin Invest* 2011; **121**: 4409–4419.
- Volckaert T, Campbell A, Dill E, et al. Localized Fgf10 expression is not required for lung branching morphogenesis but prevents differentiation of epithelial progenitors. *Development* 2013; **140**: 3731–3742.
- Nyeng P, Norgaard GA, Kobberup S, et al. FGF10 maintains distal lung bud epithelium and excessive signaling leads to progenitor state arrest, distalization, and goblet cell metaplasia. *BMC Dev Biol* 2008; **8**: 2.
- Barkauskas CE, Cbron MJ, Rackley CR, et al. Type 2 alveolar cells are stem cells in adult lung. *J Clin Invest* 2013; **123**: 3025–3036.
- Hokuto I, Perl AK, Whitsett JA. FGF signaling is required for pulmonary homeostasis following hyperoxia. *Am J Physiol Lung Cell Mol Physiol* 2004; **286**: L580–L587.
- Hadchouel A, Durrmeyer X, Bouzigon E, et al. Identification of *SPOCK2* as a susceptibility gene for bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 2011; **184**: 1164–1170.
- Prince LS, Karp PH, Moninger TO, et al. KGF alters gene expression in human airway epithelia: potential regulation of the inflammatory response. *Physiol Genomics* 2001; **6**: 81–89.
- Ware LB, Matthay MA. Keratinocyte and hepatocyte growth factors in the lung: roles in lung development, inflammation, and repair. *Am J Physiol Lung Cell Mol Physiol* 2002; **282**: L924–L940.
- Tichelaar JW, Wesselkamper SC, Chowdhury S, et al. Duration-dependent cytoprotective versus inflammatory effects of lung epithelial fibroblast growth factor-7 expression. *Exp Lung Res* 2007; **33**: 385–417.
- Gupte VV, Ramasamy SK, Reddy R, et al. Overexpression of fibroblast growth factor-10 during both inflammatory and fibrotic phases attenuates bleomycin-induced pulmonary fibrosis in mice. *Am J Respir Crit Care Med* 2009; **180**: 424–436.
- Tong L, Zhou J, Rong L, et al. Fibroblast growth factor-10 (FGF-10) mobilizes lung-resident mesenchymal stem cells and protects against acute lung injury. *Sci Rep* 2016; **6**: 21642.
- Klar J, Blomstrand P, Brunmark C, et al. Fibroblast growth factor 10 haploinsufficiency causes chronic obstructive pulmonary disease. *J Med Genet* 2011; **48**: 705–709.
- *38. McGrath-Morrow SA, Cho C, Soutiere S, et al. The effect of neonatal hyperoxia on the lung of p21Waf1/Cip1/Sdi1-deficient mice. *Am J Respir Cell Mol Biol* 2004; **30**: 635–640.

- *39. Woyda K, Koebrich S, Reiss I, et al. Inhibition of phosphodiesterase 4 enhances lung alveolarisation in neonatal mice exposed to hyperoxia. *Eur Respir J* 2009; **33**: 861–870.
- *40. Seimetz M, Parajuli N, Pichl A, et al. Inducible NOS inhibition reverses tobacco-smoke-induced emphysema and pulmonary hypertension in mice. *Cell* 2011; **147**: 293–305.
- *41. Gentleman RC, Carey VJ, Bates DM, et al. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 2004; **5**: R80.
- *42. Smyth GK. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 2004; **3**: Article 3.
- *43. McQualter JL, Yuen K, Williams B, et al. Evidence of an epithelial stem/progenitor cell hierarchy in the adult mouse lung. *Proc Natl Acad Sci U S A* 2010; **107**: 1414–1419.
- *Cited only in supplementary material.

SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Supplementary figure legends

Figure S1. Validation of the HOX injury model to trigger hypoalveologenesis

Figure S2. Greater magnification of the heatmap shown in Figure 2C

Figure S3. Immunofluorescence for phospho-Smad3

Figure S4. Differentially expressed genes in HOX versus NOX in AECII cells isolated from lungs of WT and *Fgf10*^{+/-} mice

Figure S5. Greater magnification of the graphs shown in Figure 3B, a, b

Figure S6. Greater magnification of the graphs shown in Figure 3D, E

Figure S7. Impact of HOX versus NOX on the epithelium and mesenchyme of *Fgf10*^{+/-} and WT lungs at P3

Table S1. Primers used for RT-qPCR

Table S2. Antibodies used for western blotting

Table S3. Antibodies used for FACS

Table S4. Antibodies used for immunofluorescence

50 Years ago in the *Journal of Pathology*...

Colonial pigmentation of *Staphylococcus aureus*

A. T. Willis, J. Jean O'Connor and J. A. Smith

Fimbriae and adhesive properties in salmonellae

J. P. Duguid, E. S. Anderson and I. Campbell

To view these articles, and more, please visit:

www.thejournalofpathology.com

Click 'ALL ISSUES (1892 - 2016)', to read articles going right back to Volume 1, Issue 1.

The Journal of Pathology
Understanding Disease



GENERAL ARTICLE

Impact of *Fgf10* deficiency on pulmonary vasculature formation in a mouse model of bronchopulmonary dysplasia

Cho-Ming Chao^{1,2,3,†}, Alena Moiseenko², Djuro Kosanovic², Stefano Rivetti², Elie El Agha^{1,2}, Jochen Wilhelm², Marian Kampschulte⁴, Faady Yahya², Harald Ehrhardt³, Klaus-Peter Zimmer³, Guillermo Barreto^{5,6}, Albert A. Rizvanov⁶, Ralph T. Schermuly², Irwin Reiss⁷, Rory E. Morty⁵, Robbert J. Rottier⁸, Saverio Bellusci^{1,2,6,*} and Jin-San Zhang^{1,*}

¹International Collaborative Center on Growth Factor Research, School of Pharmaceutical Sciences, Wenzhou Medical University and Institute of Life Sciences, Wenzhou University, Wenzhou, Zhejiang 325035, China,

²Member of the German Lung Research Center (DZL), Department of Internal Medicine II, Universities of Gießen and Marburg Lung Center, Excellence Cluster Cardio-Pulmonary System (ECCPS), 35392 Gießen, Germany, ³University Children's Hospital Gießen, Department of General Pediatrics and Neonatology, Justus-Liebig-University, 35392 Gießen, Germany. Member of the German Lung Research Center (DZL), Universities of Gießen and Marburg Lung Center (UGMLC), 35392 Gießen, Germany, ⁴Department of Radiology, Justus-Liebig-University, University Hospital Gießen, 35392 Gießen, Germany, ⁵Max-Planck-Institute for Heart and Lung Research, Member of the German Lung Research Center (DZL), 61231 Bad Nauheim, Germany,

⁶Institute of Fundamental Medicine and Biology, Kazan (Volga Region) Federal University, Kazan 420021, Russian Federation, ⁷Division of Neonatology, Erasmus Medical Center–Sophia Children's Hospital,

3015 Rotterdam, The Netherlands and ⁸Department of Pediatric Surgery, Erasmus Medical Center–Sophia Children's Hospital, 3015 Rotterdam, The Netherlands; Department of Cell Biology, Erasmus Medical Center, 3015 Rotterdam, The Netherlands

*To whom correspondence should be addressed. Saverio Bellusci, Tel: 0049-641-9946730; Fax: 0049-641-9946739;

Email: saverio.bellusci@innere.med.uni-giessen.de and Jin-San Zhang, Tel: 0086-577-86599693; Fax: (+86)-577-86689983; Email: zhang_jinsan@163.com

Abstract

Bronchopulmonary dysplasia (BPD), characterized by alveoli simplification and dysmorphic pulmonary microvasculature, is a chronic lung disease affecting prematurely born infants. Pulmonary hypertension (PH) is an important BPD feature associated with morbidity and mortality. In human BPD, inflammation leads to decreased fibroblast growth factor 10 (FGF10) expression but the impact on the vasculature is so far unknown. We used lungs from *Fgf10*^{+/-} versus *Fgf10*^{+/+} pups to investigate the effect of *Fgf10* deficiency on vascular development in normoxia (NOX) and hyperoxia (HOX, BPD mouse model). To assess the role of fibroblast growth factor receptor 2b (Fgfr2b) ligands independently of early developmental

[†]Cho-Ming Chao, <http://orcid.org/0000-0002-0034-8264>

Received: October 17, 2018. Revised: December 11, 2018. Accepted: December 14, 2018

© The Author(s) 2018. Published by Oxford University Press. All rights reserved.

For Permissions, please email: journals.permissions@oup.com

defects, we used an inducible double transgenic system in mice allowing inhibition of Fgfr2b ligands activity. Using vascular morphometry, we quantified the pathological changes. Finally, we evaluated changes in FGF10, surfactant protein C (SFTPC), platelet endothelial cell adhesion molecule (PECAM) and alpha-smooth muscle actin 2 (α -SMA) expression in human lung samples from patients suffering from BPD. In NOX, no major difference in the lung vasculature between *Fgf10*^{+/-} and control pups was detected. In HOX, a greater loss of blood vessels in *Fgf10*^{+/-} lungs is associated with an increase of poorly muscularized vessels. Fgfr2b ligands inhibition postnatally in HOX is sufficient to decrease the number of blood vessels while increasing the level of muscularization, suggesting a PH phenotype. BPD lungs exhibited decreased FGF10, SFTPC and PECAM but increased α -SMA. *Fgf10* deficiency-associated vascular defects are enhanced in HOX and could represent an additional cause of morbidity in human patients with BPD.

Introduction

Fibroblast growth factor 10 (FGF10) protein deficiency has been reported in patients with bronchopulmonary dysplasia (BPD) (1). BPD is a chronic lung disease of prematurely born infants and remains a leading cause of morbidity and mortality. In humans, inflammation is known to increase risk for BPD (2,3). Supporting a functional link between inflammation and FGF10-messenger ribonucleic acid (mRNA) expression, inflammatory mediators activated by bacterial-derived lipopolysaccharides (LPS) (4) such as NF- κ B, Sp1 and Sp3 were found to inhibit *Fgf10* transcription in mouse lung explants (5,6). Indeed, the inhibition of *Fgf10* expression is mediated by LPS receptors (toll-like receptors 2 and 4) activation (1). In addition to inflammation-induced FGF10 deficiency, congenital FGF10 expression deficiency [as well as deficiency in the FGF10 receptor, fibroblast growth factor receptor 2b (FGFR2b)] has been reported in humans suffering from aplasia of lacrimal and salivary glands (ALSG) and lacrimo-auriculo-dento-digital (LADD) syndromes (7–10). Patients with heterozygous FGF10 loss-of-function exhibit significant decrease in inspiratory vital capacity (IVC), forced expiratory volume 1 (FEV1) and FEV1/IVC ratio compared to non-carrier siblings and to predicted reference values (11). These data are consistent with chronic obstructive pulmonary disease. As both patients with BPD and ALSG or LADD syndrome are characterized by developmental abnormalities, we hypothesized that dysregulation of FGF10 expression contributes to the associated lung pathogenesis in these patients.

Fgf10 has been shown using animal models to be a key developmental regulator during lung development. This is illustrated by the fact that *Fgf10* null pups die at birth due to lung agenesis (12). Fgf10 is localized in the mesenchyme of the developing lung from the onset of organogenesis and encodes a secreted protein, which acts in a paracrine fashion mainly through the receptor Fgfr2b, expressed in the lung epithelium (13–15). During the initial phases of the pseudoglandular stage of lung development [embryonic day (E) 9.5 through 16.5 in mice], Fgf10 expression adjacent to the nascent epithelial buds as well as its activity *in vitro* on isolated lung epithelium grown in matrigel suggested that Fgf10 plays a crucial role in controlling branching morphogenesis (16). We reported that congenital *Fgf10* deficiency (mice heterozygous for *Fgf10*, *Fgf10*^{+/-}) disrupts the formation of the alveolar lineage in a quantitative and qualitative manner leading to increased alveolar epithelial cell type I and decreased alveolar epithelial cell type II (AT2). Furthermore, surfactant protein B and C (Sftpb and Sftpc) levels were reduced in the mutants and such decreases are associated with the observed fatal hyperoxia (HOX)-induced lung injury in a mouse model of BPD (17).

During the pseudoglandular stage, Fgf10 also indirectly orchestrates the development of the adjacent mesenchyme, which contains the progenitors of multiple important lung cell types, including the endothelium, the vascular and airway

smooth muscle cells, the alveolar myofibroblasts, the lipofibroblasts, the interstitial fibroblasts and the nerve cells (for a review, see (18,19)). Its impact on the mesenchyme is not surprising as Fgf10 signalling is embedded in an interactive signalling network comprising major pathways such as retinoic acid, sonic hedgehog, bone morphogenetic protein 4, transforming growth factor beta-1, Wnt, platelet-derived growth factor and vascular endothelial growth factor. In addition to a developmental role, Fgf10 is a major player in regeneration of the lung after injury. In bleomycin-induced lung fibrosis, *Fgf10* overexpression is both protective and therapeutic. Fgf10 is also involved in the regeneration of the bronchial lung epithelium after naphthalene injury (20,21).

The evidence confirming the key role of Fgf10 in embryonic lung development is strong. However, less is known about the impact of constitutive *Fgf10* deficiency on the formation and maintenance of the pulmonary vasculature and in the context of normoxia (NOX) and HOX-induced lung injury.

To demonstrate the effect of *Fgf10* deficiency on postnatal pulmonary vasculature in normoxic condition, we used a constitutive heterozygous *Fgf10*^{+/-} mouse line. Since oxygen toxicity is one of the major risk factors contributing to BPD, we used the HOX-induced BPD mouse model (85% oxygen from P0–P8) to investigate the impact of *Fgf10* deficiency in hyperoxic condition. As 100% of the *Fgf10*^{+/-} mutant pups are dead at P8 during HOX exposure (17), we chose P3 as a time point for further analysis of the lung vasculature. The analysis of lung vascular morphometry to identify changes in lung vascular structure consists of quantification of vessel count and extent of vessel muscularization. We also used gene arrays on *Fgf10*^{+/-} versus *Fgf10*^{+/+} control littermate whole lungs exposed to NOX or HOX to identify transcriptomic changes potentially linked to vascular formation. We also performed immunofluorescence (IF) and reverse transcriptase-quantitative polymerase chain reaction on experimental and control lungs at different stages of development.

Finally, in human lung samples of patients suffering from BPD, we used immunohistochemistry (IHC) and IF to evaluate the changes in FGF10, SFTPC, platelet endothelial cell adhesion molecule (PECAM) and ACTA2 expressions. For the first time, we report the consequence of *Fgf10* deficiency in mice on postnatal lung vasculature and during HOX-induced lung injury and suggest that defective FGF10 signalling in human patients with BPD is causative for some of the vascular defects.

Results

FGF10 and its receptor FGFR2 are expressed in lungs from patients with BPD

Using IHC, we found that FGF10 and its receptor FGFR2 are expressed in the lung parenchyma of patients with BPD.

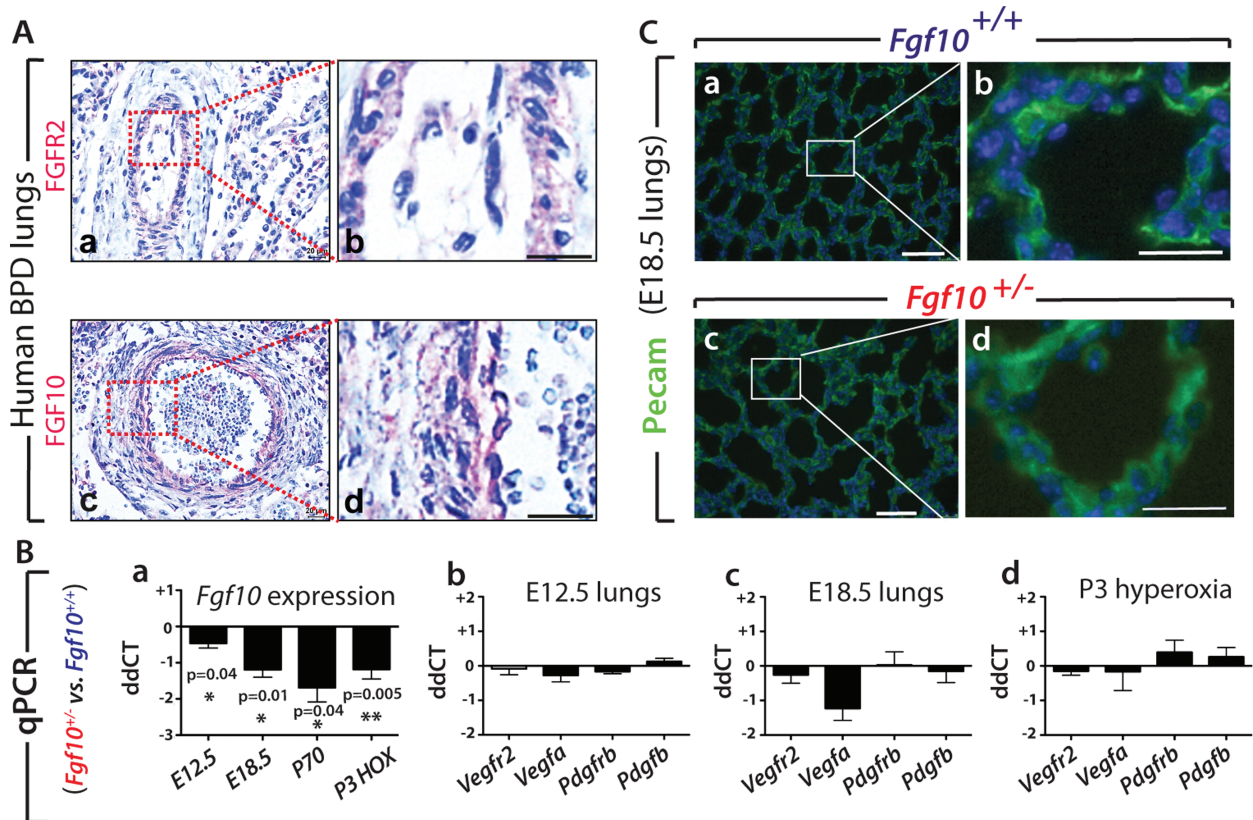


Figure 1. Expression of FGF10 and associated targets in human and mouse lungs. (A) FGFR2 receptor and its ligand FGF10 are expressed in lungs from patients with BPD ($n = 4$). (Aa) FGFR2 and (Ac) FGF10 expression in remodelled pulmonary vasculature. (Ab) and (Ad) respective high magnification. Scale bar: 20 μ m. (B–C) Analysis of vascular smooth muscle cell and endothelial cell markers did not show changes in *Fgf10*^{+/-} versus *Fgf10*^{+/+} control lungs at E12.5, E18.5 and P3 (HOX). (Ba) qPCR analysis showed significant decrease of *Fgf10* expression in *Fgf10*^{+/-} versus *Fgf10*^{+/+} lungs at E12.5, E18.5, P70 and P3 HOX, $n = 4$ each. (Bb–Bd) Comparative expression by qPCR analysis in *Fgf10*^{+/-} versus *Fgf10*^{+/+} control lungs at (b) E12.5, (c) E18.5 and (d) P3 HOX of *Vegfr2*, *Vegfa*, *Pdgfrb* and *Pdgfb*. No significant difference is observed even though a trend towards a decrease in *Vegfa* is observed at E18.5. (C) IF for PECAM1 staining on E18.5 (a,b) *Fgf10*^{+/+} and (c,d) *Fgf10*^{+/-} lungs ($n = 4$ each) showed slightly lower immunoreactivity in *Fgf10*^{+/-} lungs. Scale bar: a, c, 100 μ m; b, d, 30 μ m.

In particular, FGF10 and FGFR2 positive immunoreactivity are localized in relevant lung histopathological features of the disease, such as remodelled pulmonary vasculature (Fig. 1A) as well as in alveolar septa and interstitial fibrotic regions (Supplementary Material, Fig. S1A and C) and in distorted conducting airways (Supplementary Material, Fig. S1B and D).

Expression levels of vascular smooth muscle cell and endothelial cell markers are indistinguishable between *Fgf10*^{+/-} versus control *Fgf10*^{+/+} lungs in NOX and HOX

The formation of the vascular system during development was first investigated by examining the expression levels of key vascular genes, previously reported to be regulated in the lungs of *Fgf10* hypomorphic (*Fgf10*^{LacZ/-}) embryos displaying one copy of the *Fgf10*^{LacZ} hypomorphic allele and one copy of the deleted (*Fgf10*⁻) allele (22). We compared the expression of these genes in *Fgf10*^{+/-} versus control lungs. The decrease in *Fgf10* expression in experimental lungs was validated by qPCR at different time points (Fig. 1Ba). At all the stages considered [embryonic day (E) 12.5, E18.5, P70], *Fgf10* was reduced but at different levels indicating potential compensatory mechanisms at work allowing to regulate *Fgf10* transcription from the remaining *Fgf10* wild-type allele. Decrease in *Fgf10* expression in the P3 lungs between the

two genotypes was also detected in the context of HOX exposure (Fig. 1Ba).

Next, we determined by qPCR in *Fgf10*^{+/-} versus control lungs at the time points mentioned before (Fig. 1Bb–d) the expressions of *Vegfr2*, its associated ligand *Vegfa*, as well as the vascular smooth muscle receptor platelet derived growth factor receptor beta (*Pdgfrb*) and its associated ligand *Pdgfb*. For all these time points and conditions, we found that the expression of these genes was not severely affected by loss of one copy of the *Fgf10* allele, even though a decrease was observed at E18.5 for *Vegfa*. IF in lungs at E18.5 for PECAM1 to label the endothelial cells indicated a strong and very defined signal in control lungs (Fig. 1Ca, b; $n = 4$) and an equally strong but more diffuse signal in mutant lung (Fig. 1Cc, d; $n = 4$). As the samples were processed under the same conditions, this difference could reflect potential endothelial defects in *Fgf10*^{+/-} lungs. Interestingly, a significant decrease in PECAM1 mRNA expression was detected in BPD versus control lungs (Supplementary Material, Fig. S2A).

Morphometry analysis of mutant versus control lungs in NOX suggests only minor defects in the status of the vasculature upon reduced *Fgf10* expression

To further examine the status of the vasculature in mutant versus control lungs in NOX, we quantified at P3 the number of

vessels and extent of muscularization of these vessels. The analysis was based on detection of muscle tissue (α -SMA staining) surrounding the vessel endothelium (von Willebrand staining, vWF) (Fig. 2A). Vessels were subdivided into four groups based on their diameters (10–20 μ m, 20–70 μ m, 70–150 μ m and >150 μ m). The number of blood vessels in *Fgf10*^{+/-} versus control lungs in NOX indicated no difference in total vessel count. However, a significant reduction was observed in the number of 70–150 μ m vessels ($P = 0.049$) (Fig. 2B). No difference in muscularization was noted (Fig. 2Ca–d).

Decreased number of blood vessels and decreased muscularization is observed in *Fgf10*^{+/-} versus control lungs exposed to HOX

Next, we compared the vascular response of mutant and control lungs to HOX. In both genotypes, we observed a drop in total blood vessel count upon HOX injury, the 20–70 μ m blood vessels being the most affected (Fig. 2Da and b). Interestingly, in control lungs, HOX significantly increased the number of 70–150 μ m blood vessels (Fig. 2Da, $P < 0.001$). In *Fgf10*^{+/-} lungs, HOX significantly reduced the total blood vessel count ($P = 0.01$) (Fig. 2Db). As for the control lungs, we observed a decrease in the number of 20–70 μ m blood vessels ($P = 0.008$) and an increase in the number of 70–150 μ m blood vessels ($P = 0.02$).

The direct comparison of the *Fgf10*^{+/-} versus control lungs in HOX (Fig. 2E) indicated decreased vessel number in mutant lungs ($P = 0.07$), which is mostly affecting the 20–70 μ m blood vessels ($P = 0.06$). A significant loss of muscularization (decrease in partial muscularized but increase in non-muscularized vessels) was seen for the 20–70 μ m blood vessel group (Fig. 2Fb) and reduced muscularization was observed for the 70–150 μ m blood vessels (Fig. 2Fc). Interestingly, despite these defects, we failed to see any evidence of lung hemorrhage in *Fgf10*^{+/-} lungs in HOX at P3 (Fig. 2A), suggesting that in our conditions, the contribution of the vascular system to the previously observed lethal phenotype is likely minor (17).

FGF10 expression is reduced in patients with BPD and is associated with epithelial and vascular defects

We further validated in human lungs the observations made in mice. We obtained samples from either patients with BPD ($n = 4$) or neonates who died from causes unrelated to lung disease ($n = 2$). In non-BPD control lungs, FGF10 protein was detected around the conducting airways and within the lung interstitial tissue. However, lungs from patients with BPD displayed lower FGF10 protein expression (Fig. 2Ga and b), confirming the previously reported decrease in FGF10 in BPD lungs (1). qRT-PCR analysis was carried out for FGF10 and associated ligands as well as *FGFR2b* in human BPD ($n = 3$) versus control ($n = 4$) lungs (Supplementary Material, Fig. S2A). We confirmed the decrease in FGF10 expression as well as FGF7 and *FGFR2b*. No changes in the expression of FGF1 and FGF3 were detected. In mouse, the expression of *Fgf1*, 3 and 7 is not changed between *Fgf10*^{+/-} and wild type (WT) lungs ($n = 4$ each group) (Supplementary Material, Fig. S2B).

Mature SFTPC, a secreted protein expressed by AT2 cells, which was strongly detected in control lungs, was much reduced in BPD samples (Fig. 2Gc and d). PECAM, an endothelial marker, was also reduced in BPD versus control lungs (Fig. 2Ge and f). In line with the known pulmonary hypertension (PH) phenotype, α -smooth muscle actin (α -SMA) expression was increased in patients with BPD (Fig. 2Gg and h).

Transcriptomic analysis of the *Fgf10*^{+/-} and control lungs at P3 in NOX or HOX

Next, we compared the gene arrays from HOX- versus NOX-exposed *Fgf10*^{+/-} and control P3 lungs (Fig. 3A). Volcano plots revealed that HOX treatment was indeed effective in triggering changes in gene expression profiles in both control and experimental lungs (Fig. 3B and C, respectively). However, changes between the two genotypes, either in NOX or HOX, were more modest (Fig. 3D and E, respectively). The expression changes in response to HOX correlated between the two genotypes (Fig. 3F). The light blue points within the central circle represent genes that are not considerably regulated by HOX in either genotype. The dark blue points represent genes that are similarly regulated by HOX in both genotypes. The red points represent genes that show a more than 2-fold differential regulation between the genotypes. To support this result, we identified sets of genes differentially expressed in HOX versus NOX (selected according to their P -value) in control P3 lungs (Fig. 3G). As expected from the correlation analysis, these genes were similarly differentially regulated in HOX versus NOX in *Fgf10*^{+/-} P3 lungs (Fig. 3G). (See Supplementary Material, Fig. S3 for higher magnification of the heatmaps shown in Fig. 3G)

In contrast, the differential expression profiles due to the genotype did not correlate between the hyperoxic and normoxic conditions (Fig. 3H). To further define the genes affected by the decrease in *Fgf10* expression in NOX, we identified the top 100 genes regulated between *Fgf10*^{+/-} versus control in NOX (Fig. 3I). Interestingly, these genes respond to HOX by being globally up-regulated (top part of the heatmap) or down-regulated (low part of the heatmap) (Fig. 3I, right heatmap). (See Supplementary Material, Fig. S4 for higher magnification of the heatmaps shown in Fig. 3I.)

Next, we compared by qPCR the changes in *Fgf* signalling and the associated epithelial markers between *Fgf10*^{+/-} and control lungs in NOX (Fig. 3Ja and b). As expected, the decrease in *Fgf10* expression is associated with decreased *Fgfr2b*, *Etv5* and *Spry2*, which support impaired *Fgf10* signalling in NOX. The decrease in epithelial markers, particularly significant for *Epcam*, was also observed (Fig. 3Jb). *Fgf10*^{+/-} lungs at P3 do not exhibit significantly increased *Tgf β* signalling (Fig. 3Jc). Nonetheless, we observe a modest increase in collagen expression (Fig. 3Jd) and except for *Cnab2*, no significant change in cell cycle gene expression was detected (Fig. 3Je).

The same analysis in HOX showed significant decreased expression of *Fgf10* and an increase of epithelial markers (Fig. 3Ka and b). Further, we found significant increase in *Tgf β 1* and *Tgf β 3* and drastic increase in all the collagen genes (Fig. 3Kc and d).

These results are supported by our previous report (17). Increased phospho-SMAD3 indicating activated *Tgf- β* signalling was detected in *Fgf10*^{+/-} versus control mice in HOX P3 lung. In addition, our western blot results show a trend towards increase collagen levels in *Fgf10*^{+/-} versus control mice (Supplementary Material, Fig. S5A).

Finally, cell cycle genes observed in the gene array, which regulate cell cycle either negatively (cyclin-dependent kinase inhibitor 1A, *Cdkn1a*) or positively (*Ccnb1*, *Ccnb2*, *Ccna2*) are significantly up-regulated (Fig. 3Ke).

In order to identify the significant genes/pathways differentially affected by HOX versus NOX between *Fgf10*^{+/-} versus control, we carried out an analysis of the interaction HOX $\times$ genotype (Fig. 4). This analysis relies on the comparison of the genes differentially expressed in (HOX versus NOX in *Fgf10*^{+/-}

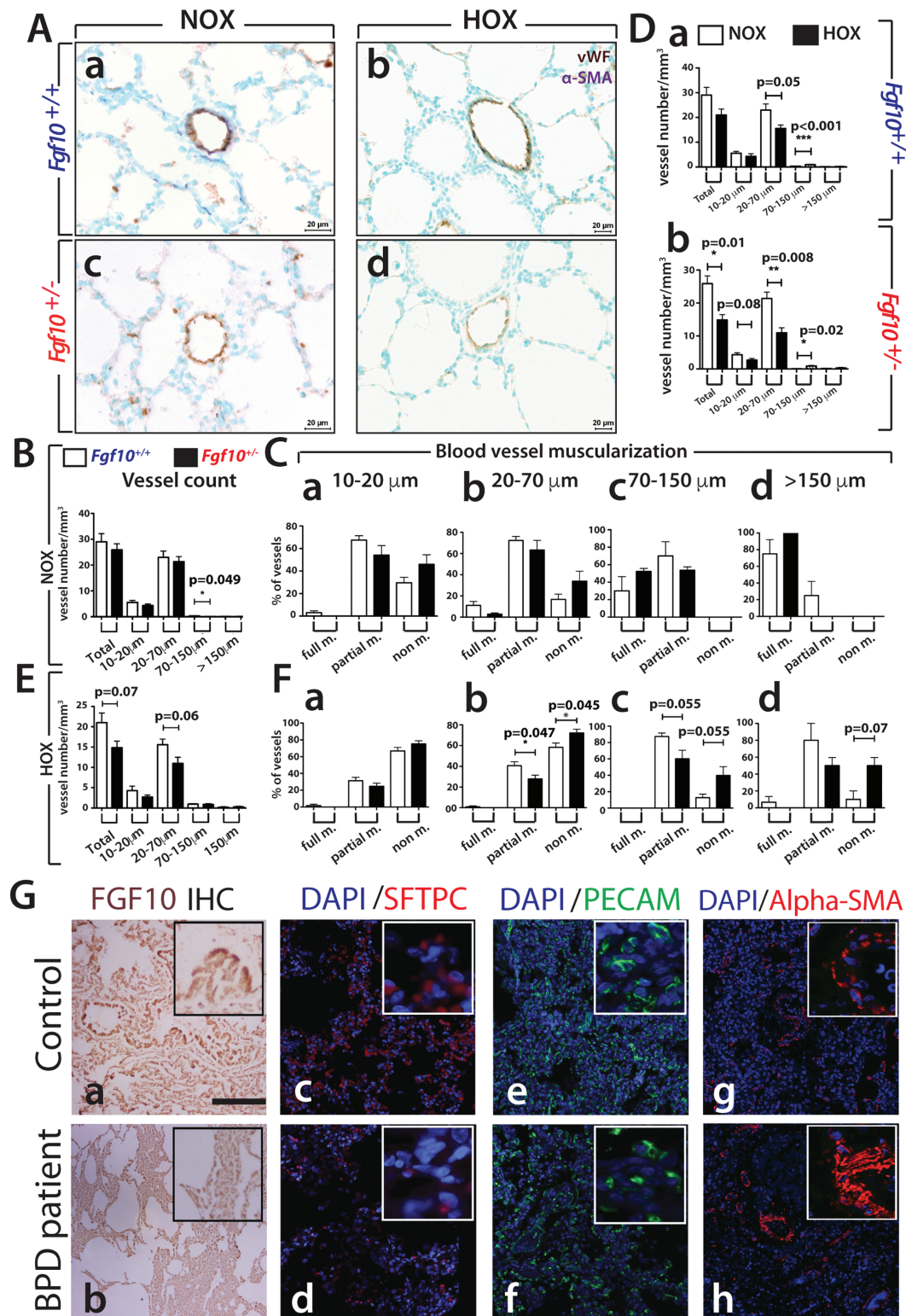


Figure 2. Analysis of the vascular defects in mouse and human lungs. (A–F) Analysis of the vascular defects in P3 $Fgf10^{+/-}$ versus $Fgf10^{+/+}$ control lungs in HOX. (A) IHC for vWF and α -SMA of (a, b) $Fgf10^{+/+}$ and (c, d) $Fgf10^{+/-}$ lungs between NOX ($Fgf10^{+/+}$ $n = 6$, $Fgf10^{+/-}$ $n = 3$) and HOX ($n = 4$ each). (B) Total vessel count in NOX between P3 $Fgf10^{+/+}$ and $Fgf10^{+/-}$ lungs. (C) Blood vessel muscularization in NOX between $Fgf10^{+/+}$ and $Fgf10^{+/-}$ lungs for blood vessels at (a) 10–20 μ m, (b) 20–70 μ m, (c) 70–150 μ m and (d) >150 μ m. (D) Total vessel count between NOX and HOX in (a) $Fgf10^{+/+}$ and (b) $Fgf10^{+/-}$ lungs. (E) Total vessel count in HOX between P3 $Fgf10^{+/+}$ and $Fgf10^{+/-}$ lungs. (F) Blood vessel muscularization in HOX between $Fgf10^{+/+}$ and $Fgf10^{+/-}$ control lungs for blood vessels at (a) 10–20 μ m, (b) 20–70 μ m, (c) 70–150 μ m and (d) >150 μ m. Scale: Aa–d, 20 μ m. (G) Immunofluorescence for FGF10, SFTPC, PECAM and ACTA2 in lungs of BPD ($n = 4$) versus control non-BPD ($n = 2$) patients. (a, b) FGF10 expression, (c, d) mature-SFTPC expression, (e, f) PECAM expression and (g, h) ACTA2 expression. Scale bars A–F: 100 μ m, insets in (A–F): 25 μ m.

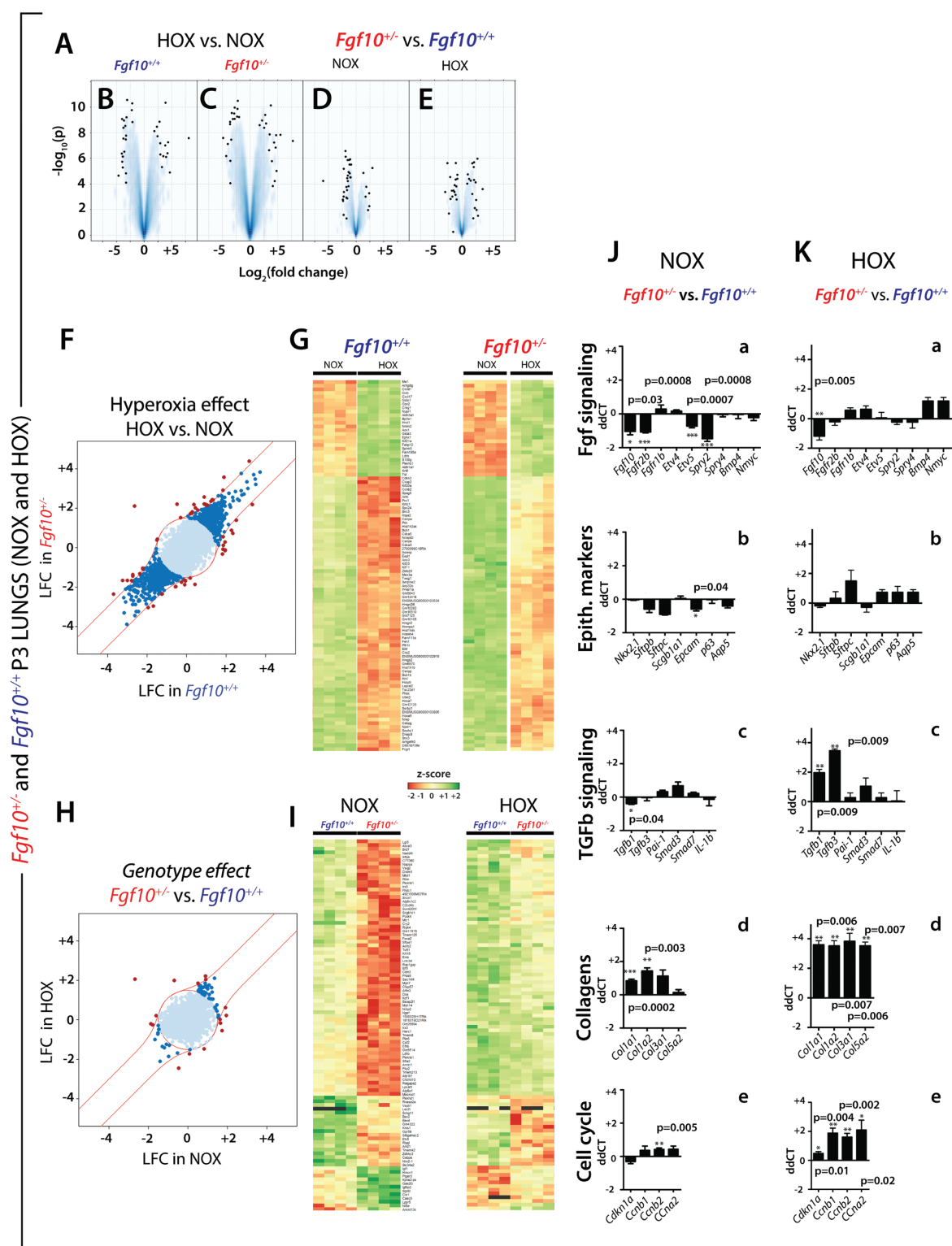


Figure 3. Identification of sets of genes differentially expressed in HOX versus NOX (selected according to their P-value) in whole lungs from WT or *Fgf10*^{+/-} (A) volcano plots. (B) Volcano plot for HOX versus NOX in *Fgf10*^{+/+} (control) lungs (n = 4 each). (C) Volcano plot for HOX versus NOX in *Fgf10*^{+/-} lungs (n = 4 each). (D) Volcano plot from *Fgf10*^{+/-} versus control lungs in NOX. (E) Volcano plot in *Fgf10*^{+/-} versus control lungs in HOX. (F) HOX effect HOX versus NOX. (G) Left heat map: top 100 regulated genes selected based on their P-value between NOX and HOX in *Fgf10*^{+/+} lungs (n = 4 each), right heat map: analysis of the same top 100 genes between NOX and HOX in *Fgf10*^{+/-} lungs (n = 4 each). (H) Genotype effect *Fgf10*^{+/-} versus control lungs. (I) Left heat map: top 100 regulated genes between *Fgf10*^{+/-} versus control lungs in NOX (n = 4 each), right heat map: analysis of the same top 100 genes between *Fgf10*^{+/-} versus control lungs in HOX (n = 4 each). (J–K) qPCR validation of gene expression in *Fgf10*^{+/-} versus control lungs in NOX (n = 4 each) (J) and HOX (n = 4 each) (K) for (a) Fgf signalling, (b) Epithelial markers, (c) Tgfb signalling, (d) collagens and (e) cell cycle.

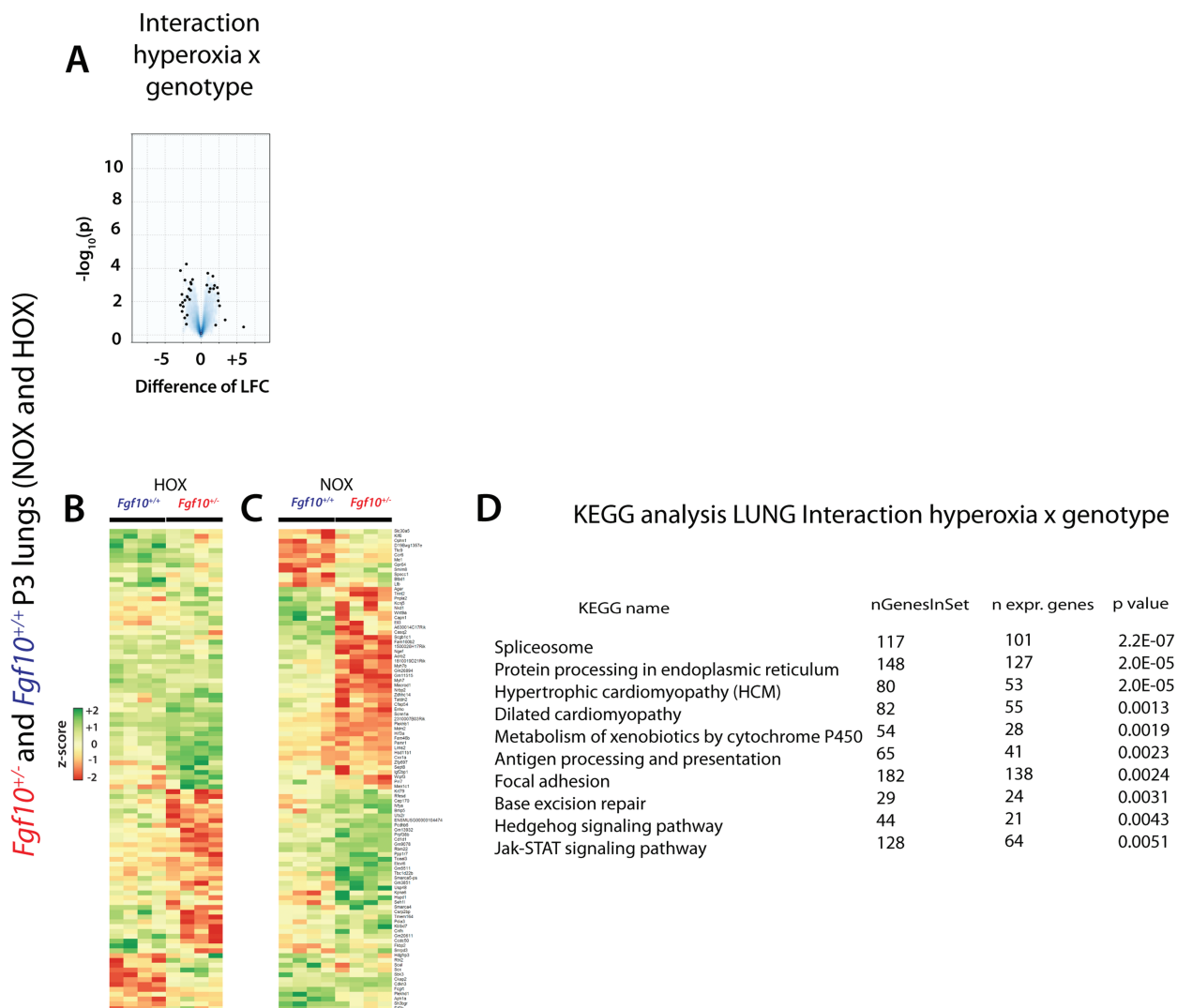


Figure 4. Interaction HOX × genotype analysis to identify the significant genes/pathways differentially affected by HOX in *Fgf10*^{+/+} versus control P3 lungs. (A) Volcano plot. (B) Top 100 regulated genes in HOX in *Fgf10*^{+/+} versus control lungs (n = 4 each). (C) Analysis of the same top 100 genes in NOX between *Fgf10*^{+/+} and control lungs (n = 4 each). (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis between *Fgf10*^{+/+} versus control lungs in HOX versus NOX.

lungs) with those differentially expressed in (HOX versus NOX in control lungs). The volcano plot identified a set of genes, which are up- and down-regulated (Fig. 4A). The top 100 regulated genes according to their *P*-values are represented in the corresponding heat maps (Fig. 4B and C). As expected from the interaction analysis, the differential regulation observed in HOX (Fig. 4B) is no longer maintained in NOX (Fig. 4C). (Please see higher magnification of the heatmaps in the [Supplementary Material, Fig. S6](#)). For example, genes, which expression was not drastically changed in HOX between the two genotypes (located in the top part of the heatmap) are differentially regulated in NOX conditions. Furthermore, genes that were down-regulated in HOX conditions are up-regulated in NOX conditions. These sets of genes, which do not show a parallel increase or decrease in HOX versus NOX, are therefore likely to contribute to the lethal phenotype observed in the *Fgf10*^{+/+} mice in HOX. KEGG analysis reveals that the main processes altered differentially are spliceosomes, protein processing in endoplasmic reticulum, metabolism of xenobiotics by cytochrome P450, antigen processing and presentation (Fig. 4D).

Regarding the changes in the Hedgehog (Hh) pathway ([Supplementary Material, Fig. S7](#)), our data show an increase of expression in the *Fgf10*^{+/+} versus control mice in some elements of the Hh pathway. As reported previously (23,24), we have indications that Hh signalling to the lung mesenchyme regulates capillary network formation of the developing distal lung and through the regulation of expression of *Vegfa* in sub-mesothelial lung mesenchyme. The main difference observed in NOX and HOX is an increase in the expression of the transcriptional factor *Gli2* and 1, respectively. Interestingly, we previously reported that Sonic Hedgehog (Shh) down-regulates *Fgf10* expression in the mesenchyme (16) and it was recently shown that *Fgf10* induces Shh expression in the epithelium (25). From the developmental studies, it is therefore not surprising that *Fgf10* could impact Shh expression and a down-regulation of Shh expression and activity should be expected in the *Fgf10*^{+/+} versus control lungs. However, our data are not sufficient to prove a substantial repression of the pathway, further analysis of the protein localization or post translational modification would be needed. The changes in the focal adhesion ([Supplementary Material,](#)

Fig. S8) and Janus kinases (Jak)-Signal Transducer and Activator of Transcription proteins (STAT) (Supplementary Material, Fig. S9) signalling pathways appear more marked in the *Fgf10*^{+/-} versus control mice, but once again we cannot determine with absolute certainty the exact contribution of each of these pathways to the observed phenotype, especially when the main change in downstream elements of the pathway involves cyclin D1, which is a common target for multiple effector pathways. We also investigated the expression of different elements of the Wnt pathway using KEGG pathway analyses in NOX and HOX, finding a significant alteration of expression in many of them (Supplementary Material, Fig. S10A and B). In particular, the Wnt canonical pathway in HOX shows an up-regulation through the increased expression of *Wnt10b*, a gene encoding an activator of the pathway, and the down-regulation of *adenomatous polyposis coli* (*Apc*), a gene encoding a negative regulator of β -catenin activity. These findings suggest an activation of the β -catenin nuclear activity and are supported by an increase expression of the T-cell factor/lymphoid enhancer factor (TCF/LEF) downstream targets in the analyzed sample, in particular, *Fos1*, *Wisp1* and *Cyclin D1*. However, western blot analysis using whole lung protein extract shows no major difference in phospho- β -catenin between *Fgf10*^{+/-} versus WT lungs (Supplementary Material, Fig. S5B).

Transcriptomic analysis of *Fgf10*^{+/-} versus control lungs supports the correlation between decrease *Fgf10* expression and vascular defects

Next, a KEGG pathway analysis for the genes involved in vascular smooth muscle cell contraction and Vegf signalling between the two genotypes in NOX (Fig. 5) and HOX (Supplementary Material, Fig. S11) was carried out. Figure 5A indicates that 60.9% of the genes controlling vascular smooth muscle cell contraction were differentially expressed between the two genotypes in NOX (41.3% up-regulated, 19.6% down-regulated). Among the down-regulated genes, we found genes encoding for transmembrane proteins [e.g. *Ramp1* (receptor activity modifying protein 1), *Kcnmb2*, *Adora2*] and cytosolic proteins [e.g. *Itpr3* (inositol 1,4,5-trisphosphate receptor type 3), *Pla2g1* (phospholipase A2 group I), *Arhgef*, *Adcy8*]. In particular, *Ramp1* is a receptor compound for intermedin/adrenomedullin leading to stabilization of endothelial barrier function and attenuation of ventilator-induced lung injury in mice (26). Furthermore, adrenomedullin acts through *Ramp1* and is proposed to have an antifibrotic effect in human lung fibroblast (27). *Ramp1*, as part of the CGRP1 receptor, regulates the pulmonary circulation (28). In addition, Caveolin-1 interacts with *Itpr3* to regulate Ca²⁺ entry in endothelial cells (29). Interestingly, *Pla2g1* inactivation reduces atherosclerosis in mice (30).

Among the genes up-regulated, we found genes encoding for transmembrane proteins [e.g. *Ptgir* (prostaglandin I2 receptor), *Cacna1*, *Adra1a*, *Agtr1b*, *Npr1*] and cytosolic proteins (e.g. *Rock1*, *Plcb2*, *Prkaca*). In particular, *Ptgir*, a major regulator in the prostacyclin pathway, has been shown to be dysregulated in congenital diaphragmatic hernia leading to PH (31). Rho A/rho kinase (Rock) pathway plays an important role in the pathogenesis of human PH (32). Furthermore, *Rock1* contributes to the profibrotic response of endothelial cells in experimental pulmonary fibrosis (33). Figure 5B indicates that 42.9% of the genes involved in Vegf signalling were differentially expressed (17.9% up-regulated, 25% down-regulated). Among the down-regulated genes, we found genes encoding for cytosolic proteins [e.g. *Src*, *Sphk1* (sphingosine kinase 1), *Pla2g4*, *Rac3*]. Src-family kinases, a family of non-receptor tyrosine kinases, are major regulators

of vascular smooth muscle function in health and disease (34). *Sphk1* is involved in endothelial cell motility and angiogenesis (35). *Sphk1* deficiency increased pulmonary vascular hyperresponsiveness, which is one aspect in pulmonary arterial hypertension pathogenesis (36). *Pla2* (phospholipase A2) activity plays a substantial role in protecting pulmonary microvascular endothelial cells against oxidative stress (37). Among the genes up-regulated, we found genes encoding for cytosolic proteins [e.g. *Pik3cd* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta), *Shc2*, *Prkcb*, *Hspb1*]. In particular, inhibition of *Pik3cd* has been shown to attenuate antigen-induced airway inflammation and hyperresponsiveness by preventing vascular leakage in mice (38). Interestingly, while the percentage of genes being differentially expressed in vascular smooth muscle cell contraction pathway are very similar in HOX (Supplementary Material, Fig. S11A) and NOX (Fig. 5A), the total number of genes being affected in the Vegf signalling pathway is much lower in HOX (Supplementary Material, Fig. S11B) compared to NOX (Fig. 5B). Especially, the number of down-regulated genes decreases from 25% to 11%. One reason is the up-regulation of genes upon HOX exposure, which are previously down-regulated in NOX, resulting in no more differential expression when comparing to the control group. For example, *Pla2g4* is down-regulated in NOX but up-regulated in HOX supporting its protective role in oxidative stress. Taken together, the transcriptomic analysis of *Fgf10*^{+/-} versus control lungs in NOX suggests that reduced *Fgf10* expression could lead to vascular defects by dysregulating genes that are playing a crucial role in endothelial barrier function, atherosclerosis, pulmonary circulation/ hypertension, vascular smooth muscle function, airway inflammation and endothelial oxidative stress.

Increased myosin light chain phosphorylation is observed in BPD versus control lungs

Given the transcriptomic data regarding smooth muscle cell contraction in NOX (Fig. 5) and HOX (Supplementary Material, Fig. S11) between *Fgf10*^{+/-} and WT lungs, we examined by IHC the extend of phosphorylation of two key myosin light chain proteins identified in our gene array, myosin light chain 9 (MYL9) and MYL6b in BPD (n = 3) versus control (n = 4) lungs. Both MYL9 and MYL6b are detected in pulmonary vessels. MYL9 expression appears to be decreased in BPD versus control (Supplementary Material, Fig. S12a and b) while the expression of MYL6b is unchanged (Supplementary Material, Fig. S12e and f). Analysis of the expression of the corresponding phosphorylated forms indicates a clear increase in the context of BPD (Supplementary Material, Fig. S12c, d, g and h), thereby supporting our transcriptomic data. A similar analysis was carried out in mice (Supplementary Material, Fig. S13). As the KEGG analysis showed that MYL9 in NOX and MYL6b in HOX could be potentially regulated, we focused on their expression by IHC in these two conditions. In NOX, MYL9 expression (as well as its corresponding phosphorylated form) does not display any difference between *Fgf10*^{+/-} and control lungs (Supplementary Material, Fig. S13Aa-d). However, in HOX, MYL6b and its phosphorylated form exhibited the same pattern as in human BPD (Supplementary Material, Fig. S13Ba-d).

Blockade of *Fgfr2b* ligands activity postnatally leads to decreased blood vessel number and increased muscularization

Fgf10^{+/-} lungs display 'build in' developmental defects (17), which could cause the vascular defects observed. While the

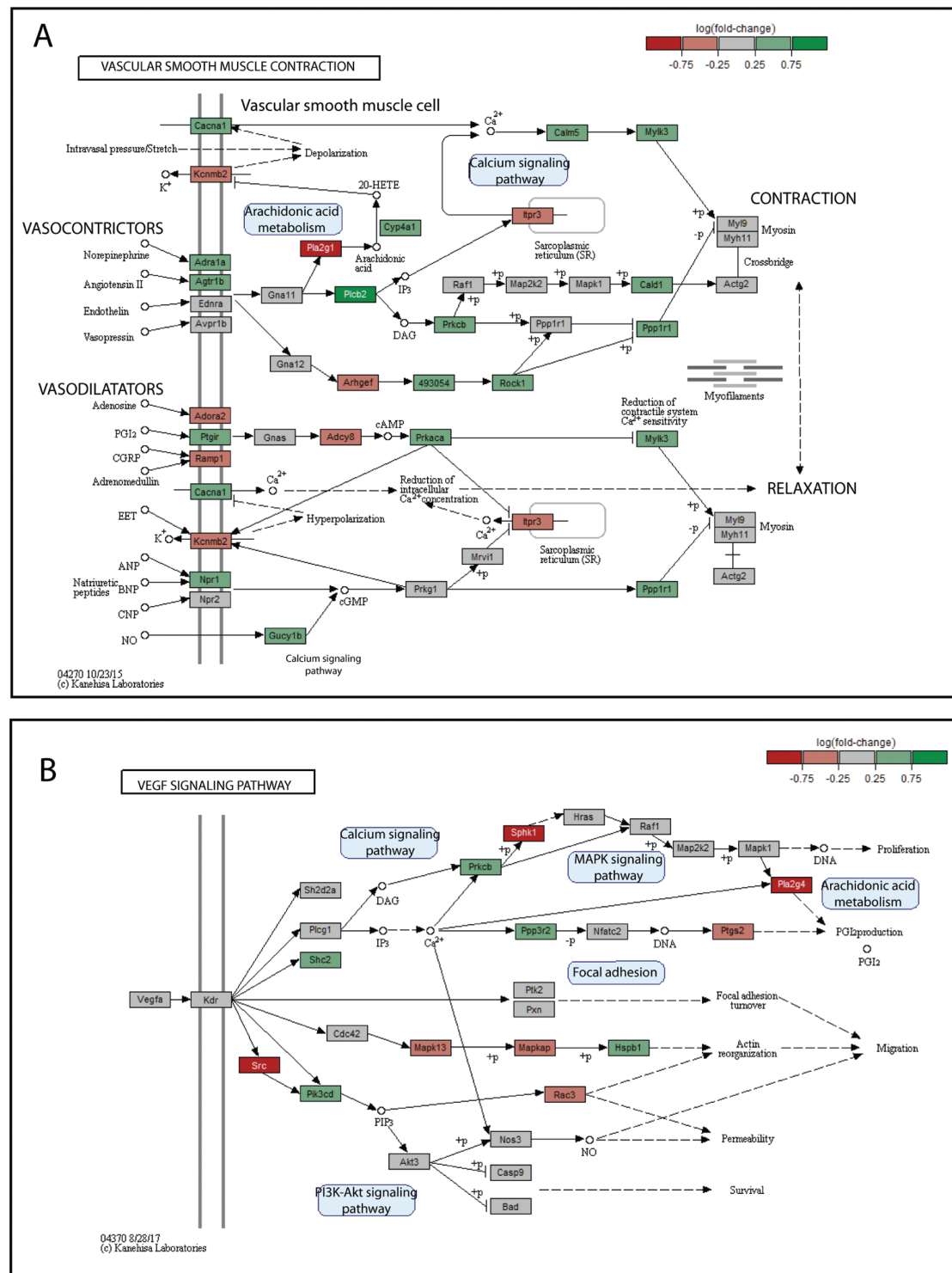
KEGG PATHWAY ANALYSIS *Fgf10*^{+/-} vs. *Fgf10*^{+/+} in NOX

Figure 5. Transcriptomic analysis of *Fgf10*^{+/-} versus control P3 lungs in NOX suggests that reduced *Fgf10* expression could lead to vascular defects. KEGG pathway analysis of the expression of genes involved in (A) vascular smooth muscle cell contraction and (B) Vegf signalling pathway in *Fgf10*^{+/-} versus control P3 lungs in NOX (*n* = 4 each). Note that 60.9% of the genes are affected for the (A) vascular smooth muscle cell contraction and 42.9% of the genes are affected for the (B) Vegf signalling pathway.

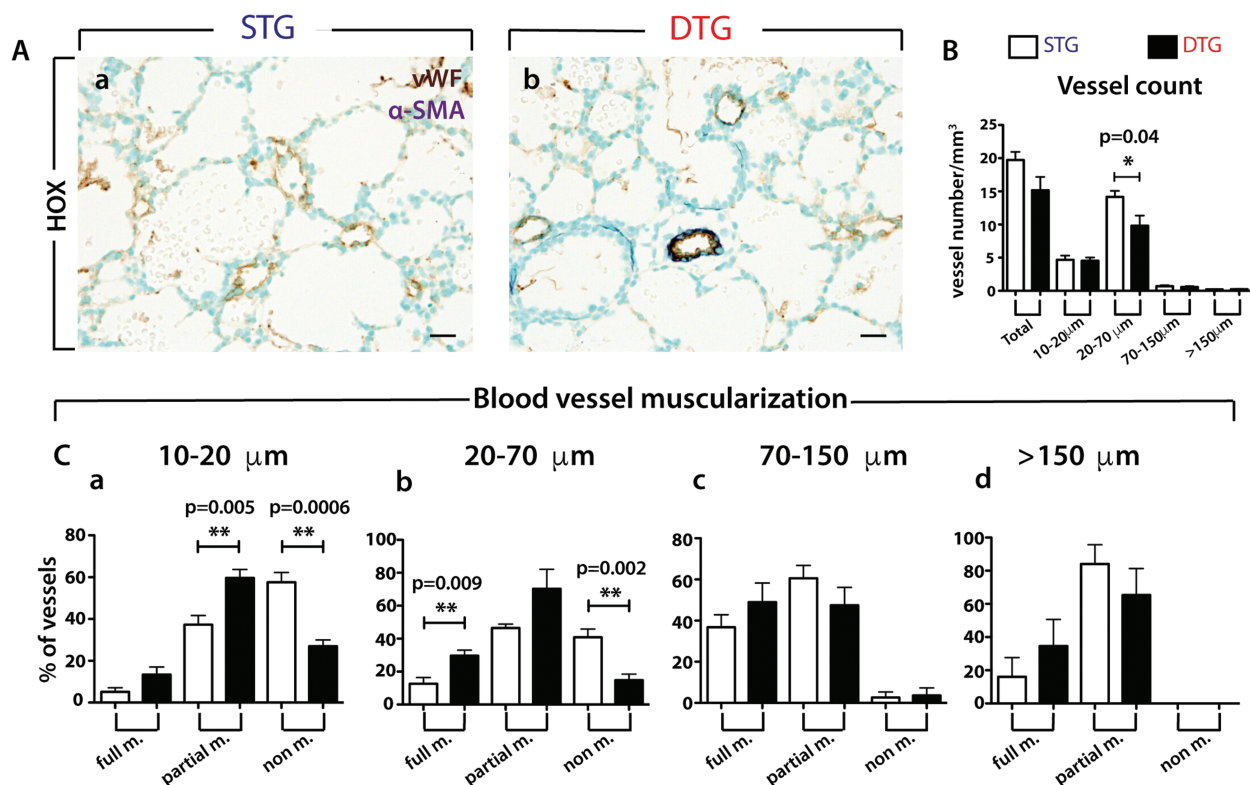


Figure 6. Analysis of the vascular defects in P3 double transgenic (DTG: *Rosa26^{rtTA/rtTA}*, *Tg(Tet(O)sFgfr2b)/+*) and control single transgenic (STG: *Rosa26^{rtTA/rtTA}*, *+/+*) lungs in HOX. (A) IHC for vWF and α-SMA of (a) STG and (b) DTG lungs (*n* = 5 each). (B) Total vessel count in STG and DTG lungs. (C) Blood vessel muscularization between STG and DTG lungs for blood vessels at (a) 10–20 μm, (b) 20–70 μm, (c) 70–150 μm and (d) >150 μm. Scale: Aa, b, 20 μm.

Fgf10^{+/-} mice are useful to understand human pathologies associated with reduced FGF10 expression such as patients with ALSG and LADD syndromes (7,10), it is very unlikely that this model is representative of the majority of cases with BPD, where due to inflammation, FGF10 expression is reduced during the saccular/alveolar stages of lung development. Supporting this conclusion, mutation in the FGF10 gene has not been reported so far in the context of BPD. In order to investigate the impact of Fgfr2b ligands inhibition postnatally, we developed a transgenic mouse model [*Rosa26^{rtTA/rtTA}*, *Tg(Tet(O)sFgfr2b)/+*] allowing the expression of a soluble form of the Fgf10 receptor Fgfr2b upon exposure of the mice to doxycycline. This system is well established in our laboratory and has been validated in many organs (21,39–42). We have therefore exposed the nursing females to dox food immediately after the birth of double transgenic [DTG, *Rosa26^{rtTA/rtTA}*, *Tg(Tet(O)sFgfr2b)/+*] and control single transgenic (STG, *Rosa26^{rtTA/rtTA}*, *+/+*) pups and analyzed the lungs for vascular defects at P3 in HOX using the vWF and α-SMA staining (Fig. 6Aa and b) as previously described. The quantification of the vessel number indicates a significant decrease of the 20–70 μm blood vessels in the DTG versus control lungs (*P* = 0.04, *n* = 5) as well as decreased total blood vessels (Fig. 6B). Further analysis of the muscularization status indicates increased muscularization of both 10–20 μm and 20–70 μm vessels (Fig. 6Ca and b). This is a feature seen in the context of PH and indicates that the blockade of Fgfr2b ligands postnatally is sufficient to elicit the PH phenotype, a characteristic defect seen in BPD.

Discussion

We have previously reported a clear link between *Fgf10* deficiency and vascular defects in *Fgf10^{LacZ/-}* hypomorphic lungs (these lungs express only 20% of the normal level of *Fgf10*) (22). We showed that *Vegfa* is a target of Fgf10 in the developing lung epithelium and that decreased *Fgf10* levels eventually translate into decreased *Vegfa* and associated vascular defects. *Vegfa* is not significantly reduced in E18.5 *Fgf10^{+/-}* versus WT lungs. Only a trend towards reduction is visible in the present study (Fig. 1Ab). In the *Fgf10^{LacZ/-}* hypomorphic lungs, the vascular tree is severely underdeveloped (22). Our quantification of the number and size of blood vessels indicates that significant vascular defects are not observed in P3 *Fgf10^{+/-}* lungs in NOX. However, such defects emerge after HOX exposure and are characterized by a significant drop in the number of blood vessels and by an increase of the more immature (smooth muscle actin-negative) blood vessels. However, these defects did not appear to lead to lung hemorrhages, either at P3 or at later time points analyzed, suggesting that the observed lethality is not due to a failing vascular system (17). Interestingly, vascular defects, primarily PH, have been proposed as the main causes of lethality in infants with BPD (43). The PH phenotype is not observed in *Fgf10^{+/-}* lungs but is present upon Fgfr2b ligands inhibition postnatally. This suggests that developmental defects upon reduction of *Fgf10* expression could be protective postnatally against PH. This could be a possibility, for example, if the smooth muscle progenitor cells are impaired and do not respond to proliferative stimuli

following HOX exposure. Interestingly, we reported defective smooth muscle cells formation in the *Fgf10* hypomorphic lung (44). Along these lines, a decrease in *Fgf10* expression starting during the saccular phase that would allow normal smooth muscle cell formation could then be causative for the PH phenotype. Alternatively, *Fgf10* decrease during the saccular phase is not sufficient to trigger a PH phenotype as additional *Fgfr2b* ligands, such as *Fgf1* and *Fgf7* could act redundantly with *Fgf10* and will have to be inhibited also for the vascular phenotype to appear. Our results allow for the first time to propose vascular defects caused by inhibition of *Fgfr2b* ligands activity postnatally. It is still unclear if these defects are the consequences of direct or indirect effect of *Fgfr2b* ligands on the endothelium and/or vascular smooth muscle cells. Interestingly, the simultaneous deletion of *Fgfr1* and *Fgfr2* in endothelial cells while allowing normal vascular homeostasis leads to impaired neovascularization of the skin and retina after injury and is associated with delayed wound healing (45). In addition, *Fgfr1* and *Fgfr2* in endothelial cells play a critical role for cardiac functional recovery and vascular remodelling following cardiac ischemia-reperfusion injury, without affecting the cardiac hypertrophic response suggesting that from the activation of the Fgf pathway in endothelial cells after ischemia injury could be beneficial (46).

The presence of more immature (smooth muscle actin-negative) blood vessels in our experimental mice in HOX raises the possibility that *Fgf10* signalling contributes to the formation of vascular smooth muscle cells during organogenesis via its action on *Pdgfr* (22) or via a more direct action on smooth muscle cell progenitors (47).

Interestingly, recombinant *Fgf10* has been shown to act directly on mesenchymal-derived cells such as cardiomyocytes (48), adipocyte stem cells (49) and lipofibroblasts (50), strengthening the concept that in the lung, *Fgf10* does not only act on the epithelium. *Fgf10* has also been shown to directly support the proliferation of endothelial cells (51).

It is therefore tempting to subdivide the effects of decreased *Fgf10* expression on the vasculature into two classes: one class linked to developmental defects where the blood vessels are more sensitive and immature and one class linked to the role of *Fgf10* in repair as a proliferative factor for the endothelium, thereby limiting the vascular damages triggered by HOX.

In conclusion, constitutive decrease in *Fgf10* mRNA levels in mice leads to lung congenital defects, which are compatible with postnatal survival, but which compromise the ability of the lungs to cope with sub-lethal hyperoxic injury. In particular, we provide evidence that *Fgf10* heterozygous mice display pulmonary vasculature defects during normal lung development and during injury. This knowledge may be critical in designing therapies to prevent lung injury in neonates at risk for BPD and in adult lung disorders characterized by *FGF10* deficiency.

Materials and Methods

Ethical approvals

Animal studies were performed in accordance with the National Institutes of Health Guidelines for the Use of Laboratory Animals and approved by the Federal Authorities for Animal Research of the Regierungspraesidium Giessen, Hessen, Germany protocols 105/2011. For the human studies written informed consent was received from participants prior to inclusion in the study at Columbia University (New York, USA). Participants were identified by number, not by name. In addition, human lung tissues were also obtained from Erasmus MC Sophia Children's Hospital

(Rotterdam, The Netherlands). Human lung tissue donation was approved by the ethics committee of the Erasmus MC Sophia Children's Hospital (Rotterdam, The Netherlands).

Mice

Fgf10^{+/-} mice were generated by crossing *Fgf10*^{fllox/fllox} mice (*Fgf10*^{tm1.2Sms/J}, Jackson laboratory stock 023729) with Cytomegalovirus (CMV)-Cre mice [B6.C-Tg(CMV-cre)1CgN/J, Jackson laboratory stock 006054]. The resulting *Fgf10*^{+/-} mice (*Fgf10*^{tm1.1Sms/J}) were crossed for at least five generations with C57BL/6 mice. *Fgf10*^{+/-} and *Fgf10*^{+/+} embryonic and newborn littermates were used.

Human samples

Lung sections from four patients with BPD were obtained. Two males (25 and 34 weeks of gestational age) and two females (23 and 24 weeks of gestational age) underwent invasive ventilation for either 1 or 4 months and died between 1 to 6 months after birth. Diagnosis of BPD was confirmed post-mortem by resident pathologists in all patients. Lung sections from two females (37 weeks and full term) who died (7 days and 13 months, respectively) from causes other than lung disease were obtained. Further samples were obtained for MYL6b and MYL9 immunostaining. Lung sections from three infants diagnosed with BPD (25, 28, 31 weeks of gestational age) were used. Lung sections from three infants (24, 26, 33 weeks of gestational age) who died from causes other than BPD were used as controls. RNA from three patients (two with 26 weeks and one with 27 weeks of gestational age) with BPD and from four controls (27, 33, 38 and 39 weeks of gestational age) were used for qRT-PCR.

Left lobe perfusion, isolation and tissue processing

The left lobe was perfused through the trachea with a pressure of 20 cm H₂O with 5 ml phosphate buffered saline (PBS) followed by 5 ml, 4% paraformaldehyde (PFA). The trachea was tied off with a string and the lung was removed and placed in 4% PFA for max. 24 h at 4°C. Lungs were then progressively dehydrated (30%, 50%, 70%, 99.6% ethanol, each 3 h) and embedded with a Leica embedding machine (EG 1150C). Paraffin blocks were kept cold and 5 µm sections were cut.

Immunostaining

Immunofluorescence staining for SFTPC, PECAM and α-SMA. Paraffin sections were deparaffinized, blocked with 3% bovine serum albumin (BSA) and 0.4% Triton X-100 [in tris-buffered saline (TBS)] at room temperature (RT) for 1 h and then incubated with primary antibodies against mature-SFTPC (Seven Hills, 1:1000), PECAM (Abcam, 1:100) and α-SMA (Sigma-Aldrich, 1:400) at RT for 1 h or at 4°C overnight. After incubation with primary antibodies, slides were washed three times in TBST (TBS buffer + 0.1% tween 20) for 5 min, incubated with secondary antibodies at RT for 1 h and then washed three times in TBST before being mounted with Prolong Gold Anti-fade Reagent with DAPI (4', 6-diamidino-2-phenylindole; Invitrogen) (Table 1).

FGFR2 and FGF10 staining. Immunohistochemistry for FGFR2 and FGF10 was performed on lung tissue sections from patients with BPD. First, paraffin sections were deparaffinized. For FGFR2 staining, antigen unmasking were done by incubation of the slides in boiling buffer solution for 15 min, then blocked with 3% BSA for 60 min in RT and incubated with the primary antibody

Table 1. Antibodies for immunohistochemistry

Antibody	Company (Catalog number)	Dilution
FGF10	Antibodies-online (ABIN360398)	1:30 (human)
FGFR2 (BEK)	Santa Cruz (sc-122)	1:300 (human)
SFTPC	Seven Hills (WRAB-9337)	1:100 (human)
PECAM/CD31	BD Pharmingen (550274)	1:100 (mouse and human)
α -SMA	Sigma-Aldrich (A2547)	1:600 (mouse)
		1:400 (human)
von-Willebrand-factor (VWF)	Dako (A0082)	1:1200(mouse)
MYL6b	Merck (AB3381)	1:100 (mouse)
		1:200 (human)
Phospho MYL6b	BosterBio (A14059)	1:600 (mouse)
		1:200 (human)
MYL9	Abcam (ab64161)	1:200 (mouse and human)
Phospho MYL9	Abcam (ab2480)	1:100 (mouse and human)

(FGFR2, 1:300) over night at 4°C. After washing steps, incubation with secondary antibody was performed before covering the slides with DAPI solution. For FGF10 staining, antigen unmasking were done by incubation of the slides in boiling buffer solution for 25 min, then treated with proteinase K for 15 min at RT. Afterwards slides were blocked with 10% BSA for 60 min in RT followed by incubation of the primary antibody (FGF10, 1:30) at 4°C overnight. After washing steps, incubation with secondary antibody was performed before covering the slides with DAPI solution (Table 1).

α -SMA/vWF double staining. The 3 μ m sections were deparaffinized, endogenous peroxidase was removed by incubation with 3% H₂O₂-methanol solution for 20 min, then washed with Aqua Dest and PBS each 2 $\times$ 5 min. For antigen retrieval, sections were trypsinized for 10 min at 37°C, then washed with PBS (4 $\times$ 5 min) and immersed in blocking buffer (10% BSA) for a further 20 min. After washing with PBS (4 $\times$ 5 min), sections were blocked with Rodent Block M (Biocare Medical MM horseradish peroxidase (HRP)-Polymer Kit, Cat. No. 1-800-799-9499) for 30 min, washed with PBS, incubated with primary antibody (alpha-actin 1:800 diluted, 30 min) and HRP polymer (20 min) before immersed in substrate solutions (Vip substrat Kit, vector, 30 s-4 min). Slides were monitored under the microscope for staining progression and washed with tap water for 5 min. Before staining for vWF, sections were immersed in blocking buffer (10% BSA) for 15 min, washed with PBS, blocked with serum (ImmPRESS Kit Anti-rabbit Ig), then sections were incubated with vWF antibody (1:1200 diluted) at 37°C for 30 min and washed again. After incubation with secondary antibody (Anti-rabbit Ig peroxidase, ImmPRESS reagent), sections were stained with diaminobenzidine (DAB) substrate Kit (SK-4100, vector) and staining progression was observed under the microscope. After washing with tap water, sections were counterstained with methyl green for nuclei on a heating plate (60°C), progressively dehydrated and coverslipped (Table 1).

Immunostaining for MYL6b, phospho-MYL6b, MYL9 and phospho-MYL9 in mouse and human lungs. The 3 μ m sections were deparaffinized. For antigen retrieval, sections were heated in a microwave for 25 min in Rodent Decloaker (Biocare/Zytomed Systems), then washed with PBS (4 $\times$ 5 min) and immersed in blocking buffer (10% BSA) for a further 10 min. After washing with PBS (4 $\times$ 5 min), sections were blocked with Rodent Block M (Biocare/Zytomed Systems) for 30 min. For human samples

Protein-Block from Zytocell-Plus AP Polymer-Kit was used instead of Rodent Block M for 5 min. Slides were washed with PBS and incubated with primary antibody overnight. After washing, slides with human samples were incubated with PostBlock solution for 20 min before incubation with AP-Polymer Reagent for 30 min. Slides with mouse samples were not incubated with PostBlock solution. Next, slides were washed and immersed in substrate solutions (RedChromogen, 2-10 min). Slides were monitored under the microscope for staining progression and washed with tap water for 5 min. After washing, sections were counterstained with hematoxylin for nuclei on a heating plate (60°C), progressively dehydrated and coverslipped (Table 1).

Image acquisition. Fluorescent images were acquired using Leica DM5500 B fluorescence microscope connected to Leica DFC360 FX camera. Lung tissue sections without immunofluorescence were analyzed under light microscope and representative photomicrographs were taken by usage of computer software for image analysis (QWin, Leica, Wetzlar, Germany).

RNA extraction

After lung function measurements were taken, the right bronchus was clamped and either cranial and accessory or caudal and medial lobes were removed, placed in TRIzol, homogenized in GentleMACs and frozen in liquid nitrogen for RNA extraction. RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

Quantitative real-time RT-PCR

RNA was reverse-transcribed (QuantiTect Reverse Transcription Kit, Cat. No. 205313, Qiagen GmbH, Deutschland, Hilden). cDNA was diluted to a concentration of 5 ng/ μ l. Primers were designed using Roche Applied Sciences online Assay Design Tool. All primers were designed to span introns and blasted using NCBI software for specificity. Sybr Green Master Mix (Invitrogen, Cat. No.11733-038) was used for RT-PCR with a Roche LightCycler 480 machine. Samples were run in triplicates using Hprt as a reference gene. The dCT and ddCT values were calculated as follows: dCT (WT) = CT (WT, reference gene) – CT (WT, gene of interest), dCT (Mutant) = CT (Mutant, reference gene) – CT (Mutant, gene of interest), ddCT = dCT (Mutant) – dCT (WT). Mouse primers are listed below (Table 2).

Western blot

Loading buffer was added to protein samples from the lungs (5% SDS in bromophenol blue and β -mercaptoethanol), denatured for 8 min at 98°C and cooled on ice. 15 μ g of protein sample was loaded on a 8–10% polyacrylamide gel and run at 35 mA per gel for ~1 h. Samples were then transferred electrically to a polyvinylidene fluoride membrane (Amersham, Germany) by semi-dry electro blotting (70 mA per gel, gel size, 7 $\times$ 9 cm) for 90 min. The membrane was blocked with 5% milk in TBS-T blocking buffer at RT on a shaker for 1 h followed by incubation with primary antibody overnight (Supplementary Material, Table S2) at 4°C. After washing with 1 $\times$ TBS-T four times for 15 min each, the membrane was incubated with the respective HRP-labelled secondary antibody (dilution 1:2000) at RT for 1 h followed by four times washing with 1 $\times$ TBS-T buffer for 15 min each. The protein bands were detected by Enhanced Chemiluminescence (Amersham, Germany) treatment and emitted signals were detected with a chemiluminescence imager. Quantification of bands was done by using ImageJ software Table 3.

HOX injury (BPD mouse model)

Newborn pups were subjected to HOX (85% O₂) injury from P0–P8 in a chamber (Proox Model 110, Biospherix). To minimize oxygen toxicity and bias, nursing dams were rotated every 24 h

between NOX and HOX. Pups and dams received food and water ad libitum.

Vascular morphometry

The degree of muscularization was determined from 3 μ m mouse lung sections. Morphological assessment of lung vessel muscularization (outer diameter: 10–20 μ m, 20–70 μ m, 70–150 μ m and >150 μ m) was performed via computer-assisted analysis (Leica Q Win Standard analyzing software) at 40 $\times$ or 63 $\times$ magnification under a microscope. The analytical software detected the vessels' endothelium that appeared in a brown color (vWF staining) and the muscle tissue surrounding the vessels that appeared in a violet color (α -SMA staining). The software distinguished between non-muscularized vessels (no smooth muscle cells detectable with α -SMA staining), partially muscularized (minimum one smooth muscle cell and maximum 75% of the vessel circumference with α -SMA staining) and fully muscularized (>75% of the vessel circumference with α -SMA staining).

Gene arrays

The data from the microarray experiment have been deposited in NCBI's Gene Expression Omnibus (GEO) and is accessible through GEO Series accession number GSE113104.

Table 2. Human and mouse primers forward/reverse

Human gene	Forward sequence (5'-3')	Reverse sequence (5'-3')
FGF7	AAGGGACCCAAGAGATGAAGA	CCTTTGATTGCCACAATTC
FGF10	GAAGGAGAACTGCCCGTACA	GGCAACAACTCCGATTCTACT
FGFR2b	GATAAATAGTTCCAAT	TGCCCTATATAATTG
	GCAGAAAGTGCT	GAGACCTTACA
PECAM1	CTGTCCCTGATGCCGTG	AGCAGGGCAGGTTTCATAAAT
PGBD	TGTCTGGTAACGGCAATGCG	CCCACGCGAATCACTCTCAT
Mouse gene	Forward sequence (5'-3')	Reverse sequence (5'-3')
Fgf1	GTAGTTTCCTAGAGCAGGTTG	TGATAAAGTGAGTGAAAGAGAGC
Fgf3	GATTACTGCGGTGGAAGTGG	CCGTTCCACAACTCACACTC
Fgf7	ACTATCTGCTTATAAAATGGCTGCT	GTGGGGCTTGATCATCTGAC
Vegfr2	CAGTGGTACTGGCAGCTAGAAG	ACAAGCATACGGGCTTGTTT
Vegfa	AAAAACGAAAGCGCAAGAAA	TTTCTCCGCTCTGAACAAGG
Pdgfb	GAGTCGGCATGAATCGCT	CAGCCCCATCTTCATCTACG
Pdgfrb	GGAGAACAGAGAGGAAACAGT	GCGGAAAACCTGAGAGAAT
Hprt	CCACAGGACTAGAACCTGCTAA	CCTAAGATGAGCGCAAGTTGAA

Table 3. Antibodies used for western blotting

Primary antibody (rabbit)	Company (Catalog number)	Dilution	Secondary antibody	Company (Catalog number)	Dilution
COL1A1	Meridian Bioscience (T40777R)	1:1000	Swine anti rabbit HRP	Dako (P0217)	1:2000
β -ACTIN	Abcam (ab8227)	1:30.000	Swine anti rabbit HRP	Dako (P0217)	1:2000
Phospho- β -CATENIN	Cell Signaling (9566)	1:1000	Anti-Rabbit IgG (H+L), HRP Conjugate	Promega W401B	1:5000
Total β -CATENIN	Cell Signaling (9582)	1:1000	Anti-Rabbit IgG (H+L), HRP Conjugate	Promega W401B	1:5000
β -ACTIN	BioLegend (664802)	1:2500	HRP Goat anti-rat IgG	BioLegend 405405	1:5000

Statistical analyses

Significance was determined by two-tailed Student's t-test using GraphPad PRISM statistical analysis software. All data are presented as mean $\pm$ SEM. Values of $P < 0.05$ were considered significant.

Supplementary Material

Supplementary Material is available at HMG online.

Acknowledgements

We would like to thank Kerstin Goth and Jana Rostkovius for managing and genotyping the mice used for experiments. We want to thank Ewa Bieniek for assistance in immunostaining. We also thank Caterina Tiozzo for giving us the human BPD samples.

Conflict of Interest statement. None declared.

Funding

National Heart, Lung, and Blood Institute (NHLBI) support [HL107307 to S.B.]; Deutsche Forschungsgemeinschaft [DFG; BE4443/1-1, BE4443/4-1, BE4443/6-1, KFO309 P7 and SFB1213-projects A02 and A04 to S.B.]; Landes-Offensive zur Entwicklung Wissenschaftlich-Ökonomischer Exzellenz (LOEWE); Universitätsklinikum Giessen & Marburg (UKGM); Universities of Giessen and Marburg Lung Center (UGMLC); DZL and COST [BM1201]; Whenzhou Medical University and the National Natural Science Foundation of China [grant number 81472601 to J.S.Z.]; Forschungsförderung of the UKGM and the Excellence Cluster Cardio-Pulmonary System (ECCPS) [to C.M.C.]; The ECCPS [to E.E.A.]; Program of Competitive Growth and State Assignment [20.5175.2017/6.7 to A.A.R.]. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Author contributions

Concept and design: CMC, SB and JSZ. Acquisition of data: CMC, AM, DK, SR, JW, MK and FY. Analysis and interpretation: CMC, AM, JW, DK, EEA, FY and SB. Drafting and editing of the manuscript: CMC, AM, EEA, HE, KPZ, GB, AR, RTS, IR, REM, RR, SB and JSZ. All authors read and approved the final manuscript.

References

- Benjamin, J.T., Smith, R.J., Halloran, B.A., Day, T.J., Kelly, D.R. and Prince, L.S. (2007) FGF-10 is decreased in bronchopulmonary dysplasia and suppressed by toll-like receptor activation. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **292**, L550–L558.
- Watterberg, K.L., Demers, L.M., Scott, S.M. and Murphy, S. (1996) Chorioamnionitis and early lung inflammation in infants in whom bronchopulmonary dysplasia develops. *Pediatrics*, **97**, 210–215.
- Klinger, G., Levy, I., Sirota, L., Boyko, V., Lerner-Geva, L. and Reichman, B. (2010) Outcome of early-onset sepsis in a national cohort of very low birth weight infants. *Pediatrics*, **125**, e736–e740.
- Stoll, B.J., Hansen, N.I., Bell, E.F., Shankaran, S., Laptook, A.R., Walsh, M.C., Hale, E.C., Newman, N.S., Schibler, K., Carlo, W.A. et al. (2010) Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. *Pediatrics*, **126**, 443–456.
- Benjamin, J.T., Carver, B.J., Plosa, E.J., Yamamoto, Y., Miller, J.D., Liu, J.H., van der Meer, R., Blackwell, T.S. and Prince, L.S. (2010) NF-kappaB activation limits airway branching through inhibition of Sp1-mediated fibroblast growth factor-10 expression. *J. Immunol.*, **185**, 4896–4903.
- Carver, B.J., Plosa, E.J., Stinnett, A.M., Blackwell, T.S. and Prince, L.S. (2013) Interactions between NF-kappaB and SP3 connect inflammatory signaling with reduced FGF-10 expression. *J. Biol. Chem.*, **288**, 15318–15325.
- Entesarian, M., Dahlqvist, J., Shashi, V., Stanley, C.S., Falahat, B., Reardon, W. and Dahl, N. (2007) FGF10 missense mutations in aplasia of lacrimal and salivary glands (ALSG). *Eur. J. Hum. Genet.*, **15**, 379–382.
- Entesarian, M., Matsson, H., Klar, J., Bergendal, B., Olson, L., Arakaki, R., Hayashi, Y., Ohuchi, H., Falahat, B., Bolstad, A.I. et al. (2005) Mutations in the gene encoding fibroblast growth factor 10 are associated with aplasia of lacrimal and salivary glands. *Nat. Genet.*, **37**, 125–127.
- Milunsky, J.M., Zhao, G., Maher, T.A., Colby, R. and Everman, D.B. et al. (2006) LADD syndrome is caused by FGF10 mutations. *Clin. Genet.*, **69**, 349–354.
- Rohmann, E., Brunner, H.G., Kayserili, H., Uyguner, O., Nurnberg, G., Lew, E.D., Dobbie, A., Eswarakumar, V.P., Uzumcu, A., Ulubil-Emeroglu, M. et al. (2006) Mutations in different components of FGF signaling in LADD syndrome. *Nat. Genet.*, **38**, 414–417.
- Klar, J., Blomstrand, P., Brunmark, C., Badhai, J., Hakansson, H.F., Brange, C.S., Bergnedal, B. and Dahl, N. (2011) Fibroblast growth factor 10 haploinsufficiency causes chronic obstructive pulmonary disease. *J. Med. Genet.*, **48**, 705–709.
- Sekine, K., Ohuchi, H., Fujiwara, M., Yamasaki, M., Yoshizawa, T., Sato, T., Yagishita, N., Matsui, D., Koga, Y., Itoh, N. et al. (1999) Fgf10 is essential for limb and lung formation. *Nat. Genet.*, **21**, 138–141.
- Arman, E., Haffner-Krausz, R., Gorivodsky, M. and Lonai, P. (1999) Fgfr2 is required for limb outgrowth and lung-branching morphogenesis. *Proc. Natl. Acad. Sci. U. S. A.*, **96**, 11895–11899.
- De Moerloose, L., Spencer-Dene, B., Revest, J.M., Hajihosseini, M., Rosewell, I. and Dickson, C. (2000) An important role for the IIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. *Development*, **127**, 483–492.
- Ohuchi, H., Hori, Y., Yamasaki, M., Harada, H., Sekine, K., Kato, S. and Itoh, N. (2000) FGF10 acts as a major ligand for FGF receptor 2 IIb in mouse multi-organ development. *Biochem. Biophys. Res. Commun.*, **277**, 643–649.
- Bellusci, S., Grindley, J., Emoto, H., Itoh, N. and Hogan, B.L. (1997) Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. *Development*, **124**, 4867–4878.
- Chao, C.M., Yahya, F., Moiseenko, A., Tiozzo, C., Shrestha, A., Ahmadvand, N., El Agha, E., Quantius, J., Dilai, S., Kheirollahi, V. et al. (2017) Fgf10 deficiency is causative for lethality in a mouse model of bronchopulmonary dysplasia. *J. Pathol.*, **241**, 91–103.
- El Agha, E. and Bellusci, S. (2014) Walking along the fibroblast growth factor 10 route: a key pathway to understand the control and regulation of epithelial and mesenchymal cell-lineage formation during lung development and repair after injury. *Scientifica (Cairo)*, **2014**, 538379.

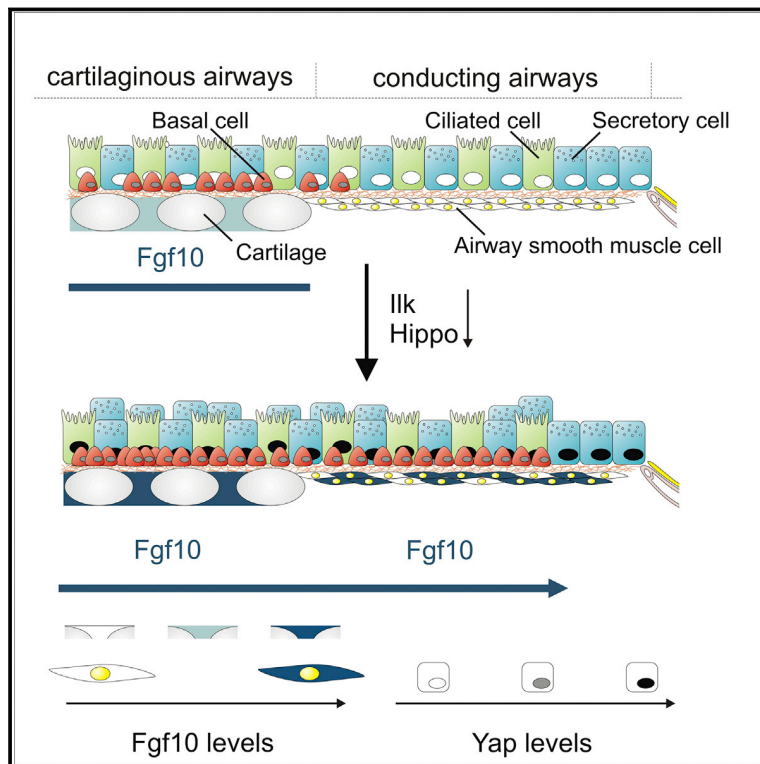
19. Chao, C.M., El Agha, E., Tiozzo, C., Minoo, P. and Bellusci, S. (2015) A breath of fresh air on the mesenchyme: impact of impaired mesenchymal development on the pathogenesis of bronchopulmonary dysplasia. *Front. Med. (Lausanne)*, **2**, 27.
20. Gupte, V.V., Ramasamy, S.K., Reddy, R., Lee, J., Weinreb, P.H., Violette, S.M., Guenther, A., Warburton, D., Driscoll, B., Minoo, P. et al. (2009) Overexpression of fibroblast growth factor-10 during both inflammatory and fibrotic phases attenuates bleomycin-induced pulmonary fibrosis in mice. *Am. J. Respir. Crit. Care Med.*, **180**, 424–436.
21. Volckaert, T., Dill, E., Campbell, A., Tiozzo, C., Majika, S., Bellusci, S. and De Langhe, S.P. (2011) Parabronchial smooth muscle constitutes an airway epithelial stem cell niche in the mouse lung after injury. *J. Clin. Invest.*, **121**, 4409–4419.
22. Ramasamy, S.K., Mailleux, A.A., Gupte, V.V., Mata, F., Sala, F.G., Veltmaat, J.M., Del Moral, P.M., De Langhe, S., Parsa, S. and Kelly, L.K. (2007) Fgf10 dosage is critical for the amplification of epithelial cell progenitors and for the formation of multiple mesenchymal lineages during lung development. *Dev. Biol.*, **307**, 237–247.
23. Yao Q., Renault M.A., Chapouly C., Vandierdonck, S., Belloc, I., Jaspard-Vinassa, B., Daniel-Lamazière, J.M., Laffargue, M., Merched, A., Desgranges, C. and Gadeau, A.P. (2014) Sonic hedgehog mediates a novel pathway of PDGF-BB-dependent vessel maturation. *Blood*, **123**(15), 2429–2437.
24. White A.C., Lavine K.J. and Ornitz D.M. (2007) FGF9 and SHH regulate mesenchymal Vegfa expression and development of the pulmonary capillary network. *Development*, **134**(20), 3743–3752.
25. Herriges J.C., Verheyden J.M., Zhang Z., Sui, P., Zhang, Y., Anderson, M.J., Swing, D.A., Zhang, Y., Lewandoski, M. and Sun, X. (2015) FGF-regulated ETV transcription factors control FGF-SHH feedback loop in lung branching. *Dev. Cell*, **35**(3), 322–332.
26. Muller-Redetzky, H.C., Kummer, W., Pfeil, U., Hellwig, K., Will, D., Paddenberger, R., Tabeling, C., Hippenstiel, S., Suttorp, N. and Witzenrath, M. (2012) Intermedin stabilized endothelial barrier function and attenuated ventilator-induced lung injury in mice. *PLoS One*, **7**, e35832.
27. Hao, S.L., Yu, Z.H., Qi, B.S., Luo, J.Z. and Wang, W.P. (2011) The antifibrosis effect of adrenomedullin in human lung fibroblasts. *Exp. Lung Res.*, **37**, 615–626.
28. Qing, X., Svaren, J. and Keith, I.M. (2001) mRNA expression of novel CGRP1 receptors and their activity-modifying proteins in hypoxic rat lung. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **280**, L547–L554.
29. Sundivakkam, P.C., Kwiatek, A.M., Sharma, T.T., Minshall, R.D. and Tirupathi, C. (2009) Caveolin-1 scaffold domain interacts with TRPC1 and IP3R3 to regulate Ca²⁺ store release-induced Ca²⁺ entry in endothelial cells. *Am. J. Physiol. Cell Physiol.*, **296**, C403–C413.
30. Hollie, N.I., Konanias, E.S., Goodin, C. and Hui, D.Y. (2014) Group 1B phospholipase A(2) inactivation suppresses atherosclerosis and metabolic diseases in LDL receptor-deficient mice. *Atherosclerosis*, **234**, 377–380.
31. Mous, D.S., Buscop-van Kempen, M.J., Wijnen, R.M.H., Tiobboel, D. and Rottier, R.J. (2017) Changes in vasoactive pathways in congenital diaphragmatic hernia associated pulmonary hypertension explain unresponsiveness to pharmacotherapy. *Respir. Res.*, **18**, 187.
32. Ishikura, K., Yamada, N., Ito, M., Ota, S., Nakamura, M., Isaka, N. and Nakano, T. (2006) Beneficial acute effects of rho-kinase inhibitor in patients with pulmonary arterial hypertension. *Circ. J.*, **70**, 174–178.
33. Knipe, R.S., Probst, C.K., Lagares, D., Franklin, A., Spinney, J.J., Brazee, P.L., Grasberger, P., Zhang, L., Black, K.E., Sakai, N. et al. (2018) The rho kinase isoforms ROCK1 and ROCK2 each contribute to the development of experimental pulmonary fibrosis. *Am. J. Respir. Cell Mol. Biol.*, **58**, 471–481.
34. MacKay, C.E. and Knock, G.A. (2015) Control of vascular smooth muscle function by Src-family kinases and reactive oxygen species in health and disease. *J. Physiol.*, **593**, 3815–3828.
35. Fu, P., Ebenezer, D.L., Berdyshev, E.V., Bronova, I.A., Shaaya, M., Harijith, A. and Natarajan, V. (2016) Role of sphingosine kinase 1 and S1P transporter Spns2 in HGF-mediated lamellipodia formation in lung endothelium. *J. Biol. Chem.*, **291**, 27187–27203.
36. Haberberger, R.V., Tabeling, C., Runciman, S., Gutbier, B., König, P., Andratsch, M., Schutte, H., Suttorp, N., Gibbins, I. and Witzenrath, M. (2009) Role of sphingosine kinase 1 in allergen-induced pulmonary vascular remodeling and hyperresponsiveness. *J. Allergy Clin. Immunol.*, **124**, 933–941 e931–939.
37. Lien, Y.C., Feinstein, S.I., Dodia, C. and Fisher, A.B. (2012) The roles of peroxidase and phospholipase A2 activities of peroxiredoxin 6 in protecting pulmonary microvascular endothelial cells against peroxidative stress. *Antioxid. Redox Signal.*, **16**, 440–451.
38. Lee, K.S., Kim, S.R., Park, S.J., Min, K.H., Lee, K.Y., Choe, Y.H., Park, S.Y., Chai, O.H., Zhang, X., Song, C.H. et al. (2008) Mast cells can mediate vascular permeability through regulation of the PI3K-HIF-1 α -VEGF axis. *Am. J. Respir. Crit. Care Med.*, **178**, 787–797.
39. Parsa, S., Kuremoto, K., Seidel, K., Tabatabai, R., Mackenzie, B., Yamaza, T., Akiyama, K., Branch, J., Koh, C.J., Al Alam, D. et al. (2010) Signaling by FGFR2b controls the regenerative capacity of adult mouse incisors. *Development*, **137**, 3743–3752.
40. Parsa, S., Ramasamy, S.K., De Langhe, S., Gupte, V.V., Haigh, J.J., Medina, D. and Bellusci, S. (2008) Terminal end bud maintenance in mammary gland is dependent upon FGFR2b signaling. *Dev. Biol.*, **317**, 121–131.
41. Volckaert, T., Campbell, A., Dill, E., Li, C., Minoo, P. and De Langhe, S. (2013) Localized Fgf10 expression is not required for lung branching morphogenesis but prevents differentiation of epithelial progenitors. *Development*, **140**, 3731–3742.
42. MacKenzie, B., Henneke, I., Hezel, S., Al Alam, D., El Agha, E., Chao, C.M., Quantius, J., Wilhelm, J., Jones, M., Goth, K. et al. (2015) Attenuating endogenous Fgfr2b ligands during bleomycin-induced lung fibrosis does not compromise murine lung repair. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **308**, L1014–L1024.
43. Kool, H., Mous, D., Tibboel, D., de Klein, A. and Rottier, R.J. (2014) Pulmonary vascular development goes awry in congenital lung abnormalities. *Birth Defects Res. C Embryo Today*, **102**, 343–358.
44. Mailleux, A.A., Kelly, R., Veltmaat, J.M., De Langhe, S.P., Zaffran, S., Thiery, J.P. and Bellusci, S. (2005) Fgf10 expression identifies parabronchial smooth muscle cell progenitors and is required for their entry into the smooth muscle cell lineage. *Development*, **132**, 2157–2166.
45. Oladipupo, S.S., Smith, C., Santeford, A., Park, C., Sene, A., Wiley, L.A., Osei-Owusu, P., Hsu, J., Zapata, N., Liu, F. et al. (2014) Endothelial cell FGF signaling is required for injury response but not for vascular homeostasis. *Proc. Natl. Acad. Sci. U. S. A.*, **111**, 13379–13384.

46. House SL, Castro, A.M., Lupu, T.S., Weinheimer, C., Smith, C., Kovacs, A. and Ornitz, D.M. (2016) Endothelial fibroblast growth factor receptor signaling is required for vascular remodeling following cardiac ischemia-reperfusion injury. *Am. J. Physiol. Heart Circ. Physiol.*, **310**, H559–H571.
47. Moiseenko, A., Kheirollahi, V., Chao, C.M., Ahmadvand, N., Quantius, J., Wilhelm, J., Herold, S., Ahlbrecht, K., Morty, R.E., Rizvanov, A.A. et al. (2017) Origin and characterization of alpha smooth muscle actin-positive cells during murine lung development. *Stem Cells*, **35**, 1566–1578.
48. Rochais, F., Sturny, R., Chao, C.M., Mesbah, K., Bennett, M., Mohun, T.J., Bellusci, S. and Kelly, R.G. (2014) FGF10 promotes regional foetal cardiomyocyte proliferation and adult cardiomyocyte cell-cycle re-entry. *Cardiovasc. Res.*, **104**, 432–442.
49. Sakaue, H., Konishi, M., Ogawa, W., Asaki, T., Mori, T., Yamasaki, M., Takata, M., Ueno, H., Kato, S., Kasuga, M. et al. (2002) Requirement of fibroblast growth factor 10 in development of white adipose tissue. *Genes Dev.*, **16**, 908–912.
50. Al Alam, D., El Agha, E., Sakurai, R., Kheirollahi, V., Moiseenko, A., Danopoulos, S., Shrestha, A., Schmoldt, C., Quantius, J., Herold, S. et al. (2015) Evidence for the involvement of fibroblast growth factor 10 in lipofibroblast formation during embryonic lung development. *Development*, **142**, 4139–4150.
51. Sugimoto, K., Yoshida, S., Mashio, Y., Toyota, N., Xing, Y., Xu, H., Fujita, Y., Huang, Z., Touma, M. and Wu, Q. (2014) Role of FGF10 on tumorigenesis by MS-K. *Genes Cells*, **19**, 112–125.

Developmental Cell

Fgf10-Hippo Epithelial-Mesenchymal Crosstalk Maintains and Recruits Lung Basal Stem Cells

Graphical Abstract



Authors

Thomas Volckaert, Tingting Yuan, Cho-Ming Chao, ..., Victor J. Thannickal, Reinhard Fässler, Stijn P. De Langhe

Correspondence

sdelanghe@uabmc.edu

In Brief

Volckaert et al. demonstrate a novel mode of stem cell regulation in which basal stem cells during homeostasis or differentiated airway epithelial cells after injury downregulate their Hippo signaling to generate their own localized *Fgf10*-expressing stromal niche, which maintains or amplifies the stem/progenitor cell population via Fgf10-Fgfr2b signaling.

Highlights

- Basal stem cells generate their own *Fgf10*-expressing stromal niche via Yap-Wnt7b
- ILK-mediated Hippo signaling actively maintains adult airway epithelial quiescence
- Hippo inactivation in mature daughter cells activates a basal stem cell niche
- Integrin-linked kinase senses airway epithelial injury to drive regeneration



Volckaert et al., 2017, Developmental Cell 43, 48–59
October 9, 2017 © 2017 Elsevier Inc.
<http://dx.doi.org/10.1016/j.devcel.2017.09.003>

CellPress

Fgf10-Hippo Epithelial-Mesenchymal Crosstalk Maintains and Recruits Lung Basal Stem Cells

Thomas Volckaert,^{1,2,7} Tingting Yuan,^{1,2,7} Cho-Ming Chao,³ Harold Bell,¹ Alina Sitaula,¹ Luisa Szimtmtenings,² Elie El Agha,³ Diptiman Chanda,² Susan Majka,⁴ Saverio Bellusci,³ Victor J. Thannickal,² Reinhard Fässler,⁵ and Stijn P. De Langhe^{1,2,6,8,9,*}

¹Department of Pediatrics, Division of Cell Biology, National Jewish Health, Denver, CO 80206, USA

²Department of Medicine, Division of Pulmonary, Allergy & Critical Care Medicine, University of Alabama at Birmingham, THT 422, 1720 2nd Avenue South, Birmingham, AL 35294-2182, USA

³German Center for Lung Research, Excellence Cluster Cardio-Pulmonary System, Universities of Giessen and Marburg Lung Center, 35392 Giessen, Germany

⁴Department of Medicine, Division of Allergy, Pulmonary and Critical Care Medicine or Division of Cardiovascular Medicine, Vanderbilt University Medical Center, Nashville, TN 37232, USA

⁵Department of Molecular Medicine, Max Planck Institute of Biochemistry, 82152 Martinsried, Germany

⁶Department of Cellular and Developmental Biology, School of Medicine, University of Colorado Denver, Aurora, CO 80045, USA

⁷These authors contributed equally

⁸Senior author

⁹Lead Contact

*Correspondence: sdelanghe@uabmc.edu

<http://dx.doi.org/10.1016/j.devcel.2017.09.003>

SUMMARY

The lung harbors its basal stem/progenitor cells (BSCs) in the protected environment of the cartilaginous airways. After major lung injuries, BSCs are activated and recruited to sites of injury. Here, we show that during homeostasis, BSCs in cartilaginous airways maintain their stem cell state by downregulating the Hippo pathway (resulting in increased nuclear Yap), which generates a localized *Fgf10*-expressing stromal niche; in contrast, differentiated epithelial cells in non-cartilaginous airways maintain quiescence by activating the Hippo pathway and inhibiting *Fgf10* expression in airway smooth muscle cells (ASMCs). However, upon injury, surviving differentiated epithelial cells spread to maintain barrier function and recruit integrin-linked kinase to adhesion sites, which leads to Merlin degradation, downregulation of the Hippo pathway, nuclear Yap translocation, and expression and secretion of Wnt7b. Epithelial-derived Wnt7b, then in turn, induces *Fgf10* expression in ASMCs, which extends the BSC niche to promote regeneration.

INTRODUCTION

Stem cells are required for tissue homeostasis and repair. They reside in a highly specialized stromal microenvironment called the stem cell niche (Scadden, 2006), where they are kept in a quiescent state during homeostasis or rapidly amplify upon tissue insults. Basal stem/progenitor cells (BSCs), located in the protected environment of the cartilaginous airways of the trachea and mainstem bronchi, were identified as progenitors

of secretory or ciliated cells (Rock et al., 2009). Secretory cells represent transit-amplifying cells with the potential to self-renew or differentiate into post-mitotic ciliated daughter cells (Pardo-Saganta et al., 2015; Rawlins et al., 2009). Secretory cells drive almost the entire cell turnover in the conducting airways during homeostasis or repair of small injuries (Rawlins et al., 2009), and give rise to alveolar epithelial cells after major lung epithelial injuries (Barkauskas et al., 2013; Kim et al., 2005; Lee et al., 2014). Interestingly, after major lung epithelial injuries, BSCs emerge at sites of injury where they aid in restoring lung architecture (Kumar et al., 2011; Vaughan et al., 2015; Zuo et al., 2015). How BSCs are maintained and restricted to cartilaginous airways during homeostasis, and how they are activated and recruited to sites of severe injury are not well understood.

The production and maintenance of BSCs during development depends on *Fgf10* secreted by the stromal tissue located in between the cartilage rings (Volckaert et al., 2013b). Furthermore, overexpression of *Fgf10* in all lung cells during late embryonic development induces the ectopic development of BSCs in conducting airways (Volckaert et al., 2013b), where they normally do not reside. Similarly, the maintenance of the adult BSC pool depends on Yap, the nuclear effector of the Hippo pathway, whereas overexpression of *Yap* in adult tracheal BSCs results in BSC hyperplasia and stratification (Tata et al., 2013; Zhao et al., 2014). These findings indicate that lung stem/progenitor cells deploy Hippo signaling to maintain their stemness, and that Hippo signaling executes these tasks by controlling *Fgf10*-*Fgfr2b* signaling. It further suggests that BSCs are absent from non-cartilaginous conducting airways because airway smooth muscle cells (ASMCs) which envelop them, do not secrete *Fgf10* during homeostasis (Volckaert et al., 2011, 2013b).

Interestingly, upon injury-induced loss of airway epithelial cells, surviving differentiated cells spread out to seal the wound and maintain barrier function and secrete Wnt7b to induce ectopic *Fgf10* expression in ASMCs (Volckaert et al., 2011, 2013a). This mesenchymal-derived *Fgf10* then acts back on

the airway epithelium to drive regeneration by expanding secretory transit-amplifying cells and inhibiting their differentiation into ciliated cells (Volckaert et al., 2011, 2013b). Airway epithelial injury leads to the inactivation of the Hippo pathway in differentiated epithelial cells of the conducting airway (Lange et al., 2015), and genetic inactivation of the Hippo pathway or overexpressing *Yap* in secretory cells breaks their quiescence (Lange et al., 2015; Zhao et al., 2014).

In the present paper, we tested how the Hippo pathway regulates *Fgf10* expression in stromal cell niches. We report the existence of a mode of stem cell regulation in which tracheal BSCs during homeostasis or differentiated cells after injury down-regulate their Hippo signaling (resulting in increased nuclear *Yap*) to generate their own localized *Fgf10*-expressing stromal niche, which maintains or amplifies the stem/progenitor cell population via *Fgf10*-*Fgfr2b* signaling. Mechanistically, we also show that surviving epithelial cells spread out after airway epithelial injury and recruit integrin-linked kinase (*Ilk*) to adhesion sites, which results in the destabilization of *Merlin*, an upstream activator of the Hippo pathway. An increase in nuclear *Yap* in surviving and spreading airway epithelial cells then leads to the expression and secretion of *Wnt7b*, which acts on the ASMCs to induce the release of *Fgf10*. *Fgf10* subsequently activates *Fgfr2b* signaling on BSCs to drive their expansion, mobilization, and/or differentiation. This intricate epithelial-mesenchymal reciprocal signaling pathway between stem/progenitor cells or differentiated cells and their niche cells ensures maintenance of stemness during homeostasis and promotes regeneration in response to injury.

RESULTS

Tracheal Basal Stem/Progenitor Cells Maintain and Restrict Their Cell Pool during Homeostasis by Generating a Localized *Fgf10*-Expressing Stromal Niche

Our previous studies have demonstrated that the development and maintenance of BSCs during embryogenesis depends on *Fgf10* secreted by stromal cells in between cartilaginous rings of the upper airway (Volckaert et al., 2013b). To test whether the *Fgf10*-*Fgfr2b* signaling axis controls BSC amplification and maintenance in adult tracheas, we induced the expression of *Fgf10* in adult *Rosa26-rtTA;Tet-Fgf10* mice (hereafter, referred to as *rtTa-Fgf10*), or inactivated *Fgfr2b* in all adult airway epithelial cells using *Sox2-CreERT2;Fgfr2b^{fl/fl}* or in BSC specifically using *Krt5-CreERT2;Fgfr2b^{fl/fl}* mice (hereafter, referred to as *Sox2-Fgfr2b^{fl/fl}* and *K5-Fgfr2b^{fl/fl}*, respectively). While overexpression of *Fgf10* for 2 weeks caused a significant expansion of Keratin-5 (K5)- and p63-positive BSCs in the trachea, conditional deletion of *Fgfr2b* resulted in depletion of BSCs (Figures 1A–1C, S1A, and S1B). BSCs were lost to both differentiation (Figure S1A) and apoptosis (Figure S1C) due to the loss of *Fgfr2b* signaling within the basal cell compartment but not upon inactivation of *Fgfr2b* in secretory daughter cells using *Scgb1a1-CreER;Fgfr2b^{fl/fl}* mice (Figure S1D). These data, together with our previous findings during development (Volckaert et al., 2013b), indicate that *Fgfr2b* signaling is required for BSC maintenance during pre- and post-natal life.

It has been shown that inactivation of the Hippo pathway or overexpression of *Yap*, the transcriptional effector of the Hippo pathway, in secretory and/or tracheal BSCs drives their amplifi-

cation. In addition, *Yap* ablation in BSCs results in their loss over a period of 12 weeks (Lange et al., 2015; Zhao et al., 2014), supporting the concept that lung stem/progenitor cells deploy Hippo signaling to control their fate. To test whether Hippo signaling executes this task by controlling *Fgf10*-*Fgfr2b* signaling, we disrupted the *Mst1/2* genes, which results in inactivation of the Hippo pathway and increased nuclear *Yap*. This was accomplished by either conditional deletion of *Mst1/2* genes in all airway epithelial cells using *Sox2-CreERT2;Mst1/2^{fl/fl}* mice, or only in secretory/ciliated daughter cells using *Scgb1a1-Cre;Mst1/2^{fl/fl}* mice (referred to as *Sox2-Mst1/2^{fl/fl}* and *Scgb1a1-Mst1/2^{fl/fl}*, respectively) (Figure 1D). These experiments revealed increased *Fgf10* expression in the tracheal stromal niche (Figures 1E, S1E, and S1F), and amplification of the tracheal BSC population in both mouse strains (Figures 1D, 1F, and 1G). Lineage tracing using the *Confetti* allele in tracheas of *Scgb1a1-Cre;Mst1/2^{fl/fl};Rosa26R-Confetti* mice (hereafter, referred to as *Scgb1a1-Mst1/2^{fl/fl}-Confetti*) indicated that the expansion of BSCs in *Scgb1a1-Mst1/2^{fl/fl}-Confetti* tracheas was not caused by the dedifferentiation of secretory cells but rather by amplification of existing BSCs (Figure 1F). To test the hypothesis that this BSC expansion was due to the increase in stromal *Fgf10* expression, we simultaneously deleted *Mst1/2* and *Fgfr2b* in all tracheal epithelial cells using *Sox2-CreERT2;Mst1/2^{fl/fl};Fgfr2b^{fl/fl}* mice (referred to as *Sox2-Mst1/2^{fl/fl}-Fgfr2b^{fl/fl}*), and found that BSCs failed to expand and were lost (Figures 1D, 1F, 1G, and S1C), indicating that the Hippo pathway in epithelial cells promotes BSC quiescence by repressing *Fgf10* expression in a non-cell autonomous manner within the stromal niche. To further confirm this finding, we deleted *Yap* in all tracheal epithelial cells and simultaneously overexpressed *Fgf10* by intercrossing *Sox2-CreERT2;Yap^{fl/fl}* and *rtTa-Fgf10* mice (referred to as *Sox2-Yap^{fl/fl}-rtTa-Fgf10*) (Figure 1D) or intercrossing *Sox2-CreERT2;Yap^{fl/fl}* and *Rosa26-LSL-rtTa;Tet-Fgf10* mice (referred to as *Sox2-Yap^{fl/fl}-rtTa^{fl/fl}-Fgf10*) (Figure S1G). The *Rosa26-LSL(Lox-STOP-Lox)-rtTA* (referred to as *rtTa^{fl/fl}*) cassette carries a loxP-flanked stop codon, which is removed in the presence of active Cre recombinase allowing for cell-type-specific *rtTa* expression. Consistent with previous studies (Zhao et al., 2014), the majority of BSCs were lost 10–12 weeks after tamoxifen-induced ablation of *Yap* when *Fgf10* expression was not induced (Figures 1D and 1G–1I). In sharp contrast, however, induction of *Fgf10* expression after tamoxifen-induced *Yap* ablation efficiently triggered BSC proliferation and halted further BSC loss (Figures 1D and 1G–1I), suggesting that epithelial *Yap* is critical for generating a localized *Fgf10*-expressing stromal niche. Indeed, inactivation of *Yap* in *Sox2-CreERT2;Yap^{fl/fl}* mice resulted in decreased *Fgf10* expression in the tracheal stromal niche (Figures S1F and S1H).

Next, we wanted to address how increased nuclear *Yap* induces *Fgf10* expression in the tracheal stromal niche. Intriguingly, an interaction between *Yap* and p63 has been suggested to indirectly regulate basal stem cell proliferation and maintenance in squamous cell carcinoma by inducing *Fgf10* expression in the adjacent stroma (Ramsey et al., 2013; Zhao et al., 2014). Expression of *Wnt7b*, which is one of the main upstream regulators of *Fgf10* expression in the lung, has been shown to be driven by p63 in other tissues (Trink et al., 2007). In the lung, *Wnt7b* expression is regulated in part by the transcription factors

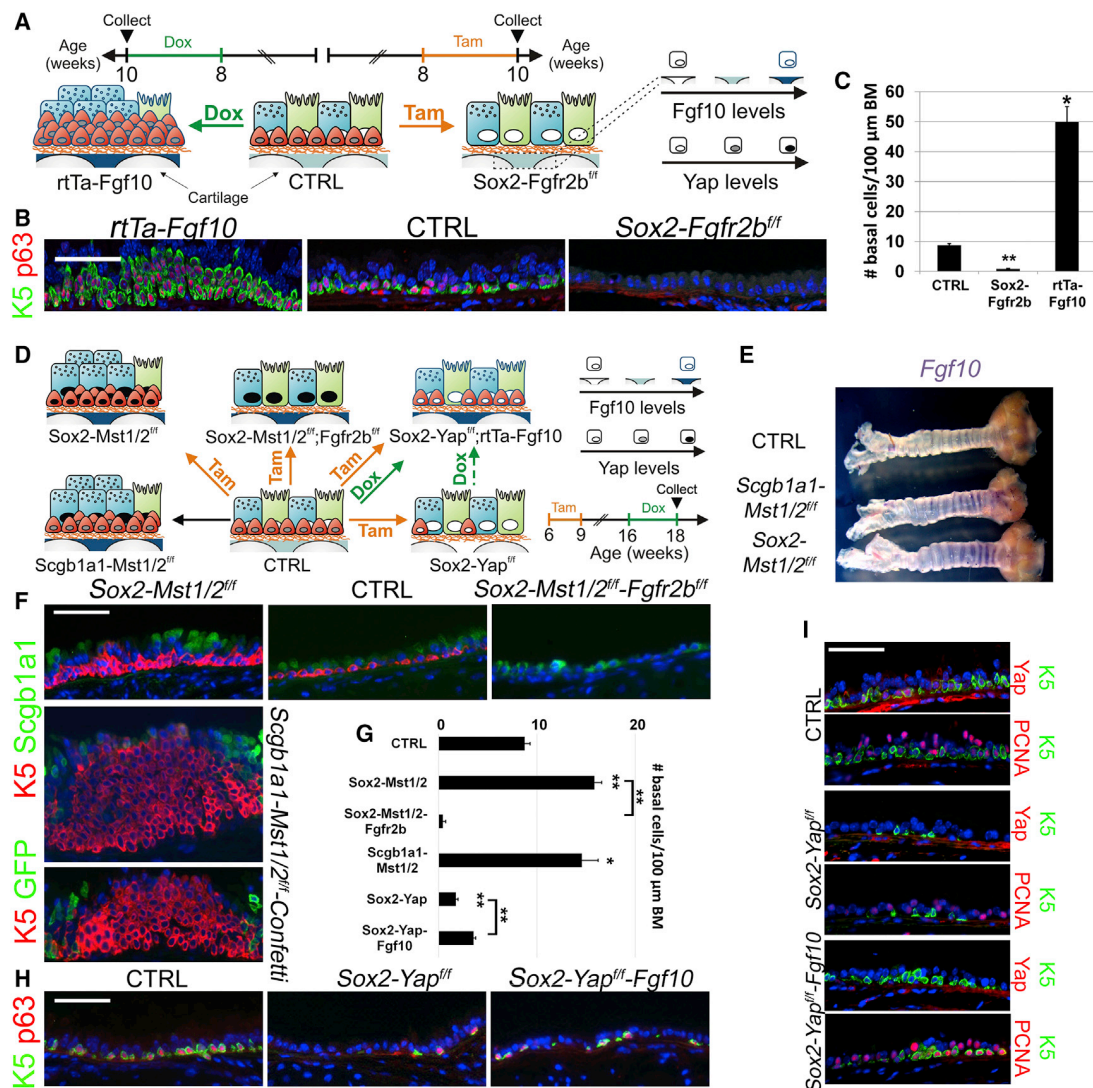


Figure 1. The Inactive Hippo Pathway in Basal Stem/Progenitor Cells Generates the *Fgf10*-Expressing Tracheal Stromal Niche Required to Maintain Their Cell Pool

(A) Experimental strategy and schematic representation of tracheal BSC amplification after *Fgf10* overexpression or tracheal BSC loss after *Fgfr2b* ablation in airway epithelial cells. Ciliated, secretory, and BSCs are shown in green, blue, and red, respectively.

(B) Immunostaining on *rtTa-Fgf10*, control, and *Sox2-Fgfr2b^{fl/fl}* tracheas for the BSC markers Keratin 5 (K5) (green) and p63 (red) 14 days after doxycycline or tamoxifen induction.

(C) Quantification of the number (#) of BSCs per 100 μm of basement membrane for the pictures represented in (B).

(D) Experimental strategy and schematic representation of *Mst1/2* ablation in all airway epithelial cells or selectively in secretory/ciliated cells alone or in combination with either *Fgfr2b* or *Yap* in all airway epithelial cells with or without simultaneously inducing *Fgf10* expression.

(E) Whole-mount *in situ* hybridization for *Fgf10* in control, *Scgb1a1-Mst1/2^{fl/fl}*, and *Sox2-Mst1/2^{fl/fl}* tracheas. Note purple *Fgf10* expression between the tracheal cartilage rings.

(F) Upper panels show immunostaining on control, *Sox2-Mst1/2^{fl/fl}*, and *Sox2-Mst1/2^{fl/fl}-Fgfr2b^{fl/fl}* tracheas 2 months after tamoxifen induction as well as on a 2.5-month-old *Scgb1a1-Mst1/2^{fl/fl}-Confetti* trachea for the BSC marker K5 (red) and the secretory cell marker *Scgb1a1* (green). Lower panel shows immunostaining on *Scgb1a1-Mst1/2^{fl/fl}-Confetti* trachea for the BSC marker K5 (red) and GFP (green).

(G) Quantification of the number (#) of BSCs per 100 μm of basement membrane (BM) of pictures represented in (F and H).

(H) Immunostaining on control, *Sox2-Yap^{fl/fl}*, *Sox2-Yap^{fl/fl}-rtTa-Fgf10* tracheas for the BSC markers K5 (green) and p63 (red) after tamoxifen-induced deletion of *Yap* and/or doxycycline-induced *Fgf10* expression. Refer to (D) for the experimental strategy.

(I) Immunostaining on adjacent tracheal sections from control, *Sox2-Yap^{fl/fl}*, and *Sox2-Yap^{fl/fl}-Fgf10* mice for BSC marker K5 (green) and *Yap* (red) or proliferation marker PCNA (red) 12 weeks after tamoxifen and 2 weeks after doxycycline induction, starting at 10 weeks after tamoxifen induction.

Nuclei, DAPI (blue). * $p < 0.05$; ** $p < 0.01$; $n \geq 6$. Error bars represent means $\pm$ SEM. Scale bars, 100 μm (B, F, and H); 50 μm (I). See also Figure S1.

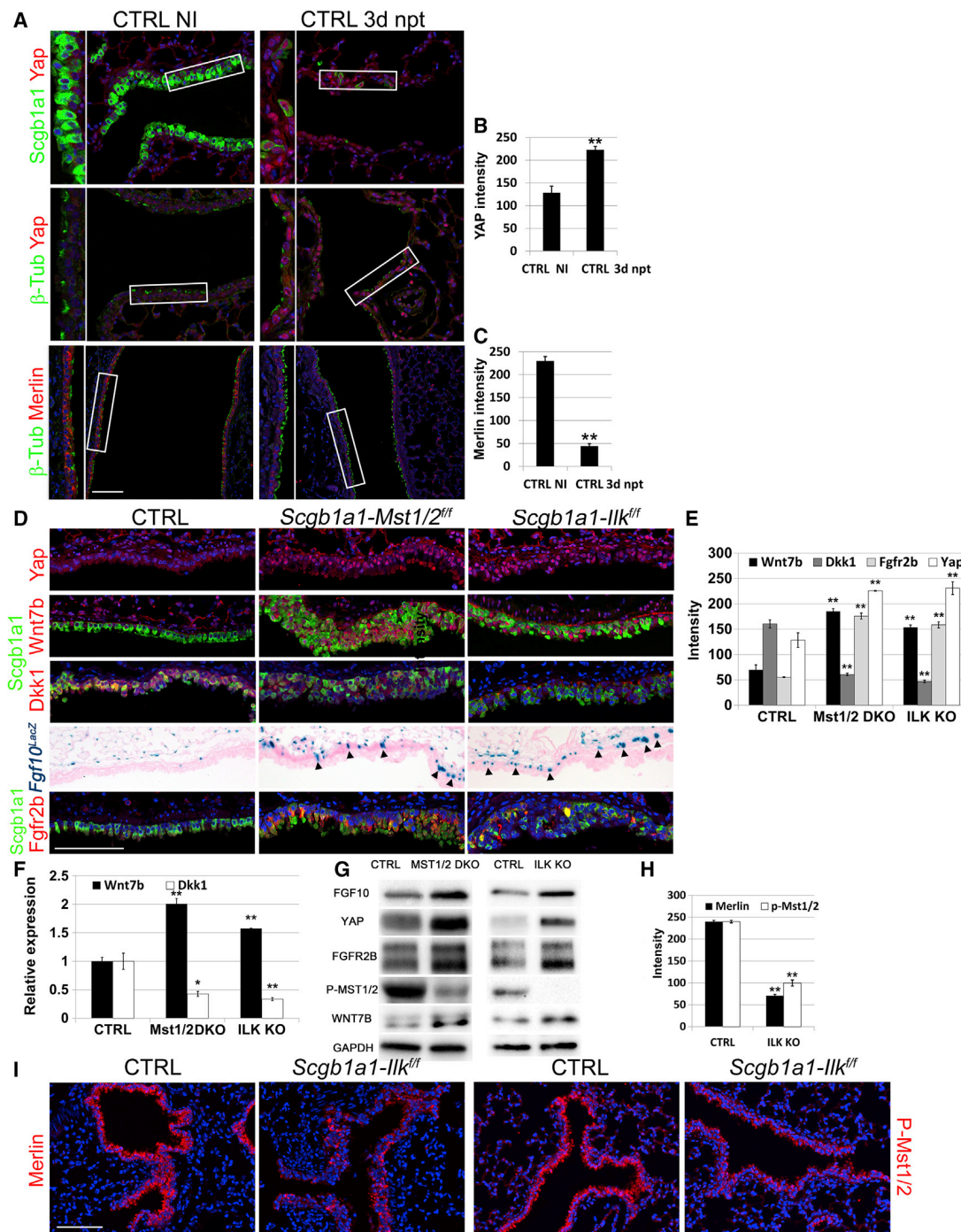


Figure 2. Inactivation of the Hippo Pathway in Differentiated Airway Epithelial Cells after Injury or after *Ilk* Inactivation Induces Epithelial *Wnt7b* Expression and *Fgf10* Secretion by ASMCs

(A) Immunostaining on non-cartilaginous airways of non-injured (NI) control lungs and lungs 3 days after naphthalene (npt)-induced injury for secretory cell marker *Scgb1a1* (green) or ciliated cell marker β -tubulin (green) and Yap or Merlin (red).
 (B) Quantification of Yap pixel intensity of pictures represented in (A) ($n \geq 6$ mice).
 (C) Quantification of Merlin pixel intensity of pictures represented in (A) ($n \geq 6$ mice).
 (D) Immunostaining on non-cartilaginous airways of control *Fgf10^{LacZ}*, *Scgb1a1-Mst1/2^{fl/fl}-Fgf10^{LacZ}*, and *Scgb1a1-Ilk^{fl/fl}-Fgf10^{LacZ}* lungs for secretory cell marker *Scgb1a1* (green) with either Yap (red) or *Wnt7b* (red) or *Dkk1* (red) or *Fgfr2b* (red) and β -gal staining of *Fgf10^{LacZ}*. Black arrowheads indicate *Fgf10* expression in ASMCs.
 (E) Quantification of pixel intensity of Yap, *Wnt7b*, *Dkk1*, and *Fgfr2b* signals represented in (D) ($n \geq 6$ mice).
 (F) Relative mRNA expression of *Wnt7b* and *Dkk1* in control, *Scgb1a1-Mst1/2^{fl/fl}*, and *Scgb1a1-Ilk^{fl/fl}* lungs.

(legend continued on next page)

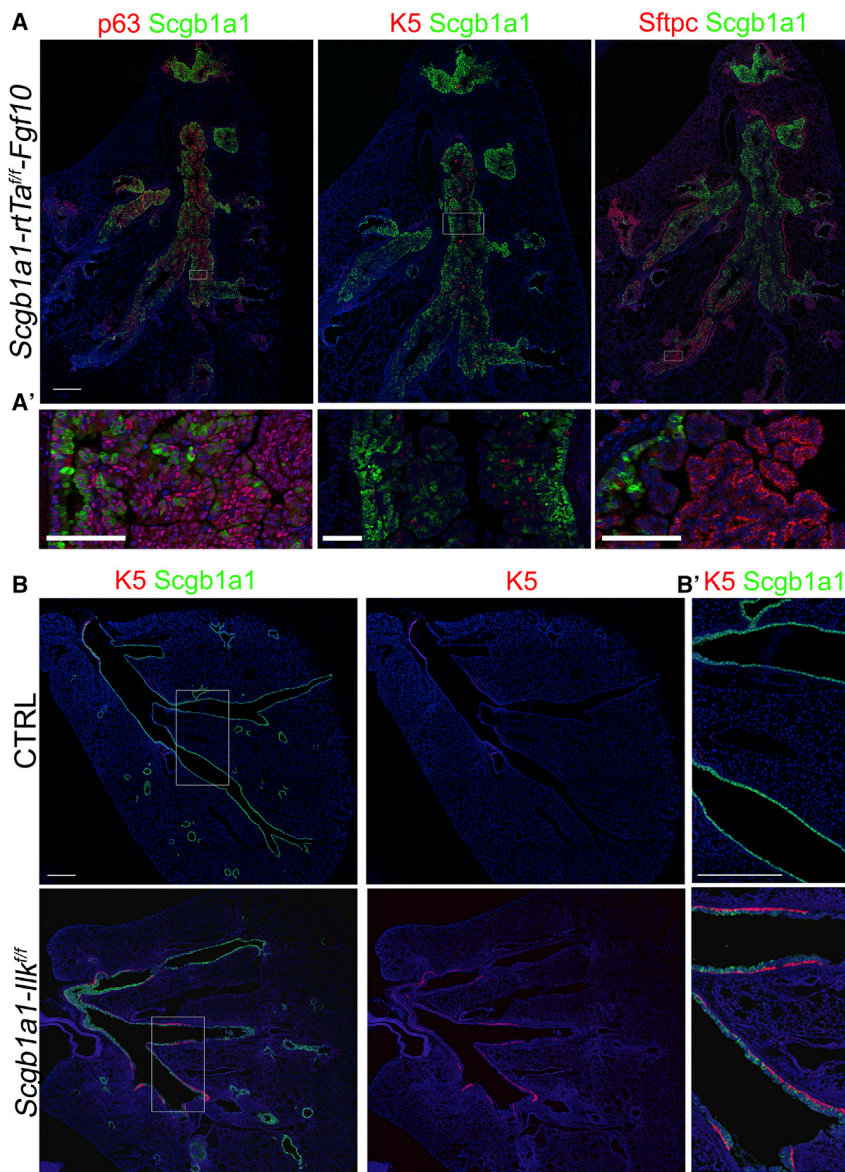


Figure 3. *Fgf10* Overexpression or *Ilk* Deletion in Secretory Cells Induces Basal Cell Recruitment to the Non-cartilaginous Conducting Airways

(A) Immunostaining for Scgb1a1 (green) and p63 (red) (left), or Scgb1a1 (green) and K5 (red) (middle), or Scgb1a1 (green) and Sftpc (red) (right) on non-cartilaginous airways of *Scgb1a1-rtTa^{flox}-Fgf10* mice, which were induced with doxycycline for 5.5 weeks starting at 3 weeks of age. (A') High magnification of selected regions in (A).

(B) Immunostaining on control and *Scgb1a1-Ilk^{flox}* non-cartilaginous airways for Scgb1a1 (green) and K5 (red) or K5 (red) by itself. (B') High magnification of selected regions in (B).

Nuclei were stained with DAPI (blue). Scale bar, 500 μ m (A and B), 100 μ m (A'), 300 μ m (B'). See also Figure S5.

a decrease in stromal *Fgf10* expression compared with control tracheas (Figure S1F) concomitant with a decrease in BSCs (Figure S1J). Interestingly, conditionally ablating *Wnt7b* in secretory cells alone using *Scgb1a1-Cre;Wnt7b^{flox}* mice did not affect the BSC population (Figure S1J), indicating that *Wnt7b* expressed by basal cells is required for their maintenance through the induction of *Fgf10* in the adjacent mesenchyme.

Inactivation of the Hippo Pathway in Differentiated Airway Epithelial Cells in Non-cartilaginous, Conducting Airways Activates *Fgf10* Expression within the ASMC Niche

We have previously shown that ASMCs provide a niche for secretory “transit-amplifying” progenitor cells by releasing *Fgf10* in response to *Wnt7b* secreted by airway epithelial cells that survive

and spread after airway epithelial injury (Volckaert et al., 2011). We observed that naphthalene-mediated lung injury inactivates Hippo signaling (i.e., increased nuclear Yap translocation) in airway epithelial cells (Figures 2A and 2B), suggesting that, similar to that observed in cartilaginous airways, the Hippo pathway may also control *Fgf10* expression within the ASMC niche. To further establish that the Hippo pathway controls *Fgf10* in the ASMC niche, we deleted *Mst1/2* in secretory and ciliated cells using *Scgb1a1-Mst1/2^{flox}* mice and intercrossed the mice with an *Fgf10^{LacZ}* reporter line to monitor *Fgf10* expression. The efficient deletion of *Mst1/2* (Figure S2A)

and spread after airway epithelial injury (Volckaert et al., 2011). We observed that naphthalene-mediated lung injury inactivates Hippo signaling (i.e., increased nuclear Yap translocation) in airway epithelial cells (Figures 2A and 2B), suggesting that, similar to that observed in cartilaginous airways, the Hippo pathway may also control *Fgf10* expression within the ASMC niche. To further establish that the Hippo pathway controls *Fgf10* in the ASMC niche, we deleted *Mst1/2* in secretory and ciliated cells using *Scgb1a1-Mst1/2^{flox}* mice and intercrossed the mice with an *Fgf10^{LacZ}* reporter line to monitor *Fgf10* expression. The efficient deletion of *Mst1/2* (Figure S2A)

(G) Western blot analysis showing FGF10, YAP, FGFR2B, P-MST1/2, and WNT7B protein expression in control, *Scgb1a1-Mst1/2^{flox}* (MST1/2 KO), and *Scgb1a1-Ilk^{flox}* (ILK KO) lungs.

(H) Quantification of pixel intensities of pictures represented in (I) ($n \geq 6$ mice).

(I) Immunostaining on control and *Scgb1a1-Ilk^{flox}* non-cartilaginous airways for Merlin (red) or phospho-Mst1/2 (red).

Nuclei, DAPI (blue). * $p < 0.05$; ** $p < 0.01$; $n \geq 6$. Error bars represent means $\pm$ SEM. Scale bar, 100 μ m. See also Figures S2, S3, S4, S6, and S7.

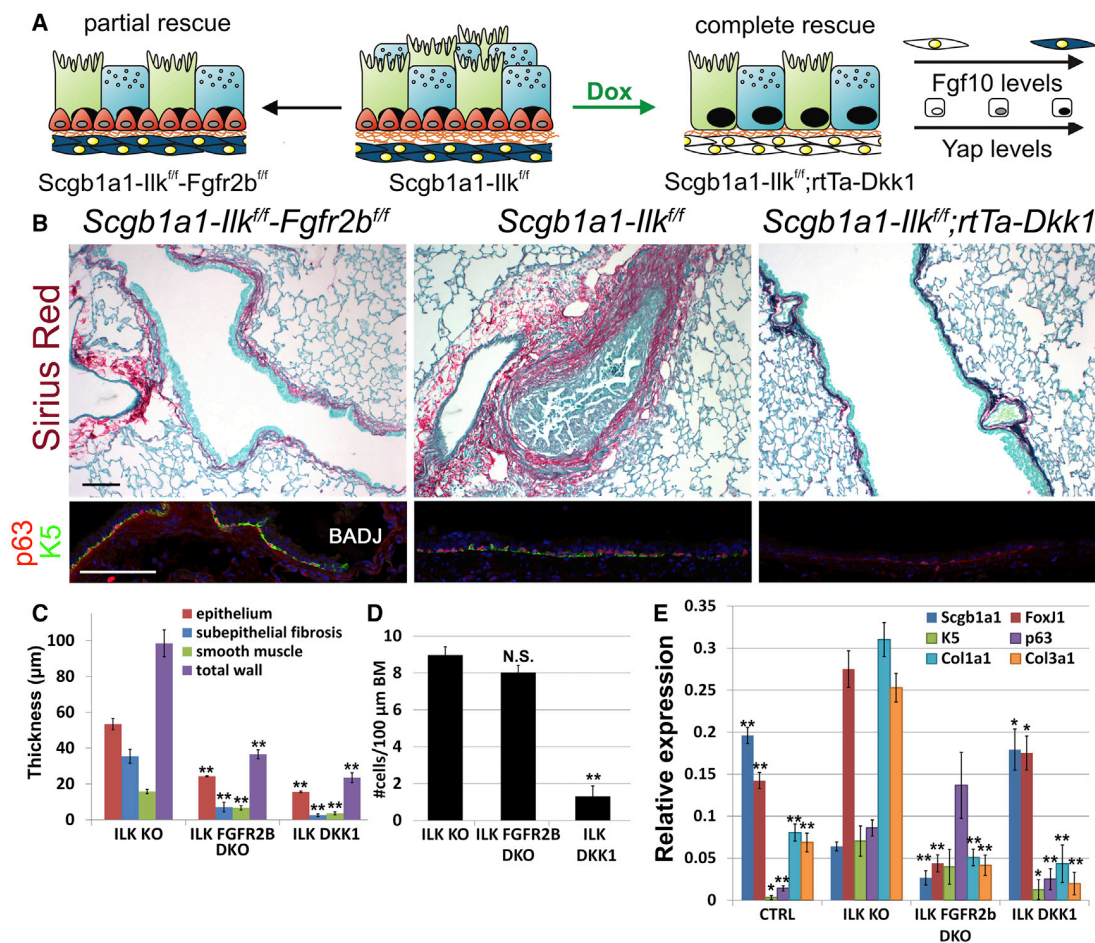


Figure 4. Fgf10 Expression in ASMCs Drives Secretory and Basal Cell Amplification in the Lower Non-cartilaginous Conducting Airways upon *Ilk* Inactivation

(A) Schematic representation of airway BSC amplification or loss, and stromal *Fgf10* or nuclear Yap levels in the different mutant strains. (B) Sirius red (collagen)/fast green staining and immunostaining for BSC markers p63 (red) and Keratin 5 (K5) (green) or secretory cell marker *Scgb1a1* (green) and Yap (red). (C and D) Morphometric analysis of airway remodeling (C) and quantification of BSC numbers (D) of images represented in (B). (E) Relative mRNA levels of *Scgb1a1* (secretory cells), *FoxJ1* (ciliated cells), *K5* (BSCs), *p63* (BSCs), *Col1a1* (collagen), and *Col3a1* (collagen) in control, *Scgb1a1-Ilk*^{fl/fl}, *Scgb1a1-Ilk*^{fl/fl}-*Fgfr2b*^{fl/fl}, and *Scgb1a1-Ilk*^{fl/fl}-*rtTa-Dkk1* lungs. Nuclei were stained with DAPI (blue). **p* < 0.05; ***p* < 0.01; N.S., not significant; *n* ≥ 6. Error bars represent means ± SEM. Scale bars, 100 μm . See also Figures S3, S4, S6F, and S7.

led to nuclear translocation of Yap in secretory cells (Figure S2B), increased *Wnt7b* with decreased *Dkk1* expression in secretory and ciliated cells, elevated *Fgf10* expression in the ASMC niche, and increased *Fgfr2b* expression in airway epithelial cells (Figures 2D–2G, S2A, S3A, S3B, and S3D). To investigate whether the expansion of airway epithelial cells upon conditional *Mst1/2* ablation is caused by the sustained secretion of *Fgf10* by the ASMC niche, we simultaneously inactivated the *Mst1/2* and *Fgfr2b* genes in all airway epithelial cells using *Sox2-Mst1/2*^{fl/fl}-*Fgfr2b*^{fl/fl} mice (Figure S2). These studies revealed that, despite the accumulation of Yap in their nuclei (Figure S2B), secretory cell hyperplasia did not occur (Figures S2 and S4; Figure S6C shows the specificity of the *Sox2-CreERT2* line). This confirms that ASMC niche-derived *Fgf10* is induced downstream of epithelial Yap and acts in a

non-cell autonomous and paracrine manner on neighboring secretory cells.

Fgf10 Overexpression or Inactivation of Ilk in Secretory Cells Extends the Basal Stem Cell Niche and Induces Secretory and Basal Stem/Progenitor Cell Hyperplasia in Conducting Airways

Based on our observation that ectopic BSCs do not populate the non-cartilaginous airways upon *Mst1/2* inactivation in secretory cells (data not shown), despite induction of *Fgf10* in ASMCs, we suspected that *Fgf10* levels did not reach the threshold required to recruit BSCs to the non-cartilaginous airways. To determine whether *Fgf10* expression alone is sufficient to induce secretory and BSC amplification in the non-cartilaginous airways in the absence of injury, we overexpressed *Fgf10* in all

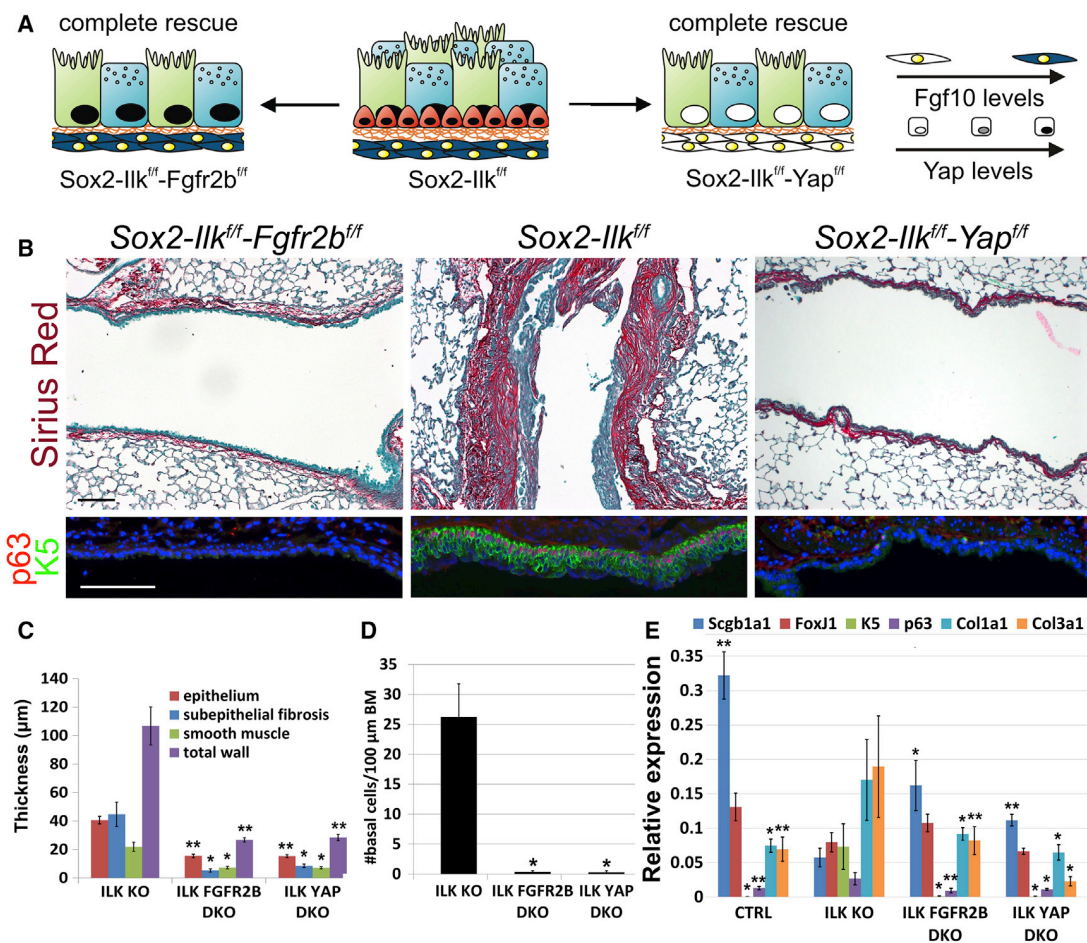


Figure 5. Fgf10-Fgfr2b Signaling Downstream of Yap Drives Secretory and Basal Cell Amplification in the Lower Non-cartilaginous Conducting Airways upon *Ilk* Inactivation

(A) Schematic representation of non-cartilaginous airway secretory/ciliated amplification and BSC amplification or loss, and stromal Fgf10 or nuclear Yap levels upon airway epithelial *Ilk*/Fgfr2b ablation, *Ilk* ablation, and *Ilk*/Yap ablation. All lungs were collected at 2 months after tamoxifen induction.

(B) Sirius red (collagen)/fast green staining and immunostaining on Sox2-Ilk^{fl/fl}-Fgfr2b^{fl/fl}, Sox2-Ilk^{fl/fl}, and Sox2-Ilk^{fl/fl}-Yap^{fl/fl} non-cartilaginous airways for BSC markers p63 (red) and Keratin 5 (K5) (green).

(C and D) Morphometric analysis of airway remodeling (C) and quantification of BSC numbers (#) per 100 μm of basement membrane (D) from images represented in (B).

(E) Relative mRNA levels of *Scgb1a1* (secretory cells), *FoxJ1* (ciliated cells), *K5* (BSCs), *p63* (BSCs), *Col1a1* (collagen), and *Col3a1* (collagen) in control, Sox2-Ilk^{fl/fl} (ILK KO), Sox2-Ilk^{fl/fl}-Fgfr2b^{fl/fl} (ILK FGFR2b DKO), and Sox2-Ilk^{fl/fl}-Yap^{fl/fl} (ILK YAP DKO) lungs.

Nuclei were stained with DAPI (blue). *p < 0.05; **p < 0.01; n ≥ 6. Error bars represent means ± SEM. Scale bars, 100 μm. See also Figures S3, S4, and S6C.

secretory cells for 5.5 weeks starting at 3 weeks of age using *Scgb1a1-Cre;Rosa26R-LSL-rtTa;Tet-Fgf10* mice or in ASMCs for 10 weeks starting at 9 weeks of age using *Acta2-CreERT2;Rosa26R-LSL-rtTa;Tet-Fgf10* mice, which were first placed on tamoxifen chow for 3 weeks starting at 6 weeks of age (referred to as *Scgb1a1-rtTa^{fl/fl}-Fgf10* and *Acta2-rtTa^{fl/fl}-Fgf10*, respectively). These studies demonstrate that *Fgf10* overexpression is sufficient to allow for BSCs to populate the non-cartilaginous airways (Figures 3A, S4, and S5A).

Interestingly, Merlin activates the Hippo pathway by regulating Mst1/2 and sequestering Yap in the cytoplasm (Yin et al., 2013). Merlin has been shown to stabilize by binding integrin-linked kinase (Ilk) and Ilk-associated proteins such as paxillin and Rsu (Apte et al., 2009; Donthamsetty et al., 2013; Manetti et al.,

2013; Serrano et al., 2013); it is degraded during cell spreading by mechanisms that are not fully understood (Cooper and Giancotti, 2014; Das et al., 2015; Okada et al., 2009). Intriguingly, we observed high expression of Merlin levels in secretory and ciliated cells of non-injured lungs and low expression in surviving, spreading epithelial cells after naphthalene-induced airway epithelial injury (Figures 2A and 2C). To determine whether robust activation of the ASMC niche with higher and more sustained levels of Fgf10 permits BSCs to populate the non-cartilaginous conducting airways, we deleted *Ilk* from secretory cells using *Scgb1a1-Cre;Ilk^{fl/fl}* mice (referred to as *Scgb1a1-Ilk^{fl/fl}*). We found that Merlin protein levels as well as Mst1/2 phosphorylation are decreased (Figures 2H and 2I), while nuclear Yap and Wnt7b are increased in secretory and ciliated daughter cells of

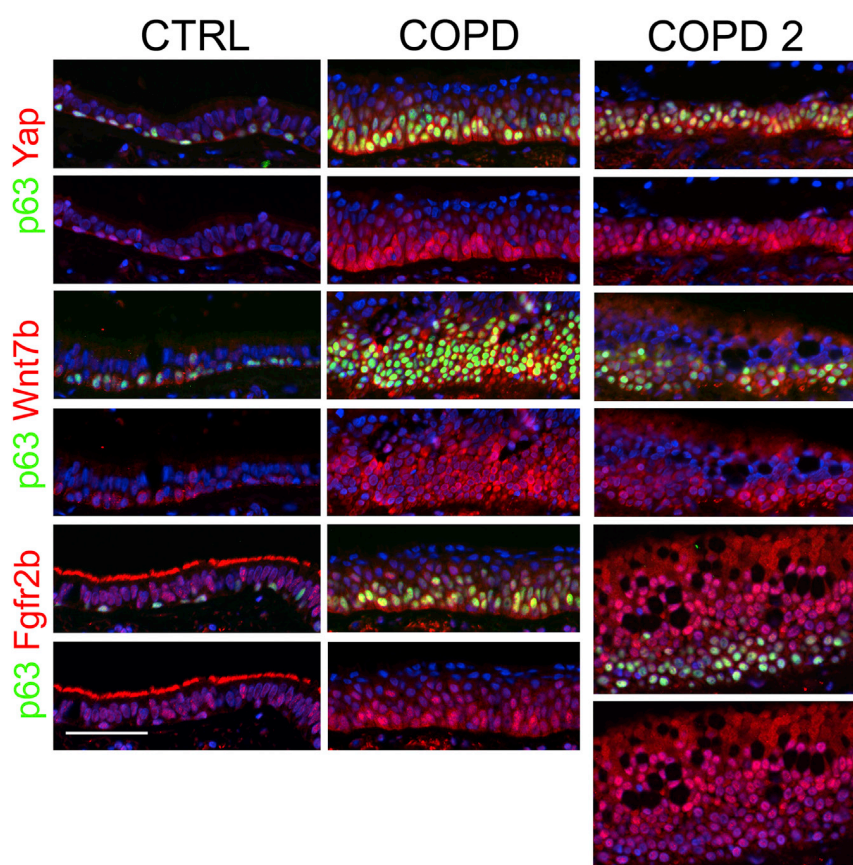


Figure 6. The Hippo Pathway Regulates *Fgf10* Signaling to Induce Basal Stem/Progenitor Cell Amplification in COPD

Immunostaining on control and COPD distal airways for BSC marker p63 (green) and Yap (red) or Wnt7b (red) or Fgfr2b (red). (n = 5). Note staining of cilia for Fgfr2b in control but not in COPD airways, indicating a loss of ciliated cells in COPD. Scale bar, 100 μ m. See also Table S1.

secretion by the ASMC niche, we simultaneously inactivated *Ilk* and *Fgfr2b* in secretory cells, but not BSCs, using *Scgb1a1-Cre;Ilk^{fl/fl};Fgfr2b^{fl/fl}* mice (referred to as *Scgb1a1-Ilk^{fl/fl}-Fgfr2b^{fl/fl}*). Despite the rescue of secretory cell hyperplasia and the subepithelial fibrosis phenotype, we found that the non-cartilaginous airways still showed aberrant accumulation of BSCs (Figures 4A–4E, S4, and S7). However, when we blocked Wnt7b-induced *Fgf10* expression in the ASMC niche by overexpressing *Dkk1* or deleting *Wnt7b* simultaneously with *Ilk* in secretory cells using *Scgb1a1-Cre;Ilk^{fl/fl};Rosa26-rtTA;Tet-Dkk1;Fgf10^{LacZ}* (referred to as *Scgb1a1-Ilk^{fl/fl}-rtTA-Dkk1-Fgf10^{LacZ}*) or *Scgb1a1-Cre;Ilk^{fl/fl};Wnt7b^{fl/fl}* (referred to as *Scgb1a1-Ilk^{fl/fl}-Wnt7b^{fl/fl}*) mice, we rescued both the amplification of BSCs/secretory cells and subepithe-

Scgb1a1-Ilk^{fl/fl} mice (Figures 2D–2G). Importantly, we found a significant increase in *Fgf10* expression in the ASMC niche of *Scgb1a1-Ilk^{fl/fl}* lungs compared with controls as well as *Scgb1a1-Cre;Mst1/2^{fl/fl}* mice (Figures 2D, 2G, S3A, S3B, and S3D) and elevated numbers of secretory cells and BSCs (Figures 2D, 3B, and S6E) in conducting airways by 1 month after *Ilk* deletion. Interestingly, the airways of *Scgb1a1-Ilk^{fl/fl}* mice demonstrated subepithelial fibrosis, with increased stromal SMAD2/3 phosphorylation, and airway obstruction (Figures S4 and S5B). The recruitment of BSCs to the conducting airways in *Scgb1a1-Ilk^{fl/fl}* lungs mostly occurred within the upper and middle non-cartilaginous airways; although it should be noted that BSCs were occasionally found along the entire length of the non-cartilaginous conducting airways all the way down to the distal broncho-alveolar duct junctions (Figure 3B). Lineage tracing using the *Rosa26-mTmG* allele in the airways of *Scgb1a1-CreER;Ilk^{fl/fl};Rosa26-mTmG* mice supports the concept that the aberrant accumulation of BSCs and subepithelial fibrotic myofibroblasts in these airways is not due to dedifferentiation of secretory cells (Figures S6A, S6B, and S6D).

Integrin-Linked Kinase Inactivation Increases *Fgf10*-*Fgfr2b* Signaling Downstream of Yap to Drive Secretory and Basal Stem/Progenitor Cell Amplification in Non-cartilaginous Conducting Airways

To establish whether the secretory and BSC amplification in *Scgb1a1-Ilk^{fl/fl}* non-cartilaginous airways depends on *Fgf10*

secretion by the ASMC niche, we simultaneously inactivated *Ilk* and *Fgfr2b* in secretory cells, but not BSCs, using *Scgb1a1-Cre;Ilk^{fl/fl};Fgfr2b^{fl/fl}* mice (referred to as *Scgb1a1-Ilk^{fl/fl}-Fgfr2b^{fl/fl}*). Despite the rescue of secretory cell hyperplasia and the subepithelial fibrosis phenotype, we found that the non-cartilaginous airways still showed aberrant accumulation of BSCs (Figures 4A–4E, S4, and S7). Evidence of inactivation of the Hippo pathway was provided by the nuclear localization of Yap in airway epithelial cells from rescued lungs (Figure S7).

To further confirm that the aberrant accumulation of BSCs upon secretory cell-specific *Ilk* deletion is due to a non-cell autonomous effect mediated by *Fgf10* secreted by the ASMC niche downstream of Yap, we generated *Sox2-CreERT2;Ilk^{fl/fl}*, *Sox2-CreERT2;Ilk^{fl/fl};Fgfr2b^{fl/fl}*, and *Sox2-CreERT2;Ilk^{fl/fl};Yap^{fl/fl}* mouse lines (referred to as *Sox2-Ilk^{fl/fl}*, *Sox2-Ilk^{fl/fl}-Fgfr2b^{fl/fl}*, and *Sox2-Ilk^{fl/fl}-Yap^{fl/fl}*, respectively), in which we either deleted *Ilk* alone or in combination with either *Fgfr2b* or *Yap* in all airway epithelial cells, including BSCs. We found BSC and secretory cell hyperplasia as well as subepithelial fibrosis in the non-cartilaginous airways of *Sox2-Ilk^{fl/fl}* mice in which *Fgf10* is upregulated in the ASMC niche. However, this phenotype was rescued in *Sox2-Ilk^{fl/fl}-Fgfr2b^{fl/fl}* mice, in which the airway epithelium cannot respond to upregulated *Fgf10*, as well as in *Sox2-Ilk^{fl/fl}-Yap^{fl/fl}* mice in which the ASMC niche does not secrete *Fgf10* (Figures 5A–5E, S3C, S4, and S7). These results indicate that epithelial *Ilk* efficiently activates the Hippo pathway to maintain airway epithelial cell quiescence by inhibiting *Fgf10* secretion from the ASMC niche.

The Yap-Wnt7b-Fgf10 Regenerative Pathway Is Chronically Activated in COPD Airways

Our results demonstrate that epithelial-mesenchymal interactions coordinated by Hippo signaling actively maintain

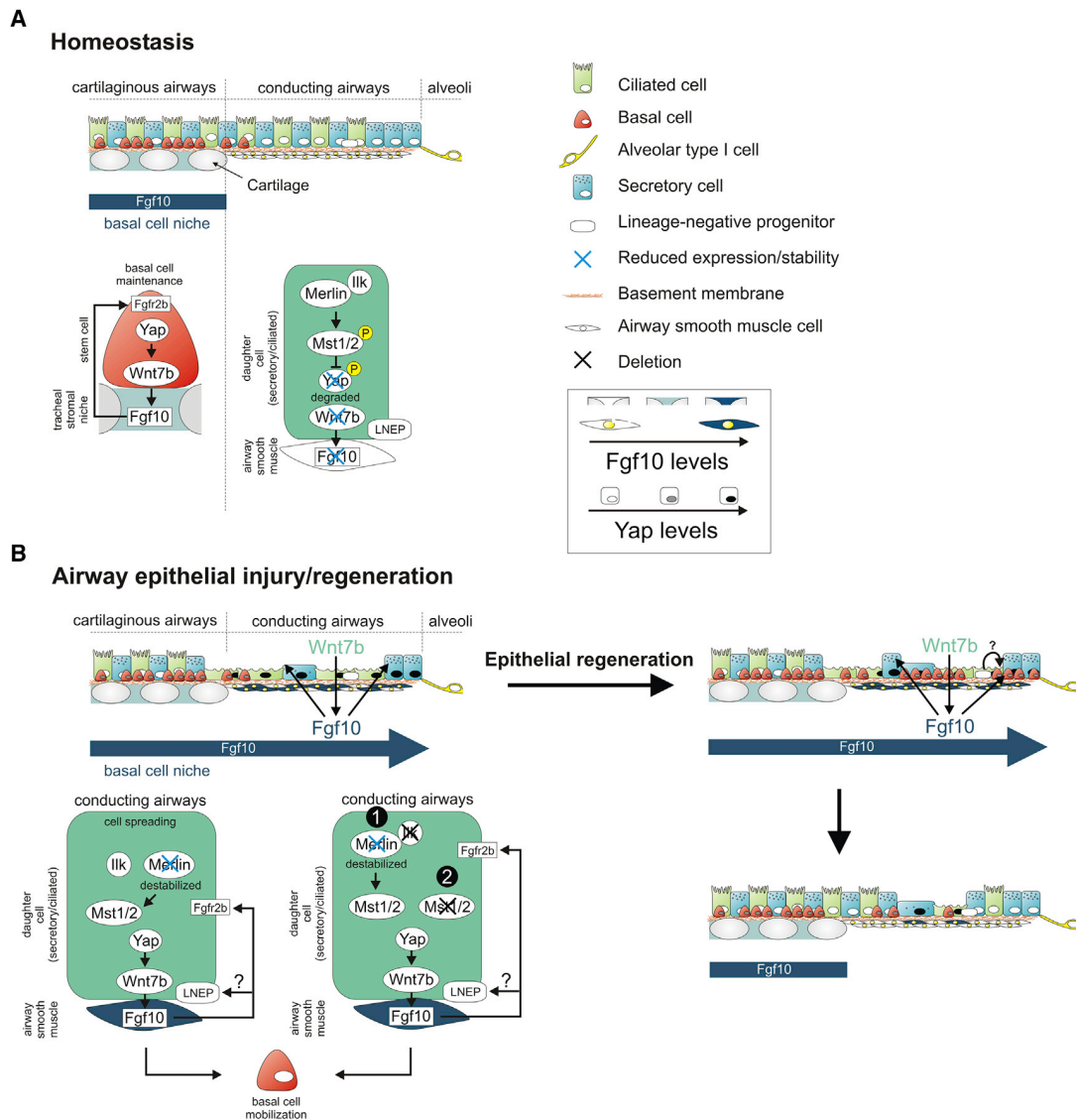


Figure 7. Model Showing How Yap Maintains and Generates Basal-like Stem Cells by Activating the Stromal Niche during Homeostasis and Epithelial Regeneration

(A) During homeostasis, basal stem/progenitor cells are only found in the trachea, where they maintain themselves by inducing *Fgf10* expression in the tracheal stromal niche downstream of Yap. Ilk positively regulates the Hippo pathway in conducting airway epithelial cells to maintain their quiescence by inhibiting Yap-mediated activation of *Fgf10* expression in ASMCs.

(B) Hippo pathway inactivation in conducting non-cartilaginous airway epithelium in response to injury or by deleting *Ilk* (1) or *Mst1/2* (2) results in nuclear Yap localization and increased epithelial Wnt7b expression and *Fgf10* expression in ASMCs, which reciprocally breaks epithelial quiescence. The *Fgf10*-expressing basal stem cell niche extends into the non-cartilaginous airways allowing for BSC mobilization into the lower conducting airways upon airway epithelial *Ilk* deletion. Alternatively, *Fgf10* released by the activated ASMC niche could drive the differentiation of LNEPs, which are scattered along the lower conducting airways, into the basal stem/progenitor cell lineage.

post-natal tissue homeostasis, and downregulation of Hippo signaling during injury is required to initiate regeneration and recruitment of stem cells to the site of injury. However, activation of the Yap-Wnt7b-Fgf10 regenerative pathway may need to be delicately controlled to maintain airway epithelial quiescence and prevent airway remodeling; indeed, our data in the preceding animal modeling studies support the concept that deregulation of Hippo signaling leads to aberrant repair and regeneration in the lung. We examined whether this repair

cascade is deregulated in the airways of human subjects with chronic obstructive pulmonary disease (COPD), a chronic lung disease associated with airway remodeling and obstructive physiology. Increased nuclear YAP levels and FGFR2b and WNT7b expression were observed in squamous metaplastic areas within the airway epithelium of COPD subjects (Figure 6 and Table S1). These findings provide proof of concept that the Hippo pathway is inactivated to induce *Fgf10* expression and BSC amplification in human COPD.

DISCUSSION

We report a mode of stem cell regulation in which BSCs generate and maintain their own localized stem cell niche by regulating the expression of *Fgf10* within the stromal compartment of the cartilaginous airways (Figure 7A); loss of this tonic Fgf10 signaling leads to BSC depletion. We further demonstrate that the Hippo signaling pathway in either tracheal BSCs themselves during homeostasis or in sparsely surviving and spread out differentiated epithelial cells after injury controls the maintenance and neo-generation of *Fgf10*-expressing stromal stem cell niches in the lung. We previously reported that upon airway epithelial injury, ASMCs that envelop the non-cartilaginous airways are activated to release Fgf10 (Volckaert et al., 2011). Fgf10 then binds Fgfr2b on BSCs to mobilize and expand this stem/progenitor pool by inhibiting their premature differentiation, thus facilitating efficient lung regeneration (Figure 7B). The source of recruited BSCs to sites of injury may depend on the level and type of injury and may include the recently identified lineage-negative epithelial progenitors (LNEPs) (Ray et al., 2016; Vaughan et al., 2015). In our model, we show via lineage tracing that the ectopic BSCs lining the non-cartilaginous airways are not derived from secretory cells, supporting the concept that induction of *Fgf10* expression by the stromal niche allows for the recruitment of tracheal BSCs or the differentiation of Sox2⁺ LNEPs along the BSC lineage.

We found that Ilk and Merlin, the stability of which is controlled by its interaction with Ilk, tune the Hippo pathway and the nuclear translocation of Yap that leads to the induction of *Wnt7b* expression; this, in turn, triggers *Fgf10* expression in the airway niche to activate BSCs and/or induce differentiation of LNEPs. We demonstrate that Ilk positively regulates the Hippo pathway in non-cartilaginous airway epithelial cells to maintain their quiescence during normal homeostasis by inhibiting Yap-mediated activation of the Wnt7b-Fgf10 epithelial-mesenchymal crosstalk between airway epithelial progenitor cells and their stromal niche. Our findings suggest that integrin signaling through Ilk represents a sensor of airway epithelial injury that acts upstream of the Wnt7b-Fgf10 repair cascade and functions as a gatekeeper for airway epithelial quiescence; this may prevent sustained airway remodeling as observed in chronic lung diseases, such as COPD and asthma. Interestingly, Ilk has been shown to act as an upstream Hippo pathway activator in the liver where it is essential for the proper termination of the regenerative response (Apte et al., 2009). However, more recently, Ilk was reported to inhibit the Hippo pathway in human breast, prostate, and colon cancer cell lines (Serrano et al., 2013), suggesting that Ilk regulation of Hippo signaling may be contextual (e.g., normal versus cancer cells). A recent paper has also defined a role for epithelial Shh in the regulation of airway epithelial cell quiescence; similar to our studies, this occurs through an indirect non-cell autonomous mechanism (Peng et al., 2015). Together, these studies highlight the critical importance of developmental pathways in epithelial-mesenchymal crosstalk during injury repair in adult mammalian lungs.

Our data also demonstrate substantial aberrant signaling of these developmental pathways in chronic airway remodeling associated with human COPD. This suggests that chronic activation of the ASMC niche in COPD may drive BSC amplification or drive the differentiation of LNEPs present in the conducting

airways along the BSC lineage, possibly preventing them from contributing to alveolar epithelial homeostasis and regeneration. This may explain why airway BSC metaplasia precedes emphysema in COPD (Auerbach et al., 1961; Dye and Adler, 1994; Kennedy et al., 1984; Leopold et al., 2009; Lumsden et al., 1984; Peters et al., 1993; Shaykhiev and Crystal, 2013; Shaykhiev et al., 2011). Thus, while transient *Fgf10* expression by ASMCs is critical for proper airway epithelial regeneration in response to injury (Volckaert et al., 2011, 2013a), sustained Fgf10 secretion by the stem cell niche, in response to chronic Ilk/Hippo inactivation (i.e., chronic nuclear Yap), results in pathological airway remodeling. Finally, our studies support the therapeutic targeting of the Hippo/Fgf10/Fgfr2b signaling axis in chronic lung disease.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHODS DETAILS
 - β -gal Staining
 - Picro-Sirius/Fast Green FCF Staining
 - Immunohistochemistry and Fluorescence
 - *In Situ* Cell Death Detection (TUNEL Staining)
 - Proliferation
 - Western Blot
 - *In Situ* Hybridization
 - Microscopy and Imaging
 - Quantitative Real-Time PCR
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.devcel.2017.09.003>.

AUTHOR CONTRIBUTIONS

T.V. and T.Y. designed and performed experiments, analyzed data, and wrote and edited the manuscript. H.B., C.-M.C., A.S., and L.S. performed experiments. E.E., D.C., S.M., V.J.T., and S.B. edited the manuscript. R.F. wrote and edited the manuscript and provided floxed *Ilk* mice. S.D.L. conceived and led the project, performed experiments, analyzed data, and wrote and edited the manuscript.

ACKNOWLEDGMENTS

This study was supported by NIH (R01 HL107307), DFG (BE443/4-1 and BE443/6-1), Loewe, and UKGM to S.B., Munich Heart Alliance to R.F., and NIH (R01 HL126732, HL092967, HL132156), March of Dimes (1-FY15-352), and the Pulmonary Fibrosis Foundation Albert Rose established investigator award to S.D.L.

Received: February 17, 2017

Revised: July 20, 2017

Accepted: September 8, 2017

Published: October 9, 2017

REFERENCES

- Apte, U., Gkretsi, V., Bowen, W.C., Mars, W.M., Luo, J.H., Donthamsetty, S., Orr, A., Monga, S.P., Wu, C., and Michalopoulos, G.K. (2009). Enhanced liver regeneration following changes induced by hepatocyte-specific genetic ablation of integrin-linked kinase. *Hepatology* 50, 844–851.
- Auerbach, O., Stout, A.P., Hammond, E.C., and Garfinkel, L. (1961). Changes in bronchial epithelium in relation to cigarette smoking and in relation to lung cancer. *N. Engl. J. Med.* 265, 253–267.
- Barkauskas, C.E., Counce, M.J., Rackley, C.R., Bowie, E.J., Keene, D.R., Stripp, B.R., Randell, S.H., Noble, P.W., and Hogan, B.L. (2013). Type 2 alveolar cells are stem cells in adult lung. *J. Clin. Invest.* 123, 3025–3036.
- Bellusci, S., Grindley, J., Emoto, H., Itoh, N., and Hogan, B.L. (1997). Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. *Development* 124, 4867.
- Belteki, G., Haigh, J., Kabacs, N., Haigh, K., Sison, K., Costantini, F., Whitsett, J., Quaggin, S.E., and Nagy, A. (2005). Conditional and inducible transgene expression in mice through the combinatorial use of Cre-mediated recombination and tetracycline induction. *Nucleic Acids Res.* 33, e51.
- Clark, J.C., Tichelaar, J.W., Wert, S.E., Itoh, N., Perl, A.K., Stahlman, M.T., and Whitsett, J.A. (2001). FGF-10 disrupts lung morphogenesis and causes pulmonary adenomas in vivo. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 280, L705–L715.
- Cooper, J., and Giancotti, F.G. (2014). Molecular insights into NF2/Merlin tumor suppressor function. *FEBS Lett.* 588, 2743–2752.
- Das, T., Safferling, K., Rausch, S., Grabe, N., Boehm, H., and Spatz, J.P. (2015). A molecular mechanotransduction pathway regulates collective migration of epithelial cells. *Nat. Cell Biol.* 17, 276–287.
- De Moerloose, L., Spencer-Dene, B., Revest, J.M., Hajihosseini, M., Rosewell, I., and Dickson, C. (2000). An important role for the IIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. *Development* 127, 483–492.
- Donthamsetty, S., Bhawe, V.S., Mars, W.M., Bowen, W.C., Orr, A., Haynes, M.M., Wu, C., and Michalopoulos, G.K. (2013). Role of PINCH and its partner tumor suppressor Rsu-1 in regulating liver size and tumorigenesis. *PLoS One* 8, e74625.
- Dye, J.A., and Adler, K.B. (1994). Effects of cigarette smoke on epithelial cells of the respiratory tract. *Thorax* 49, 825–834.
- Grashoff, C., Aszodi, A., Sakai, T., Hunziker, E.B., and Fassler, R. (2003). Integrin-linked kinase regulates chondrocyte shape and proliferation. *EMBO Rep.* 4, 432–438.
- Ji, H., Houghton, A.M., Mariani, T.J., Perera, S., Kim, C.B., Padera, R., Tonon, G., McNamara, K., Marconcin, L.A., Hezel, A., et al. (2006). K-ras activation generates an inflammatory response in lung tumors. *Oncogene* 25, 2105–2112.
- Kelly, R.G., Brown, N.A., and Buckingham, M.E. (2001). The arterial pole of the mouse heart forms from Fgf10-expressing cells in pharyngeal mesoderm. *Dev. Cell* 1, 435–440.
- Kennedy, S.M., Elwood, R.K., Wiggs, B.J., Pare, P.D., and Hogg, J.C. (1984). Increased airway mucosal permeability of smokers. Relationship to airway reactivity. *Am. Rev. Respir. Dis.* 129, 143–148.
- Kim, C.F., Jackson, E.L., Woolfenden, A.E., Lawrence, S., Babar, I., Vogel, S., Crowley, D., Bronson, R.T., and Jacks, T. (2005). Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* 121, 823–835.
- Kumar, P.A., Hu, Y., Yamamoto, Y., Hoe, N.B., Wei, T.S., Mu, D., Sun, Y., Joo, L.S., Dagher, R., Zielonka, E.M., et al. (2011). Distal airway stem cells yield alveoli in vitro and during lung regeneration following H1N1 influenza infection. *Cell* 147, 525–538.
- Lange, A.W., Sridharan, A., Xu, Y., Stripp, B.R., Perl, A.K., and Whitsett, J.A. (2015). Hippo/Yap signaling controls epithelial progenitor cell proliferation and differentiation in the embryonic and adult lung. *J. Mol. Cell Biol.* 7, 35–47.
- Lee, J.H., Bhang, D.H., Beede, A., Huang, T.L., Stripp, B.R., Bloch, K.D., Wagers, A.J., Tseng, Y.H., Ryeom, S., and Kim, C.F. (2014). Lung stem cell differentiation in mice directed by endothelial cells via a BMP4-NFATc1-thrombospondin-1 axis. *Cell* 156, 440–455.
- Leopold, P.L., O'Mahony, M.J., Lian, X.J., Tilley, A.E., Harvey, B.G., and Crystal, R.G. (2009). Smoking is associated with shortened airway cilia. *PLoS One* 4, e8157.
- Lumsden, A.B., McLean, A., and Lamb, D. (1984). Goblet and Clara cells of human distal airways: evidence for smoking induced changes in their numbers. *Thorax* 39, 844–849.
- Manetti, M.E., Geden, S., Bott, M., Sparrow, N., Lambert, S., and Fernandez-Valle, C. (2013). Stability of the tumor suppressor merlin depends on its ability to bind paxillin LD3 and associate with beta1 integrin and actin at the plasma membrane. *Biol. Open* 1, 949–957.
- Okada, M., Wang, Y., Jang, S.W., Tang, X., Neri, L.M., and Ye, K. (2009). Akt phosphorylation of merlin enhances its binding to phosphatidylinositols and inhibits the tumor-suppressive activities of merlin. *Cancer Res.* 69, 4043–4051.
- Pardo-Saganta, A., Tata, P.R., Law, B.M., Saez, B., Chow, R., Prabhu, M., Gridley, T., and Rajagopal, J. (2015). Parent stem cells can serve as niches for their daughter cells. *Nature* 523, 597–601.
- Park, K.S., Whitsett, J.A., Di Palma, T., Hong, J.H., Yaffe, M.B., and Zannini, M. (2004). TAZ interacts with TTF-1 and regulates expression of surfactant protein-C. *J. Biol. Chem.* 279, 17384–17390.
- Peng, T., Frank, D.B., Kadzik, R.S., Morley, M.P., Rathi, K.S., Wang, T., Zhou, S., Cheng, L., Lu, M.M., and Morrissey, E.E. (2015). Hedgehog actively maintains adult lung quiescence and regulates repair and regeneration. *Nature* 526, 578–582.
- Peters, E.J., Morice, R., Benner, S.E., Lippman, S., Lukeman, J., Lee, J.S., Ro, J.Y., and Hong, W.K. (1993). Squamous metaplasia of the bronchial mucosa and its relationship to smoking. *Chest* 103, 1429–1432.
- Rajagopal, J., Carroll, T.J., Guseh, J.S., Bores, S.A., Blank, L.J., Anderson, W.J., Yu, J., Zhou, Q., McMahon, A.P., and Melton, D.A. (2008). Wnt7b stimulates embryonic lung growth by coordinately increasing the replication of epithelium and mesenchyme. *Development* 135, 1625–1634.
- Ramsey, M.R., Wilson, C., Ory, B., Rothenberg, S.M., Faquin, W., Mills, A.A., and Ellisen, L.W. (2013). FGFR2 signaling underlies p63 oncogenic function in squamous cell carcinoma. *J. Clin. Invest.* 123, 3525–3538.
- Rawlins, E.L., Okubo, T., Xue, Y., Brass, D.M., Auten, R.L., Hasegawa, H., Wang, F., and Hogan, B.L. (2009). The role of Scgb1a1+ Clara cells in the long-term maintenance and repair of lung airway, but not alveolar, epithelium. *Cell Stem Cell* 4, 525–534.
- Ray, S., Chiba, N., Yao, C., Guan, X., McConnell, A.M., Brockway, B., Que, L., McQualter, J.L., and Stripp, B.R. (2016). Rare SOX2+ airway progenitor cells generate KRT5+ cells that repopulate damaged alveolar parenchyma following influenza virus infection. *Stem Cell Reports* 7, 817–825.
- Rock, J.R., Onaitis, M.W., Rawlins, E.L., Lu, Y., Clark, C.P., Xue, Y., Randell, S.H., and Hogan, B.L. (2009). Basal cells as stem cells of the mouse trachea and human airway epithelium. *Proc. Natl. Acad. Sci. USA* 106, 12771–12775.
- Scadden, D.T. (2006). The stem-cell niche as an entity of action. *Nature* 441, 1075–1079.
- Serrano, I., McDonald, P.C., Lock, F., Muller, W.J., and Dedhar, S. (2013). Inactivation of the Hippo tumour suppressor pathway by integrin-linked kinase. *Nat. Commun.* 4, 2976.
- Shaykhiev, R., and Crystal, R.G. (2013). Basal cell origins of smoking-induced airway epithelial disorders. *Cell Cycle* 13, 341–342.
- Shaykhiev, R., Otaki, F., Bonsu, P., Dang, D.T., Teater, M., Strulovici-Barel, Y., Salit, J., Harvey, B.G., and Crystal, R.G. (2011). Cigarette smoking reprograms apical junctional complex molecular architecture in the human airway epithelium in vivo. *Cell. Mol. Life Sci.* 68, 877–892.
- Simon, D.M., Arkan, M.C., Srisuma, S., Bhattacharya, S., Andalcio, T., Shapiro, S.D., and Mariani, T.J. (2006). Epithelial cell PPARgamma is an endogenous regulator of normal lung maturation and maintenance. *Proc. Am. Thorac. Soc.* 3, 510–511.
- Tata, P.R., Mou, H., Pardo-Saganta, A., Zhao, R., Prabhu, M., Law, B.M., Vinarsky, V., Cho, J.L., Breton, S., Sahay, A., et al. (2013). Dedifferentiation of committed epithelial cells into stem cells in vivo. *Nature* 503, 218–223.

- Trink, B., Osada, M., Ratovitski, E., and Sidransky, D. (2007). p63 transcriptional regulation of epithelial integrity and cancer. *Cell Cycle* 6, 240–245.
- Vaughan, A.E., Brumwell, A.N., Xi, Y., Gotts, J.E., Brownfield, D.G., Treutlein, B., Tan, K., Tan, V., Liu, F.C., Looney, M.R., et al. (2015). Lineage-negative progenitors mobilize to regenerate lung epithelium after major injury. *Nature* 517, 621–625.
- Volckaert, T., Dill, E., Campbell, A., Tiozzo, C., Majka, S., Bellusci, S., and De Langhe, S.P. (2011). Parabronchial smooth muscle constitutes an airway epithelial stem cell niche in the mouse lung after injury. *J. Clin. Invest.* 121, 4409–4419.
- Volckaert, T., Campbell, A., and De Langhe, S. (2013a). c-Myc regulates proliferation and Fgf10 expression in airway Smooth muscle after airway epithelial injury in mouse. *PLoS One* 8, e71426.
- Volckaert, T., Campbell, A., Dill, E., Li, C., Minoo, P., and De Langhe, S. (2013b). Localized Fgf10 expression is not required for lung branching morphogenesis but prevents differentiation of epithelial progenitors. *Development* 140, 3731–3742.
- Weidenfeld, J., Shu, W., Zhang, L., Millar, S.E., and Morrisey, E.E. (2002). The WNT7b promoter is regulated by TTF-1, GATA6, and Foxa2 in lung epithelium. *J. Biol. Chem.* 277, 21061–21070.
- Wendling, O., Bornert, J.M., Chambon, P., and Metzger, D. (2009). Efficient temporally-controlled targeted mutagenesis in smooth muscle cells of the adult mouse. *Genesis* 47, 14–18.
- Winnier, G., Blessing, M., Labosky, P.A., and Hogan, B.L. (1995). Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev.* 9, 2105–2116.
- Xin, M., Kim, Y., Sutherland, L.B., Murakami, M., Qi, X., McAnally, J., Porrello, E.R., Mahmoud, A.I., Tan, W., Shelton, J.M., et al. (2013). Hippo pathway effector Yap promotes cardiac regeneration. *Proc. Natl. Acad. Sci. USA* 110, 13839–13844.
- Yin, F., Yu, J., Zheng, Y., Chen, Q., Zhang, N., and Pan, D. (2013). Spatial organization of Hippo signaling at the plasma membrane mediated by the tumor suppressor Merlin/NF2. *Cell* 154, 1342–1355.
- Zhao, R., Fallon, T.R., Saladi, S.V., Pardo-Saganta, A., Villoria, J., Mou, H., Vinarsky, V., Gonzalez-Celeiro, M., Nunna, N., Hariri, L.P., et al. (2014). Yap tunes airway epithelial size and architecture by regulating the identity, maintenance, and self-renewal of stem cells. *Dev. Cell* 30, 151–165.
- Zuo, W., Zhang, T., Wu, D.Z., Guan, S.P., Liew, A.A., Yamamoto, Y., Wang, X., Lim, S.J., Vincent, M., Lessard, M., et al. (2015). p63Krt5 distal airway stem cells are essential for lung regeneration. *Nature* 517, 616–620.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
mouse anti- β -tubulin (3F3-G2)	Seven Hills Bioreagents	Cat# LMAB-3F3; RRID: AB_451728
goat anti-Scgb1a1 (clone T-18)	Santa Cruz Biotechnology Inc.	Cat# sc-9772; RRID: AB_2238819
rabbit anti-Scgb1a1	Seven Hills Bioreagents	Cat# WRAB-CCSP; RRID: AB_451716
rabbit anti-beta-galactosidase	Rockland Immunochemicals Inc	Cat# 100-4136; RRID: AB_219904
rabbit anti-Fgfr2 (Bek) (clone C-17)	Santa Cruz Biotechnology Inc.	Cat# sc-122; RRID: AB_631509
goat anti- p-SMAD2/3 (Ser423/425)	Santa Cruz Biotechnology Inc.	Cat# sc-11769; RRID: AB_2193189
mouse anti- α -Actin (clone 1A4)	Santa Cruz Biotechnology Inc.	Cat# sc-32251; RRID: AB_262054
chicken anti-GFP	Aves Labs Inc.	Cat# GFP-1020; RRID: AB_10000240
rabbit anti-Keratin 5 (clone EP1601Y)	Thermo Fisher Scientific	Cat# MA5-14473; RRID: AB_10979451
rabbit anti-FGF10	Acris	Cat# AP14882PU-N; RRID: AB_1752406
rabbit anti-FGF10	Millipore	Cat# ABN44; RRID: AB_11204345
rabbit anti-p63 (Δ N) (clone poly6190)	BioLegend	Cat# 619002; RRID: AB_2207170
mouse anti-p63 (clone 4A4)	Biocare Medical	Cat# CM163B; RRID: AB_10583039
rabbit anti-ILK	Cell Signaling Technology Inc.	Cat# 3862S; RRID: AB_10695593
rabbit anti-phospho-Mst1/2	Cell Signaling	Cat# 3681S; RRID: AB_330269
rabbit anti-RFP	Rockland Immunochemicals Inc.	Cat# 600-401-379; RRID: AB_2209751
rabbit anti-Sitpc	Seven hills bioreagents	Cat# WRAB-9337; RRID: AB_2335890
rabbit anti-YAP	Cell Signaling Technology Inc.	Cat# 4912S; RRID: AB_10694682
mouse anti-Yap (clone 63.7)	Santa Cruz Biotechnology Inc.	Cat# sc-101199; RRID: AB_1131430
rabbit anti-DKK1 (clone H-120)	Santa Cruz Biotechnology Inc.	Cat# sc-25516; RRID: AB_2091346
mouse anti-E-Cadherin	BD Transduction Laboratories	Cat# 610181; RRID: AB_397580
goat anti-Wnt7b	R&D systems	Cat# AF3460; RRID: AB_2304437
rabbit anti-Merlin (Nf2) (clone A-19)	Santa Cruz Biotechnology Inc.	Cat# sc-331; RRID: AB_2298548
rabbit anti-Mst1/2	Abcam	Cat# ab87322; RRID: AB_2042395
mouse anti-PCNA	Zymed Laboratories Inc.	Cat# 93-1143;
goat anti-chicken FITC	Aves Labs, Inc.	Cat# F-1005; RRID: AB_2313516
anti-GAPDH	Abcam	Cat# ab9485; RRID: AB_307275
Mouse anti-BrdU (clone BU-1)	GE Healthcare	Cat# RPN202; RRID: AB_2314032
sheep Anti-Digoxigenin-AP	Sigma-Aldrich	Cat# 11093274910; RRID: AB_514497
goat anti-chicken FITC	Aves Labs, Inc.	Cat# F-1005; RRID: AB_2313516
Cy3 AffiniPure F(ab') ₂ Fragment Donkey Anti-Goat IgG (H+L)	Jackson ImmunoResearch	Cat# 705-166-147; RRID: AB_2340413
Cy3 AffiniPure F(ab') ₂ Fragment Goat Anti-Rat IgG (H+L)	Jackson ImmunoResearch	Cat# 112-166-003; RRID: AB_2338253
Cy3 AffiniPure F(ab') ₂ Fragment Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	Cat# 715-166-150; RRID: AB_2340816
Cy3 AffiniPure F(ab') ₂ Fragment Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch	Cat# 711-166-152; RRID: AB_2313568
Alexa Fluor 647 AffiniPure F(ab') ₂ Fragment Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	Cat# 715-606-150; RRID: AB_2340865
Alexa Fluor 488 AffiniPure F(ab') ₂ Fragment Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	Cat# 715-546-150; RRID: AB_2340849
Alexa Fluor 488 AffiniPure F(ab') ₂ Fragment Donkey Anti-Goat IgG (H+L)	Jackson ImmunoResearch	Cat# 705-546-147; RRID: AB_2340430
Alexa Fluor 647 AffiniPure F(ab') ₂ Fragment Donkey Anti-Goat IgG (H+L)	Jackson ImmunoResearch	Cat# 705-606-147; RRID: AB_2340438

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Alexa Fluor 488 AffiniPure F(ab') ₂ Fragment Donkey Anti-Chicken IgY (IgG) (H+L)	Jackson ImmunoResearch	Cat# 703-546-155; RRID: AB_2340376
Alexa Fluor 488 AffiniPure F(ab') ₂ Fragment Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch	Cat# 711-546-152; RRID: AB_2340619
Alexa Fluor 647 AffiniPure F(ab') ₂ Fragment Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch	Cat# 711-606-152; RRID: AB_2340625
Alexa Fluor 488 AffiniPure F(ab') ₂ Fragment Donkey Anti-Rat IgG (H+L)	Jackson ImmunoResearch	Cat# 712-546-150; RRID: AB_2340685
Cy3 AffiniPure F(ab') ₂ Fragment Donkey Anti-Rat IgG (H+L)	Jackson ImmunoResearch	Cat# 712-166-150; RRID: AB_2340668
Peroxidase AffiniPure F(ab') ₂ Fragment Donkey Anti-Goat IgG (H+L)	Jackson ImmunoResearch	Cat# 705-036-147; RRID: AB_2340392
Peroxidase AffiniPure F(ab') ₂ Fragment Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch	Cat# 711-036-152; RRID: AB_2340590
Peroxidase AffiniPure F(ab') ₂ Fragment Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	Cat# 715-036-150; RRID: AB_2340773
Chemicals, Peptides, and Recombinant Proteins		
Sirius Red in saturated Picric acid	Rowley Biochemical Institute	Cat# F-357-2
Fast green FCF solution	Fisher Scientific	Cat# F99-10
X-gal	Research Products International	Cat# B71800
BrdU	GE Healthcare	Cat# RPN201
RNase A	Roche	Cat# 109169
Proteinase K solution	Invitrogen	Cat# 25530-049
Critical Commercial Assays		
In Situ Cell Death Detection Kit, Fluorescein	Roche	Cat# 11 684 795 910
PCNA staining kit	Zymed Laboratories Inc.	Cat# 93-1143
Maxima First Strand cDNA Synthesis	Thermo Fisher Scientific	Cat# FERK1642
Amersham anti-BrdU antibody kit	GE healthcare	Cat# RPN202
Taqman Gene Expression Assays	Applied Biosystems	Cat# 4369016
Experimental Models: Organisms/Strains		
<i>Tet-Dkk1</i>	De Langhe Lab	(Volckaert et al., 2011)
<i>Tet-Fgf10</i>	Jeffrey Whitsett	(Clark et al., 2001)
<i>Rosa26-rtTa</i>	De Langhe Lab	(Volckaert et al., 2011)
<i>Scgb1a1-Cre</i>	Thomas Mariani	(Ji et al., 2006; Simon et al., 2006)
<i>Fgfr2b^{flf}</i>	Clive Dickson	(De Moerlooze et al., 2000)
<i>Fgf10^{LacZ}</i>	Saverio Bellusci	(Kelly et al., 2001)
<i>Sox2-CreERT2</i>	The Jackson Laboratory	JAX 017593
<i>Rosa26R-Confetti</i>	The Jackson Laboratory	JAX 017492
<i>Scgb1a1-CreER</i>	The Jackson Laboratory	JAX 016225
<i>Rosa26R-LSL-rtTa</i>	The Jackson Laboratory	JAX 005670
<i>Rosa26-mTmG</i>	The Jackson Laboratory	JAX 007676
<i>Wnt7b^{flf}</i>	The Jackson Laboratory	JAX 008467
<i>Acta2-CreERT2</i>	Pierre Chambon	Wendling et al., 2009
<i>Krt5-CreERT2</i>	The Jackson Laboratory	JAX 018394
<i>Yap^{flf}</i>	Eric Olson	Xin et al., 2013
<i>Ilk^{flf}</i>	Reinhard Fassler	(Grashoff et al., 2003)
<i>Mst1/2^{flf}</i>	The Jackson Laboratory	JAX 017635
Software and Algorithms		
ImageJ	NIH	https://imagej.nih.gov/ij/

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
β -glucuronidase (qPCR primer)	Life technologies	Mm00446953_m1
<i>Scgb1a1</i> (qPCR primer)	Life technologies	Mm00442046_m1
<i>FoxJ1</i> (qPCR primer)	Life technologies	Mm00807215_m1
<i>Keratin 5</i> (qPCR primer)	Life technologies	Mm01305291_g1
<i>p63</i> (<i>Trp63</i>) (qPCR primer)	Life technologies	Mm00495788_m1
<i>Muc5a/c</i> (qPCR primer)	Life technologies	Mm01276718_m1
<i>Col1a1</i> (qPCR primer)	Life technologies	Mm00801666_g1
<i>Col3a1</i> (qPCR primer)	Life technologies	Mm01254476_m1
<i>Wnt7b</i> (qPCR primer)	Life technologies	Mm00437358_m1
<i>Dkk1</i> (qPCR primer)	Life technologies	Mm00438422_m1

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Stijn P. De Langhe (sdelanghe@uabmc.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All mice were bred and maintained in a pathogen-free environment with free access to food and water. Both male and female mice were used for all experiments. *Tet-Dkk1* (Volckaert et al., 2011), *Tet-Fgf10* (Clark et al., 2001), *Rosa26-rtTa* (Volckaert et al., 2011), *Scgb1a1-Cre* (Ji et al., 2006; Simon et al., 2006), *Fgfr2b^{flf}* (De Moerloose et al., 2000), *Fgf10^{LacZ}* (Kelly et al., 2001), *Ilk^{flf}* (Grashoff et al., 2003), *Sox2-CreERT2* (JAX 017593), *Rosa26R-Confetti* (JAX 017492), *Scgb1a1-CreER* (JAX 016225), *Rosa26R-LSL-rtTa* (Beltke et al., 2005), *Rosa26-mTmG* (JAX 007676), *Mst1/2^{flf}* (JAX 017635), *Wnt7b^{flf}* (Rajagopal et al., 2008), *Acta2-CreERT2* (Wendling et al., 2009), *Krt5-CreERT2* (JAX 018394) and *Yap^{flf}* (Xin et al., 2013) mice were previously described. All experiments were approved by the National Jewish Health and University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC). Naphthalene (Sigma-Aldrich) was dissolved in corn oil at 30 mg/ml and administered intraperitoneally at 8 weeks of age, with doses adjusted to achieve a 95% decrease in the abundance of *Scgb1a1* mRNA in total lung RNA of WT mice at 3 days after injection. For tamoxifen induction, mice were placed on tamoxifen containing food (rodent diet with 400 mg/kg tamoxifen citrate; Harlan Teklad TD.130860) at 6-8 weeks of age for 2 to 3 weeks and lungs were isolated 8-13 weeks later. For doxycycline induction: mice were placed on doxycycline containing food (rodent diet with 625 mg/kg dox; Harlan Teklad TD.09761). Lungs from non tamoxifen treated mice were isolated at 4-10 weeks of age.

METHODS DETAILS **β -gal Staining**

Fgf10^{LacZ} lungs were dissected and fixed in 4% formalin in PBS at room temperature for 5 minutes and rinsed in PBS. For staining, X-Gal (Research Products International, B71800) was dissolved in Dimethylformamide and freshly added to LacZ staining solution (5mM Potassium hexacyanoferrate(III), 5mM Potassium hexacyanoferrate(II) trihydrate, 2mM MgCl₂, 81mM Na₂HPO₄ and 20mM NaH₂PO₄) at a final concentration of 1mg/ml. Lungs were inflated with freshly prepared LacZ staining/X-gal solution and stained overnight at 37°C in the dark with gentle agitation. After rinsing with PBS, lungs were postfixed in 4% formalin in PBS at room temperature overnight. For microtome sections, after 4% formalin fixation, lungs were washed in PBS, dehydrated, and paraffin embedded.

Picro-Sirius/Fast Green FCF Staining

5 μ m paraffin sections were stained for collagen as follows. In brief, after deparaffinization, slides were rehydrated through a series of decreasing ethanol concentrations. Slides were then incubated in Sirius red/Fast green FCF solution (0.1% Fast green FCF (F99-10; Fisher) dissolved in 0.1 % Sirius Red in saturated Picric acid (F-357-2; Rowley Biochemical Institute)) for 1 h. After incubation, slides were washed in 0.01 N Hydrochloric Acid for 2 min, rinsed in dH₂O, dehydrated, cleared in toluene and mounted.

Immunohistochemistry and Fluorescence

All staining was done on paraffin sections of formalin-fixed lungs or tracheas. Immunofluorescent staining was performed with the following primary antibodies: mouse anti- β -tubulin (1:500; 3F3-G2; Seven Hills Bioreagents), goat anti-Scgb1a1 (1:200; clone T-18; sc-9772; Santa Cruz Biotechnology Inc.), rabbit anti-Scgb1a1 (1:500; WRAB-CCSP; Seven Hills Bioreagents), rabbit anti-beta-galactosidase (1:500; 100-4136; Rockland Immunochemicals Inc), rabbit anti-Fgfr2 (Bek) (1:500; clone C-17; sc-122; Santa

Cruz Biotechnology Inc.), mouse anti- α -Actin (1:500; clone 1A4; sc-32251; Santa Cruz Biotechnology Inc.), chicken anti-GFP (1:250; GFP-1020; Aves Labs Inc.), rabbit anti-Keratin 5 (1:200; clone EP1601Y; MA5-14473, Thermo Fisher Scientific), rabbit anti-FGF10 (1:1000; AP14882PU-N; Acris) (1:1000, ABN44, Millipore), rabbit anti-p63 (Δ N) (1:500; clone poly6190, 619002; BioLegend), rabbit anti-ILK (1:50; 3862S; Cell Signaling Technology Inc.), mouse anti-p63 (1:200; clone 4A4; CM163B; Biocare Medical), rabbit anti-phospho-Mst1/2 (1:250; 3681S; Cell Signaling), goat anti-p-SMAD2/3 (Ser423/425) (1:100; sc-11769; Santa Cruz Biotechnology Inc.), rabbit anti-RFP (1:200; 600-401-379; Rockland Immunochemicals Inc), rabbit anti-Sftpc (1:200; WRAB-9337; Seven hills bioreagents), rabbit anti-YAP (1:50; 4912S; Cell Signaling Technology Inc.), mouse anti-Yap (1:100; clone 63.7; sc-101199; Santa Cruz Biotechnology Inc.), rabbit anti-DKK1 (1:200; clone H-120; sc-25516; Santa Cruz Biotechnology Inc.), mouse anti-E-Cadherin (1:200; 610181; BD Transduction Laboratories), goat anti-Wnt7b (1:25; AF3460; R&D systems), rabbit anti-Merlin (Nf2) (1:250; clone A-19; sc-331; Santa Cruz Biotechnology Inc.), rabbit anti-Mst1/2 (1:200; ab87322; Abcam), mouse anti-PCNA (PCNA staining kit; 93-1143; Zymed Laboratories Inc.). After deparaffinization, slides were rehydrated through a series of decreasing ethanol concentrations, antigen unmasked by either microwaving in citrate-based antigen unmasking solution (Vector Labs, H-3000) or by incubating sections with proteinase K (7.5 μ g/ml) (Invitrogen, 25530-049) for 7 min at 37°C. Tissue sections were then washed in TBS with 0.1% Tween-20 and blocked with 3% Bovine Serum Albumin (BSA), 0.4% Triton in TBS for 30 min at room temperature followed by overnight incubation of primary antibodies diluted in 3% BSA, 0.1% Triton in TBS. The next day, slides were washed in TBS with 0.1% Tween-20 and incubated with secondary antibodies diluted in 3% BSA, 0.1% Triton in TBS for 3h at room temperature. All fluorescent staining was performed with appropriate secondary antibodies from Jackson ImmunoResearch, except for goat anti-chicken FITC (1:200; F-1005; Aves Labs, Inc.). Slides were mounted using Vectashield with (Vector Labs, H-1200) or without DAPI (Vector Labs, H-1000) depending on immunostaining.

In Situ Cell Death Detection (TUNEL Staining)

After deparaffinization, slides were rehydrated through a series of decreasing ethanol concentrations and permeabilized using 0.5% Triton X-100 in TBS for 8 minutes. TUNEL staining was performed according to the manufacturer's instructions (In Situ Cell Death Detection Kit, Fluorescein; 11 684 795 910; Roche). After completion of TUNEL staining, slides were subjected to immunofluorescence staining using goat anti-Scgb1a1 (1:100; clone T-18; sc-9772; Santa Cruz Biotechnology Inc.). Slides were mounted using Vectashield with DAPI (Vector Labs, H-1200).

Proliferation

Mice were given intraperitoneal injections of 10 μ l BrdU (GE Healthcare; RPN201) per gram body weight 4 hours before sacrifice. Lungs were fixed in 4% paraformaldehyde, dehydrated, and paraffin embedded. Sections were treated with monoclonal mouse anti-BrdU (clone BU-1; RPN202; GE Healthcare) according to the manufacturer's instructions. FITC-labeled anti-mouse secondary antibodies were used (Jackson ImmunoResearch). All slides were mounted using Vectashield with DAPI.

Western Blot

Left lobes of lung tissue were lysed in NP40 lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% NP40, 10% Glycerol, supplemented with protease and phosphatase inhibitors) and lysates were separated on a 4%–12% acrylamide gel and transferred to PVDF membranes (Bio-Rad). Antibodies used for immunoblotting were: rabbit anti-GAPDH (1:3000; ab9485; Abcam), rabbit anti-FGF10 (1:1000; AP14882PU-N; Acris) (1:1000, ABN44, Millipore), rabbit anti-FGFR2 (1:500; sc-122; Santa Cruz Biotechnology Inc.), rabbit anti-YAP (1:500; Cell Signaling Technology Inc.), goat anti-WNT7B (1:500; AF3460; R&D systems), rabbit anti-p-MST1/MST2 (1:500; 3681S; Cell Signaling Technology Inc.), and HRP-conjugated secondary antibodies (Jackson ImmunoResearch).

In Situ Hybridization

The whole-mount *in situ* hybridization protocol used was based on a previously described method (Winnier et al., 1995). Tracheas were dissected and fixed in 4% formalin in PBS overnight. The next day, tracheas were rinsed in PBS and dehydrated to 100% ethanol. Tracheas were then bleached in 4 parts ethanol, 1 part 30% hydrogen peroxide for 1h at room temperature. Tracheas were sequentially washed in PBS containing 0.1% Tween-20 (PBST), treated with 1ml of 7.5 μ g/ml proteinase K (Invitrogen, 25530-049) in PBST for 5 min at room temperature, washed in PBST containing 2mg/ml glycine, washed in PBST, fixed in 4% formaldehyde/0.2% glutaraldehyde in PBS for 20 min at room temperature and washed in PBST. Tracheas were then placed in hybridization solution (prewarmed to 70°C) (50% deionized formamide, 5x SSC buffer, 0.05mg/ml tRNA, 1% SDS and 0.05 mg/ml Heparin), replaced with fresh hybridization solution and prehybridized at 70°C for 1h with gentle agitation. Tracheas were then hybridized in probe/hybridization solution overnight at 70°C with gentle agitation. The next day, tracheas were washed in Solution I (50% deionized formamide, 5x SSC buffer, 1% SDS) for 2x 30 min at 70°C. After solution I washes, tracheas were washed in 1:1 Solution I: Solution II (0.5M NaCl, 10mM Tris pH7.5, 0.1% Tween-20) for 10 min at 70°C, washed in Solution II for 3x 5min at room temperature, 2x 30min in 100 μ g/ml RNase A (Roche, 109169) in Solution II at 37°C, 2x 30min in Solution III (50% formamide, 2X SSC) for 30min at 65°C and washed with TBST/0.5mg/ml Levamisole. Tracheas were then blocked in blocking solution containing 2% Blocking Reagent (11096176001, Sigma-Aldrich), 10% Heat inactivated Fetal Bovine Serum (HI-FBS) in TBST/0.5mg/ml Levamisole for 1h at room temperature and then incubated with sheep Anti-Digoxigenin-AP, (1:2000, 11093274910, Sigma-Aldrich) in 1% Blocking Reagent, 1% HI-FBS in TBST/0.5mg/ml Levamisole at 4°C overnight. Blocking Reagent was prepared by dissolving in Malic acid buffer (100mM Maleic acid, 150mM NaCl, pH7.5). The next day, samples were washed 5-6 times with TBST/0.5mg/ml Levamisole and washed in the

same solution overnight at 4°C. The following day, tracheas were washed with NTMT /Alkaline Phosphatase buffer (100mM NaCl, 100mM Tris pH9.5, 50mM MgCl₂, 0.1% Tween-20 and 0.5mg/ml Levamisole) 2x for 20min at room temperature. Tracheas were then incubated in BM purple solution (11442074001, Sigma-Aldrich) at 37° degrees in the dark until the signal appears. Finally, tracheas were washed in PBS, post-fixed in 4% formalin in PBS at room temperature. A 584-bp fragment of mouse *Fgf10* cDNA was used as template for the synthesis of digoxigenin (DIG)-labeled riboprobe (Bellusci et al., 1997) using DIG RNA labeling mix (Roche, 11277073910).

Microscopy and Imaging

Tissue was imaged using a micrometer slide calibrated Zeiss Axioimager microscope using axiovision software, a Leica MZ16FA stereomicroscope, a Zeiss LSM700 confocal microscope using ZEN imaging software as well as a Nikon A1R confocal with NIS-elements software. Subepithelial collagen thickness was quantified on Picro-sirius red stained sections. Structural changes to the airway were morphometrically analyzed as follows: the thickness of the bronchial epithelial layer, subepithelial fibrosis, smooth muscle layer, and total wall was measured on H&E and picro-sirius red stained sections. A minimum of 5 bronchi, measuring 150–350 μ m in luminal diameter, were analyzed per mouse ($n \geq 6$). BSCs in trachea were quantified covering the entire length of the trachea from cartilage ring 1 to 10 of each mouse using 10-20X magnification fields and was performed on the basis of nuclear staining with DAPI (nuclei) and immunostaining for the BSC specific markers K5/p63 and expressed as the mean number of BSCs per 100 μ m of basement membrane or per section. In the lung, cells were counted using at least 5-10 non-overlapping 10-20X magnification fields from ≥ 6 different lungs. Images were processed and analyzed using ImageJ/Fiji (NIH) and Adobe Photoshop Creative Suite 3 (Adobe) software. Immunostaining intensity was quantified after thresholding of pixel intensities.

Quantitative Real-Time PCR

Total RNA was extracted from lung accessory lobes using RNeasy (Invitrogen, AM7021) and Total RNA Kit I (Omega Biotek, R6834-02) according to the manufacturer's instructions. RNA concentration was determined by spectrophotometry. cDNA was generated using Maxima™ First Strand cDNA Synthesis (Fisher Scientific, FERK1642) according to the manufacturer's instructions. Gene expression was analyzed by quantitative RT-PCR using Taqman Gene Expression Assays (Applied Biosystems, 4369016) directed against the mouse targets β -glucuronidase (Mm00446953_m1), *Scgb1a1* (Mm00442046_m1), *FoxJ1* (Mm00807215_m1), keratin 5 (*K5*) (Mm01305291_g1), *p63* (*Trp63*) (Mm00495788_m1), *Muc5a/c* (Mm01276718_m1), *Col1a1* (Mm00801666_g1), *Col3a1* (Mm01254476_m1), *Wnt7b* (Mm00437358_m1), *Dkk1* (Mm00438422_m1). Quantitative real-time PCR was performed using a StepOne Plus system (Applied Biosystems). Data were presented as expression relative to the housekeeping gene β -glucuronidase $\pm$ standard error of mean (SEM). Each experiment was repeated with samples obtained from at least 3 different lungs preparations.

QUANTIFICATION AND STATISTICAL ANALYSIS

All results are expressed as mean values $\pm$ SEM. The 'n' represents biological replicates and can be found in the figure legends. The significance of differences between 2 sample means was determined by unpaired two-tailed student's t-test. P values less than 0.05 were considered statistically significant.

Developmental Cell, Volume 43

Supplemental Information

Fgf10-Hippo Epithelial-Mesenchymal Crosstalk

Maintains and Recruits Lung Basal Stem Cells

Thomas Volckaert, Tingting Yuan, Cho-Ming Chao, Harold Bell, Alina Sitaula, Luisa Szimmlenings, Elie El Agha, Diptiman Chanda, Susan Majka, Saverio Bellusci, Victor J. Thannickal, Reinhard Fässler, and Stijn P. De Langhe

Supplemental figures

Figure S1

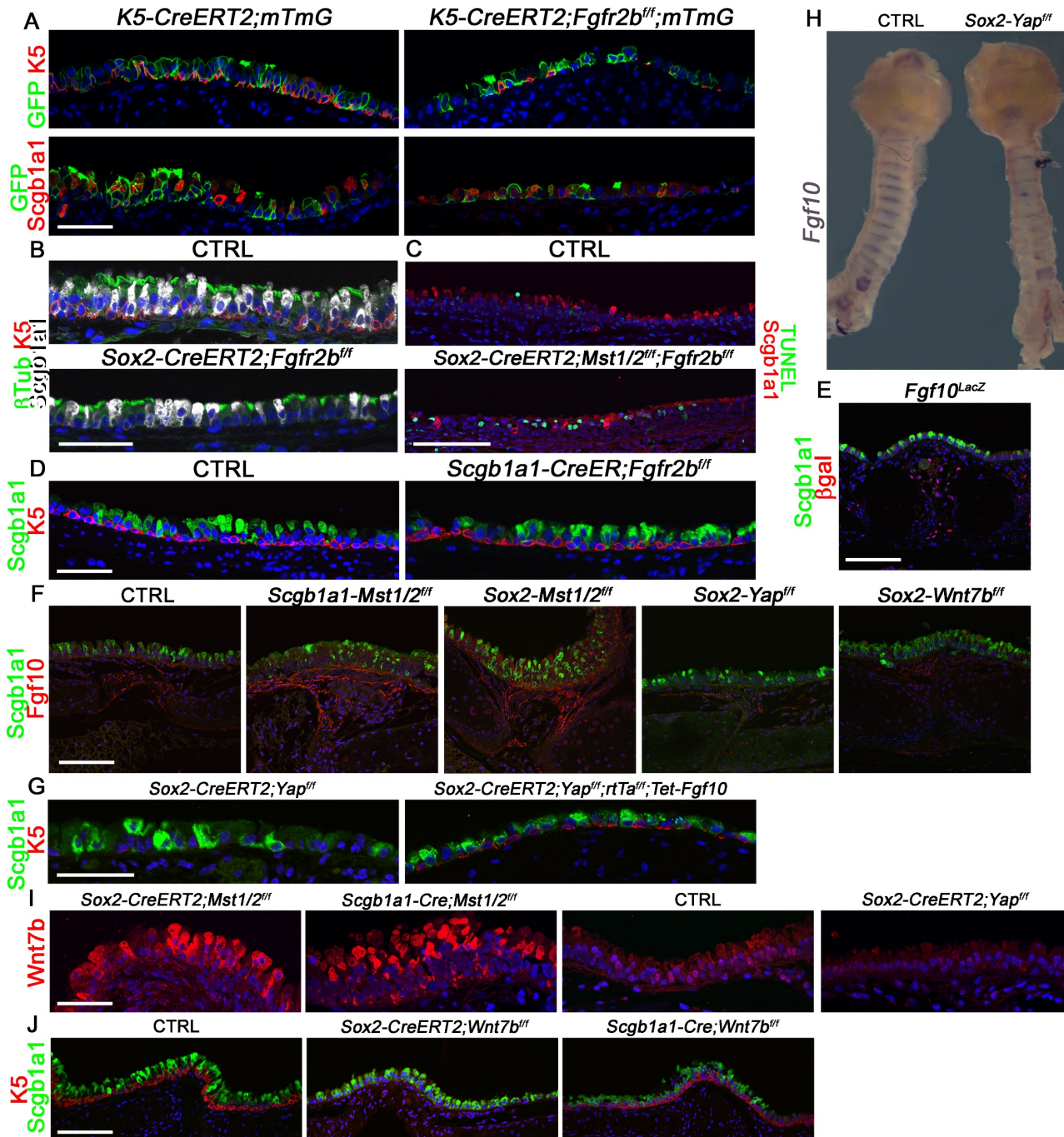


Figure S1. Related to Figure 1. Epithelial Hippo signaling controls *Fgf10* expression in the stromal tracheal niche.

- (A) Immunostaining for GFP (green) and Keratin 5 (K5) (red) or Scgb1a1 (red) on tracheas from control and *Krt5-CreERT2;Fgfr2b^{ff}* mice.
- (B) Immunostaining for β -tubulin (green), K5 (red) and Scgb1a1 (white) on tracheas from control and *Sox2-CreERT2;Fgfr2b^{ff}* mice.
- (C) TUNEL (red) together with Scgb1a1 (green) immunostaining on control and *Sox2-Mst1/2^{ff}-Fgfr2b^{ff}* tracheas.
- (D) Immunostaining for K5 (red) and Scgb1a1 (green) on control and *Scgb1a1-Cre-Fgfr2b^{ff}* tracheas.

- (E) Immunostaining for β -galactosidase (β -gal) (red) and Scgb1a1 (green) on *Fgf10-LacZ* trachea showing localization of *Fgf10*-expressing cells in the stromal niche (i.e. in between cartilage rings).
- (F) Immunostaining for Fgf10 (red) and Scgb1a1 (green) on control, *Scgb1a1-Mst1/2^{ff}*, *Sox2-Mst1/2^{ff}*, *Sox2-Yap^{ff}* and *Sox2-Wnt7b^{ff}* tracheas.
- (G) Immunostaining for K5 (red) and Scgb1a1 (green) on *Sox2-Yap^{ff}* and *Sox2-Yap-rtta^{ff}-Tet-Fgf10* tracheas.
- (H) Whole-mount in situ hybridization for *Fgf10* on control and *Sox2-Yap^{ff}* tracheas.
- (I) Immunostaining for Wnt7b (red) on *Sox2-Mst1/2^{ff}*, *Scgb1a1-Mst1/2^{ff}*, control and *Sox2-Yap^{ff}* mice.
- (J) Immunostaining for K5 (red) and Scgb1a1 (green) on control, *Sox2-Wnt7b^{ff}* and *Scgb1a1-Wnt7b^{ff}* tracheas.

Krt5-CreERT2;Fgfr2b^{ff} tracheas were collected 6 weeks after tamoxifen induction. *Sox2-Mst1/2^{ff}*, *Sox2-Wnt7b^{ff}*, *Sox2-Mst1/2^{ff}-Fgfr2b^{ff}*, *Scgb1a1ER-Fgfr2b^{ff}* tracheas were collected 8 weeks after tamoxifen induction. *Sox2-Fgfr2b^{ff}* tracheas were collected 2 weeks after tamoxifen induction. *Sox2-Yap^{ff}* and *Sox2-Yap-rtta^{ff}-Tet-Fgf10* tracheas were collected 12 weeks after tamoxifen induction, after being placed on dox for 3 weeks starting 9 weeks after tamoxifen induction. Scale bars, 50 μ m (A,B,D,G,I); 100 μ m (C,E,F,J).

Figure S2

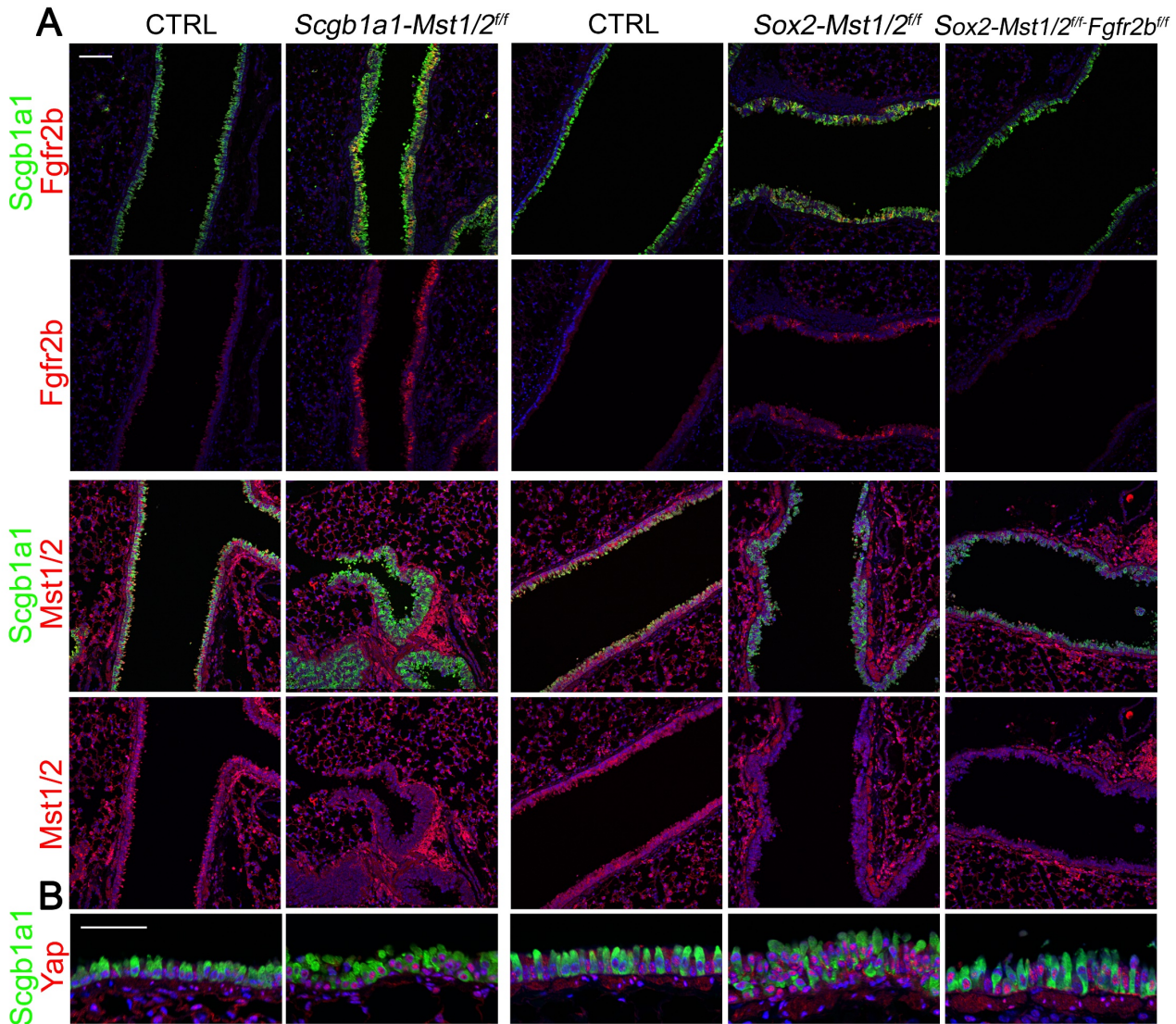


Figure S2. Related to Figure 2. Verification of recombination efficiency of floxed alleles in *Scgb1a1-Mst1/2^{fl/fl}*, *Sox2-Mst1/2^{fl/fl}*, and *Sox2-Mst1/2^{fl/fl}-Fgfr2b^{fl/fl}* compound mutant non-cartilaginous airways.

(A,B) Immunostaining for Scgb1a1 (green) and Fgfr2b (red) or Scgb1a1 (green) and Mst1/2 (red) or Scgb1a1 (green) and Yap (red) on *Scgb1a1-Mst1/2^{fl/fl}*, *Sox2-Mst1/2^{fl/fl}*, and *Sox2-Mst1/2^{fl/fl}-Fgfr2b^{fl/fl}* non-cartilaginous airways showing efficient deletion of *Fgfr2b* and *Mst1/2* as well as nuclear localization Yap after *Mst1/2* ablation. *Sox2-Mst1/2^{fl/fl}* and *Sox2-Mst1/2^{fl/fl}-Fgfr2b^{fl/fl}* lungs were harvested 2 months after tamoxifen administration. Nuclei were stained with DAPI (blue). Scale bars, 100 μ m (A); 50 μ m (B).

Figure S3

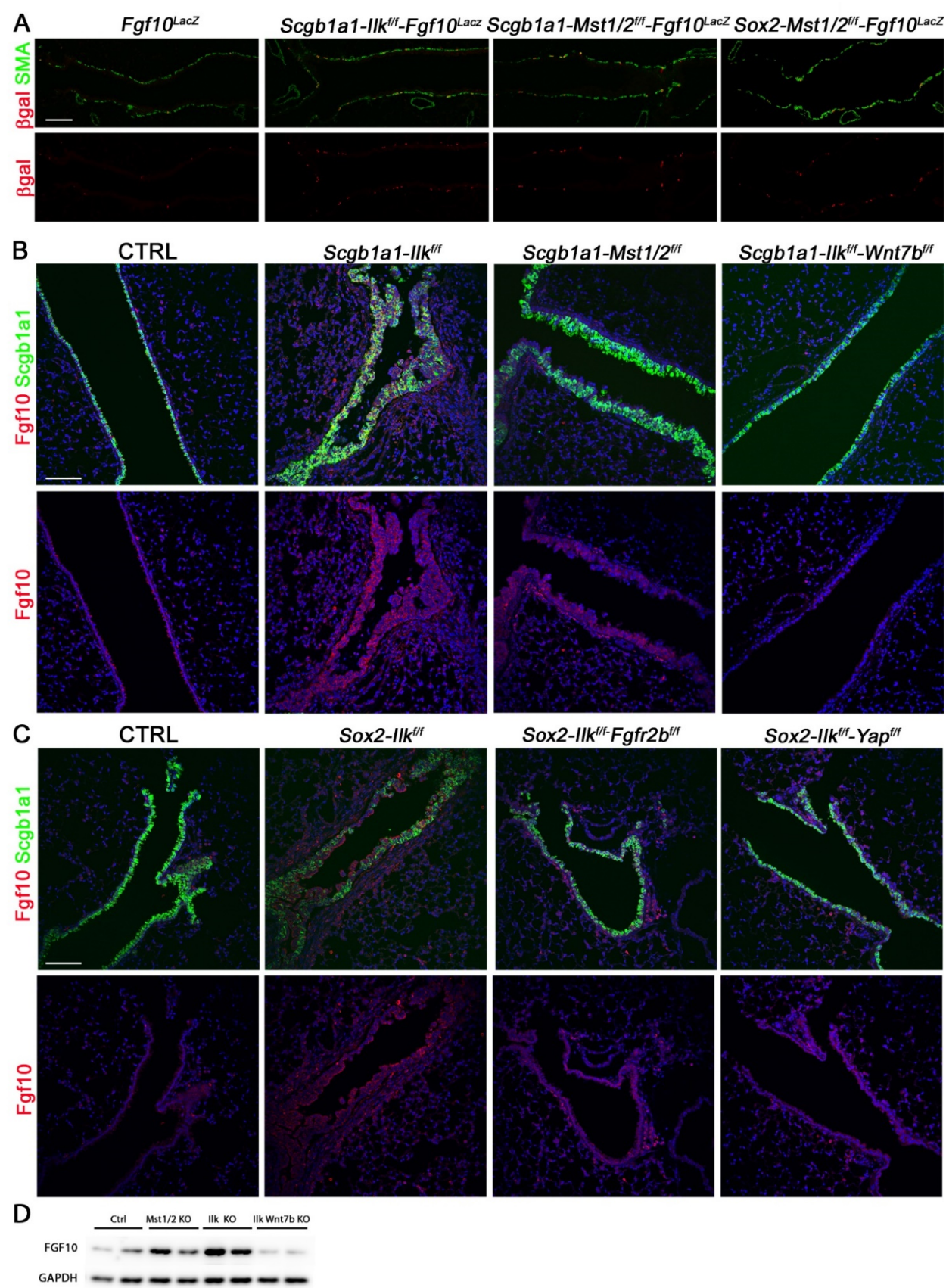


Figure S3. Related to Figures 2, 4 and 5. Fgf10 expression in the stromal niche is increased upon inactivation of Ilk or Mst1/2 in non-cartilaginous airway epithelium.

(A) Immunostaining for SMA (green) and β -galactosidase (β -gal) (red) on *Fgf10^{LacZ}*, *Scgb1a1-Ilk^{ff}-Fgf10^{LacZ}*, *Scgb1a1-Mst1/2^{ff}-Fgf10^{LacZ}* and *Sox2-Mst1/2^{ff}-Fgf10^{LacZ}* non-cartilaginous airways 2 months after tamoxifen induction.

(B) Immunostaining for Fgf10 (red) and Scgb1a1 (green) on control, *Scgb1a1-Ilk^{ff}*, *Scgb1a1-Mst1/2^{ff}* and *Scgb1a1-Ilk^{ff}-Wnt7b^{ff}* non-cartilaginous airways.

(C) Immunostaining for Fgf10 (red) and Scgb1a1 (green) on control, *Sox2-Ilk^{ff}*, *Sox2-Ilk^{ff}-Fgfr2b^{ff}* and *Sox2-Ilk^{ff}-Yap^{ff}* non-cartilaginous airways 2 months after tamoxifen induction.

(D) Western blot analysis showing Fgf10 protein expression in control, *Scgb1a1-Mst1/2^{ff}* (Mst1/2 KO), *Scgb1a1-Ilk^{ff}* (Ilk KO) and *Scgb1a1-Ilk^{ff}-Wnt7b^{ff}* (Ilk Wnt7b KO) lungs. Note the increased Fgf10 levels in Ilk KO compared to Mst1/2 KO non-cartilaginous airways.

Scale bars, 100 μ m (A,B,C)

Figure S4

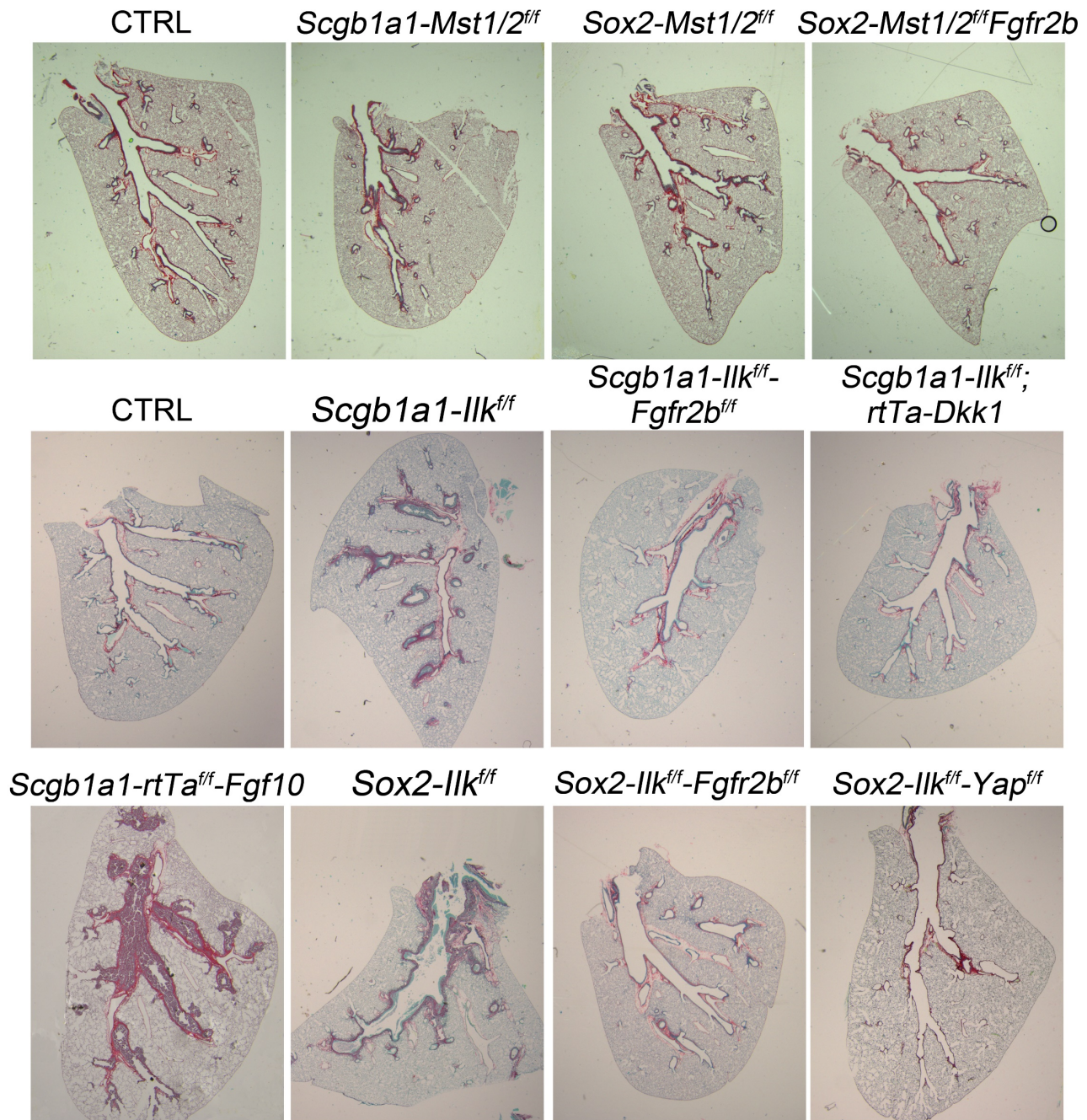


Figure S4. Related to Figures 2-5. Whole lobe view showing airway remodeling in the different mouse strains.

Picro-sirius red (collagen)/fast green staining and immunostaining on control, *Scgb1a1-Mst1/2^{f/f}*, *Sox2-Mst1/2^{f/f}*, *Sox2-Mst1/2^{f/f}-Fgfr2b^{f/f}*, *Scgb1a1-Ilk^{f/f}*, *Scgb1a1-Ilk^{f/f}-Fgfr2b^{f/f}*, *Scgb1a1-Ilk^{f/f}-rtTa-Dkk1*, *Scgb1a1-rtTa^{f/f}-Fgf10*, *Sox2-Ilk^{f/f}*, *Sox2-Ilk^{f/f}-Fgfr2b^{f/f}* and *Sox2-Ilk^{f/f}-Yap^{f/f}* lungs.

Figure S5

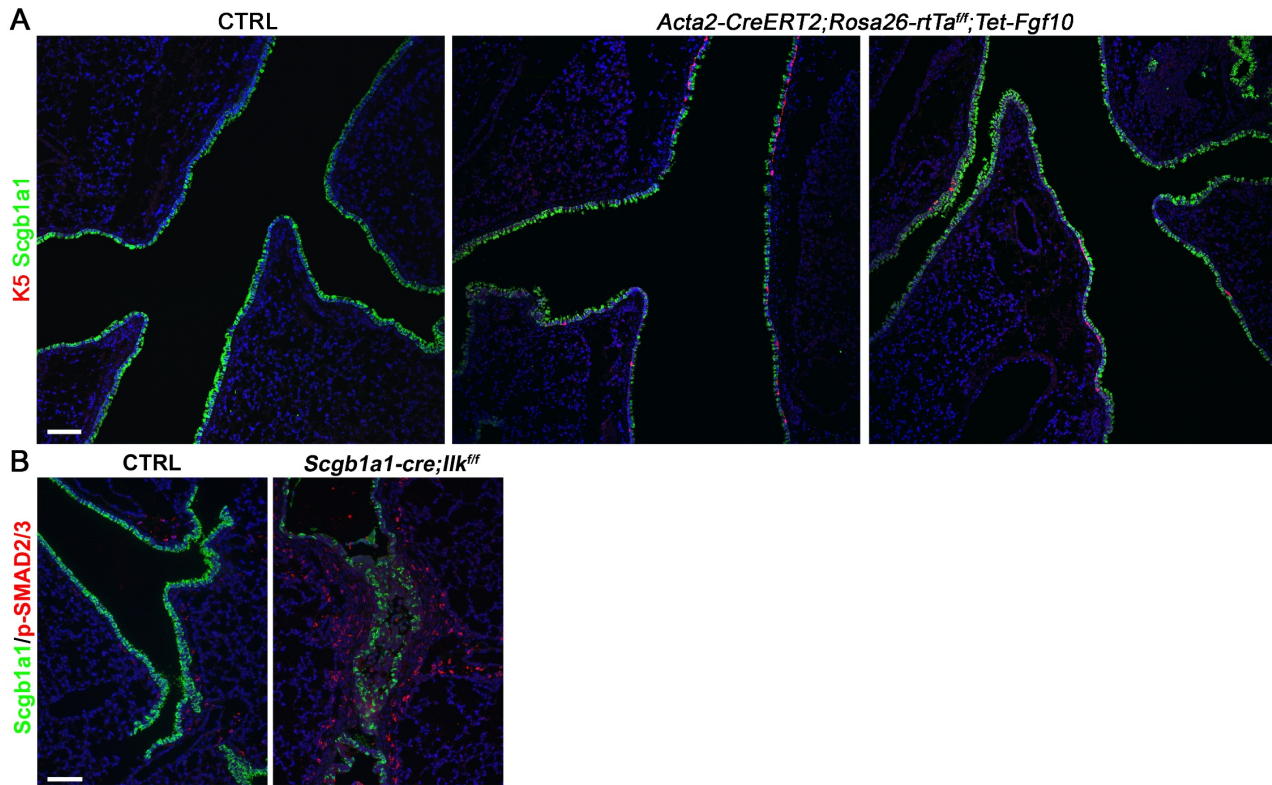


Figure S5. Related to Figure 3. Overexpression of *Fgf10* in ASMCs recruits basal cells to the non-cartilaginous conducting airways.

(A) Immunostaining for K5 (red) and Scgb1a1 (green) on non-cartilaginous airways of control and *Acta2-rtTa^{fl}-Tet-fgf10* mice which were put on tamoxifen food for a period of 3 weeks, followed by 10 weeks of doxycycline induction.

(B) Immunostaining for Scgb1a1 (green) and p-SMAD2/3 (red) on control and *Scgb1a1-Ilk^{fl}* non-cartilaginous airways showing increased TGF- β signaling in the fibrotic tissue surrounding the airways.

Scale bars, 100 μ m (A,B)

Figure S6

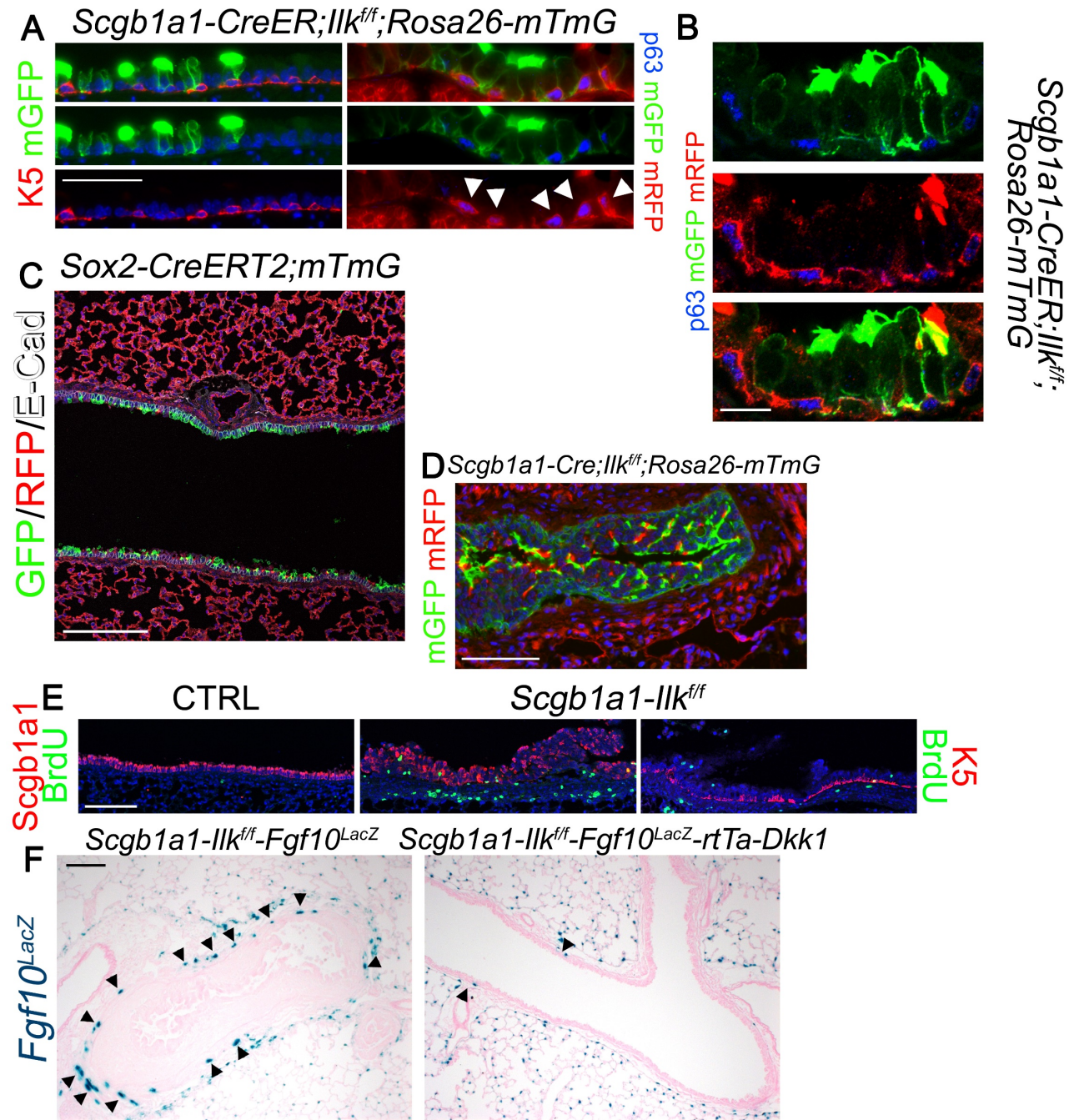


Figure S6. Related to Figures 2, 3 and 5. Basal cell accumulation in non-cartilaginous airways after secretory cell-specific *Ilk* ablation does not occur through secretory cell dedifferentiation.

(A) Immunostaining on a *Scgb1a1ER-Ilk^{fl/fl}-Rosa26-mTmG* non-cartilaginous airway for Keratin 5 (K5) and GFP (green) or RFP (red), GFP (green) and p63 (blue) 2 months after tamoxifen induction. White arrowheads indicate RFP⁺ GFP⁻ BSCs and are therefore not derived from secretory cells.

(B) Immunostaining on a *Scgb1a1ER-Ilk^{fl/fl}-Rosa26-mTmG* non-cartilaginous airway for RFP (red), GFP (green) and p63 (blue) 2 months after tamoxifen induction.

(C) Immunostaining on a *Sox2-CreERT2-Rosa26-mTmG* non-cartilaginous airway for RFP (red), GFP (green) and E-Cadherin (white) 2 months after tamoxifen induction.

(D) Immunostaining on a *Scgb1a1-Ilk^{fl/fl}-Rosa26-mTmG* non-cartilaginous airway for GFP (green) and RFP (red) showing that subepithelial fibrotic myofibroblasts are not derived from secretory cells.

(E) Immunostaining for the proliferation marker BrdU (green) together with Scgb1a1 (red) or K5 (red) on BrdU labeled control and *Scgb1a1-Ilk^{fl/fl}* non-cartilaginous airways.

(F) β -gal (arrowheads) and eosin staining of *Scgb1a1-Ilk^{fl/fl}-Fgf10^{LacZ}* and *Scgb1a1-Ilk^{fl/fl}-Fgf10^{LacZ}-rtTa-Dkk1* non-cartilaginous airways showing that *Dkk1* overexpression inhibits *Fgf10* expression in ASMCs upon deletion of *Ilk* in secretory cells.

Nuclei were stained with DAPI (blue) in (A, C, D, E). Scale bars, 50 μ m (A), 10 μ m (B), 100 μ m (C,D,E,F).

Figure S7

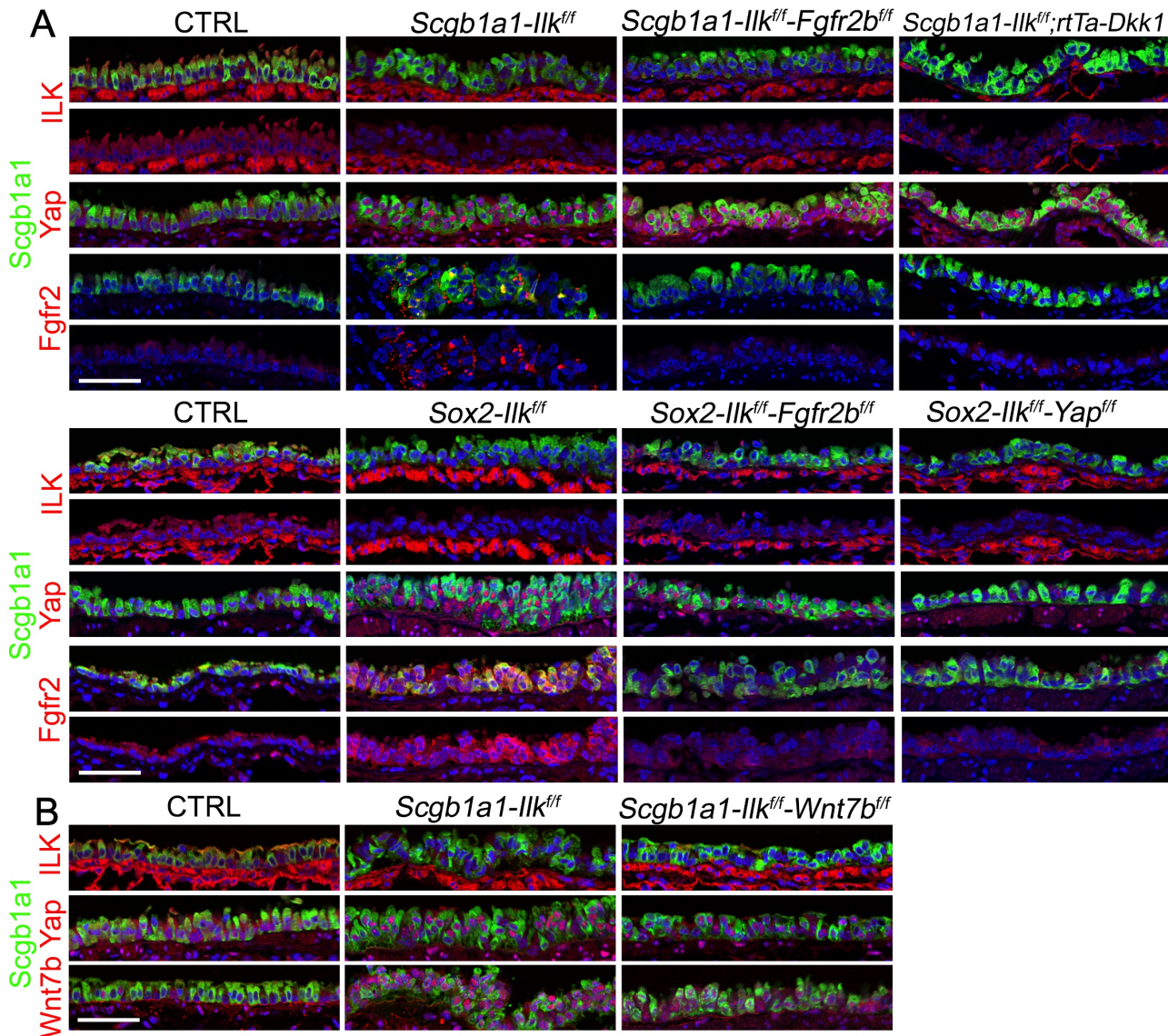


Figure S7. Related to Figures 2-5. Verification of recombination efficiency of floxed alleles in *Ilk*-deficient non-cartilaginous airway epithelium and the different rescue models.

(A) Immunostaining for Scgb1a1 (green) together with ILK or Yap or Fgfr2b (red) on non-cartilaginous airways of control, *Scgb1a1-Ilk^{fl/fl}*, *Scgb1a1-Ilk^{fl/fl}-Fgfr2b^{fl/fl}* and *Scgb1a1-Ilk^{fl/fl};rtTa-Dkk1* mice (upper panels) and control, *Sox2-Ilk^{fl/fl}*, *Sox2-Ilk^{fl/fl}-Fgfr2b^{fl/fl}* and *Sox2-Ilk^{fl/fl}-Yap^{fl/fl}* mice harvested 2 months after tamoxifen induction (lower panels).

(B) Immunostaining for Scgb1a1 (green) together with ILK or Yap or Wnt7b (red) on control, *Scgb1a1-Ilk^{fl/fl}* and *Scgb1a1-Ilk^{fl/fl}-Wnt7b^{fl/fl}* non-cartilaginous airways.

Nuclei were stained with DAPI (blue). Scale bar 100 μ m.

Table S1. Related to Figure 6. COPD sample information. All COPD samples were obtained from lung transplant patients. Py, pack year: Number of pack-years = (packs smoked per day) × (years as a smoker).

Patient info	Therapy
Patient 1 Smoker, stopped 2009 Gold stage IV COPD	Spiriva 1x1, Seretide DA 50/500 2x1, Berodual, Berdualos Inhalation 3x/day, Theospirex 300mg; Furosemid, Spiron, Normodipine 2,5mg, Medrol 4mg O2-Therapy
Patient 2 Smoker, 32py Gold stage IV COPD	Symbicort forte 2 x 1 Hb, Respicur ret. 300mg in the evening, Berodual bis 3x2 puffs, Nomexor 5mg ½ Tabl, Spiriva 18µg. O2-Therapy
Patient 3 Smoker, 30py Gold stage IV COPD	Spiriva 18µg, Foster DA, Berodual bis max 8 puffs, Respicur 400mg. O2-Therapy
Patient 4 Never smoked (inhalative gas-exposition in a shoe-factory for about 20 years) Gold stage IV COPD	Spiriva 18µg, Seretide Diskurs Forte 50/500 1-0-1, Berotec DA 4x2 Hb; in case of shortness of breath: Unifyl 1x1 and Aprednisolon 25mg. O2-Therapy
Patient 5 Smoker, 50py Gold stage IV COPD	Spiriva Respimat 2-0-0, Seretide standard DA + Volumatik 2-0-2, Berodual DA if needed, Daxas 500mg. O2- Therapy
Patient 6 Smoker Gold stage IV COPD	Spiriva Capsule. + Inhalation. 1-0-0, Seretide standard Discus with Volumatik 1-0-1, Combivent DA + Spacer if needed, Aprednisolon 5mg ½ or ¼. O2- Therapy

FGF10-FGFR2B Signaling Generates Basal Cells and Drives Alveolar Epithelial Regeneration by Bronchial Epithelial Stem Cells after Lung Injury

Tingting Yuan,^{1,8} Thomas Volckaert,^{1,8} Elizabeth F. Redente,^{2,3} Seantel Hopkins,¹ Kylie Klinkhammer,¹ Roxana Wasnick,⁴ Cho-Ming Chao,⁴ Jie Yuan,¹ Jin-San Zhang,⁵ Changfu Yao,⁶ Susan Majka,⁷ Barry R. Stripp,⁶ Andreas Günther,⁴ David W.H. Riches,^{2,3} Saverio Bellusci,⁴ Victor J. Thannickal,¹ and Stijn P. De Langhe^{1,*}

¹Department of Medicine, Division of Pulmonary, Allergy & Critical Care Medicine, University of Alabama at Birmingham, THT 422, 1720 2nd Avenue S., Birmingham, 35294-2182 AL, USA

²Department of Pediatrics, Division of Cell Biology, National Jewish Health, Denver, CO 80206, USA

³Denver Veteran Affairs Medical Center, Denver, CO 80206, USA

⁴German Center for Lung Research, Excellence Cluster Cardio-Pulmonary System, Universities of Giessen and Marburg Lung Center, 35392 Giessen, Germany

⁵School of Pharmaceutical Sciences, First Affiliated Hospital, Wenzhou Medical University, Wenzhou, Zhejiang 325035, China

⁶Women's Guild Lung Institute, Department of Medicine, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA

⁷Department of Medicine, National Jewish Health, Denver, CO 80206, USA

⁸Co-first author

*Correspondence: sdelanghe@uabmc.edu

<https://doi.org/10.1016/j.stemcr.2019.04.003>

SUMMARY

Idiopathic pulmonary fibrosis is a common form of interstitial lung disease resulting in alveolar remodeling and progressive loss of pulmonary function because of chronic alveolar injury and failure to regenerate the respiratory epithelium. Histologically, fibrotic lesions and honeycomb structures expressing atypical proximal airway epithelial markers replace alveolar structures, the latter normally lined by alveolar type 1 (AT1) and AT2 cells. Bronchial epithelial stem cells (BESCs) can give rise to AT2 and AT1 cells or honeycomb cysts following bleomycin-mediated lung injury. However, little is known about what controls this binary decision or whether this decision can be reversed. Here we report that inactivation of *Fgfr2b* in BESCs impairs their contribution to both alveolar epithelial regeneration and honeycomb cysts after bleomycin injury. By contrast overexpression of *Fgf10* in BESCs enhances fibrosis resolution by favoring the more desirable outcome of alveolar epithelial regeneration over the development of pathologic honeycomb cysts.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a common form of interstitial lung disease resulting in alveolar remodeling and progressive loss of pulmonary function, respiratory failure, and death, often within 5 years of diagnosis (King et al., 2011; Steele and Schwartz, 2013). IPF pathogenesis encompasses fibrotic remodeling, inflammation, and loss of lung architecture (Barkauskas and Noble, 2014). Although the underlying causes of IPF remain elusive, genetic, and experimental evidence support the concept that chronic alveolar injury and failure to properly repair the respiratory epithelium represent key pathogenic mechanisms (Camelo et al., 2014; Thannickal et al., 2004; Yang et al., 2013). Histologically, respiratory epithelial cells of the lung parenchyma express atypical proximal airway epithelial and indeterminate cell-type markers (Plantier et al., 2011; Seibold et al., 2013), including goblet and basal cell (BC) characteristics that are normally restricted to conducting airways. Fibrotic lesions and bronchiolized or honeycomb structures replace alveolar structures, the latter normally lined by alveolar type 1 (AT1) and AT2 cells. Genome-wide transcriptome analyses of lung tissue and isolated epithelial cells from IPF patients demonstrate dramatic changes in ciliated, basal, and goblet cell-associated

gene expression and loss of normal alveolar epithelial cells, reflecting profound changes in epithelial cell differentiation and function in IPF (Kropski et al., 2015; Seibold et al., 2013; Xu et al., 2016).

It is now generally accepted that insults to the alveolar epithelium, resulting in a loss of AT2 stem cells or mutations in alveolar epithelial cells that either impair their self-renewal and/or impair their differentiation into AT1 cells, can serve as a trigger of pulmonary fibrosis (Barkauskas and Noble, 2014). However, functional abnormalities in bronchiolar epithelial cells (and not just alveolar epithelial cells) can predispose patients to development of IPF. A gain-of-function mutation in mucin 5B (*MUC5B*), which is specifically expressed by club, mucous, and goblet cells (Evans et al., 2004; Ostedgaard et al., 2017; Roy et al., 2011, 2014), and which results in a 30-fold increase in *MUC5B* expression, is associated with increased risk of both familial and sporadic IPF (Seibold et al., 2011; Zhang et al., 2011). Overexpression of *Muc5b* also causes mucociliary dysfunction and enhances lung fibrosis in mice (Hancock et al., 2018). The precise mechanism by which this genetic variant leads to fibrosis is unknown, but one possibility is that trapped harmful agents cause damage to this stem cell population and therefore affect bronchial epithelial-mediated alveolar epithelial regeneration (Li et al., 2012; Roy et al., 2014).





Several studies have shown that subsets of SCGB1A1⁺ bronchial epithelial club cells can give rise to AT1 and AT2 cells following bleomycin-mediated lung injury, yet these findings remain somewhat controversial in the field largely because of the reliance on single CreER(T2) lines (Barkauskas et al., 2013; Guha et al., 2017; Kim et al., 2005; Kumar et al., 2011; Lee et al., 2014; McQualter et al., 2010; Rock et al., 2011; Zheng et al., 2012, 2013). In the mouse bleomycin model, ~50% of new AT2 cells which appear after bleomycin injury are thought to be derived from subsets of bronchial epithelial cells, whereas ~40% are thought to be derived from pre-existing AT2 cells (Barkauskas et al., 2013). However, the contribution of each stem cell population largely depends on the severity of the injury. *p63*⁺/*Scgb1a1*⁺/*Sox2*⁺ bronchial epithelial stem cell (BESCs) have been shown to invade the lung parenchyma after severe influenza-mediated lung injury and give rise to neo-BCs in response to HIF1 α -mediated Notch signaling, or AT2 cells in response to increased β -catenin signaling (Kumar et al., 2011; Ray et al., 2016; Vaughan et al., 2015; Xi et al., 2017; Yang et al., 2018; Zuo et al., 2015). Whether these cells respond in a similar fashion to other types of injury such as bleomycin injury has not yet been investigated. BESCs are thought to be a reserve stem cell population that helps in regeneration of the alveolar epithelium when most AT2 stem cells have been exhausted. However, in IPF it appears that this backup repair mechanism is severely impaired, resulting instead in the development of bronchiolized regions in the lung parenchyma lined with BCs that are referred to as “honeycomb cysts.”

We have previously shown that FGFR2B signaling is required for tracheal BC maintenance and that *Fgf10* overexpression can induce p63 (BC marker) and SFTPC (AT2 cell marker) expression in club cells (Balasooriya et al., 2017; Volckaert et al., 2013, 2017). Importantly, FGF10 levels are reduced in lungs from aged mice before and after injury as well as in IPF subjects with progressive compared with stable disease (Chanda et al., 2016). In addition, we previously demonstrated that *Fgf10*-expressing lipofibroblasts (LIFs), which form the AT2 stem cell niche, differentiate into myofibroblasts upon bleomycin injury (El Agha et al., 2017). In the present study, we demonstrate that FGF10-FGFR2B signaling also actively maintains AT2 stem cells. We now demonstrate, using single-cell RNA sequencing (RNA-seq), that these *Fgf10*-expressing fibroblasts downregulate *Fgf10* expression upon injury. Furthermore, we show that loss of FGF10-FGFR2B signaling in bronchial epithelial cells impairs the generation of both neo-BCs and alveolar epithelial (AT1 and AT2) cells after bleomycin injury. By contrast, *Fgf10* overexpression in bronchial epithelial cells promotes their differentiation along the AT2 lineage

rather than the BC lineage after injury. Taken together, our findings suggest that reduced levels of *Fgf10* compromise alveolar epithelial regeneration by BESCs and we demonstrate that exogenous FGF10 can promote the differentiation of BESCs along more desirable reparative alveolar epithelial lineages.

RESULTS

FGF10-FGFR2B Signaling Is Required for Maintaining AT2 Cells

We have recently shown that FGF10-FGFR2B signaling drives BC development and is also required for adult BC maintenance (Volckaert et al., 2013, 2017). However, BCs are not the only stem cell population that depends on an *Fgf10*-expressing niche in the adult lung. In the adult lung, every AT2 cell sits adjacent to an *Fgf10*-expressing LIF, which forms the AT2 stem cell niche (Figures 1A and 1B). However, these *Fgf10*-expressing AT2 niche cells differentiate into myofibroblasts upon bleomycin injury (Figures 1C and 1D) (El Agha et al., 2017). Interestingly, while lung FGF10 levels increase after bleomycin injury, it is unclear whether this increase is caused by an increase in the number of *Fgf10*-expressing fibroblasts or whether individual fibroblasts express more *Fgf10*. To investigate this question, we reanalyzed single-cell RNA-seq data on isolated EPCAM⁺CD31⁺CD45⁺ stromal cells from uninjured or injured α SMA-GFP;*Tbx4*-Cre;*Rosa26*-tdTomato lungs 3 weeks after bleomycin treatment (Xie et al., 2018). We found that individual *Fgf10*-expressing stromal cells expressed lower levels of *Fgf10* in injured compared with uninjured lungs (Figure 1E), but that the total number of *Fgf10*-expressing fibroblasts increased after injury. Interestingly, haploinsufficiency for *FGF10* has been linked to the development of emphysema in humans (Klar et al., 2011) suggesting that tonic FGF10 signaling may not only be required for BCs but also for AT2 cell maintenance. As each AT2 cell is closely juxtaposed to an *Fgf10*-expressing LIF (Figures 1A and 1B), we hypothesized that AT2 stem cells require high local levels of FGF10 for their maintenance.

To investigate this we inactivated the FGF10 receptor, *Fgfr2b*, in mature AT2 cells of adult *Sftpc*^{CreERT2};*Fgfr2b*^{f/f}; *mTmG* mice and found that this resulted in a nearly complete loss of AT2 cells (Figure 1F) and have a reduced capacity to regenerate alveolar epithelium after bleomycin injury (Figure 1G) (note that Cre activity before and after tamoxifen treatment of all the different mouse lines used in this manuscript is documented in Figures S1 and S2). In conclusion, our data demonstrate that FGFR2B signaling is required to actively maintain AT2 and BCs during adult life.

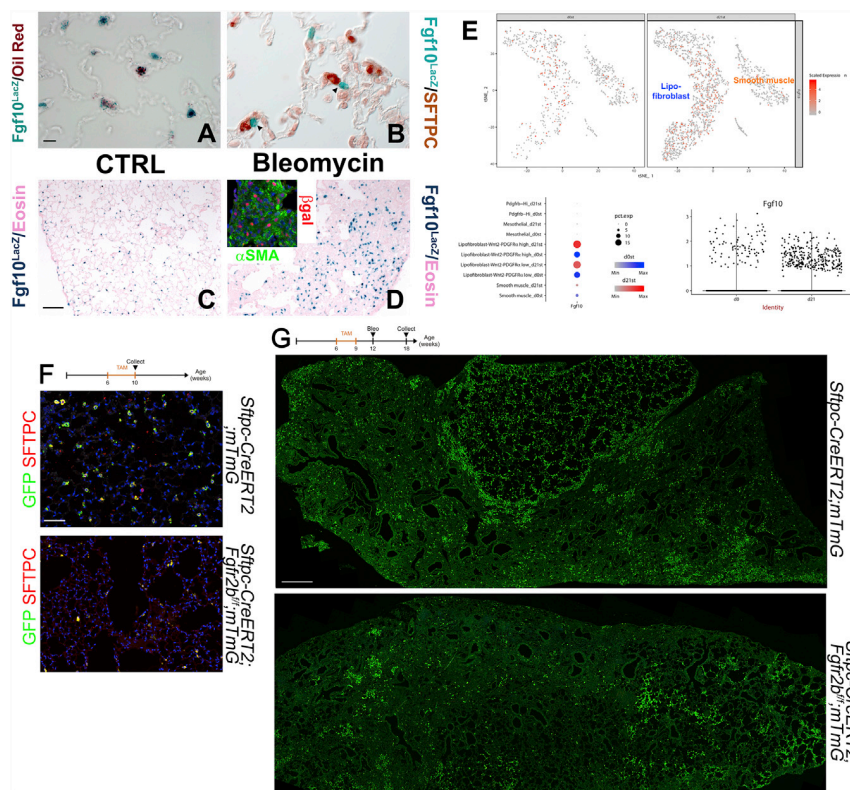


Figure 1. Fgf10-Fgfr2b Signaling Is Required for AT2 Stem Cell Maintenance

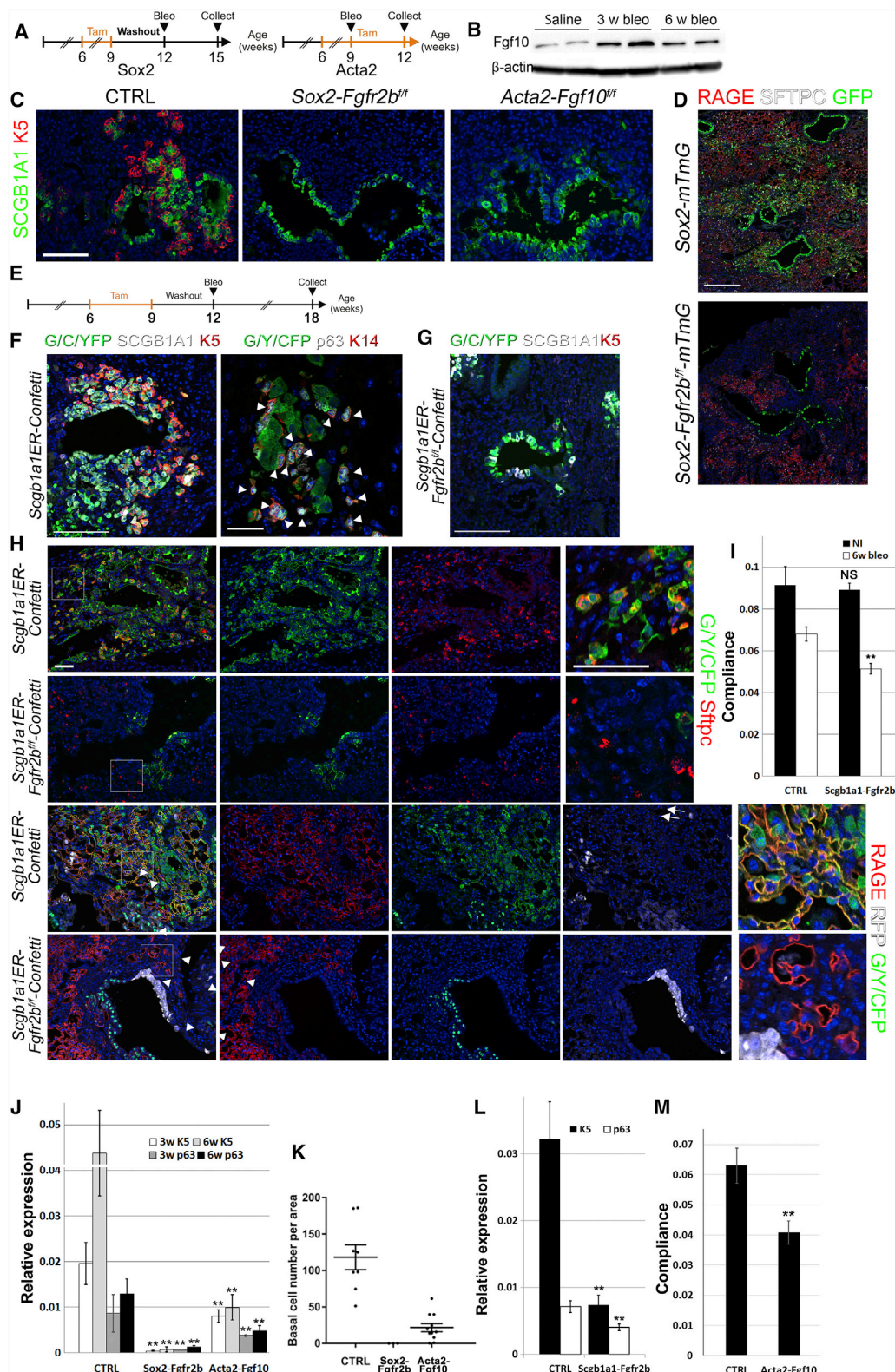
(A) β -Gal (blue) and oil red (LIF marker) staining on frozen sections of 3-month-old *Fgf10^{LacZ}* lungs shows that LIFs express *Fgf10*. (B) β -Gal (blue) and immunostaining for SFTPC (brown) on paraffin sections of 3-month-old *Fgf10^{LacZ}* lungs shows LIFs located adjacent to AT2 cells. (C and D) β -Gal and eosin staining on *Fgf10^{LacZ}* lungs 21 days after saline (C) and bleomycin treatment (D). Inset in (D) shows coimmunostaining for α -SMA (MYF marker) and β -gal (FGF10), demonstrating that LIFs transdifferentiate into MYFs after bleomycin injury. (E) Single-cell RNA-seq analysis on GFP⁺, EPCAM⁺, CD45⁺, TER119⁺ sorted fibroblasts isolated from *Tbx4^{CreERT2};mTmG* mice show fewer LIFs expressing higher levels of *Fgf10* in noninjured lungs compared with lungs 3 weeks after bleomycin injury. (F) Immunostaining for GFP and SFTPC (AT2 cell) on *Sftpc^{CreERT2};mTmG* and *Sftpc^{CreERT2};Fgfr2b^{f/f};mTmG* lungs 4 weeks after being placed on tamoxifen chow. (G) Immunostaining for GFP (AT2 cell and descendant) on *Sftpc^{CreERT2};mTmG* and *Sftpc^{CreERT2};Fgfr2b^{f/f};mTmG* lungs 6 weeks after bleomycin treatment. Scale bars, 10 μ m (A), 100 μ m (C), 50 μ m, and (F) 500 μ m (G).

FGF10-FGFR2B Signaling in Bronchial Epithelium Generates Neo-Basal and Alveolar Epithelial Cells after Bleomycin Injury

We recently reported that overexpression of *Fgf10* is sufficient to induce the expression of basal and AT2 cell markers in club cells (Volckaert et al., 2017). The dependence of both BCs and AT2 cells on FGF10-FGFR2B signaling raises the question of whether different levels of FGF10-FGFR2B signaling in bronchial epithelial cells may be required for alveolar epithelial regeneration versus BC expansion that leads to distal bronchiolization and generation of honeycomb cysts after bleomycin injury. To investigate this, we inactivated *Fgfr2b* in all bronchial epithelial cells using *Sox2^{CreERT2};Fgfr2b^{f/f}* mice or in club cells specifically using *Scgb1a1^{CreERT2};Fgfr2b^{f/f}* mice while simultaneously lineage tracing them with an mTmG or Confetti reporter. Our data suggest that both alveolar epithelial regeneration and the generation of neo-BCs at sites of injury is impaired in these mice (Figures 2A, 2C–2L, and S3A–S3E). This incapacity of BECs to invade the lung parenchyma after bleomycin injury resulted in large holes near the BADJ of injured mutant lungs (Figure S3E). Inactivation of *Fgf10*

in the stromal niche, airway smooth muscle cells (ASMCs) and myofibroblasts, using *Acta2^{CreERT2};Fgf10^{f/f}* mice, also blocks the generation of neo-BCs at the site of injury (Figures 2A, 2C, 2J, and 2K). Furthermore, we find a significant reduction in lung compliance 5–6 weeks after bleomycin injury in both *Scgb1a1^{CreERT2};Fgfr2b^{f/f}* and *Acta2^{CreERT2};Fgf10^{f/f}* mice compared with control littermates (Figures 2I, 2M, and S3E), indicating an impaired resolution of pulmonary fibrosis.

To verify that bronchial epithelial cells give rise to bona fide BCs and alveolar epithelial cells we performed single-cell transcriptome analysis on lineage-traced GFP⁺, Tomato⁺, CD45⁺, and TER119⁺ isolated cells from uninjured and bleomycin injured *Sox2^{CreERT2};mTmG* lungs, which were treated with tamoxifen food for 3 weeks followed by a 2-month washout period. We identified GFP⁺ tuft, hill, ionocyte, club, goblet, neuroendocrine, UPK3a, and ciliated cells in both uninjured and injured lungs, yet only found basal, AT2 and AT1 cells in injured *Sox2^{CreERT2};mTmG* lungs (Figure 3). A heatmap for gene expression levels of the latter three cell types clearly shows segregation and enrichment for classical basal, AT2, and AT1 cell markers in each



(legend on next page)



population, indicating that bronchial epithelial cells give rise to bona fide basal, AT2, and AT1 cells after bleomycin injury. Intriguingly, we found that AT2 cells express higher levels of *Fgfr2* than BCs suggesting a stronger dependence on FGF10 signaling. Unexpectedly, injured lungs also featured a distinct *de novo* previously undescribed ciliated cell population, which was *Gfp*⁺, *Sox2*⁺, *Foxj1*⁺, but *Epcam*[−] and *E-cadherin*[−] (*Cdh1*). Together, our data demonstrate for the first time, to our knowledge, the crucial contribution of BECs to alveolar epithelial regeneration and the resolution of pulmonary fibrosis after bleomycin injury, suggesting that BECs may not merely be considered as a reserve stem cell population.

Overexpression of *Fgf10* in Bronchial Epithelium Promotes the Generation of AT2 Cells over BCs

As our data suggest that FGF10-FGFR2B signaling is involved in BC generation and bronchiolization, as well as in alveolar epithelial regeneration, we questioned if the fate decision to either BCs or AT2 cells might depend on FGF10-FGFR2B signal strength. To investigate whether increased FGF10 signaling specifically in bronchial epithelial cells but not AT2 cells, promotes alveolar epithelial regeneration instead of bronchiolization, we generated *Sox2*^{CreERT2};*Rosa26-LSL-rtTA*;*Tet-Fgf10* mice, in which we can inducibly overexpress *Fgf10* specifically in bronchial epithelial cells and their descendants by placing mice on doxycycline chow. We found that overexpressing *Fgf10* in BECs for 3 weeks starting 2 weeks after bleomycin injury pushed BECs along the AT2 cell lineage (Figure 4A) result-

ing in a significant reduction in neo-BCs (Figure 4D). We further hypothesized that these new AT2 cells would differentiate into AT1 cells once *Fgf10* induction is terminated. To investigate this, we overexpressed *Fgf10* in BECs for 3 weeks starting 2 weeks after bleomycin injury and harvested the lungs 1 week after *Fgf10* induction was stopped (i.e., 6 weeks after bleomycin injury). Our findings indeed showed that bronchial epithelial cells acquire an AT2 fate as long as *Fgf10* is overexpressed (Figure 4A). However, once *Fgf10* expression was stopped, some of the new AT2 cells spontaneously differentiated into AT1 cells (Figure 4B). Thus, our data highlight the importance of a strict temporally regulated regimen of *Fgf10* induction and suggest that FGF10 could be exploited as a novel therapeutic approach to promote alveolar epithelial regeneration in IPF patients by quickly and transiently boosting their endogenous stem cell population. Interestingly, our findings also suggest that overexpression of *Fgf10* results in a decrease in collagen deposition as measured by hydroxyproline levels (Figure 4C), and a significant reduction in neo-BCs (Figure 4D). These data are consistent with the idea that fibrosis is a wound-healing response that occurs when epithelial stem cells are exhausted.

Neo-BCs Exhibit Indeterminate States of Differentiation

Our findings suggest that FGF10-FGFR2B signaling in distal airway SCGB1A1⁺ cells drives both alveolar epithelial regeneration and bronchiolization following bleomycin injury. These findings further suggest the possibility that

Figure 2. Distal Bronchial Epithelial Cells Reprogram into Neo-BCs and AT1/2 Cells upon Bleomycin Injury in Response to *Fgf10*-*Fgfr2b* Signaling

- (A) Experimental strategy of (C, J, and K).
- (B) Western blotting for FGF10 on wild-type (wt) saline-treated lungs and wt lungs 3 and 6 weeks after bleomycin injury.
- (C) Immunostaining on ctrl, *Sox2-Fgfr2b*^{f/f}, and *Acta2-Fgf10*^{f/f} lungs for SCGB1A1 (green) and keratin 5 (K5) (red) at 3 weeks after bleomycin injury.
- (D) Immunostaining on *Sox2-mTmG* and *Sox2-Fgfr2b*^{f/f}-*mTmG* lungs for SFTPC (white), RAGE (red), and GFP (green) at 6 weeks after bleomycin injury.
- (E) Experimental strategy of (D and F–H).
- (F) Immunostaining on *Scgb1a1ER-Confetti* lung for K5 (red), SCGB1A1 (white), or K14 (red), p63 (white), and G/Y/CFP (green) at 6 weeks after bleomycin injury. White arrowheads show K14, p63, and G/Y/CFP triple-positive cells, indicating the dedifferentiation of distal airway club cells into neo-BCs.
- (G) Immunostaining on *Scgb1a1ER-Fgfr2b*^{f/f}-*Confetti* lung for *Scgb1a1ER-Confetti* lung for K5 (red), SCGB1A1 (white), and G/Y/CFP (green), showing no basal cells near injury.
- (H) Immunostaining on *Scgb1a1ER-Confetti* and *Scgb1a1ER-Fgfr2b*^{f/f}-*Confetti* lungs for SFTPC (red), or RAGE (red) and RFP (white) and G/Y/CFP (green) (RFP and G/Y/CFP label the four different confetti clone cells) at 6 weeks after bleomycin injury.
- (I) Flexivent pulmonary function analysis (static compliance) on ctrl and *Scgb1a1ER-Fgfr2b*^{f/f} lungs 6 weeks after bleomycin injury.
- (J) Relative mRNA levels of *K5* and *p63* in ctrl, *Sox2-Fgfr2b*^{f/f}, and *Acta2-Fgf10*^{f/f} lungs 3 and 6 weeks after bleomycin injury.
- (K) Quantification of BC numbers in ctrl, *Sox2-Fgfr2b*^{f/f}, and *Acta2-Fgf10*^{f/f} lungs at 3 weeks after bleomycin injury.
- (L) qPCR of relative mRNA levels of *K5* and *p63* in ctrl and *Scgb1a1ER-Fgfr2b*^{f/f} lungs 6 weeks after bleomycin injury.
- (M) Flexivent pulmonary function analysis (static compliance) on ctrl and *Acta2*^{CreERT2}-*Fgf10*^{f/f} lungs 5 weeks after bleomycin injury. Nuclei were stained with DAPI (blue).

**p < 0.01, *p < 0.05; n ≥ 14; error bars mean ± SEM. Scale bars, 100 μm (C and H), 200 μm (F, left and G), 20 μm (F, right), 250 μm (D).

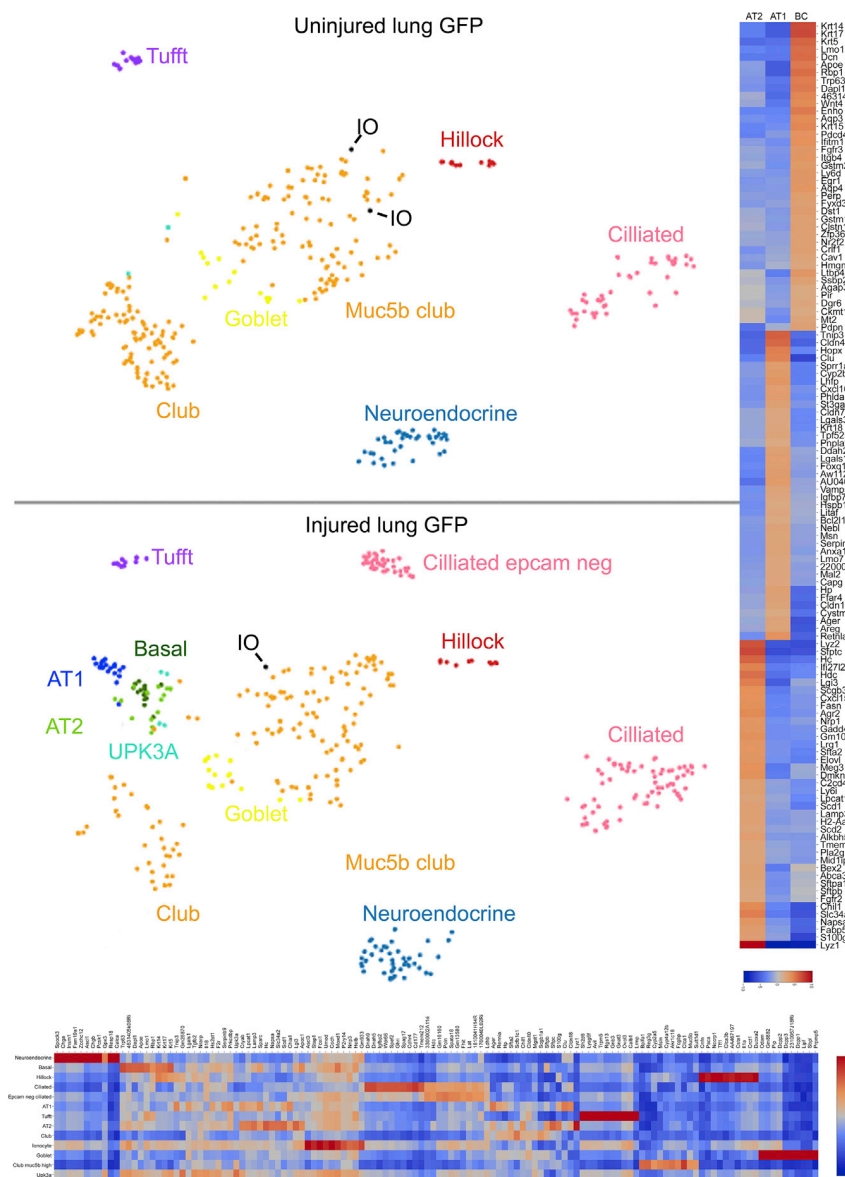


Figure 3. Single-Cell RNA-Seq Analysis of Lineage-Traced *Sox2^{CreERT2};mTmG* Cells 6 Weeks after Saline or Bleomycin Treatment
t-SNE plots show neuroendocrine cells, ciliated cells, hillock cells, tuft cells, ionocytes (IO), distal and proximal club cells, goblet cells, UPK3A cells, AT2, AT1, and BCs. Heatmaps show differential gene expression between the different subpopulations and/or AT1, AT2, and neo-BCs specifically highlighting cell-type signatures among both injured and uninjured lungs.

actin [α -SMA]) markers indicating that the same indeterminate states of differentiation observed in IPF also occur in mice after bleomycin injury (Figures 4E–4H and S4C).

It could be argued that these neo-BCs are derived from (broncho-alveolar stem cells [BASCs]) (Kim et al., 2005), which are club cells that co-express SCGB1A1⁺ and SFTPC⁺. To explore this possibility we lineage-labeled AT2 cells and BASCs using an *Sftpc^{CreERT2};mTmG* mouse line. We found that labeling the BASCs required high doses of tamoxifen, likely because *Sftpc* expression is lower in these cells than in AT2 cells. With lower doses of tamoxifen administration only AT2 cells were labeled (Figures S4A and S4B). Interestingly, we found that, when only AT2 cells were labeled before bleomycin injury, none of the neo-BCs that arose after injury were GFP⁺ (Figure S4A). However, when BASCs

some bronchial epithelial cells may initially, transiently reprogram into neo-BCs before giving rise to alveolar epithelial cells. Interestingly, recent single-cell transcriptome analyses show that individual epithelial cells within bronchiolized honeycomb regions of IPF lungs frequently co-express AT1, AT2, basal, and other conducting airway selective markers, and even mesenchymal or epithelial-mesenchymal transition (EMT) markers, demonstrating “indeterminate” states of differentiation not seen during normal lung development or homeostasis (Gokey et al., 2018; Xu et al., 2016). Our data also suggest that some of the neo-BCs in bronchiolized regions in IPF or mouse lungs after bleomycin injury co-express basal (p63), and AT2 cell (SFTPC) markers or mesenchymal (alpha smooth muscle

were labeled before injury neo-BCs were also lineage labeled (Figures 4I and 4J). Note that the lineage-labeled cells are SCGB1A1⁺ and K5⁺.

Our data further suggest that some neo-BCs that appear after bleomycin injury transiently express the mesenchymal marker α -SMA (Figure 4H) approximately 2 weeks after injury. This is indicative of a partial EMT-like migratory phenotype, and is remarkably similar to myoepithelial cells, a stem cell population located in the submucosal glands (Anderson et al., 2017; Lynch et al., 2016; Volckert and De Langhe, 2014), which also co-express BC (K5, p63) and mesenchymal markers (α -SMA) (Lynch et al., 2018; Tata et al., 2018). Interestingly, we previously reported how FGF10-FGFR2B signaling induces a partial

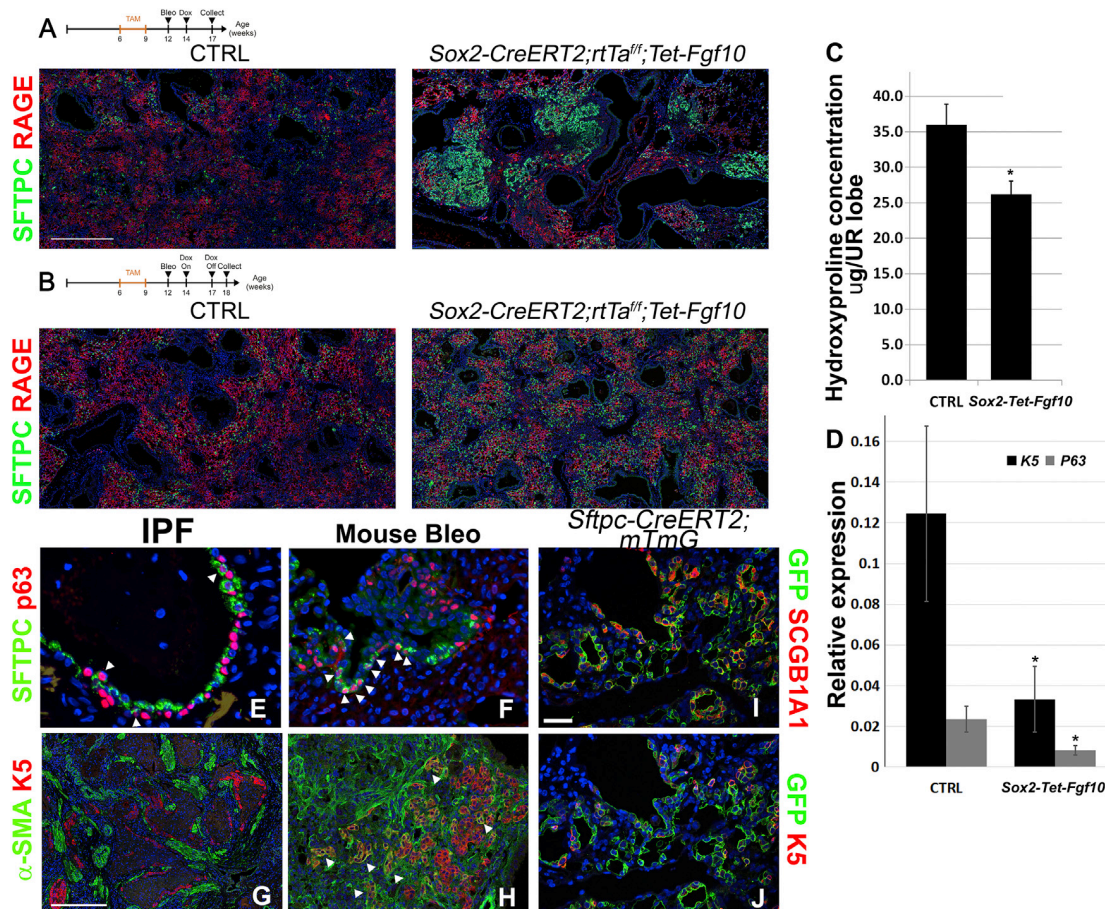


Figure 4. Overexpression of *Fgf10* in Bronchial Epithelial Cells after Bleomycin Injury Promotes AT2 Cell Differentiation

(A and B) Coimmunostaining for RAGE (AT1 cell) and/or SFTPC (AT2 cell) on *Sox2*^{CreERT2};LSL-rtTa;Tet-Fgf10 lungs that received intratracheal bleomycin at 2 months of age and were placed on doxycycline-containing chow 2 weeks after bleomycin injury for 3 weeks and were harvested either at 5 (A) or 6 (B) weeks after injury.

(C) Hydroxyproline measurements for collagen content in ctrl and Sox2CreERT2; LSL-rtTa;Tet-Fgf10 mice 6 weeks after bleomycin injury (n ≥ 15).

(D) Relative mRNA levels of K5, p63 in ctrl and Sox2^{CreERT2};LSL-rtTa;Tet-Fgf10 mice 6 weeks after bleomycin injury (n ≥ 15).

(E–J) Coimmunostaining for SFTPC (green) and p63 (red) (E and F) or α-SMA (green) and K5 (red) (G and H) on honeycomb regions in lungs from IPF patients and mouse lungs 18 days after bleomycin injury. White arrowheads (E and F) show SFTPC and p63 double-positive cells. (I and J) Immunostaining on *Sftpc*^{CreERT2};mTmG lungs for SCGB1A1 (red) or K5 (red) and GFP (green) at 3 weeks after bleomycin injury. Nuclei were stained with DAPI (blue). White arrowheads (H) show α-SMA (green) and K5 (red) double-positive cells.

*p < 0.05; n ≥ 14; error bars mean ± SEM. Scale bars, 500 μm (A and B), 100 μm (G and H), and 50 μm (E, F, I, and J).

EMT-like phenotype in a subset of club cells that drive airway epithelial regeneration after naphthalene injury (Volckaert et al., 2011). While single-cell transcriptomics has identified a population of indeterminate epithelial cells with mesenchymal traits in IPF lungs (Xu et al., 2016), we have not yet been able to visualize K5⁺ α-SMA⁺ double-positive cells in IPF lungs (Figure 4G). This suggests the possible transient nature of these “elusive” cells, which may be more abundant during the early stages of disease.

FGFR2B Signaling Is Required for Neo-BCs to Differentiate into AT2 Cells during the Resolution of Bleomycin IPF

To investigate the possibility that some neo-BCs might be able to differentiate into alveolar epithelial cells during the resolution of bleomycin-mediated injury in mice, we lineage-traced neo-BCs after bleomycin injury using *Krt5*^{CreERT2};mTmG mice (with tamoxifen treatment starting at 17 days after bleomycin injury; because the first neo-BCs only appear 14 days after injury). When we followed the

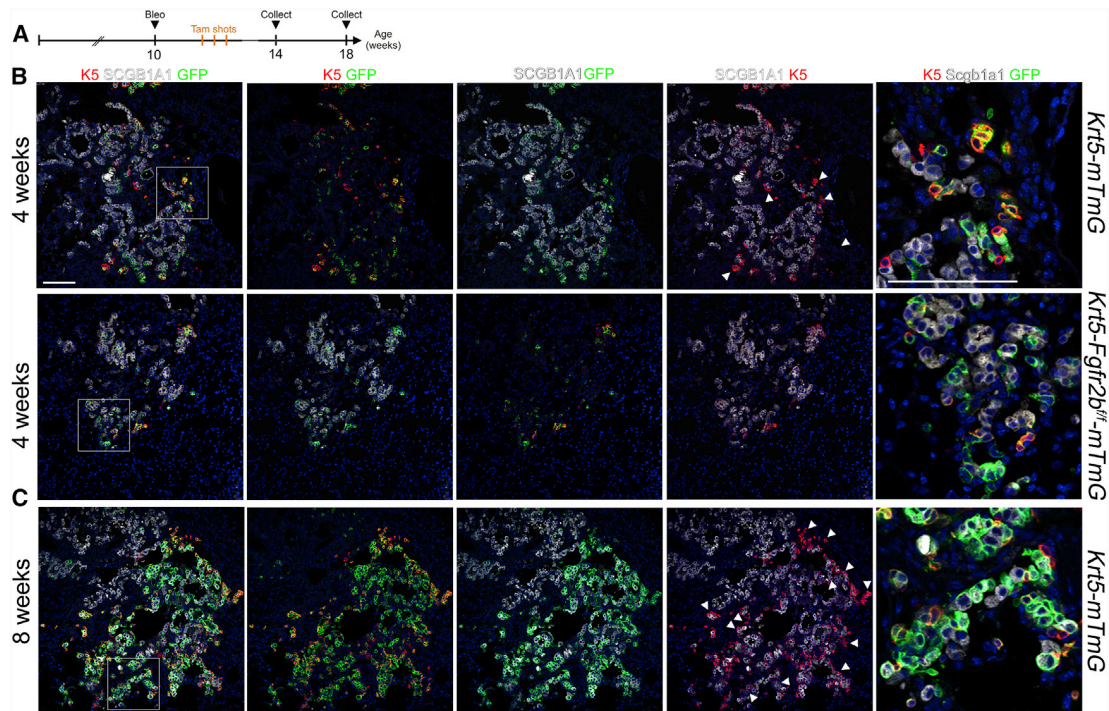


Figure 5. Neo-BCs Require Fgfr2b Signaling for their Maintenance

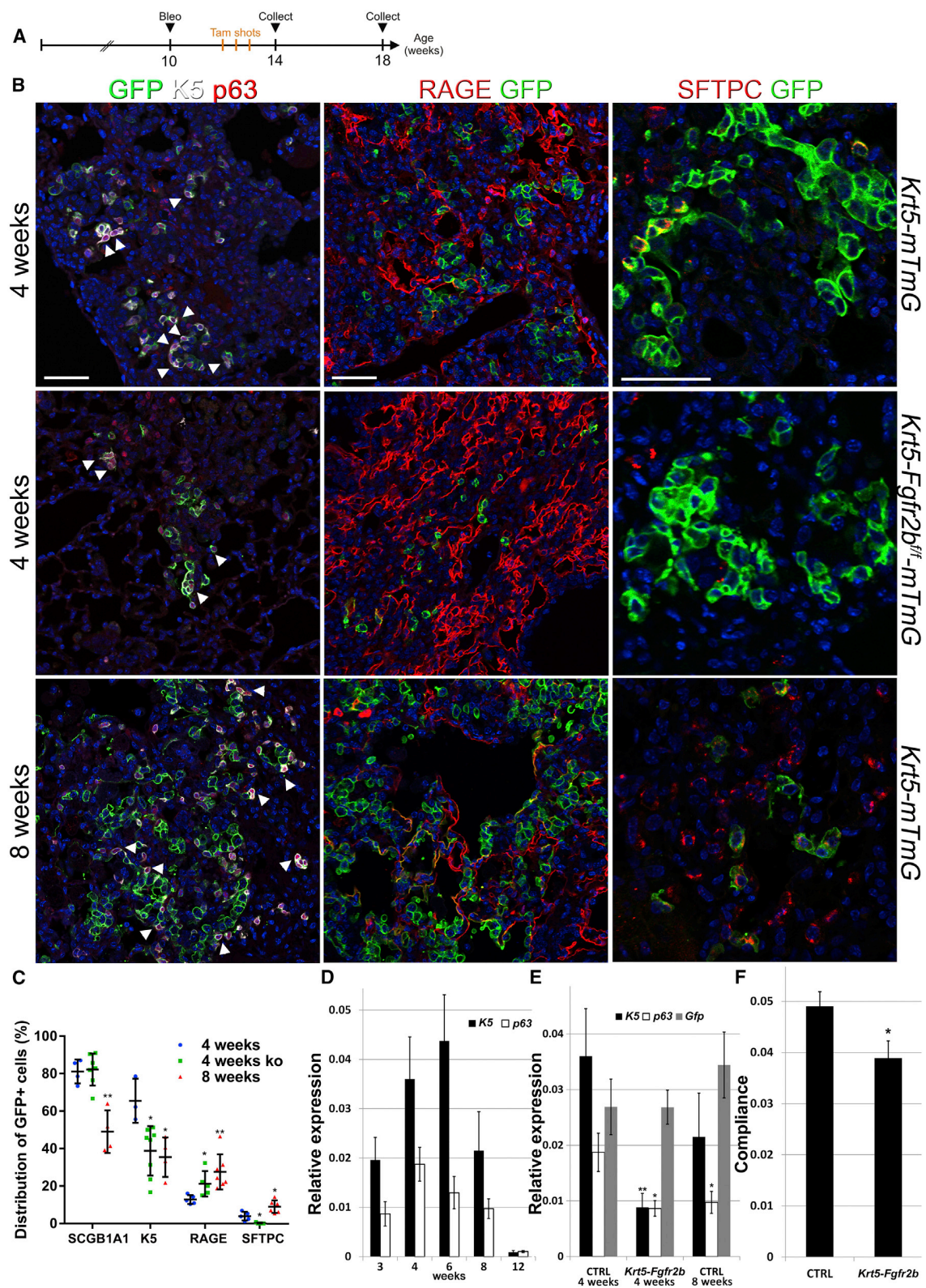
(A) Experimental strategy of (B). Induction with tamoxifen was performed at days 17, 19, and 21 after bleomycin injury. (B and C) Immunostaining on *Krt5-mTmG* lungs and *Krt5-Fgfr2b^{fl/fl}-mTmG* lungs to localize keratin 5 (K5) (red), SCGB1A1 (white), and GFP (green) (left) at 4 (B) or 8 weeks (C) after bleomycin injury. White arrowheads (B and C) show K5 (red) positive and SCGB1A1 (white) negative cells. Nuclei were stained with DAPI (blue). Scale bars, 100 μ m (B and C).

fate of these cells to 11 days or 4 weeks after bleomycin injury, we found that $\sim 65\%$ of the GFP-labeled cells were still positive for KRT5 and p63; however, $\sim 35\%$ of GFP-labeled cells no longer expressed these BC markers. Interestingly, we found that, at 4 weeks after bleomycin injury, $\sim 13\%$ of GFP⁺ cells were positive for the AT1 cell marker RAGE, whereas about $\sim 4\%$ stained positive for SFTPC, an AT2 cell marker (Figures 5A, 5B, and 6A–6C). Intriguingly, we also found that $\sim 81\%$ of GFP⁺ cells were positive for SCGB1A1, a club cell marker (Figures 5A, 5B, and 6A–6C), again indicating that neo-BCs that appear after bleomycin injury are derived from *Sox2⁺/Scgba1a1⁺* bronchial epithelial cells or that these BCs also give rise to club cells. As fibrosis resolution is not complete by 4 weeks after bleomycin injury, we lineage traced these cells until 8 weeks after injury. At 8 weeks after bleomycin injury, only $\sim 35\%$ of GFP⁺ cells still expressed BC markers, whereas $\sim 28\%$ of GFP⁺ cells had differentiated into RAGE⁺ AT1 cells, and $\sim 9\%$ had differentiated in SFTPC⁺ AT2 cells (Figures 5A, 5C, 6A–6C, and S5). Interestingly, $\sim 49\%$ of GFP⁺ cells stained for SCGB1A1 at 8 weeks after bleomycin injury.

A time course of BC abundance after bleomycin injury further indicated that neo-BCs first appeared in the paren-

chyma around 2 weeks after injury (not shown) and peaked at around 4–6 weeks after injury after which their numbers slowly declined (Figure 6D). However, when total lung *Gfp* expression levels were measured we observed a trend toward increased *Gfp* levels (and GFP⁺ cells) at 8 weeks after bleomycin injury compared with 4 weeks after bleomycin injury (Figure 6E) suggesting that the lineage-labeled neo-BCs can differentiate and that their offspring persists, contributing to alveolar epithelial regeneration.

We next hypothesized that FGF10-FGFR2B signaling could also be required for the maintenance of these neo-BCs after bleomycin injury as well as their subsequent differentiation into AT1 and AT2 cells. To investigate this we inactivated *Fgfr2b* in bleomycin injury-induced neo-BCs using *Krt5^{CreERT2};Fgfr2b^{fl/fl};Rosa26^{mTmG}* mice. Interestingly, these mice die around 30 days after bleomycin injury or 13 days after inactivation of FGFR2B. By 4 weeks after injury we found a strong reduction in *Krt5* and *p63* expression, suggesting decreased BCs, but not in lineage-labeled *Gfp* expression, suggesting that FGFR2B signaling is also required for neo-BC maintenance (Figure 6E). Consistently, we found that at 4 weeks after bleomycin injury, only $\sim 39\%$ of the GFP-labeled cells in *Krt5-Fgfr2b^{fl/fl}-mTmG* lungs



(legend on next page)



remain positive for KRT5, whereas ~21% of GFP⁺ cells differentiated into RAGE⁺ AT1 cells, while none of the GFP⁺ cells had differentiated into AT2 cells (Figures 6A–6C). The lack of AT2 cell differentiation is consistent with a requirement for FGF10-FGFR2B signaling in AT2 cell maintenance (Figure 1F). Interestingly, similar to control mice, ~82% of GFP-labeled cells were positive for SCGB1A1 (Figures 5A, 5B, and 6A–6C). Furthermore, pulmonary function testing indicated that *Krt5^{CreERT2};Fgfr2b^{fl/f};mTmG* mice, whereby we effectively inactivated *Fgfr2b* in neo-BCs after bleomycin injury, showed a significant reduction in static lung compliance compared with control mice 4 weeks after bleomycin injury, suggestive of impaired resolution of pulmonary fibrosis (Figure 6F). These data, therefore, suggest that neo-BCs play a regenerative role following lung injury, by slowly giving rise to AT1 and AT2 cells and that FGFR2B signaling is required for their maintenance and proper differentiation.

In summary, our data suggest that FGF10-FGFR2B signaling drives the reprogramming of *p63⁺/Scgb1a1⁺/Sox2⁺* BECs in the distal airway into neo-BCs after bleomycin injury and their further differentiation into AT1 and AT2 cells. When FGF10-FGFR2B signaling is inhibited before injury, neo-BCs are not generated, leaving behind large cystic structures arising as a result of impaired alveolar epithelial regeneration by conducting airway epithelium (Figure S3E). Our data also suggest that FGF10-FGFR2B signaling is required for the maintenance of these injury-induced neo-BCs, some of which differentiate back into club cells or slowly give rise to AT1 but not AT2 cells when FGF10-FGFR2B signaling is abrogated.

Translation Significance of FGF10 Signaling in Human IPF Lung Slices in which FGF10 Promotes the Differentiation of BCs in Honeycomb Cysts along the AT2 Lineage

Based on our findings in mice we further validated a role for FGF10 signaling in the reprogramming of BCs within honeycomb cysts in IPF tissue along the AT2 cell lineage. To assess the potential for FGF10 to reprogram the BCs in the honeycomb cysts of IPF patients into AT2 cells we cultured

live precision cut IPF lung slices in the presence or absence of FGF10. Treatment with FGF10 resulted in a ~1.6-fold in CK5⁺ HTII-280⁺ AT2 cells and a 1.5-fold increase in CK5⁺ HTII-280⁺ double-positive cells versus a 13% decrease in CK5⁺ HTII-280[−] BCs after FGF10 treatment, supporting the concept that FGF10 signaling can push BCs in IPF tissue along the AT2 cell lineage (Figure 7A).

DISCUSSION

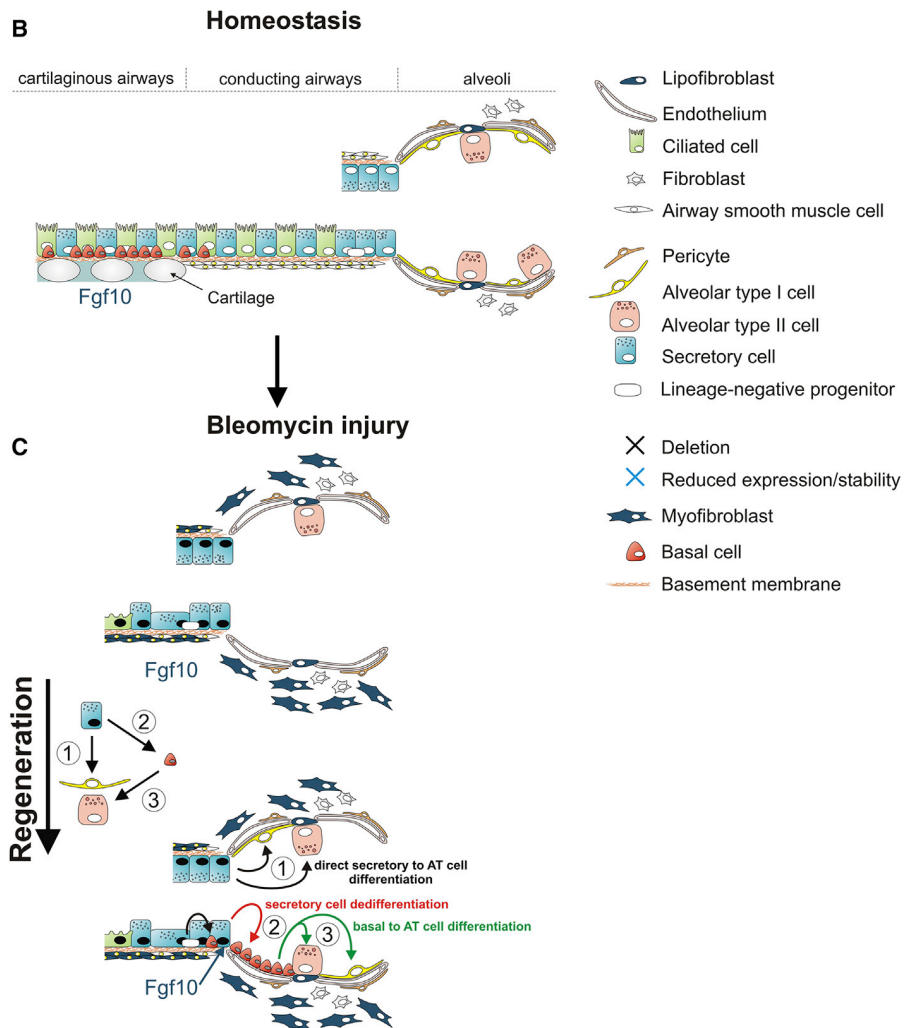
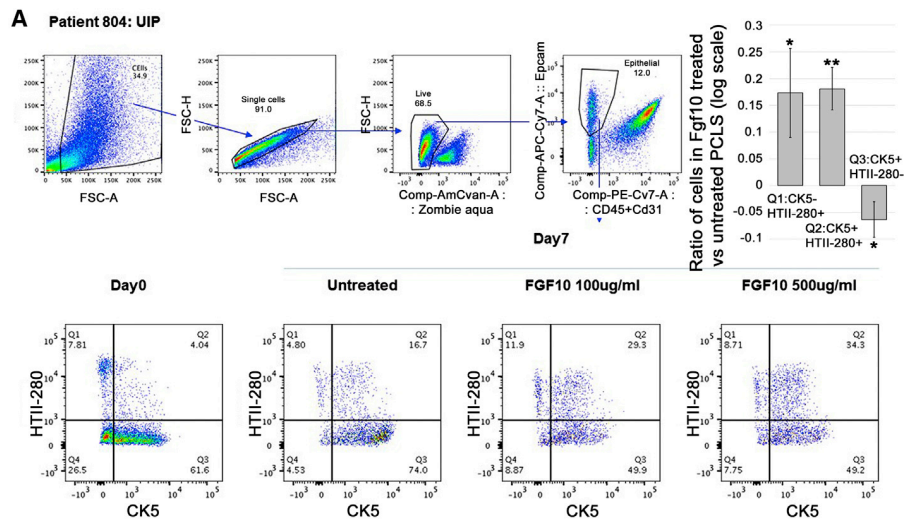
The major finding of our study is that upon acute bleomycin-mediated lung injury, FGF10-FGFR2B signaling generates BCs and drives alveolar epithelial regeneration by BECs to promote the resolution of pulmonary fibrosis (Figures 7B and 7C). Upon airway epithelial injury, ASMCs, which wrap the conducting airways, as well as myofibroblasts, are activated and release FGF10 to become an injury-induced stem/progenitor cell niche. FGF10 from the stromal niche drives the dedifferentiation of distal airway club cells into neo-BCs and alveolar epithelial cells. Interestingly our data suggest that FGF10-FGFR2B signaling is necessary to maintain and amplify the pool of neo-BCs and that it is critical to drive their differentiation into AT2 cells, whereas in the absence of FGFR2B signaling these neo-BCs primarily give rise to AT1 cells. This is consistent with our findings that FGF10-FGFR2B signaling is essential for AT2 stem cell maintenance.

Our lineage-tracing data argue that the induction of *Fgf10* expression by the stromal niche allows for the dedifferentiation of a subset of distal airway club cells. As such, we found that basal stem/progenitor cells were lineage labeled within each clonal expansion and dispersal of labeled club daughter cells into the alveoli of *Scgb1a1^{CreER};Confetti* lungs. In addition, when neo-BCs were lineage tagged after bleomycin injury using *Krt5-mTmG* mice, ~80% were found to co-express the club cell marker SCGB1A1. These findings are consistent with recent reports (Yang et al., 2018).

Our finding that FGF10-FGFR2B signaling is critical for club cell dedifferentiation and subsequent differentiation into alveolar epithelial cells allowed us for the first time

Figure 6. BCs that Appear through Reprogramming of *Scgb1a1⁺* Cells after Bleomycin Injury Require *Fgfr2b* Signaling for their Maintenance and to Differentiate into Alveolar Epithelial Cells

- (A) Experimental strategy of (B–F). Tamoxifen induction was performed on days 17, 19, and 21 after bleomycin injury.
 (B) Immunostaining on *Krt5-mTmG* lungs and *Krt5-Fgfr2b^{fl/f}-mTmG* lungs for p63 (red), K5 (white), and GFP (green) or RAGE (red) and GFP (green) (middle) or SFTPC (red) and GFP (green) (right) at 4 and/or 8 weeks after bleomycin injury. White arrowheads show p63 (red), K5 (white), and GFP (green) triple-positive cells.
 (C) Quantification of the distribution of GFP⁺ cells in (B).
 (D) Relative mRNA levels of *K5* and *p63* at 3, 4, 6, 8, and 12 weeks after bleomycin injury showing time course of BC abundance.
 (E) Relative mRNA levels of *K5*, *p63*, and *Gfp* in control and *Krt5-Fgfr2b^{fl/f}* lungs at 4 weeks after bleomycin injury.
 (F) Flexivent pulmonary function analysis measured static compliance on control and *Krt5-Fgfr2b^{fl/f}* lungs at 4 weeks after bleomycin injury. Nuclei were stained with DAPI (blue). **p < 0.01, *p < 0.05; n ≥ 14; error bars mean ± SEM. Scale bars, 50 μm (B).



(legend on next page)



to investigate the importance of these distal airway club cells or BASCs (Kim et al., 2005) in alveolar epithelial regeneration. Our data suggest for the first time that under normal conditions BSCs do play an essential role in alveolar epithelial regeneration. This is particularly intriguing because these cells are also the source of Muc5b, which has been associated with the development of pulmonary fibrosis (Nakano et al., 2016; Seibold et al., 2011).

With age, *Fgf10* expression goes down and our data strongly suggest that in young lungs FGF10-FGFR2B signaling drives the reprogramming of bronchial epithelial cells into alveolar epithelial cells, and that FGF10 treatment can possibly help misguided BCs in honeycomb cysts in IPF tissue along the right path. However, we believe that this process does not take place in patients with IPF likely because *Fgf10* expression goes down with age. It is to be hoped that these novel findings will result in new therapeutic options for the treatment of IPF.

Finally, the bleomycin mouse model for modeling human IPF has its pros and cons. For a long time, the mouse bleomycin model was not thought to generate honeycomb cysts-like structures. However, bronchiolarization and the appearance of BCs only occurs upon severe or repetitive bleomycin injury and is not observed under conditions of mild injury. The bleomycin mouse model in young mice also resolves over time, which allows us to study the regenerative capacity of the different epithelial stem cells. While human IPF is a fatal disease that does not resolve, it is also a disease of aging and stem cell exhaustion. It is therefore likely that the capacity to recover from injury also diminishes with time in humans.

EXPERIMENTAL PROCEDURES

Experimental Model and Subject Details

All mice were bred and maintained in a pathogen-free environment with free access to food and water. Both male and female mice were used for all experiments. *Fgfr2b^{fl/fl}* (De Moerloose et al., 2000), *Sox2^{CreERT2}* (JAX 017593), *Tet-Fgf10* (Clark et al., 2001), *Rosa26-rtTa* (Volckaert et al., 2011), *Rosa26-rtTa^{fl/fl}* (JAX 005670), *Rosa26⁺ Rosa26R-Confetti* (JAX 017492), *Scgb1a1^{CreER}* (JAX 016225), *Rosa26-mTmG* (JAX 007676), *Krt5^{CreERT2}* (Van Keymeulen et al., 2011), *Acta2^{CreERT2}* (Wendling et al., 2009), *Fgf10^{fl/fl}*

(Urness et al., 2010), *Sftpc^{CreERT2}* (Chapman et al., 2011), and *Fgf10^{LacZ}* (Kelly et al., 2001) mice have been described.

For bleomycin injury, adult 8- to 12-week-old mice were intratracheally instilled with 50 μ L bleomycin (0.8–2 U/kg body weight optimized for each strain, batch of bleomycin, and gender) as described previously (Redente et al., 2014). Lung function was analyzed using lung measurements of static compliance by Flexivent (SQUIRE). Bleomycin injury was induced after a 21-day washout period following tamoxifen induction. For tamoxifen induction, mice were placed on tamoxifen-containing food (rodent diet with 400 mg/kg tamoxifen citrate; Harlan Teklad TD.130860) for 2 to 3 weeks. *Scgb1a1^{CreER};Fgfr2b^{fl/fl};Rosa26R-Confetti* and control mice also received two additional intraperitoneal tamoxifen citrate shots (0.20 mg/g body weight, Enzo Life Sciences) in the last week of tamoxifen citrate feed. *Krt5CreER;Fgfr2b^{fl/fl};Rosa26-mTmG* and control mice received intraperitoneal tamoxifen citrate shots (0.125 mg/g body weight) on day 17, 19, and 21 after bleomycin injury. For doxycycline induction: mice were placed on doxycycline-containing food (rodent diet with 625 mg/kg dox; Harlan Teklad TD.09761). All experiments were approved by the National Jewish Health and University of Alabama at Birmingham Institutional animal care and use committee.

Single-Cell RNA-Seq

Sox2^{CreERT2};mTmG mice were placed on tamoxifen food for 3 weeks starting at 2 months of age, followed by a 2-month washout period before saline or bleomycin treatment. Whole lung tissues were collected, from lineage-traced mice 6 weeks after saline or bleomycin treatment. Single cells were processed through the GemCode Single Cell Platform using the GemCode Gel Bead, Chip and Library Kits (V1) or single-cell suspensions were loaded onto 3' library chips for the Chromium Single Cell 3' Library (V2, PN-120267) according to the manufacturer's recommendations (10X Genomics). Libraries were sequenced on an Illumina NextSeq 500 at >190,000 reads per cell. Further details on single-cell RNA-seq analysis and the fluorescence-activated cell sorting (FACS) protocol can be found in the Supplemental Experimental Procedures.

Immunofluorescence Microscopy and qRT-PCR

Details of all immunostaining protocols, antibodies employed, and quantitation of gene expression by qRT-PCR are provided in the Supplemental Experimental Procedures.

Microscopy and Imaging

Tissue was imaged using a micrometer slide calibrated Zeiss LSM800 Laser scanning confocal microscope using ZEN imaging software. In

Figure 7. BCs in Precision Cut IPF Lung Slices Treated with Fgf10 Differentiate into AT2 Cells

(A) Flow sorting for alveolar AT2 marker HTII-280 and CK5 on precision-cut lung slices cultured in the absence or presence of FGF10. Graph displayed as the log of the fold change shows an increase in CK5⁺ HTII-280⁺ and CK5⁺ HTII-280⁺ double-positive cells but a decrease in CK5⁺ HTII-280[−] cells after FGF10 treatment. **p < 0.01, *p < 0.05; n = 5; error bars mean $\pm$ SEM.

(B and C) Model showing how FGF10-FGFR2B maintains and generates basal-like stem cells by activating the stromal niche during homeostasis and epithelial regeneration. (B) During homeostasis, BCs are only found in the trachea, where they depend on FGF10 secreted by the intercartilage stromal tissue. (C) The dedifferentiation of distal airway club cells after major lung epithelial injury critically depends on FGF10-FGFR2B signaling. Note that, in addition to *Fgf10*-expressing ASMCs, activated myofibroblasts may further provide a strong additional source of FGF10 to allow for BC mobilization to injured regions. At injured sites, neo-BCs give rise to AT cells.



the lung, cells were counted using tiled stitched 20× images covering the entire cross-section of the left lung lobe from ≥ 6 different lungs. Images were processed and analyzed using ZEN blue (Zeiss) and Adobe Photoshop Creative Suite 3 (Adobe) software. Differentiation of GFP⁺ cells was determined by quantifying the total number of GFP⁺ cells that also showed a DAPI-stained nucleus, and how many of those were RAGE, K5, p63, or SFTPC⁺. Image quantification and analysis were performed in a double-blinded fashion.

Precision Cut Lung Slices

Human lung tissues from non-IPF donors and IPF patients undergoing lung transplantation were obtained from the Giessen biobank. The study protocol was approved by the ethics committee of the University of Giessen that conforms to the principles outlined in the declaration of Helsinki.

Vibratome slices (400 μ m) were made from low melting agarose filled de-identified human IPF transplant lung tissue. Precision cut lung slices were then cultured at the air-liquid interface in RPMI +10% Human Serum (Seraclot), ITS, Pen/Strep, Amphotericin B in the presence or absence of 500 ng/mL FGF10 for 3 to 7 days. Subsequently, slices were analyzed via FACS for basal (Abcam and AT2; Terrace Biotech, HT2-280, 1:150) cell markers.

Quantification and Statistical Analysis

All results are expressed as mean values $\pm$ SEM. The “n” represents biological replicates and can be found in the figure legends. The significance of differences between two sample means was determined by unpaired Student’s t-test (assuming unequal or equal variances as determined by the F test of equality of variances). All datasets followed a normal distribution and p values < 0.05 were considered statistically significant. The number of samples to be used was based on the number of experimental paradigms multiplied by the number in each group that is necessary to yield statistically significant results based on power analysis, to reject the null hypothesis with 80% power (type I error = 0.05).

ACCESSION NUMBERS

The accession number for the raw data files of RNA-seq analyses reported in this paper is GEO: GSE129079.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.stemcr.2019.04.003>.

AUTHOR CONTRIBUTIONS

T.Y. and T.V. designed and performed the experiments, analyzed the data, and wrote and edited the manuscript. E.F.R., S.H., K.K., R.W., J.Y., C.-M.C., B.R.S., and C.Y. performed the experiments. E.F.R., J.-S.Z., S.M., B.R.S., A.G., D.W.H.R., S.B., and V.J.T. edited the manuscript. S.P.D.L. conceived and led the project, performed experiments, analyzed data, and wrote and edited the manuscript.

ACKNOWLEDGMENTS

This study was supported by NIH R01 HL107307, DFG (BE443/4-1 and BE443/6-1), Loewe and UKGM (to S.B.), VA-CDA

11K2BX002401-01A2 (to E.R.), NIH R01 HL140595 and VA Merit I01 BX003471 (to D.R.), NIH RO1 HL135163 and PO1 HL108793 (to B.R.S.), and NIH R01 HL126732, HL132156, HL146461, and the Pulmonary Fibrosis Foundation Albert Rose established investigator award to S.D.L.

Received: March 3, 2017

Revised: April 1, 2019

Accepted: April 2, 2019

Published: May 2, 2019

REFERENCES

- Anderson, P.J., Lynch, T.J., and Engelhardt, J.F. (2017). Multipotent myoepithelial progenitor cells are born early during airway submucosal gland development. *Am. J. Respir. Cell Mol. Biol.* **56**, 716–726.
- Balasooriya, G.I., Goschorska, M., Piddini, E., and Rawlins, E.L. (2017). FGFR2 is required for airway basal cell self-renewal and terminal differentiation. *Development* **144**, 1600–1606.
- Barkauskas, C.E., and Noble, P.W. (2014). Cellular mechanisms of tissue fibrosis. 7. New insights into the cellular mechanisms of pulmonary fibrosis. *Am. J. Physiol. Cell Physiol.* **306**, C987–C996.
- Barkauskas, C.E., Crouce, M.J., Rackley, C.R., Bowie, E.J., Keene, D.R., Stripp, B.R., Randell, S.H., Noble, P.W., and Hogan, B.L. (2013). Type 2 alveolar cells are stem cells in adult lung. *J. Clin. Invest.* **123**, 3025–3036.
- Camelo, A., Dunmore, R., Sleeman, M.A., and Clarke, D.L. (2014). The epithelium in idiopathic pulmonary fibrosis: breaking the barrier. *Front. Pharmacol.* **4**, 173.
- Chanda, D., Kurundkar, A., Rangarajan, S., Locy, M., Bernard, K., Sharma, N.S., Logsdon, N.J., Liu, H., Crossman, D.K., Horowitz, J.C., et al. (2016). Developmental reprogramming in mesenchymal stromal cells of human subjects with idiopathic pulmonary fibrosis. *Sci. Rep.* **6**, 37445.
- Chapman, H.A., Li, X., Alexander, J.P., Brumwell, A., Lorzio, W., Tan, K., Sonnenberg, A., Wei, Y., and Vu, T.H. (2011). Integrin $\alpha 6 \beta 4$ identifies an adult distal lung epithelial population with regenerative potential in mice. *J. Clin. Invest.* **121**, 2855–2862.
- Clark, J.C., Tichelaar, J.W., Wert, S.E., Itoh, N., Perl, A.K., Stahlman, M.T., and Whitsett, J.A. (2001). FGF-10 disrupts lung morphogenesis and causes pulmonary adenomas in vivo. *Am. J. Physiol. Lung Cell Mol. Physiol.* **280**, L705–L715.
- El Agha, E., Moiseenko, A., Kheirollahi, V., De Langhe, S., Crnkovic, S., Kwapiszewska, G., Kusanovic, D., Schwind, F., Schermuly, R.T., Henneke, I., et al. (2017). Two-way conversion between lipogenic and myogenic fibroblastic phenotypes marks the progression and resolution of lung fibrosis. *Cell Stem Cell* **20**, 261–273.e3.
- Evans, C.M., Williams, O.W., Tuvim, M.J., Nigam, R., Mixides, G.P., Blackburn, M.R., DeMayo, F.J., Burns, A.R., Smith, C., Reynolds, S.D., et al. (2004). Mucin is produced by Clara cells in the proximal airways of antigen-challenged mice. *Am. J. Respir. Cell Mol. Biol.* **31**, 382–394.



- Gokey, J.J., Sridharan, A., Xu, Y., Green, J., Carraro, G., Stripp, B.R., Perl, A.T., and Whitsett, J.A. (2018). Active epithelial Hippo signaling in idiopathic pulmonary fibrosis. *JCI Insight* 3. <https://doi.org/10.1172/jci.insight.98738>.
- Guha, A., Deshpande, A., Jain, A., Sebastiani, P., and Cardoso, W.V. (2017). Uroplakin 3a+ cells are a distinctive population of epithelial progenitors that contribute to airway maintenance and post-injury repair. *Cell Rep.* 19, 246–254.
- Hancock, L.A., Hennessy, C.E., Solomon, G.M., Dobrinskikh, E., Estrella, A., Hara, N., Hill, D.B., Kissner, W.J., Markovetz, M.R., Grove Villalon, D.E., et al. (2018). Muc5b overexpression causes mucociliary dysfunction and enhances lung fibrosis in mice. *Nat. Commun.* 9, 5363.
- Kelly, R.G., Brown, N.A., and Buckingham, M.E. (2001). The arterial pole of the mouse heart forms from Fgf10-expressing cells in pharyngeal mesoderm. *Dev. Cell* 1, 435–440.
- Van Keymeulen, A., Rocha, A.S., Ousset, M., Beck, B., Bouvencourt, G., Rock, J., Sharma, N., Dekoninck, S., and Blanpain, C. (2011). Distinct stem cells contribute to mammary gland development and maintenance. *Nature* 479, 189–193.
- Kim, C.F., Jackson, E.L., Woolfenden, A.E., Lawrence, S., Babar, I., Vogel, S., Crowley, D., Bronson, R.T., and Jacks, T. (2005). Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* 121, 823–835.
- King, T.E., Jr., Pardo, A., and Selman, M. (2011). Idiopathic pulmonary fibrosis. *Lancet* 378, 1949–1961.
- Klar, J., Blomstrand, P., Brunmark, C., Badhai, J., Hakansson, H.F., Brange, C.S., Bergendal, B., and Dahl, N. (2011). Fibroblast growth factor 10 haploinsufficiency causes chronic obstructive pulmonary disease. *J. Med. Genet.* 48, 705–709.
- Kropski, J.A., Blackwell, T.S., and Loyd, J.E. (2015). The genetic basis of idiopathic pulmonary fibrosis. *Eur. Respir. J.* 45, 1717–1727.
- Kumar, P.A., Hu, Y., Yamamoto, Y., Hoe, N.B., Wei, T.S., Mu, D., Sun, Y., Joo, L.S., Dagher, R., Zielonka, E.M., et al. (2011). Distal airway stem cells yield alveoli in vitro and during lung regeneration following H1N1 influenza infection. *Cell* 147, 525–538.
- Lee, J.H., Bhang, D.H., Beede, A., Huang, T.L., Stripp, B.R., Bloch, K.D., Wagers, A.J., Tseng, Y.H., Ryeom, S., and Kim, C.F. (2014). Lung stem cell differentiation in mice directed by endothelial cells via a BMP4-NFATc1-thrombospondin-1 axis. *Cell* 156, 440–455.
- Li, S., Wang, Y., Zhang, Y., Lu, M.M., DeMayo, F.J., Dekker, J.D., Tucker, P.W., and Morrissey, E.E. (2012). Foxp1/4 control epithelial cell fate during lung development and regeneration through regulation of anterior gradient 2. *Development* 139, 2500–2509.
- Lynch, T.J., Anderson, P.J., Xie, W., Crooke, A.K., Liu, X., Tyler, S.R., Luo, M., Kusner, D.M., Zhang, Y., Neff, T., et al. (2016). Wnt signaling regulates airway epithelial stem cells in adult murine submucosal glands. *Stem Cells* 34, 2758–2771.
- Lynch, T.J., Anderson, P.J., Rotti, P.G., Tyler, S.R., Crooke, A.K., Choi, S.H., Montoro, D.T., Silverman, C.L., Shahin, W., Zhao, R., et al. (2018). Submucosal gland myoepithelial cells are reserve stem cells that can regenerate mouse tracheal epithelium. *Cell Stem Cell* 22, 653–667.e5.
- McQualter, J.L., Yuen, K., Williams, B., and Bertoncello, I. (2010). Evidence of an epithelial stem/progenitor cell hierarchy in the adult mouse lung. *Proc. Natl. Acad. Sci. U S A* 107, 1414–1419.
- De Moerloose, L., Spencer-Dene, B., Revest, J.M., Hajihosseini, M., Rosewell, I., and Dickson, C. (2000). An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. *Development* 127, 483–492.
- Nakano, Y., Yang, I.V., Walts, A.D., Watson, A.M., Helling, B.A., Fletcher, A.A., Lara, A.R., Schwarz, M.I., Evans, C.M., and Schwartz, D.A. (2016). MUC5B promoter variant rs35705950 affects MUC5B expression in the distal airways in idiopathic pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* 193, 464–466.
- Ostegda, L.S., Moninger, T.O., McMenimen, J.D., Sawin, N.M., Parker, C.P., Thornell, I.M., Powers, L.S., Gansemer, N.D., Bouzek, D.C., Cook, D.P., et al. (2017). Gel-forming mucins form distinct morphologic structures in airways. *Proc. Natl. Acad. Sci. U S A* 114, 6842–6847.
- Plantier, L., Crestani, B., Wert, S.E., Dehoux, M., Zwegtick, B., Guenther, A., and Whitsett, J.A. (2011). Ectopic respiratory epithelial cell differentiation in bronchiolised distal airspaces in idiopathic pulmonary fibrosis. *Thorax* 66, 651–657.
- Ray, S., Chiba, N., Yao, C., Guan, X., McConnell, A.M., Brockway, B., Que, L., McQualter, J.L., and Stripp, B.R. (2016). Rare SOX2+ airway progenitor cells generate KRT5+ cells that repopulate damaged alveolar parenchyma following influenza virus infection. *Stem Cell Reports* 7, 817–825.
- Redente, E.F., Keith, R.C., Janssen, W., Henson, P.M., Ortiz, L.A., Downey, G.P., Bratton, D.L., and Riches, D.W. (2014). Tumor necrosis factor- α accelerates the resolution of established pulmonary fibrosis in mice by targeting profibrotic lung macrophages. *Am. J. Respir. Cell Mol. Biol.* 50, 825–837.
- Rock, J.R., Barkauskas, C.E., Counce, M.J., Xue, Y., Harris, J.R., Liang, J., Noble, P.W., and Hogan, B.L. (2011). Multiple stromal populations contribute to pulmonary fibrosis without evidence for epithelial to mesenchymal transition. *Proc. Natl. Acad. Sci. U S A* 108, E1475–E1483.
- Roy, M.G., Rahmani, M., Hernandez, J.R., Alexander, S.N., Ehre, C., Ho, S.B., and Evans, C.M. (2011). Mucin production during pre- and post-natal mouse lung development. *Am. J. Respir. Cell Mol. Biol.* 44, 755–760.
- Roy, M.G., Livraghi-Buttrico, A., Fletcher, A.A., McElwee, M.M., Evans, S.E., Boerner, R.M., Alexander, S.N., Bellinghausen, L.K., Song, A.S., Petrova, Y.M., et al. (2014). Muc5b is required for airway defence. *Nature* 505, 412–416.
- Seibold, M.A., Wise, A.L., Speer, M.C., Steele, M.P., Brown, K.K., Loyd, J.E., Fingerlin, T.E., Zhang, W., Gudmundsson, G., Groshong, S.D., et al. (2011). A common MUC5B promoter polymorphism and pulmonary fibrosis. *N. Engl. J. Med.* 364, 1503–1512.
- Seibold, M.A., Smith, R.W., Urbanek, C., Groshong, S.D., Cosgrove, G.P., Brown, K.K., Schwarz, M.I., Schwartz, D.A., and Reynolds, S.D. (2013). The idiopathic pulmonary fibrosis honeycomb cyst contains a mucociliary pseudostratified epithelium. *PLoS One* 8, e58658.



- Steele, M.P., and Schwartz, D.A. (2013). Molecular mechanisms in progressive idiopathic pulmonary fibrosis. *Annu. Rev. Med.* **64**, 265–276.
- Tata, A., Kobayashi, Y., Chow, R.D., Tran, J., Desai, A., Massri, A.J., McCord, T.J., Gunn, M.D., and Tata, P.R. (2018). Myoepithelial cells of submucosal glands can function as reserve stem cells to regenerate airways after injury. *Cell Stem Cell* **22**, 668–683.e6.
- Thannickal, V.J., Toews, G.B., White, E.S., Lynch, J.P., 3rd, and Martinez, F.J. (2004). Mechanisms of pulmonary fibrosis. *Annu. Rev. Med.* **55**, 395–417.
- Urness, L.D., Paxton, C.N., Wang, X., Schoenwolf, G.C., and Mansour, S.L. (2010). FGF signaling regulates otic placode induction and refinement by controlling both ectodermal target genes and hindbrain Wnt8a. *Dev. Biol.* **340**, 595–604.
- Vaughan, A.E., Brumwell, A.N., Xi, Y., Gotts, J.E., Brownfield, D.G., Treutlein, B., Tan, K., Tan, V., Liu, F.C., Looney, M.R., et al. (2015). Lineage-negative progenitors mobilize to regenerate lung epithelium after major injury. *Nature* **517**, 621–625.
- Volckaert, T., and De Langhe, S. (2014). Lung epithelial stem cells and their niches: Fgf10 takes center stage. *Fibrogenesis Tissue Repair* **7**, 8.
- Volckaert, T., Dill, E., Campbell, A., Tiozzo, C., Majka, S., Bellusci, S., and De Langhe, S.P. (2011). Parabronchial smooth muscle constitutes an airway epithelial stem cell niche in the mouse lung after injury. *J. Clin. Invest.* **121**, 4409–4419.
- Volckaert, T., Campbell, A., Dill, E., Li, C., Minoo, P., and De Langhe, S. (2013). Localized Fgf10 expression is not required for lung branching morphogenesis but prevents differentiation of epithelial progenitors. *Development* **140**, 3731–3742.
- Volckaert, T., Yuan, T., Chao, C.M., Bell, H., Sitaula, A., Szimmtenings, L., El Agha, E., Chanda, D., Majka, S., Bellusci, S., et al. (2017). Fgf10-Hippo epithelial-mesenchymal crosstalk maintains and recruits lung basal stem cells. *Dev. Cell* **43**, 48–59.e5.
- Wendling, O., Bornert, J.M., Chambon, P., and Metzger, D. (2009). Efficient temporally-controlled targeted mutagenesis in smooth muscle cells of the adult mouse. *Genesis* **47**, 14–18.
- Xi, Y., Kim, T., Brumwell, A.N., Driver, I.H., Wei, Y., Tan, V., Jackson, J.R., Xu, J., Lee, D.K., Gotts, J.E., et al. (2017). Local lung hypoxia determines epithelial fate decisions during alveolar regeneration. *Nat. Cell Biol.* **19**, 904–914.
- Xie, T., Wang, Y., Deng, N., Huang, G., Taghavifar, F., Geng, Y., Liu, N., Kulur, V., Yao, C., Chen, P., et al. (2018). Single-cell deconvolution of fibroblast heterogeneity in mouse pulmonary fibrosis. *Cell Rep.* **22**, 3625–3640.
- Xu, Y., Mizuno, T., Sridharan, A., Du, Y., Guo, M., Tang, J., Wikenheiser-Brokamp, K.A., Perl, A.T., Funari, V.A., Gokey, J.J., et al. (2016). Single-cell RNA sequencing identifies diverse roles of epithelial cells in idiopathic pulmonary fibrosis. *JCI Insight* **1**, e90558.
- Yang, J., Wheeler, S.E., Velikoff, M., Kleaveland, K.R., LaFemina, M.J., Frank, J.A., Chapman, H.A., Christensen, P.J., and Kim, K.K. (2013). Activated alveolar epithelial cells initiate fibrosis through secretion of mesenchymal proteins. *Am. J. Pathol.* **183**, 1559–1570.
- Yang, Y., Riccio, P., Schotsaert, M., Mori, M., Lu, J., Lee, D.K., Garcia-Sastre, A., Xu, J., and Cardoso, W.V. (2018). Spatial-temporal lineage restrictions of embryonic p63(+) progenitors establish distinct stem cell pools in adult airways. *Dev. Cell* **44**, 752–761.e4.
- Zhang, Y., Noth, I., Garcia, J.G., and Kaminski, N. (2011). A variant in the promoter of MUC5B and idiopathic pulmonary fibrosis. *N. Engl. J. Med.* **364**, 1576–1577.
- Zheng, D., Limmon, G.V., Yin, L., Leung, N.H., Yu, H., Chow, V.T., and Chen, J. (2012). Regeneration of alveolar type i and ii cells from scgb1a1-expressing cells following severe pulmonary damage induced by bleomycin and influenza. *PLoS One* **7**, e48451.
- Zheng, D., Yin, L., and Chen, J. (2013). Evidence for Scgb1a1 cells in the generation of p63 cells in the damaged lung parenchyma. *Am. J. Respir. Cell Mol. Biol.* **50**, 595–604.
- Zuo, W., Zhang, T., Wu, D.Z., Guan, S.P., Liew, A.A., Yamamoto, Y., Wang, X., Lim, S.J., Vincent, M., Lessard, M., et al. (2015). p63Krt5 distal airway stem cells are essential for lung regeneration. *Nature* **517**, 616–620.

RESEARCH ARTICLE

miR-142-3p balances proliferation and differentiation of mesenchymal cells during lung development

Gianni Carraro¹, Amit Shrestha¹, Jana Rostkovius¹, Adriana Contreras^{2,3}, Cho-Ming Chao¹, Elie El Agha¹, Breanne MacKenzie¹, Salma Dilai¹, Diego Guidolin⁴, Makoto Mark Taketo⁵, Andreas Günther¹, Maya E. Kumar⁶, Werner Seeger^{1,3}, Stijn De Langhe^{7,8}, Guillermo Barreto^{2,3} and Saverio Bellusci^{1,9,*}

ABSTRACT

The regulation of the balance between proliferation and differentiation in the mesenchymal compartment of the lung is largely uncharacterized, unlike its epithelial counterpart. In this study, we determined that *miR-142-3p* contributes to the proper proliferation of mesenchymal progenitors by controlling the level of WNT signaling. *miR-142-3p* can physically bind to adenomatous polyposis coli mRNA, functioning to regulate its expression level. In *miR-142-3p* loss-of-function experiments, proliferation of parabronchial smooth muscle cell progenitors is significantly impaired, leading to premature differentiation. Activation of WNT signaling in the mesenchyme, or *Apc* loss of function, can both rescue *miR-142-3p* knockdown. These findings show that in the embryonic lung mesenchyme, the microRNA machinery modulates the level of WNT signaling, adding an extra layer of control in the feedback loop between FGFR2C and β -catenin-mediated WNT signaling.

KEY WORDS: WNT signaling, Mesenchymal cell, miRNA

INTRODUCTION

During lung organogenesis, mesenchymal cells undergo active proliferation to support the continuous growth of the organ. Simultaneously, many cells are actively differentiating into the specific cell types that will populate the adult lung – such as parabronchial and vascular smooth muscle cells, pericytes, nerve cells, lipofibroblasts and interstitial fibroblasts. A specific balance between proliferation and differentiation must exist to guarantee the formation of a functional lung. For example, parabronchial smooth muscle cell (PBSMC) progenitors are kept undifferentiated in the sub-mesothelial mesenchyme while starting to differentiate more proximally (Mailleux et al., 2005). It is crucial to understand this delicate balance in order to better

characterize lung diseases involving altered mesenchymal proliferation and differentiation.

WNT signaling plays a crucial role in proper proliferation of mesenchymal cells during lung development (De Langhe et al., 2008; Yin et al., 2008). In particular, through β -catenin (CTNNB1), it promotes G1 phase progression by activation of downstream target genes such as *Myc* and cyclin D1 (*Ccnd1*) (Niehrs and Acebron, 2012). High WNT signaling and *Myc* activation are associated with the hyper-proliferative state of cancer of many organs, including the lung (He et al., 1998; van de Wetering et al., 2002; Van Scoyk et al., 2008).

A main regulator of WNT signaling is adenomatous polyposis coli (APC), which can directly bind to CTNNB1, antagonizing the interaction with T-cell factor (TCF). In combination with AXIN and GSK3B, APC induces ubiquitylation and degradation of CTNNB1 (Clevers and Nusse, 2012). Loss of *Apc* leads to accumulation of CTNNB1 in the nucleus and hyperactivation of WNT signaling. *Apc* was first identified as a tumor suppressor gene that, upon mutation, causes intestinal cancer (Grodin et al., 1991). APC can also control cytoskeleton structure and cell migration by binding microtubules and actin filaments. For example, loss of *Apc* in the small intestine reduces the migration of epithelial cells and promotes the formation of polyps (Oshima et al., 1997).

Specific microRNAs (miRNAs) have been implicated in both lung development and disease (Jiang et al., 2010; Ornitz and Yin, 2012). In the epithelium, mice with loss of function of members of the *miR-17* family show early lethality and hypoplastic lungs, whereas overexpression results in hyperproliferation and inhibition of differentiation of epithelial progenitors (Lu et al., 2007; Ventura et al., 2008). The *miR-302* family regulates epithelial progenitor proliferation and differentiation, as well as apical-basal polarity (Tian et al., 2011). Recently, *miR-375* has been shown to target WNT signaling and regulate the differentiation of alveolar epithelial cells by controlling the expression of the frizzled 8 gene (Wang et al., 2013). However, miRNAs regulating WNT signaling in the lung mesenchyme have yet to be reported. *miR-142-3p* is a miRNA first identified for its function in the development of the lymphoid system (Neilson et al., 2007), and was subsequently implicated in leukemia (Lv et al., 2012). In the lung, *miR-142-3p* is involved in malignancy and has been reported to be an early marker for aggressive and recurrent lung adenocarcinomas (Kaduthanam et al., 2013).

Herein, we detail *miR-142-3p* as a specific regulator of *Apc* expression in the mesenchyme. Using a loss-of-function approach, we analyzed the role of the *miR-142-3p-Apc* axis in mesenchymal cells. Using both pharmacological and genetic tools, we tested whether WNT signaling upregulation is sufficient to rescue *miR-142-3p* loss-of-function and whether *Apc* is a crucial target of this miRNA.

¹University of Giessen Lung Center, Excellence Cluster in Cardio-Pulmonary Systems, member of the German Lung Center (DZL), Department of Internal Medicine II, Aulweg 130, 35392 Giessen, Germany. ²LOEWE Research Group Lung Cancer Epigenetic, Max Planck Institute for Heart and Lung Research, Member of the German Lung Center (DZL), 61231 Bad Nauheim, Germany. ³Department of Lung Development and Remodeling, Max Planck Institute for Heart and Lung Research, Member of the German Lung Center (DZL), 61231 Bad Nauheim, Germany. ⁴University of Padova, Department of Molecular Medicine, 35121 Padova, Italy. ⁵Department of Pharmacology, Kyoto University Graduate School of Medicine, Yoshida-Konoé-cho, Sakyo-ku, Kyoto 606-8501, Japan. ⁶Department of Biochemistry and HHMI, Stanford University School of Medicine, Stanford, CA 94305, USA. ⁷Department of Pediatrics, National Jewish Health, Denver, CO 80206, USA. ⁸Department of Cellular and Developmental Biology, University of Colorado Denver, Aurora, CO 80045, USA. ⁹Developmental Biology Program, Saban Research Institute of Children's Hospital Los Angeles, Los Angeles, CA 90027, USA.

*Author for correspondence (saverio.bellusci@innere.med.uni-giessen.de)

Received 12 November 2013; Accepted 15 January 2014

RESULTS

***miR-142-3p* regulates mesenchymal cell proliferation and differentiation**

In order to identify miRNAs with a specific function in the lung mesenchyme, we performed a microarray analysis on embryonic mouse lung tissue (Fig. 1A). We observed that 5 and 14 miRNAs were highly expressed, respectively, in the embryonic lung mesenchyme and the epithelium. Among those in the epithelium, we found several members of the miR-200 family. These miRNAs are involved in epithelial to mesenchymal transition (Brabletz and Brabletz, 2010), and their high expression in the embryonic epithelium suggests they may play a role in the plasticity of the branching tips during lung morphogenesis.

An *in vitro* loss of function (LOF) assay was optimized to functionally characterize the newly identified miRNAs in the mesenchyme. Embryonic day (E) 11.5 lung explants were grown in the presence of *vivo*-morpholino against either a scrambled sequence or a specific miRNA. We selected *miR-126* as a positive control for our LOF assay because it was previously described to regulate vasculature formation. Using *vivo*-morpholino against *miR-126* (*mo126*), we were able to impair vasculature formation (supplementary material Fig. S1A-I,J-M,R; $n=3$, $P \leq 0.05$), thus mimicking the phenotype observed in the *miR-126* knockdown zebrafish (Fish et al., 2008). As a negative control we selected *miR-144*, because its inactivation does not lead to a specific lung phenotype in mice (Rasmussen et al., 2010). In our LOF assay,

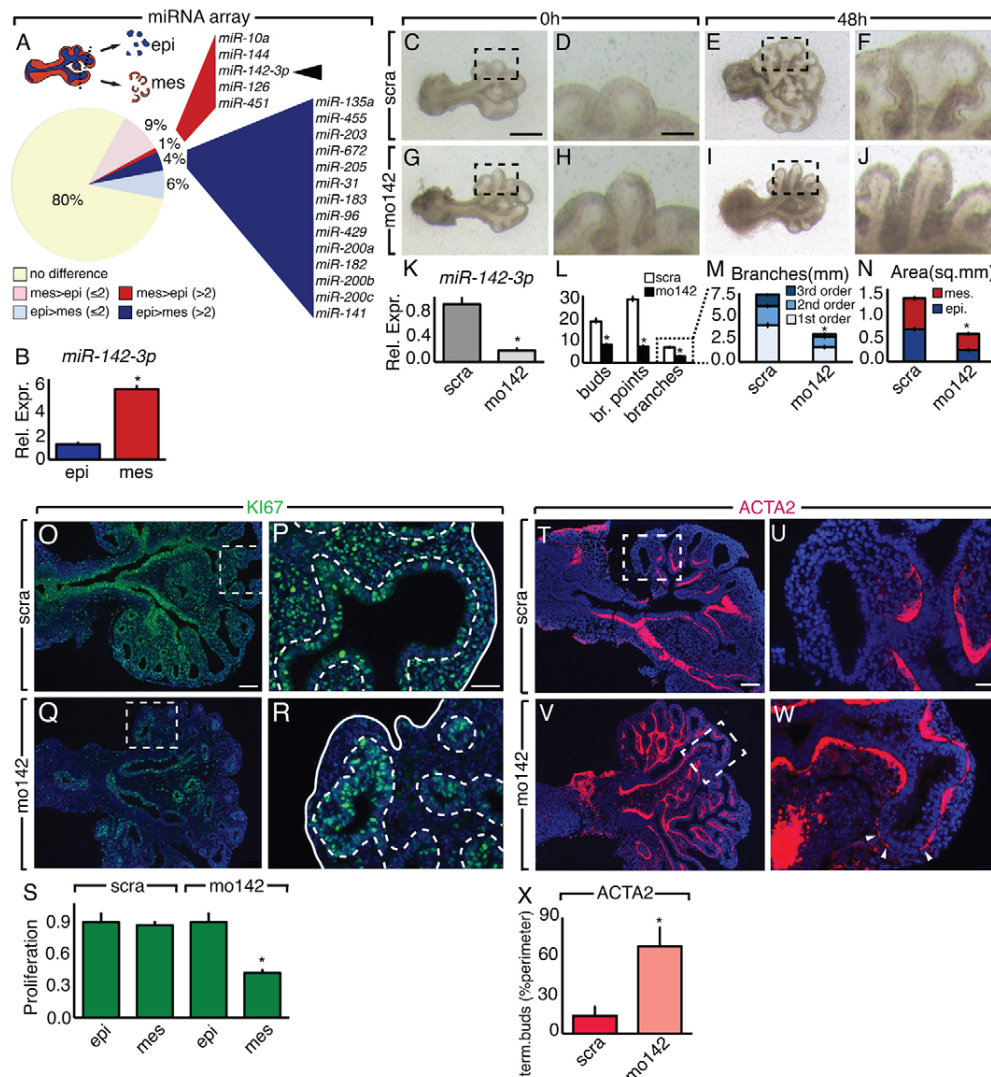


Fig. 1. *miR-142-3p* regulates mesenchymal cells proliferation and differentiation. (A) MicroRNA microarray shows miRNAs differentially expressed in E12.5 lung epithelium (blue) versus mesenchyme (red). *miR-142-3p* (arrowhead) is specifically upregulated in the embryonic lung mesenchyme. (B) Expression of *miR-142-3p* in the epithelium and mesenchyme of E12.5 lungs analyzed by qPCR. (C–J) *miR-142-3p* LOF assay on E11.5 lung explants treated with scramble *vivo*-morpholino (scra; C–F) and *miR-142-3p* *vivo*-morpholino (mo142; G–J). (K) Expression of *miR-142-3p* detected by qPCR 48 hours after treatment with mo142. (L–N) Morphometric analysis 48 hours after *miR-142-3p* LOF assay on E11.5 lung explants. (O–R) Immunostaining for Ki67 showing cell proliferation in scra- (O, P) and mo142 (Q, R)-treated E11.5 lung explants, and quantification of proliferation in the epithelium and mesenchyme (S). (T–W) Immunostaining for ACTA2 showing smooth muscle cells at the tips of scra- (T, U) and mo142 (V, W)-treated E11.5 lung explants. Dashed boxes in O, Q, T and V indicate the magnified areas in P, R, U and W, respectively. White arrowheads indicate ectopic ACTA2 expression. (X) Quantification of ACTA2 expression at the tip of the lung explants from the previous experiment. Scale bars: 250 μ m (C, E, G, I, J); 50 μ m (D, F, H, J); 100 μ m (O, Q, T, V); 25 μ m (P, R, U, W). Data are means $\pm$ s.d. See also supplementary material Fig. S1 and Movie 1.

mol144 did not produce any branching defect or alteration of proliferation (supplementary material Fig. S1A-I,N-Q,S; $n \geq 3$, $P < 0.05$). These results indicate that our LOF assay is an effective tool for the identification of functionally important miRNAs.

Using this assay we tested whether *miR-142-3p* plays a functional role in the lung mesenchyme. We first confirmed that *miR-142-3p* is highly expressed in the embryonic lung mesenchyme by qPCR (Fig. 1B; $n = 3$, $P < 0.05$). Then the LOF assay for *miR-142-3p* was conducted. qPCR showed reduced *miR-142-3p* levels (Fig. 1K; $n = 3$, $P < 0.05$) associated with impaired branching (Fig. 1C-J). The number of branching points was decreased (Fig. 1L; $n = 10$, $P < 0.05$) and the lung tips had the tendency to elongate instead of bifurcate, thus resulting in a decreased number of terminal buds (Fig. 1M; $n = 10$, $P < 0.05$; supplementary material Movie 1). Reduction of lung growth, illustrated by a diminished total lung area (Fig. 1N; $n = 10$, $P < 0.05$), was observed. Immunostaining for KI67 at 24 hours post-treatment showed a striking reduction of proliferation that, interestingly, was evident in the mesenchyme but not in the epithelium (Fig. 1O-S; $n = 4$, $P < 0.05$). Furthermore, inhibition of *miR-142-3p* led to ectopic expression of the SMC marker alpha-SMA (ACTA2) in the distal mesenchyme (Fig. 1T-X; $n = 3$, $P < 0.05$). Together, these results suggest a function for *miR-142-3p* in the control of proliferation and differentiation of the lung mesenchyme.

miR-142-3p regulates WNT signaling by directly controlling *Apc* expression

To determine whether specific signaling pathways are involved in *miR-142-3p* regulation of mesenchymal cell proliferation, we utilized the prediction software miRTar and grouped the candidate genes by pathway. Interestingly, WNT signaling was the first hit (Table 1). To verify that WNT signaling was regulated by *miR-142-3p* in the mesenchyme, we performed a LOF assay on E11.5 lung explants obtained from a WNT reporter mouse line carrying β -galactosidase (β -Gal) expression under the endogenous control of the *Axin2* promoter (*Axin2^{lacZ}* mice). Staining of β -Gal after 24 hours showed a specific inhibition of WNT signaling in the distal mesenchyme (Fig. 2A-E; $n = 3$, $P < 0.05$). Because miRNAs are negative regulators of gene expression, we aimed to identify WNT genes upregulated upon *miR-142-3p* knockdown. A qPCR screening for members of the WNT signaling pathway expressed in the lung was carried out at 12 hours and at 24 hours during our LOF assay. At 12 hours, we observed that *Apc* – a gene encoding a negative regulator of WNT signaling – was highly expressed. At 24 hours, *Apc* was still upregulated while other members were downregulated (Fig. 2F,G). Analysis of LEF1 expression by immunofluorescence confirmed a strong reduction of mesenchymal WNT signaling 24 hours after LOF assay (Fig. 2H-K). We reasoned that since the effect on proliferation, branching and WNT signaling were visible starting at 24 hours, the upregulation of *Apc* at 12 hours is an early event that could contribute to the observed phenotype. Immunofluorescence

analysis for APC at 12 hours (data not shown) and at 24 hours after our LOF assay confirmed an increase of mesenchymal APC at protein level (Fig. 2L-O). At 12 hours epithelial APC was unchanged (data not shown), but at 24 hours there was a reduction in APC. Considering the timing of this reduction and the fact that *miR-142-3p* is expressed in the mesenchyme, we assume this effect was secondary to the mesenchymal phenotype. Interestingly, *Apc* was among the five most highly ranked WNT signaling genes (*Apc*, *Tblx1*, *Rock2*, *Rac1*, *Senp2*) that were predicted as targets of *miR-142-3p* (Table 1). As *Tblx1*, *Rock2* and *Rac1* were not included in our first screening, we analyzed their expression 12 hours after the LOF assay. Although the expression of *Apc* and *Senp2* was enhanced, expression of *Tblx1*, *Rock2* and *Rac1* showed no significant change (Fig. 2P; $n = 3$, $P < 0.05$). The expression of *Senp2* was reduced at 24 hours (Fig. 2G), suggesting that the modulation of its expression by *miR-142-3p* may not be as specific as for *Apc*.

We then wanted to determine whether *miR-142-3p* was able to physically bind the mRNA of its predicted target genes. For this purpose, we performed a pull-down experiment using a biotinylated *miR-142-3p* followed by qPCR to detect specific binding. The biotinylated *miR-142-3p* was able to cause overexpression of WNT signaling target genes such as *Myc* and *Fgfr2* (supplementary material Fig. S2A,B), suggesting that biotinylation was not altering the property of *miR-142-3p*. In this assay, performed on a lung mesenchymal cell line (MLg cells), *Apc* was enriched more than six times compared with the control (Fig. 2M; $n = 3$, $P < 0.05$). The same assay was reproduced in MFLM4 and HEK293 cells, suggesting that indeed *miR-142-3p* directly interact with *Apc* to regulate its expression (supplementary material Fig. S2C; $n = 3$, $P < 0.05$). We further wanted to determine if *miR-142-3p* was binding *Apc* by specifically interacting with the predicted binding sites located at the 3'-UTR of *Apc* mRNA. For this purpose, we cloned *Apc* seed sites into two luciferase (Luc) reporter plasmids containing either a mutated binding site or a wild-type locus. We observed that the ability of *miR-142-3p* to target *Apc* was compromised in the presence of the mutated 3'UTR (Fig. 2N; $n = 3$, $P < 0.05$).

miR-142-3p downregulation leads to parabronchial smooth muscle cell progenitor differentiation

APC is a negative regulator of WNT signaling and, as a direct target of *miR-142-3p*, its increase can explain the drop in WNT signaling following *miR-142-3p* knockdown. In the embryonic lung mesenchyme, WNT, in combination with FGF signaling, creates a positive feedback loop. In fact, lack of contribution from either FGF (specifically FGF9-FGFR2c) or WNT disrupts this delicate equilibrium (Yin et al., 2011). When this system is operational, WNT signaling maintains *Fgfr2c* expression in the mesenchyme allowing mesenchymal growth through the regulation of G1 to S phase transition (De Langhe et al., 2008; Yin et al., 2008). Therefore, we decided to test whether the inhibition of WNT

Table 1. miR-142-3p targets pathway enrichment

Target genes	Pathway	P value	Number of matched genes	Number of genes in pathway
APC, TBL1X, SENP2, ROCK2, RAC1	Wnt signaling	4.50E-08	5	182
APC, ROCK2, RAC1	Regulation of actin cytoskeleton	9.19E-04	3	246
APC, RAC1	Colorectal cancer	1.82E-03	2	73
ROCK2, RAC1	Axon guidance	9.25E-03	2	167
ROCK2, RAC1	Leukocyte transendothelial migration	1.15E-02	2	187
ROCK2, RAC1	Chemokine signaling	1.20E-02	2	191
ROCK2, RAC1	Focal adhesion	1.32E-02	2	201
APC, RAC1	Pathways in cancer	5.21E-02	2	415

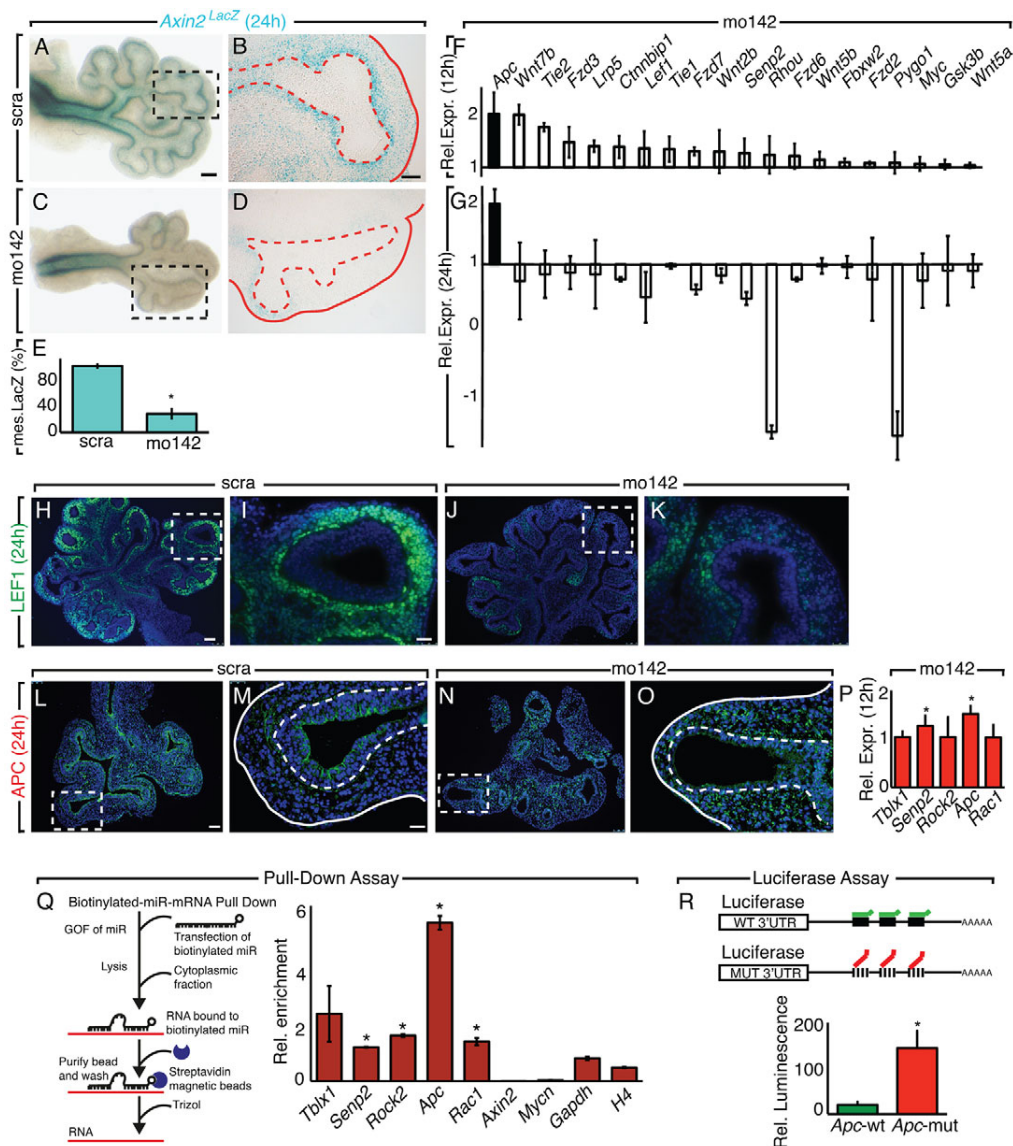


Fig. 2. *miR-142-3p* regulates WNT signaling by direct control of *Apc* expression. (A-E) *miR-142-3p* LOF assay on *Axin2^{LacZ}* E11.5 lung explants. Lung explants (A,C) and vibratome sections (B,D) are shown 24 hours after *miR-142-3p* LOF assay. Mesenchymal β-gal is quantified in E. (F,G) Members of the WNT signaling pathway were quantified by qPCR 12 and 24 hours after the *miR-142-3p* LOF assay. (H-K) LEF1 expression 24 hours after the *miR-142-3p* LOF assay. (L-O) APC expression after the *miR-142-3p* LOF assay. Immunofluorescence for APC 24 hours after scra (L,M) and mo142 (N,O) treatment. Dashed boxes indicate the magnified area shown in the panels to the right. Dashed lines in M and O demarcate the epithelium-mesenchyme boundary. (P) Quantification by qPCR of *miR-142-3p* predicted targets 12 hours after *miR-142-3p* LOF assay. (Q) Quantification by qPCR of *miR-142-3p* predicted targets after biotin mRNA-miRNA pull down assay. (R) Luciferase assay for *Apc* seed sequences on MLg cells. Scale bars: 150 μm (A,C); 100 μm (B,D); 75 μm (H,I,J,L,N); 25 μm (I,K,M,O). Data are means ± s.d. See also supplementary material Fig. S2.

signaling observed in our *miR-142-3p* LOF assay was associated with perturbation of the FGF-WNT sustained regulation. Indeed, qPCR on E11.5 lung explants showed decreased *Fgfr2c* expression beginning 24 hours into the assay (Fig. 3D-F; $n=3$, $P<0.05$). At 48 h, *Fgfr2c* reduction was associated with a specific decrease in the mesenchyme of phosphorylated ERK (pERK; Fig. 3M-Q; $n=4$, $P<0.05$) and phosphorylated MEK (pMEK; Fig. 3R-V; $n=3$, $P<0.05$). One of the functions of FGF9-FGFR2c signaling in the lung is to keep mesenchymal PBSMC progenitors undifferentiated and proliferating (Yi et al., 2009). Furthermore, we have previously shown that FGF10 is expressed in, and identifies PBSMC progenitors (Mailleux et al., 2005). Analysis by qPCR after the *miR-142-3p* LOF assay showed that *Fgfr2b* expression remained stable,

suggesting that epithelial FGF signaling was unaffected (Fig. 3J-L; $n=3$, $P<0.05$). However, *Fgf10* started decreasing at 24 hours and was significantly reduced at 48 hours (Fig. 3G-I; $n=3$, $P<0.05$). This result suggests that *Fgf10*-positive PBSMC progenitors were specifically affected by the imbalance of FGF-WNT regulation. We therefore decided to analyze the effect of our *miR-142-3p* LOF assay on SMC differentiation. To directly analyze PBSMC progenitors during *miR-142-3p* downregulation, conditional red fluorescent protein (RFP) reporter mice (*RFP^{fl}*) were crossed with mice carrying *Fgf10^{CreERT2}*. We will refer to these mice with genotype *Fgf10^{CreERT2/+},RFP^{fl/+}* as *RFP^{Fgf10}*.

Live imaging of *RFP^{Fgf10}* E11.5 lung explants treated with *miR-142-3p* vivo-morpholino (mo142; supplementary material Movie 2)

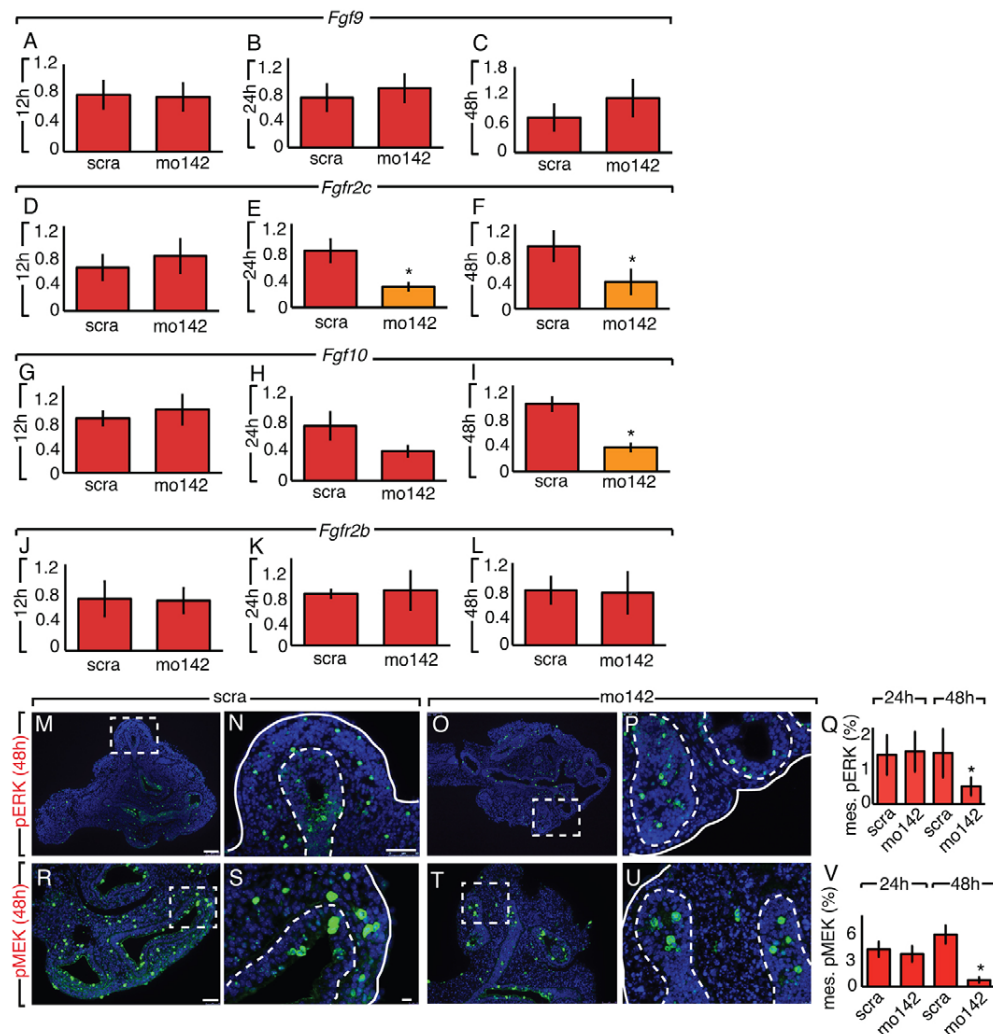


Fig. 3. Loss of *miR-142-3p* affects mesenchymal FGF signaling. (A-L) FGF signaling genes were quantified by qPCR. The expression of *Fgf9* (A-C), *Fgfr2c* (D-F), *Fgf10* (G-I) and *Fgfr2b* (J-L) was analyzed at 12, 24 and 48 hours after LOF assay on E11.5 lung explants. (M-Q) Phosphorylated ERK (pERK) expression was analyzed 24 and 48 hours after LOF assay on E11.5 lung explants. pERK expression in the epithelium and mesenchyme is shown by immunofluorescence at 48 hours (M-P) and was quantified at 24 and 48 hours (Q). pMEK expression in the epithelium and mesenchyme is shown by immunofluorescence at 48 hours (R-U) and quantified at 24 and 48 hours (V). Dashed boxes indicate the magnified areas in the adjacent panels. Dashed lines in N, P, S and U demarcate the epithelium-mesenchyme boundary. Scale bars: 100 μm (M, O, R, T); 50 μm (N, P, S, U). Data are means ± s.d.

showed that labeled PBSMC progenitors formed ring-like structures, reminiscent of smooth muscle cell phenotype, at the tip of the epithelial buds (Fig. 4A-D). These data are in agreement with the ectopic alpha-SMA (ACTA2) expression at the tip of the epithelial buds during *mo142* treatment (Fig. 1T-W). Additionally, in *mo142*-treated explants, labeled cells displayed reduced motility and proliferation, but in scrambled-treated explants, they were highly motile and proliferative (supplementary material Movie 2; Fig. 4E-G; $n=3$, $P<0.05$). Our results suggest that PBSMC progenitors prematurely differentiate upon *mo142* treatment. To further demonstrate that this was due to loss of FGF9-FGFR2c signaling, primary embryonic lung mesenchymal cells were cultured *in vitro* in different experimental conditions. Scrambled-treated primary mesenchymal cells grown in the presence of FGF9 were prevented from differentiating into SMCs (Fig. 4H,I). After *miR-142-3p* knockdown, FGF9 was no longer effective and thus differentiation into SMCs occurred (Fig. 4J-L). The role of *miR-142-3p* in SMC differentiation was further evaluated using a gain of function

approach. We used mesenchymal cells from a mouse lung mesenchymal cell line (MLg cells). Under normal *in vitro* culture conditions, MLg cells differentiate into ACTA2-positive cells. Overexpression of *miR-142-3p* by a synthetic form of *miR-142-3p* that mimics its function (*mi142*) led to reduced ACTA2 expression and diminished elongation (Fig. 4M-O). Because *Apc* is a specific target for *miR-142-3p* (Fig. 2H-N), we wanted to determine if *Apc* overexpression is sufficient to mimic *miR-142-3p* knockdown. Primary mesenchymal cells isolated from *RFP^{Fgf10}* lungs were electroporated with either control plasmids (pGFP) or experimental plasmids overexpressing APC (pAPC). *Apc* overexpression was sufficient to mimic loss of *miR-142-3p*, producing reduced proliferation and motility of PBSMC progenitors (Fig. 4P-V; $n=3$, $P<0.05$; supplementary material Movie 3).

Constitutive WNT signaling rescues *miR-142-3p* knockdown

The inhibition of WNT signaling in the *miR-142-3p* LOF assay prompted us to attempt a rescue experiment in which WNT

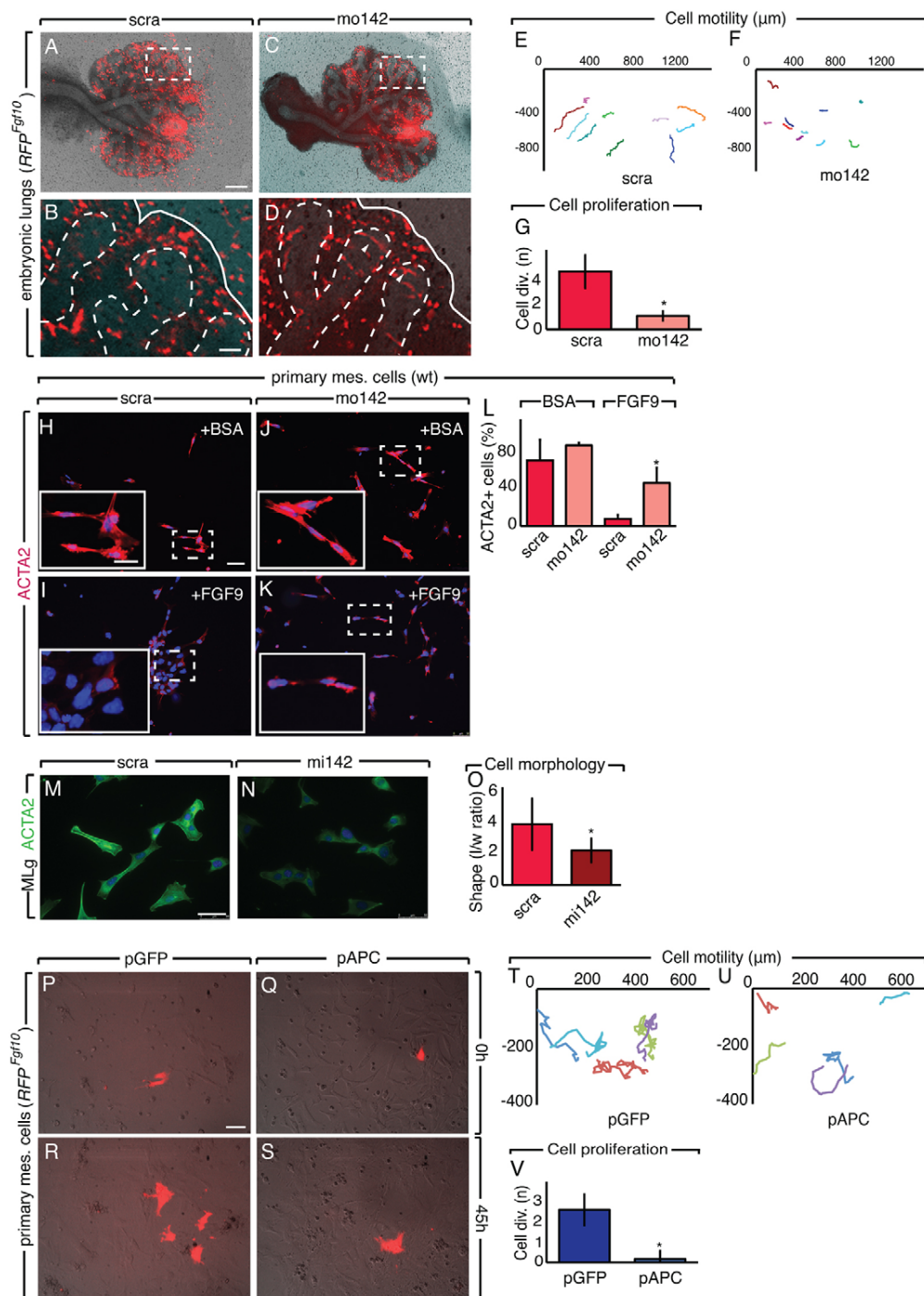


Fig. 4. Loss of *miR-142-3p* leads to lung mesenchyme differentiation. (A-D) Analysis of *RFP^{Fgf10}* lung explants after scra (A,B) and mo142 (C,D) administration, and quantification of cell motility (E,F) and proliferation (G). (H-K) Immunostaining for ACTA2 showing lung primary mesenchymal cells after administration of BSA or FGF9 in the presence of scra (H,I) or mo142 (J,K). (L) Quantification of ACTA2-positive cells. (M,N) MLg cells were analyzed for ACTA2 expression by immunostaining after scra (M) or mi142 (N) administration. (O) Quantification of cellular morphology as a ratio of length to width. (P-S) *RFP^{Fgf10}* primary mesenchymal cells were electroporated with a plasmid expressing GFP (pGFP) or APC (pAPC) and analyzed between 0 and 45 hours. (T-V) Quantification of the cell motility (T,U) and proliferation (V) for pGFP- and pAPC-treated cells. Dashed boxes indicate the magnified areas shown in the insets. Dashed lines demarcate the epithelium-mesenchyme boundary. Scale bars: 250 μm (A,C); 50 μm (B,D); 50 μm (H-K insets); 20 μm (H-K main image); 50 μm (M,N); 75 μm (P-S). Data are means $\pm$ s.d. See also supplementary material Movies 2 and 3.

signaling was stimulated at different levels of the pathway. We began with WNT3a, a commercially available WNT ligand. Lungs treated with WNT3a display expansion of the epithelium and mesenchyme compared with BSA-treated lungs (data not shown and

supplementary material Fig. S3C,D). Upon *miR-142-3p* attenuation, WNT3a was unable to affect mesenchymal growth (supplementary material Fig. S3A,B,D; $n=3$, $P<0.05$). Similar results were obtained by using FGF9 (Fig. 5A-D; $n=3$, $P<0.05$) and FGF9 in combination

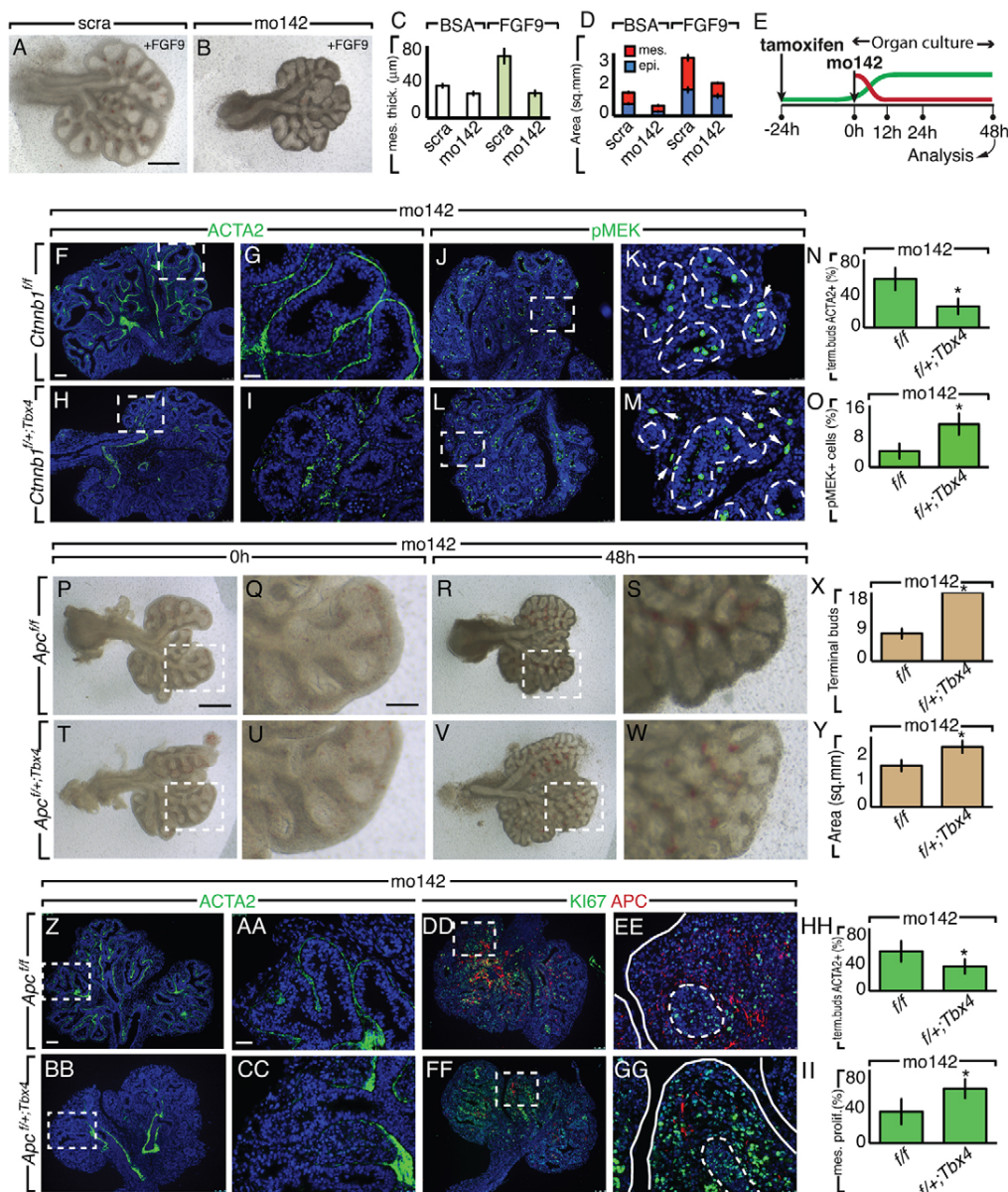


Fig. 5. Stabilized *Ctnnb1* expression or decreased *Apc* expression in the mesenchyme counteracts the effect of *miR-142-3p* inhibition on proliferation and differentiation. (A-D) FGF9 (200 ng/μl) was administered to E11.5 lung explants cultured in the presence of scra (A) or mo142 (B), and mesenchyme thickness (C) and epithelial and mesenchymal surfaces (D) were measured. (E) Schematic showing the time line of the experimental treatment. The green and red lines depict tamoxifen and mo142 effects, respectively. (F-O) E11.5 lung explants harboring a mesenchymal stabilized form of *Ctnnb1* obtained by *Tbx4*-driven Cre expression, and control lungs were treated with mo142 and immunostained for ACTA2 (F-I) or pMEK (J-M). Quantification of ACTA2 and pMEK are shown in N and O, respectively. (P-II) E11.5 lung explants with mesenchymal deletion of *Apc* by *Tbx4*-driven Cre expression (P-S) and control lungs (T-W) were treated with mo142 and their morphometry was analyzed (X,Y). Immunostaining for ACTA2 and APC (Z-CC) or Ki67 and APC (DD-GG) from the previous experiment and its relative quantification (HH,II). Dashed boxes indicate the magnified areas in the adjacent panels. Dashed lines demarcate the epithelium-mesenchyme boundary. Scale bars: 250 μm (A,B); 75 μm (F,H,I,J,L); 25 μm (G,I,K,M); 250 μm (P,R,T,V); 50 μm (Q,S,U,W); 75 μm (Z,BB,DD,FF); 25 μm (AA,CC,EE,GG). Data are means ± s.d. See also supplementary material Fig. S3.

with WNT3a (supplementary material Fig. S3E-H; $n=3$, $P<0.05$). Under these conditions FGF9 still caused expansion of the epithelium. This result further confirms that when *miR-142-3p* expression is inhibited the positive feedback loop between FGF and WNT signaling in the mesenchyme is impaired. To intervene downstream of WNT signaling, we used mice harboring a floxed allele in the *Ctnnb1* gene (*Ctnnb1*^{ex3/f}) to generate a stable form of *Ctnnb1*. Crossing this line with *Tbx4LME*^{CreERT2} mice, we were able to obtain inducible constitutive activation of *Ctnnb1*, specifically

in the mesenchyme. We will refer to the genotype *Tbx4LME*^{CreERT2/+}, *Ctnnb1*^(ex3/f) as *Ctnnb1*^{f/+;Tbx4}. *Ctnnb1*^{f/+;Tbx4} and *Ctnnb1*^{f/f} lungs from the same mating represented WNT-activated and control lungs, respectively, and were used in the LOF assay (Fig. 5E). Lungs from *Tbx4LME*^{CreERT2/+} mice injected with tamoxifen intraperitoneally were also used as controls in the LOF assay, showing similar results to the *Ctnnb1*^{f/f} lungs (data not shown). Forty-eight hours after the *miR-142-3p* LOF assay, the activation of CTNNB1 signaling in *Ctnnb1*^{f/+;Tbx4} lungs, compared

to *Ctnnb1*^{lf} lungs (supplementary material Fig. S3M-P), led to the disappearance of mol42-induced MYH11 (supplementary material Fig. S3I-L) and ACTA2 ectopic expression (Fig. 5F-I,N; *n*=3, *P*<0.05) at the tip of the epithelial buds. We also observed increased pMEK expression in mol42-treated *Ctnnb1*^{lf/+;Tbx4} compared with *Ctnnb1*^{lf} lungs, suggesting recovery of FGF signaling in the mesenchyme (Fig. 5J-M,O; *n*=3, *P*<0.05).

Reduction of *Apc* expression rescues *miR-142-3p* knockdown

Having identified *Apc* as a direct target of *miR-142-3p*, we decided to test whether reduction of *Apc* by ablation of one of the two copies of the gene was able to rescue the effect of *miR-142-3p* inhibition. For this purpose we used *Apc*^{fl} mice crossed with *Tbx4LME*^{CreERT2} mice. We will refer to the genotype *Tbx4LME*^{CreERT2/+}; *Apc*^{fl/+} as *Apc*^{fl/+;Tbx4}. *Apc*^{fl} lungs were used as controls. *Tbx4LME*^{CreERT2/+} were also used as controls with similar results to *Apc*^{fl} lungs in the LOF assay (data not shown). Forty-eight hours after the *miR-142-3p* LOF assay, the reduction of APC expression in *Apc*^{fl/+;Tbx4} compared with *Apc*^{fl} lung explants was confirmed by qPCR (data not shown) and immunohistochemistry (Fig. 5DD-GG). This reduction led to the disappearance of mol42-induced ectopic expression of ACTA2 at the tip of the epithelial buds (Fig. 5Z-CC,HH; *n*=3, *P*<0.05), as well as increased KI67 expression in the mesenchyme (Fig. 5DD-GG,II; *n*=3, *P*<0.05). In addition, *Apc*^{fl/+;Tbx4} lungs were able to recover growth and branching (Fig. 5P-Y; *n*=3, *P*<0.05). These results suggest that the inhibition of *Apc* alone is sufficient to counteract *miR-142-3p* knockdown.

DISCUSSION

In this study we describe a new function of the microRNA machinery in the lung embryonic mesenchyme. We determined that *miR-142-3p* is a crucial regulator of WNT signaling in this compartment. By directly controlling *Apc* expression, *miR-142-3p* can fine-tune its inhibition of WNT signaling. Furthermore, *miR-142-3p* controls proliferation and differentiation of mesenchymal progenitor in the embryonic lung (Fig. 6A,B).

A fine balance between FGF and WNT signaling helps maintain the pool of mesenchymal progenitor cells in the lung embryonic mesenchyme. For example, evidence suggests that FGF9 from the mesothelium can signal to *Fgf10*-positive cells located in the submesothelial mesenchyme to prevent differentiation (Colvin et al., 2001). In this system, WNT signaling helps maintain proper levels of FGF signaling, because reduced WNT signaling creates disequilibrium in the feedback loop that maintains mesenchymal

FGF-WNT signaling (De Langhe et al., 2008; Yin et al., 2011). Here, we found that *miR-142-3p* is implicated in the modulation of the WNT and FGF balance, adding an extra layer of control that increases the robustness of this system.

We show that *miR-142-3p* fulfills this function by directly controlling *Apc* expression, therefore acting as a negative regulator of an inhibitor of WNT signaling. Deregulation of *miR-142-3p* expression uncouples WNT and FGF signaling in the mesenchyme, affecting mesenchymal progenitor cells differentiation. Specifically, the *Fgf10*-positive lineage which contributes to the formation of parabronchial smooth muscle cells is affected by the deregulation of *miR-142-3p* expression, resulting in premature differentiation and proximalization of the mesenchyme.

Recently, *miR-142-3p* was found to control hemangioblast specification and vasculogenesis in *Xenopus* (Nimmo et al., 2013). In our LOF assay we observed impaired angiogenesis of the lung explants, which was associated with enrichment of markers for undifferentiated hemangioblasts such as CD31 (data not shown). This suggests that a similar regulation could also contribute to the vasculogenesis in the lung. It will be interesting to determine if *miR-142-3p* controls similar pathways – for instance WNT signaling – or if the two systems are completely independent.

Although our screening identified *Apc* as a main target of *miR-142-3p*, other genes are likely to be affected. In particular, other members of WNT signaling were found to bind to biotinylated *miR-142-3p* to a lower degree than *Apc*. Nonetheless, rescue experiments with *Apc*^{Tbx4} mice showed that the inhibition of *Apc* in the mesenchyme is sufficient to rescue the lung from *miR-142-3p* downregulation, suggesting that *Apc* is a crucial molecule regulated by *miR-142-3p* in the lung mesenchyme. We also found that in the parenchyma of the adult lungs, *miR-142-3p* and APC are mutually exclusive (data not shown). This suggests that the regulation here described may not be limited to the embryonic lung. In addition, our finding is consistent with the observation that inactivation of *Apc* in the lung mesenchyme using a *Tbx4*-rtTA driver mouse line leads to reduced and disorganized smooth muscle cell differentiation in the lung (personal communication from Wei Shi). This suggests that APC per se, has a critical role in the regulation of proliferation, survival, and differentiation of smooth muscle progenitor cells.

In summary, our work shows that *miR-142-3p* is intimately linked to mesenchymal activated WNT signaling by specific regulation of *Apc*. It will be of interest to determine if the *miR-142-3p*–*Apc*– β -catenin axis plays a role in lung diseases such as idiopathic pulmonary fibrosis, in which cellular proliferation and differentiation of mesenchymal cells is unbalanced.

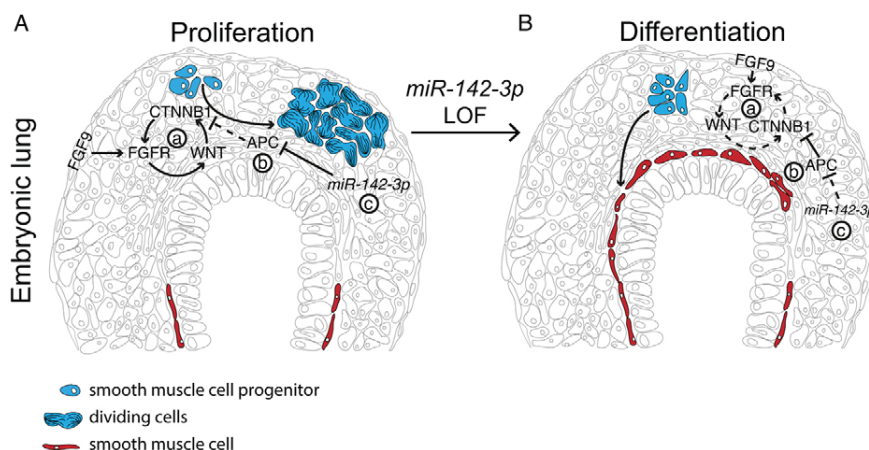


Fig. 6. Schematic of *miR-142-3p* regulation of WNT signaling by direct control of *Apc* expression. (A) A fine balance of mesenchymal FGF and WNT signaling (a) is necessary to maintain proper mesenchymal proliferation. In this context, APC (b) contributes to control the level of β -catenin (CTNNB1), and *miR-142-3p* (c) controls the inhibitory effect of APC. Changes in *miR-142-3p* expression can therefore modulate the balance between proliferation (A) and differentiation (B) of mesenchymal progenitors. Blue, mesenchymal progenitors; red, differentiated smooth muscle cells.

MATERIALS AND METHODS

Mouse strains

C57BL/6 wild-type, *Apc*^{flax/+}, *Tomato*^{flax/flax} and *Axin2*^{lacZ} mice were obtained from Jackson Laboratories. *Fgf10*^{CreERT2/+} mice were generated in our laboratory as previously described (El Agha et al., 2012). *Ctnnb1*^{(ex3)fl} mice were generated in the Taketo laboratory (Harada et al., 1999). *Tbx4*^{LME}^{CreERT2/+} mice were generated in the Krasnow laboratory (M.E.K., Patrick E. Bogard, F. Hernán Espinoza, Douglas B. Menke, David M. Kingsley and Mark A. Krasnow, unpublished data). For rescue experiments, tamoxifen was administered by intraperitoneal injection (0.1 mg/g of body weight) 24 hours before the LOF assay. Animals were housed at room temperature with a 12 hour/12 hour light/dark cycle and free access to food and water. The Federal Authorities for Animal Research of the Regierungspräsidium Giessen (Hessen, Germany) approved the study protocol (*miR-142-3p* protocol 52/2012).

MicroRNA array and qPCR

Epithelium from E12.5 lung buds was isolated as previously described (Carraro et al., 2009); mesenchyme from E12.5 lung buds was physically isolated using 0.12 mm tungsten needles (Fine Science Tools). Total RNA was extracted using a miRNeasy Mini Kit (Qiagen). The quality of the total RNA was verified using an Agilent 2100 Bioanalyzer profile. Total RNA (1000 ng) was labeled with fluorescent Hy3 and Hy5 using the miRCURY LNA Array power labeling kit (Exiqon) following the procedure described by the manufacturer. The labeled RNAs were mixed pair-wise and hybridized to the miRCURY LNA Array version 5th Generation (Exiqon). The hybridization was performed according to the miRCURY LNA array manual using an HS4800 hybridization station (Tecan). The slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies) and the image analysis was carried out using ImaGene 8.0 software (BioDiscovery). The quantified signals were background corrected (Normexp with offset value 10) (Ritchie et al., 2007), and normalized using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm. miRNAs that were more than fourfold differentially expressed were considered for validation by qPCR. Statistical analyses were performed using Student's *t*-tests. RT-PCR for mRNA was carried out using SuperScript II reverse transcriptase (Invitrogen) with random primers, and in RT-PCR for miRNA the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) was used. In both cases, reactions were assembled following the manufacturer's recommendations. qPCR was performed on a LightCycler 480 system (Roche). The universal probe library (Roche) was used for the analysis of mRNA expression. The Taqman microRNA assay (Applied Biosystems) was used for screening the differential expression of miRNAs. qPCR reactions and data analysis were performed as previously described (Carraro et al., 2009). Results are presented as relative expression compared with control. Results were collected from at least three lungs or pools of lungs from independent experiments.

Culture of embryonic lung explants

Timed-pregnant wild-type or transgenic mice were sacrificed on post-coitum (embryonic) day 11.5 (E11.5), and the embryos were harvested. Lung primordia were placed on 8 µm Nucleopore Track-Etch membranes (Whatman) and cultured in DMEM:F12 medium (Gibco) and 0.5% fetal bovine serum (FBS). Vivo-morpholinos (Gene Tools) were added at 1–4 µM to the lung explants. Recombinant FGF9 and WNT3a (R&D) were added at 200 ng/ml and 250 ng/ml, respectively. The data are representative of at least three lungs from different timed pregnant mice.

Computational targets prediction

The *miR-142-3p* targets were selected on the basis of the on-line available prediction database miRTar (mirtar.mbc.nctu.edu.tw). The 5'-UTR and the 3'-UTR of target genes were included in the screening. A minimum free energy (MFE) ≤11 Kcal/mol and Score ≥140 were used. A Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis for pathway enrichment was performed.

β-galactosidase staining

Axin2^{lacZ} lung explants were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4°C for 5 minutes with rocking, washed twice for 5 minutes in PBS at 4°C, then transferred into freshly prepared X-

gal solution and stained at 37°C until a clear precipitate formed; the method was modified from that of Hogan et al. (Hogan et al., 1994). After rinsing with PBS, epithelial explants were post-fixed in 4% PFA in PBS. For vibratome sections, samples were embedded in a mixture of 300 mg/ml albumin, 5 mg/ml gelatin and 0.6% glutaraldehyde, and sectioned. The data are representative of three lungs from independent experiments.

Immunofluorescence

Tissues were fixed in 4% PFA, gradually dehydrated in ethanol, impregnated with toluene, embedded in paraffin, and sectioned into 5 µm slices on poly-L-lysine-coated slides. Antigen retrieval was performed by boiling the sample for 10 minutes in sodium citrate buffer (10 mM, pH 6.0; Vector). The following antibodies (Abs) were used: APC Ab (Abcam), KI67 Ab (Novabios), phospho-ERK (pERK) Ab (Cell Signaling), phospho-MEK Ab (Cell Signaling), LEF1 Ab (Cell Signaling), phospho-Ser552-CTNNB1 Ab (Cell Signaling), ACTA2 Ab (Sigma), MYH11 Ab (Sigma). All antibodies were used at a 1:200 dilution. The data are representative of at least three lungs from independent experiments.

Image analysis

Photomicrographs of immunofluorescence staining were taken using a Leica DMRA fluorescence microscope with a Leica digital camera. Live-cell and lung explant time-lapse experiments were performed with a Leica AF6000 fluorescence imaging system. Digital analysis of staining intensity and distribution, and of live-imaging experiments was performed with MetaMorph (Molecular Devices) or ImageJ software. At least three different samples for each condition were analyzed. Phase-contrast images of the samples were recorded using a digital camera (Diagnostic Instruments) connected to a reversed phase-contrast microscope (Leica). The public domain software ImageJ was broadened by routines specifically developed by us and used to process and analyze the images (Guidolin et al., 2004). The number of branches, percentage of tissue area involved in branching, and branching complexity (i.e. the number of branching points of the binary skeleton and the distribution of branching orders) were the morphometric parameters estimated from each sample. Data were processed by using statistical analysis software (GraphPad Prism 3.03, GraphPad Software). The data are representative of at least three lungs from independent experiments.

miRNA pull-down assay

MLg, MFLM4 or HEK293 cells were cultured in six-well plates and transfected in triplicate with 3'-biotinylated *miR-142-3p* (Bio-miR-142) or 3'-biotinylated scramble (Bio-scramble; Dharmacon), at a final concentration of 30 nM using Lipofectamine RNAimax (Invitrogen) following the manufacturer's protocol. After 48 hours, the cells were pelleted at 1000 rpm for 5 minutes. After washing twice, cell pellets were resuspended in 0.5 ml lysis buffer [50 mM Tris-HCl, 2 mM EDTA, 0.1% NP40, 10% glycerol, 2 mM EGTA, diethylpyrocarbonate (DEPC)-treated water, 50 U RNasin (Promega) and complete mini-protease inhibitor cocktail (Roche Applied Science)], and incubated at 4°C for 10 minutes. The cytoplasmic extract was isolated by centrifugation at 10,000 rpm for 10 minutes. Streptavidin-coated magnetic beads (Invitrogen) were blocked for 1 hour at 4°C in blocking buffer (10 mM Tris-HCl pH 6.5, 1 mM EDTA, 1 mg/ml yeast tRNA and 1 mg/ml BSA) and washed twice with 1 ml washing buffer (10 mM Tris-HCl pH 6.5, 1 mM EDTA 0.5 M NaCl). Beads were resuspended in 0.5 ml washing buffer. Cytoplasmic extract was then added to the beads and incubated for 1 hour at 4°C with slow rotation. The beads were then washed five times with 1 ml washing buffer. RNA bound to the beads (pull-down RNA) or from 10% of the extract (input RNA), was isolated using Trizol reagent LS (Invitrogen). The level of mRNA in the Bio-miR-142-3p or Bio-scramble control pull-down was quantified by qPCR. mRNA levels were normalized to a housekeeping gene (*Gapdh*, *H4*). The enrichment ratio of the control-normalized pull-down RNA to the control-normalized input levels was then calculated. The data for each cell line are representative of three independent experiments.

Luciferase reporter assay

The predicted binding sequence on the 3' UTR of *Apc* was synthetically produced by PCR and transferred into a luciferase reporter vector. To

determine the specificity of the interaction, a reporter vector containing a 'seed' mutated sequence was generated using specific primers. The vector backbone was a pmiRGLO luciferase reporter (Promega) carrying a synthetic firefly luciferase (*luc2*) and a *Renilla* luciferase. Our constructs were inserted downstream of the luciferase gene into a unique *Xba*I restriction site. The *Renilla* luciferase was used as a control to normalize luminescence levels. Experiments were carried out using a dual-luciferase reporter assay system (Promega) and analyzed with a Victor³ Luminometer (PerkinElmer). The data are representative of three independent experiments.

Cell culture

For the preparation of primary mesenchymal cells, whole lungs were dissected at E14.5 and subjected to trypsin digestion to give rise to single cells. Mesenchymal cells were separated from epithelial cells by differential adhesion as previously described (Lebeche et al., 1999). Fifty minutes after plating, 200 ng/ml FGF9 was added to the mesenchymal cells. The cells were cultured in DMEM/F12 medium containing penicillin, streptomycin and 0.5% FBS in the presence or absence of FGF9. MFLM4 (Seven Hills), MLg (ATCC) and HEK293 (ATCC) cells were grown in DMEM with 10% FBS. Plasmids expressing GFP (pGFP) and APC (pAPC) were introduced into the cells by electroporation (Lonza). MLg cells were electroporated with 30 nM scramble (scra) or miR-142-3p mimic (mi142) molecules (Dharmacon). The data are representative of three independent experiments.

Statistical analysis

All data are reported as means $\pm$ s.d. Statistical analyses were performed using Student's *t*-tests, with $P \leq 0.05$ considered significant.

Acknowledgements

We thank Matthew Jones for critical reading of the manuscript.

Competing interests

The authors declare no competing financial interests.

Author contributions

G.C. developed the approach, performed experiments and prepared the manuscript. A.S., J.R., A.C., C.C., E.A. and B.M. performed experiments. S.D. and D.G. performed data analysis. M.M.T. and M.E.K. provided mouse lines. W.S. and A.G. edited the manuscript. G.B. and S.D.L. developed the approach and edited the manuscript. S.B. developed the approach and prepared the manuscript prior to submission.

Funding

This work was supported by the National Institutes of Health (NIH) [RO1 grants HL086322 and HL107307 to S.B., and HL092967 to S.D.L.]; the Excellence Cluster in Cardio-Pulmonary system, Deutsche Forschungsgemeinschaft, LOEWE (S.B.); LOEWE [III L4 – 518/15.004 2009 to G.B.]; and the Deutsche Forschungsgemeinschaft [BA 4036/1-1 to G.B.]. Deposited in PMC for release after 12 months.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.105908/-DC1>

References

- Brabletz, S. and Brabletz, T. (2010). The ZEB/miR-200 feedback loop – a motor of cellular plasticity in development and cancer? *EMBO Rep.* **11**, 670–677.
- Carraro, G., El-Hashash, A., Guidolin, D., Tiozzo, C., Turcatel, G., Young, B. M., De Langhe, S. P., Bellusci, S., Shi, W., Parnigotto, P. P. et al. (2009). miR-17 family of microRNAs controls FGF10-mediated embryonic lung epithelial branching morphogenesis through MAPK14 and STAT3 regulation of E-Cadherin distribution. *Dev. Biol.* **333**, 238–250.
- Clevers, H. and Nusse, R. (2012). Wnt/ β -catenin signaling and disease. *Cell* **149**, 1192–1205.
- Colvin, J. S., White, A. C., Pratt, S. J. and Ornitz, D. M. (2001). Lung hypoplasia and neonatal death in Fgf9-null mice identify this gene as an essential regulator of lung mesenchyme. *Development* **128**, 2095–2106.
- De Langhe, S. P., Carraro, G., Tefft, D., Li, C., Xu, X., Chai, Y., Minoo, P., Hajhosseini, M. K., Drouin, J., Kaartinen, V. et al. (2008). Formation and differentiation of multiple mesenchymal lineages during lung development is regulated by beta-catenin signalling. *PLoS ONE* **3**, e1516.
- Ei Agha, E., Al Alam, D., Carraro, G., MacKenzie, B., Goth, K., De Langhe, S. P., Voswinkel, R., Hajhosseini, M. K., Rehan, V. K. and Bellusci, S. (2012). Characterization of a novel fibroblast growth factor 10 (Fgf10) knock-in mouse line to target mesenchymal progenitors during embryonic development. *PLoS ONE* **7**, e38452.
- Fish, J. E., Santoro, M. M., Morton, S. U., Yu, S., Yeh, R. F., Wythe, J. D., Ivey, K. N., Bruneau, B. G., Stainier, D. Y. and Srivastava, D. (2008). miR-126 regulates angiogenic signaling and vascular integrity. *Dev. Cell* **15**, 272–284.
- Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M. et al. (1991). Identification and characterization of the familial adenomatous polyposis coli gene. *Cell* **66**, 589–600.
- Guidolin, D., Vacca, A., Nussdorfer, G. G. and Ribatti, D. (2004). A new image analysis method based on topological and fractal parameters to evaluate the angiostatic activity of docetaxel by using the Matrigel assay in vitro. *Microvasc. Res.* **67**, 117–124.
- Harada, N., Tamai, Y., Ishikawa, T., Sauer, B., Takaku, K., Oshima, M. and Taketo, M. M. (1999). Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. *EMBO J.* **18**, 5931–5942.
- He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B. and Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway. *Science* **281**, 1509–1512.
- Hogan, B. L., Blessing, M., Winnier, G. E., Suzuki, N. and Jones, C. M. (1994). Growth factors in development: the role of TGF-beta related polypeptide signalling molecules in embryogenesis. *Dev. Suppl.* **1994**, 53–60.
- Jiang, Q., Hao, Y., Wang, G., Juan, L., Zhang, T., Teng, M., Liu, Y. and Wang, Y. (2010). Prioritization of disease microRNAs through a human phenome-microRNAome network. *BMC Syst. Biol.* **4** Suppl. 1, S2.
- Kaduthanam, S., Gade, S., Meister, M., Brase, J. C., Johannes, M., Dienemann, H., Warth, A., Schnabel, P. A., Herth, F. J., Sültmann, H. et al. (2013). Serum miR-142-3p is associated with early relapse in operable lung adenocarcinoma patients. *Lung Cancer* **80**, 223–227.
- Lebeche, D., Malpel, S. and Cardoso, W. V. (1999). Fibroblast growth factor interactions in the developing lung. *Mech. Dev.* **86**, 125–136.
- Lu, Y., Thomson, J. M., Wong, H. Y., Hammond, S. M. and Hogan, B. L. (2007). Transgenic over-expression of the microRNA miR-17-92 cluster promotes proliferation and inhibits differentiation of lung epithelial progenitor cells. *Dev. Biol.* **310**, 442–453.
- Lv, M., Zhang, X., Jia, H., Li, D., Zhang, B., Zhang, H., Hong, M., Jiang, T., Jiang, Q., Lu, J. et al. (2012). An oncogenic role of miR-142-3p in human T-cell acute lymphoblastic leukemia (T-ALL) by targeting glucocorticoid receptor- α and cAMP/PKA pathways. *Leukemia* **26**, 769–777.
- Mailleux, A. A., Kelly, R., Veltmaat, J. M., De Langhe, S. P., Zaffran, S., Thiery, J. P. and Bellusci, S. (2005). Fgf10 expression identifies parabronchial smooth muscle cell progenitors and is required for their entry into the smooth muscle cell lineage. *Development* **132**, 2157–2166.
- Neilson, J. R., Zheng, G. X., Burge, C. B. and Sharp, P. A. (2007). Dynamic regulation of miRNA expression in ordered stages of cellular development. *Genes Dev.* **21**, 578–589.
- Niehrs, C. and Acebron, S. P. (2012). Mitotic and mitogenic Wnt signalling. *EMBO J.* **31**, 2705–2713.
- Nimmo, R., Ciaiu-Uitz, A., Ruiz-Herguido, C., Soneji, S., Bigas, A., Patient, R. and Enver, T. (2013). MiR-142-3p controls the specification of definitive hemangioblasts during ontogeny. *Dev. Cell* **26**, 237–249.
- Ornitz, D. M. and Yin, Y. (2012). Signaling networks regulating development of the lower respiratory tract. *Cold Spring Harb. Perspect. Biol.* **4**.
- Oshima, H., Oshima, M., Kobayashi, M., Tsutsumi, M. and Taketo, M. M. (1997). Morphological and molecular processes of polyp formation in *Apc(delta716)* knockout mice. *Cancer Res.* **57**, 1644–1649.
- Rasmussen, K. D., Simmini, S., Abreu-Goodger, C., Bartonicek, N., Di Giacomo, M., Bilbao-Cortes, D., Horos, R., Von Lindern, M., Enright, A. J. and O'Carroll, D. (2010). The miR-144/451 locus is required for erythroid homeostasis. *J. Exp. Med.* **207**, 1351–1358.
- Ritchie, M. E., Silver, J., Oshlack, A., Holmes, M., Diyagama, D., Holloway, A. and Smyth, G. K. (2007). A comparison of background correction methods for two-colour microarrays. *Bioinformatics* **23**, 2700–2707.
- Tian, Y., Zhang, Y., Hurd, L., Hannenhalli, S., Liu, F., Lu, M. M. and Morrissey, E. E. (2011). Regulation of lung endoderm progenitor cell behavior by miR302/367. *Development* **138**, 1235–1245.
- van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Battle, E., Coudreuse, D., Haramis, A. P. et al. (2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* **111**, 241–250.
- Van Scoyk, M., Randall, J., Sergew, A., Williams, L. M., Tennis, M. and Winn, R. A. (2008). Wnt signaling pathway and lung disease. *Transl. Res.* **151**, 175–180.
- Ventura, A., Young, A. G., Winslow, M. M., Lintault, L., Meissner, A., Erkland, S. J., Newman, J., Bronson, R. T., Crowley, D., Stone, J. R. et al. (2008). Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. *Cell* **132**, 875–886.
- Wang, Y., Huang, C., Reddy Chintagari, N., Bhaskaran, M., Weng, T., Guo, Y., Xiao, X. and Liu, L. (2013). miR-375 regulates rat alveolar epithelial cell trans-differentiation by inhibiting Wnt/ β -catenin pathway. *Nucleic Acids Res.* **41**, 3833–3844.
- Yi, L., Domyan, E. T., Lewandoski, M. and Sun, X. (2009). Fibroblast growth factor 9 signaling inhibits airway smooth muscle differentiation in mouse lung. *Dev. Dyn.* **238**, 123–137.
- Yin, Y., White, A. C., Huh, S. H., Hilton, M. J., Kanazawa, H., Long, F. and Ornitz, D. M. (2008). An FGF-WNT gene regulatory network controls lung mesenchyme development. *Dev. Biol.* **319**, 426–436.
- Yin, Y., Wang, F. and Ornitz, D. M. (2011). Mesothelial- and epithelial-derived FGF9 have distinct functions in the regulation of lung development. *Development* **138**, 3169–3177.

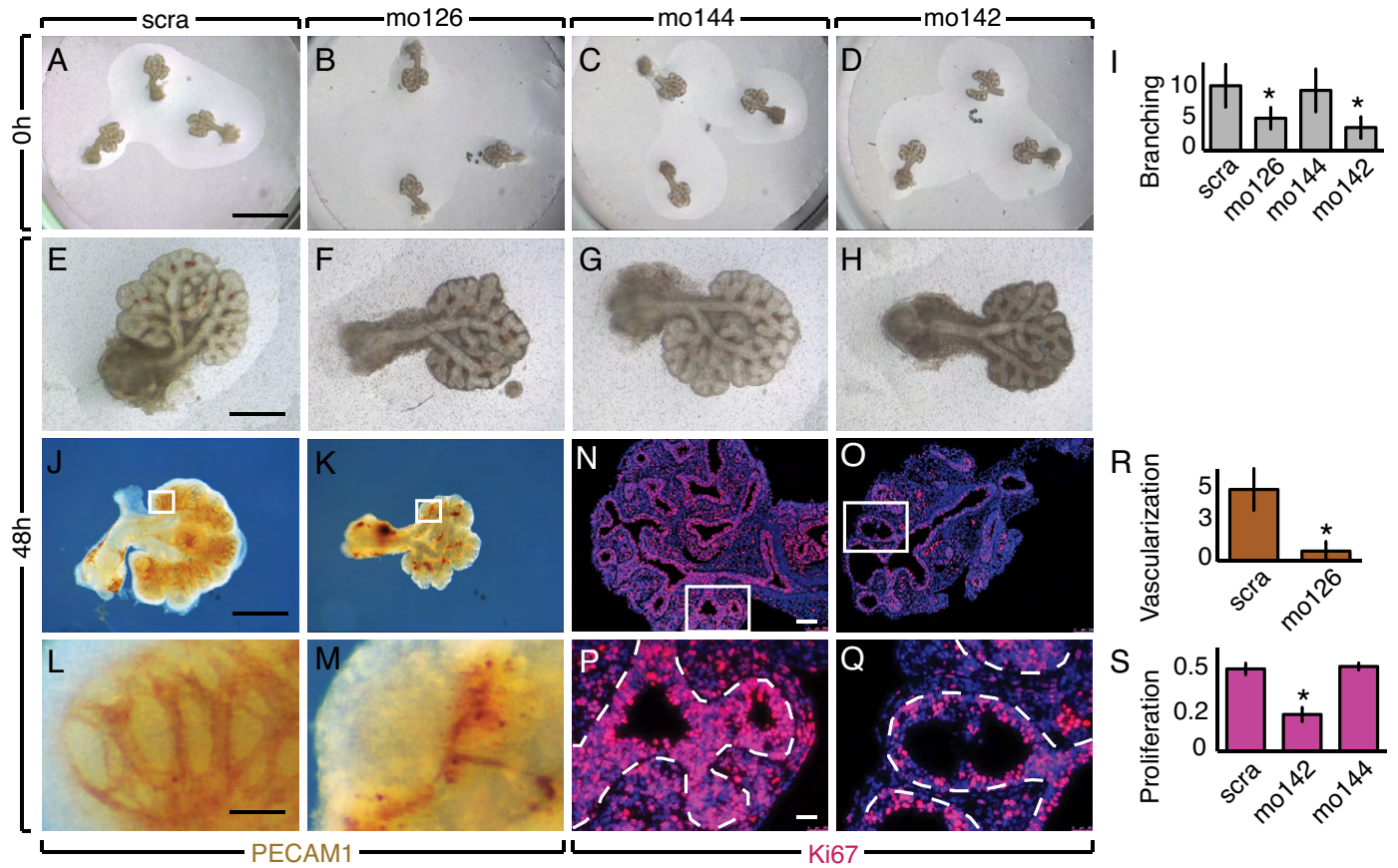


Figure S1. Validation of the vivo-morpholino LOF assay. (A-H) Morphometric changes on E11.5 lung explants administrated with scramble (scra; A,E), mo126 (B,F), mo144 (C,G), mo142 (D,H), and its relative quantification of branching (I). (J-M) Immunostaining for PECAM1 showing vasculature formation in E11.5 lung explants treated with scra (J,L) and mo126 (K,M). (R) Quantification of the vasculature ramification calculated as numbers of intercepts across terminal buds. (N-Q) Immunostaining for Ki67 showing cell proliferation in mo144 (N,P) and mo142 (O,Q) treated lungs. White boxes indicate the magnified areas in the lower panels. Dashed lines demarcate the epithelium-mesenchyme boundary. (S) Quantification of proliferation in the mesenchyme of mo144 and mo142 treated lungs. Scale bars: 1.2 mm (A-D); 250 μ m (E-H,J,K); 35 μ m (L,M); 100 μ m (N,O); 50 μ m (P,Q); Data are means $\pm$ s.d.

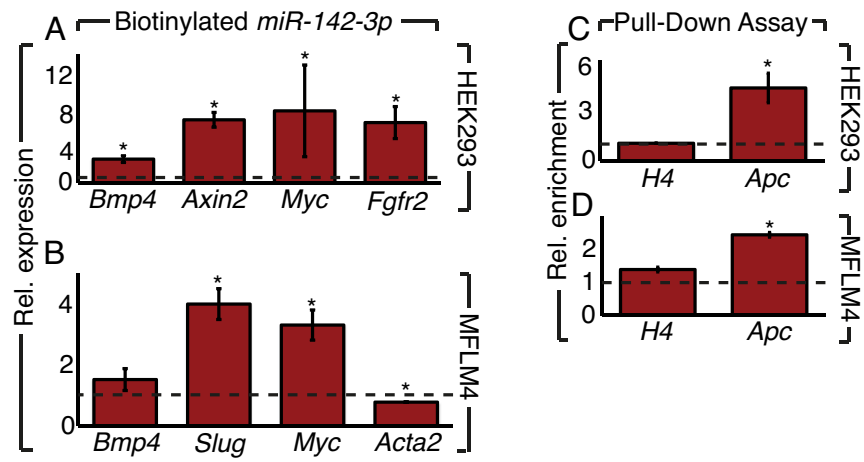
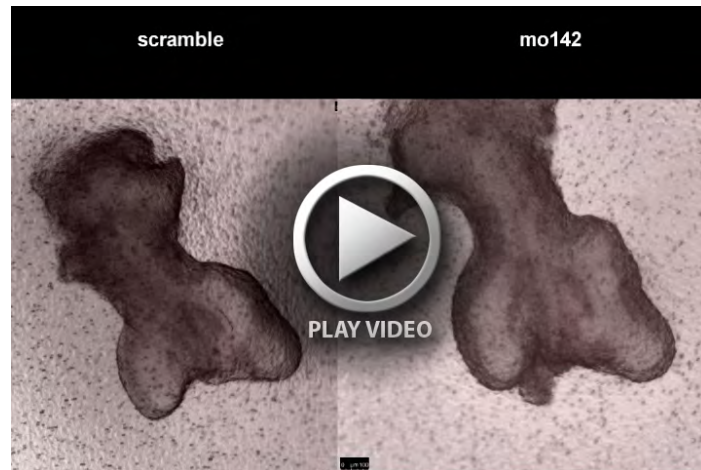
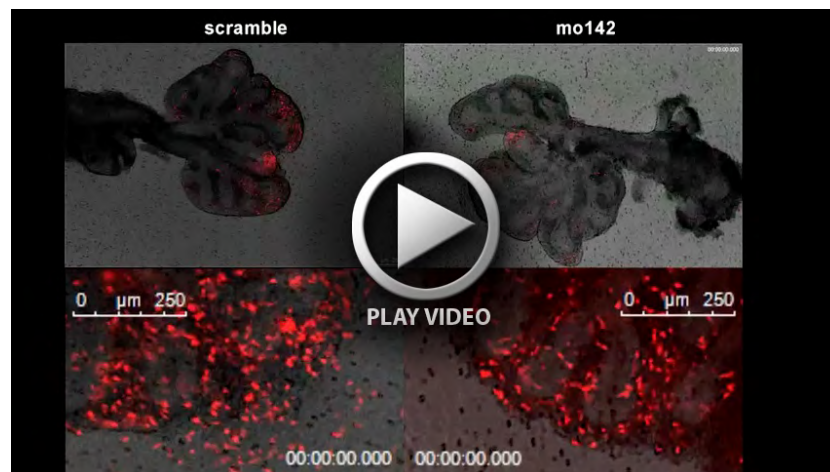


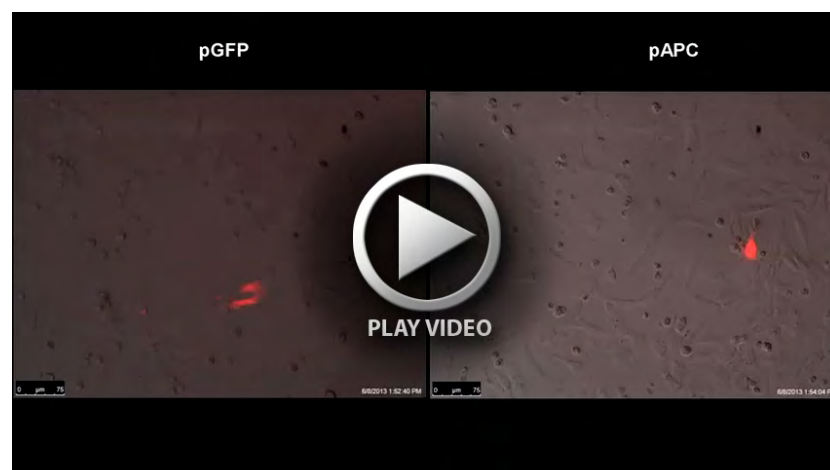
Figure S2. Pull-down assay for *miR-142-3p* on HEK293 and MFLM4 cells. (A,B) WNT signaling related gene expression is shown by qPCR after transfection of HEK293 cells (A) and MFLM4 cells (B) with biotinylated *miR-142-3p*. Data are normalized against biotinylated scramble. (C,D) *Apc* enrichment after pull-down assay on HEK293 cells and MFLM4 cells is shown by qPCR. Data are means $\pm$ s.d.



Movie 1. Bright field time-lapse microscopy of the branching lung is shown in control (scramble) and after *miR-142-3p* knockdown (mo142). Scramble and mo142 treated lungs were analyzed for 45 hours.



Movie 2. Fluorescence time-lapse microscopy of *Fgf10^{RFP}* lung explants after *miR-142-3p* LOF assay is shown. Control (scramble) and treated (mo142) lungs were analyzed for 20 hours (top row). Scramble and mo142 treated lungs are shown in high magnification for an additional 20 hours (bottom row).



Movie 3. Bright field and fluorescence time-lapse microscopy of primary culture of mesenchymal cells isolated from *Fgf10^{RFP}* lungs electroporated with *Apc* (pAPC) or control *Gfp* (pGFP) plasmids.

RESEARCH ARTICLE

Generation and Validation of *miR-142* Knock Out Mice

Amit Shrestha¹, Gianni Carraro², Elie El Agha¹, Regina Mukhametshina¹, Cho-Ming Chao¹, Albert Rizvanov³, Guillermo Barreto^{4,5}, Saverio Bellusci^{1,3,5,6*}

1 German Center for Lung Research, Excellence Cluster Cardio-Pulmonary System, Universities of Giessen and Marburg Lung Center, Giessen, Hessen, Germany, **2** Lung and Regenerative Medicine Institutes, Department of Medicine, Cedars-Sinai Medical Center, Los Angeles, California, **3** Institute of Fundamental Medicine and Biology, Kazan (Volga Region) Federal University, Kazan, Russian Federation, **4** LOEWE Research Group Lung Cancer Epigenetic Max Plank Institute, Bad Nauheim, Germany, **5** Member of the German Center for Lung Research, Giessen, Germany, **6** Developmental Biology Program, Division of Surgery, Saban Research Institute of Children's Hospital Los Angeles, University of Southern California Keck School of Medicine, Los Angeles, California

* Saverio.Bellusci@innere.med.uni-giessen.de



OPEN ACCESS

Citation: Shrestha A, Carraro G, El Agha E, Mukhametshina R, Chao C-M, Rizvanov A, et al. (2015) Generation and Validation of *miR-142* Knock Out Mice. PLoS ONE 10(9): e0136913. doi:10.1371/journal.pone.0136913

Editor: Vladimir V. Kalinichenko, Cincinnati Children's Hospital Medical Center, UNITED STATES

Received: April 21, 2015

Accepted: August 10, 2015

Published: September 1, 2015

Copyright: © 2015 Shrestha et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its supporting information files. The proprietary vector and/or the detailed map will be made available to other researchers upon reasonable request. A Material Transfer Agreement may be requested from Eve Geronimus: eve.geronimus@igbmc.fr.

Funding: SB was supported by grants from the Deutsche Forschungsgemeinschaft (BE4443/4-1 and BE4443/6-1), Landes-Offensive zur Entwicklung Wissenschaftlich-ökonomischer Exzellenz, the Universtat Klinikum Giessen Marburg, the University of Giessen Marburg Lung Center, as well as the

Abstract

microRNA-142 (miR-142) is an important regulator of many biological processes and associated signaling pathways during embryonic development, homeostasis and disease. The *miR-142* hairpin gives rise to the "guide strand" *miR-142-3p* and the sister "passenger" strand *miR-142-5p*. *miR-142-3p* has been shown to play critical, non-redundant functions in the development of the hematopoietic lineage. We have recently reported that *miR-142-3p* is critical for the control of Wnt signaling in the mesenchyme of the developing lung. *miR-142-5p* has been proposed to control adaptive growth in cardiomyocytes postnatally and its increase is associated with extensive apoptosis and cardiac dysfunction in a murine heart failure model. Using homologous recombination, we now report the generation and validation of *miR-142*-null mice. *miR-142*-null mice show a significant decrease in the expression levels of both the *3p* and *5p* isoforms. The expression of *Bzrap1*, a gene immediately flanking *miR-142* is not altered while the expression of a long non-coding RNA embedded within the *miR-142* gene is decreased. *miR-142*-null newborn pups appear normal and are normally represented indicating absence of embryonic lethality. At embryonic day 18.5, *miR-142*-null lungs display increased Wnt signaling associated with the up-regulation of *Apc* and *p300*, two previously reported targets of *miR-142-3p* and *-5p*, respectively. Adult *miR-142*-null animals display impaired hematopoietic lineage formation identical to previously reported *miR-142* gene trap knockdown mice. We report, for the first time, the homologous recombination-based *miR-142*-null mice that will be useful for the scientific community working on the diverse biological functions of *miR-142*.

program of competitive growth of Kazan Federal University. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

microRNAs (miRNAs) are 22 to 25 nucleotide-long, single stranded RNAs that are processed from hairpin transcripts. The maturation of the hairpin transcript gives rise to the 3p guide strand and 5p sister passenger strand, both of which allow the silencing of specific sets of genes through base pairing to a minimal recognition sequence [1,2]. We previously described that *miR-142-3p* is among a few miRs expressed at high level in the lung mesenchyme during early embryonic development [3]. Such restricted expression pattern suggested that *miR-142-3p* could play critical functions in controlling cell lineage formation in the mesenchyme. Using *in-vitro* approaches with embryonic lungs cultured in presence of morpholinos for *miR-142-3p*, we showed that knockdown of *miR-142-3p* leads to arrested proliferation and premature differentiation of smooth muscle progenitor cells. We established that *miR-142-3p* positively regulates Ctnnb1 (β -catenin) signaling during lung development by targeting *Adenomatous polyposis coli* (*Apc*) mRNA for degradation. *Apc* negatively regulates Ctnnb1 via direct binding to Ctnnb1 thereby antagonizing the interaction of Ctnnb1 with the transcription factor Tcf. In combination with Axin and Gsk3b, *Apc* induces ubiquitination and degradation of Ctnnb1 [4]. Using genetic tools, we showed that up-regulation of Ctnnb1 signaling specifically in the mesenchyme via the induced expression of a stable form of Ctnnb1 or the deletion of a copy of *Apc* is sufficient to rescue *miR-142-3p* morpholino-mediated loss-of-function and that *Apc* is a critical target of this miRNA.

Beyond its proposed function in lung development, *miR-142-3p* is one of the highest expressed miRs in various hematopoietic lineages [5,6]. *miR-142-3p* controls neutrophil development in zebrafish [7], orchestrates a network of actin cytoskeleton regulator during megakaryopoiesis [8] and regulates the specification of definitive hemagloblasts during organogenesis [9]. Increased levels of *miR-142-3p* in the serum are associated with recurrence of adenocarcinoma in humans [10]. *miR-142-3p* is a target of Interleukin 6 (IL6) in glioblastoma and it has been proposed that *miR-142-3p* blocks the expression of *IL6*, *Hmga2* and *Sox2* thereby suppressing the stem like properties of glioblastomas [11]. Supporting our *in-vitro* results obtained with *miR-142-3p* during lung development [3], it was reported that *miR-142-3p*, which is up-regulated in human breast cancer stem cells, activates the canonical WNT signaling pathway in a APC-suppression dependent manner [12], resulting in enhanced tumorigenicity. *miR-142-3p* regulates IL6 production upon lipopolysaccharide stimulation in dendritic cells [13]. RNA viruses can also bind *miR-142-3p* to suppress innate immunity promoting neurological disease manifestations [14]. While a significant amount of information is available for *miR142-3p*, our knowledge of *miR-142-5p* is still scarce. In the postnatal heart, *miR-142-5p* targets *p300*, a gene encoding a positive regulator of Wnt signaling and increased expression of *miR-142-5p* is associated with extensive apoptosis and cardiac dysfunction in murine heart failure model [15].

In order to unveil the role of *miR-142* in organogenesis, homeostasis and disease *in-vivo*, we generated and validated the *miR-142-null* mouse. Our validation studies confirm that both *miR-142-3p* and *miR-142-5p* are no longer expressed in these mice. We also show that *miR-142-null* embryonic lungs display increased Wnt signaling confirming the functional role of *miR-142* during lung development. *miR-142-null* adult mice display various hematological abnormalities such as decreased platelet and white blood cell count and increased mean platelet volume indicating that these mice suffer from thrombocytopenia. Similar results were obtained with the *miR-142* knockdown mice developed by Chapnik *et al.*, using exogenous gene trap technology [8]. In this paper, we report the generation and validation of a *miR-142-null* mouse line that will be useful for the scientific community working on *miR-142*.

Materials and Methods

miR-142-null mouse line establishment

The *miR-142*-null mutant mouse line was established at the MCI/ICS (Institut Clinique de la Souris, iCS, Infrastructure Nationale PHENOMIN, 1 rue Laurent Fries, 67404 Illkirch, France). A MCI proprietary vector containing a floxed neomycin resistance cassette and Protamine-Cre cassette was used (Fig A and B in [S1 Fig](#)). The use of Protamine-Cre cassette in the construction vector offers an efficient solution for the auto-excision of the floxed region in the male germ line of mice. In parallel, a 3.3kb fragment (corresponding to the 5' homology arm) and 2.6kb fragment (corresponding to the 3' homology arms) were amplified by PCR and sub-cloned into MCI proprietary vector to generate the final targeting construct. The linearized construct was electroporated in C57BL/6N mouse embryonic stem (ES) cells. After selection, targeted clones were identified by PCR using external primers and further confirmed by Southern blotting with 3' external probe. Two positive recombinant ES clones were injected into BALB/CN blastocysts, and resulting male chimeras were crossed with wild type females. The genotype of mice with germ-line transmission was confirmed using the primers described below. Generated mice were transferred to Mfd Diagnostics (Wendelsheim/Rheinland-Pfalz, Germany), and housed in a SPF environment. Harvesting organs and tissues from wild type and mutant mice following euthanasia using CO₂ was approved at Justus Liebig University Giessen by the federal authorities for animal research of the Regierungspraesidium Giessen, Hessen, Germany (Approved Protocol No. 452_M).

PCR Genotyping

miR-142 heterozygous and null mutant mice were identified by performing PCR on tail genomic DNA using four primers (P1-4). The P2/P3 primers allow detecting the *miR-142* wild type allele (174 bp). The P1/P4 primers allow detecting the presence of the LoxP cassette (105 bp) and the P1/P3 primers allow detecting the deletion of the endogenous *miR-142* gene (294 bp).

(P1) forward: GAA GAA CGA GAT CAG CAG CCT CTG TTC C; (P2) forward: ACG CTA GCA CAG TGT GTG CCC A; (P3) reverse: ACC CAT ATG ATA CAC CAG CCA CGT C; (P4) reverse: GAA GTT ATA CTA GAG CGG CCG TTC AC.

The PCR program consists of a denaturation step at 95°C for 4 min, followed by 34 cycles of denaturation (94°C for 30 s), annealing (62°C for 30 s) and extension steps (72°C for 60 s). The program ends with a completion step at 72°C for 420 s. Each PCR tube contains 2.6 U of Taq polymerase in 5μL of reaction buffer (Qiagen Master Mix), 15 pmol of each primer, 0.5 mM dNTPs and 10 ng of genomic DNA in final volume of 10 μL.

Murine Peripheral Blood Counts

In the Mfd diagnostics facility, about 250 μL of whole blood was retro-orbitally drawn from 8 weeks-old sex-matched *miR-142*-null and wild type littermates into glass capillary tubes containing 5 μL of 0.5 M EDTA, to prevent coagulation. ADVIVA2120 Hematology System (Siemens Healthcare, Germany) was used to perform complete blood count measurements. Blood count measurement was performed at Justus-Liebig Universitat Klinik für Kleintiere (Central laboratory for small animals), Giessen Germany.

miRNA computational analysis

The target prediction and pathway intersection for *miR-142-3p* and *miR-142-5p* were performed using the software mirPath. [\[16\]](#)

Quantitative Real-time PCR and Statistical Analysis

Freshly isolated embryonic lungs were lysed and total RNA was extracted using miRNeasy Mini or Micro kit (Qiagen, Hilden Germany). 1 µg of RNA was used for cDNA synthesis and RT-PCR for mRNA was carried out using Quantitative Reverse Transcription kit (Qiagen). RT-PCR for miRNA was carried out using Taqman MicroRNA Reverse Transcription kit (Applied Biosystem). In both cases, reactions were assembled following the manufacturer's recommendations. qPCR was performed using the Light Cycler 480 system (Roche Applied Science). The TaqMan microRNA assay (Applied Biosystem) was used for screening the differential expression of miRNAs whereas SYBR Green (Platinum SYBR Green qPCR Super-Mix-UDG Invitrogen) was used for the analysis of mRNA expression. *U6* and *Hprt* (Hypoxanthine phosphoribosyl transferase1) were used as reference genes for normalization of miRNA and mRNA abundance respectively. Results were collected from at least three lung samples and each reaction was run in triplicate. Data were assembled using Graph Pad Prism Software (Graph Pad software, USA) and statistical analyses were performed using Student's t-test. Data were significant if $p < 0.05$. Primers for *miR142-3p*, *miR142-5p* and *U6* were obtained from Applied Biosystems. Primers for *Bzap1*: Fwd: 5' AGA GAG CCC TGG GTA CAG C 3', Rev: 5' CCC GAA GCC TAT GTT GAA CT 3'. Primers for *LncRNA*: Fwd: 5' CTT CCT GAC CCC TGA TAC TTG 3', Rev: 5' CCC ATA TCC TCA CGG ACG 3'. Primers for *Apc*: Fwd: 5' - CAT GGA CCA GGA CAA GGA CAA AAA CC -3', Rev: 5' - GAA CAC ACA CAG CAG GAC AGA - 3'. Primers for *p300*: Fwd: 5'-ACA TGA TGC CTC GGA TGA CT -3', Rev: 5'- TAG GGG GCT GTG GCA TAT T -3'. Primers for *beta-catenin/Ctnnb1*: Fwd: 5'-GCA GCA GCA GTT TGT GGA -3, Rev: 5'-TGT GGA GAG CTC CAG TAC ACC-3.

Immunofluorescence and *In-situ* hybridization

Tissues were fixed in 4% PFA, gradually dehydrated in ethanol, impregnated with xylene, embedded in paraffin and sectioned into 5 µm slices on poly-L-Lysine-coated slides. Antigen retrieval was performed by treating the sample with Proteinase K for 3 min at 37°C. Slides were blocked twice for 5 min with Dako (DAB Emission +Dual Linksystem HRP, Life Technologies) and then incubated with digoxigenin labeled LNA probes (Exiqon, miRCURY LNA Detection probe, Vedback, Denmark) specific for *miR142-3p* and *miR142-5p*. For immunofluorescence staining the slides were de-paraffinized, blocked with 3% bovine serum albumin (BSA) and 0.4% Triton X-100 (in Tris-buffered saline (TBS) at room temperature (RT) for 1 hour and then incubated with primary antibodies against *Apc* (#Ab15270, Abcam; 1:200), p-S⁵⁵²β-catenin (#9566, Cell Signaling; 1:200) and *p300* (# sc-585, Santa Cruz; 1:200) at 4°C overnight. After incubation with primary antibodies, slides were washed three times in TBST (Tris buffer saline + 0.1% Tween 20) for 5 minutes, incubated with secondary antibodies at RT and washed three times with TBST before being mounted with Prolong Gold Anti-fade Reagent with DAPI (4',6-diamidino-2-phenylindole;Invitrogen). Photomicrographs of immunofluorescence staining were taken using a Leica DMRA fluorescence microscope with a Leica DFC360 FX camera (Leica, Wetzlar, Germany). Figures were assembled using Adobe Illustrator. The data are representative of at least three lungs from independent experiments.

Results

Generation of *miR-142*-null mice

In mice, the *miR-142* gene (ENSMUSG00000065420) is located in chromosome 11 adjacent to the second exon of a long non-coding RNA (A430104N18Rik ENSMUSG00000084796) ([Fig 1](#)). The function is still *LncRNA* is still unknown. *Benzodiazepine receptor (peripheral)*

Chromosome 11

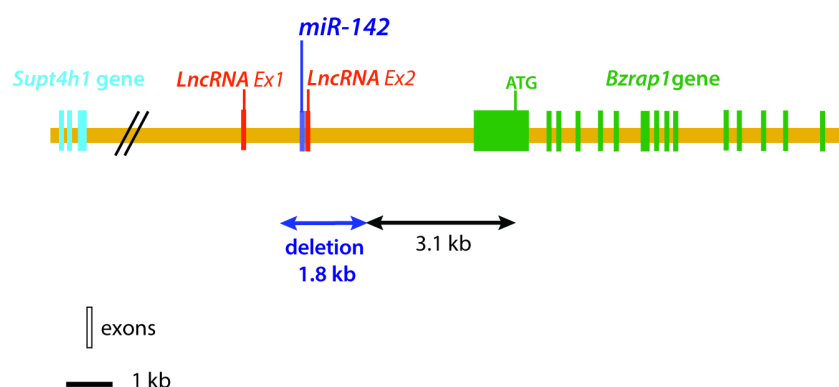


Fig 1. Schematic Representation of the *miR-142* locus in the mouse genome. *miR-142* gene is located in the vicinity of an exon belonging to a LncRNA (A430104N18Rik ENSMUSG00000084796) on chromosome 11. Deletion of *miR-142* led to the deletion of the *LncRNA* exon as well as part of the 5' UTR of *Bzap1*. LncRNA: Long non-coding RNA. *Bzap1*: Benzodiazepine receptor (peripheral) associated protein 1.

doi:10.1371/journal.pone.0136913.g001

associated protein 1 (*Bzap1*, ENSMUSG00000034156) is also another gene found downstream of *miR-142*. The *miR-142* locus also contains many GC-rich repeat regions, which render PCR amplification and screening difficult. The constitutive and complete deletion of the *miR-142* gene was therefore carried out and *miR-142* heterozygous mice were generated. Fig 2B shows the genotyping results of E18.5 embryos arising from crossing *miR-142* heterozygous mice. Fig 2C shows the validation of our primer sets to amplify either the wild type *miR-142* allele (P2/P3), the presence of the *LoxP* site (P1/P4) or the *miR-142*-null allele (P1/P3).

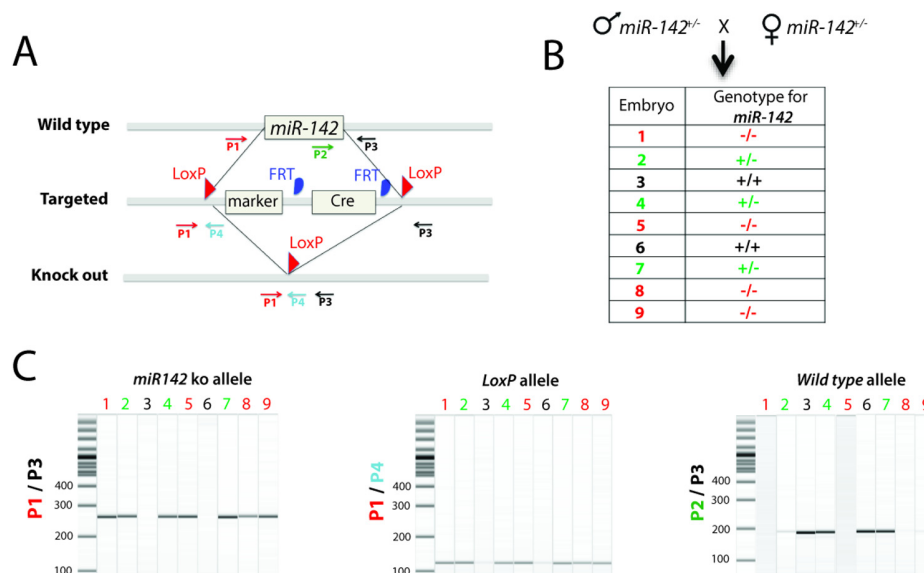


Fig 2. Genotyping Strategy for *miR-142* KO allele. **A)** Schematic diagram showing the WT, targeted and KO alleles for the *miR-142* locus. P1, P2, P3 and P4 represent the primers used to genotype wild type and KO alleles. **B-C)** PCR analysis of the genomic DNA obtained from the embryos at E18.5 from *miR-142*^{+/+} male and female intercrossing. 254 bp band size for KO allele (Primers used: P1 and P3) and 174 bp band size for WT allele (Primers used: P2 and P3) was determined. A 105 bp band was observed using *LoxP* specific primers (P1 and P4).

doi:10.1371/journal.pone.0136913.g002

Table 1. Number of embryos obtained at different stages of embryonic development.

Embryonic stages	<i>mir142</i> ^{+/+}	<i>mir142</i> ^{+/-}	<i>mir142</i> ^{-/-}	Total embryos
E12.5	3 (17%)	8 (44%)	7 (39%)	18
E18.5	3 (20%)	6 (40%)	6 (40%)	15
Post natal	34 (43%)	29 (36%) #	16 (20%) *	79

3 animals died at the age of 2–3 months

* 1 animal died at the age of 4 months

doi:10.1371/journal.pone.0136913.t001

miR-142-null pups are born alive and do not display obvious abnormalities

Litters from *miR-142* heterozygous crosses were genotyped at E12.5, E18.5 and postnatally. Although the expected Mendelian ratio for generating knockout (KO) pups from crossing two hemizygous mice is 25%, this ratio appeared higher when embryos were harvested at E12.5 (39%) and E18.5 (40%) (Table 1). The likely explanation for this observation is the low number of harvested embryos (eighteen and fifteen respectively). Another important conclusion from these data is that *miR-142*-null embryos are not dying *in utero*. In addition, individual monitoring and genotyping of all the neonates born from different litters up to 4 months of age indicate a normal representation of *miR-142*-null mice (20% of the overall postnatal mice ($n = 79$)). In the *miR-142*-null group, only one 4 month-old mouse died while three animals died in the *miR-142* heterozygous group between the ages of 3 and 4 months. None of the mice died in the control wild type group. Overall, our data indicate very low death rate in the *miR-142*-null group postnatally.

miR-142 deletion abolishes the expression of both *miR-142-3p* and *miR-142-5p*

Our experimental approach was designed to carry out the complete deletion of *miR-142*. In the process of deleting *miR-142*, one exon for the *LncRNA*, close to *miR-142*, was also deleted. In addition, part of the 5' region of *Bzrap1*, a gene encoding a regulator of synaptic transmission, was also deleted. To confirm the successful deletion of *miR-142*, RNA from E18.5 *miR-142*^{+/+}, *miR-142*^{+/-} and *miR-142*^{-/-} lungs from littermate embryos ($n = 3$ for each genotype) were extracted. qPCR analysis showed that the expression of both miRNAs, *miR-142-3p* and *miR-142-5p*, was reduced in *miR-142* heterozygous lungs compared to wild type lungs. In addition, the expression of both isoforms was completely abolished in *miR-142*-null lungs (Fig 3A and 3B). The expression levels of *Bzrap1*, a gene positioned 3.5kb downstream of *miR142*, were unchanged in *miR-142* heterozygous or null lungs compared to wild type littermates (Fig 3C), indicating that the promoter region of *Bzrap1* was not impaired for the expression in the lung. Interestingly, we observed a statistically significant decrease in *Bzrap1* expression in the liver ($n = 3$ hets vs. 3 ko, $p = 0.015$) but not in the spleen. In the future, it will therefore be important to validate the changes in *Bzrap1* expression in tissues of interest. As expected, exon 2 of the *LncRNA*, which is located within the deleted region of *miR142*, was not detected in both *miR-142* heterozygous and null embryos (Fig 3D). It still remains to be demonstrated whether the deletion of this exon leads to the complete loss of function the *LncRNA*.

Using specific digoxigenin-labeled probes for these two microRNAs, *in-situ* hybridization showed that *miR-142-3p* and *miR-142-5p* are expressed in both the mesenchyme and the epithelium of the E18.5 lung (Fig 4A, 4B, 4E and 4F). The level of expression of both microRNAs was significantly decreased in *miR142*-null samples compared to WT samples (Fig 4C, 4D, 4G,

4H vs. 4A, 4B, 4E and 4F). Blinded quantification of the expression of the respective isoforms of *miR-142* confirms the very low level of expression of both *miR-142* isoforms (Fig 4I and 4J).

Embryonic *miR-142*-null lungs display increased Wnt signaling associated with up-regulation of *Apc* and *p300*

We previously reported that attenuation of *miR-142-3p* in lung explants grown *in vitro* leads to impaired Wnt signaling in the mesenchyme via the up-regulation of *Apc* [3]. We therefore used immunofluorescence to examine the status of Wnt signaling and *Apc* in *miR-142*-null versus wild type lungs at E18.5. Surprisingly, our results indicate increased expression of the activated form of Ctnnb1/ β -catenin (p-S552) in the nuclei of *miR-142*-null (Fig 5C and 5D) versus control (Fig 5A and 5B) lungs, suggesting increased Wnt signaling upon loss of *miR-142*. However, and in agreement with our previous report [3], *miR-142*-null lungs display increased expression of *Apc* (Fig 5G and 5H vs. 5E and 5F). Next, we investigated the expression of *p300*, a previously validated target of *miR-142-5p* [15]. *p300* is a positive regulator of Ctnnb1 that synergistically activates Ctnnb1/TCF transcription [17]. Our results indicate increased expression of *p300* in *miR-142*-null versus wild type lungs (Fig 5K and 5L vs. 5I and 5J). The

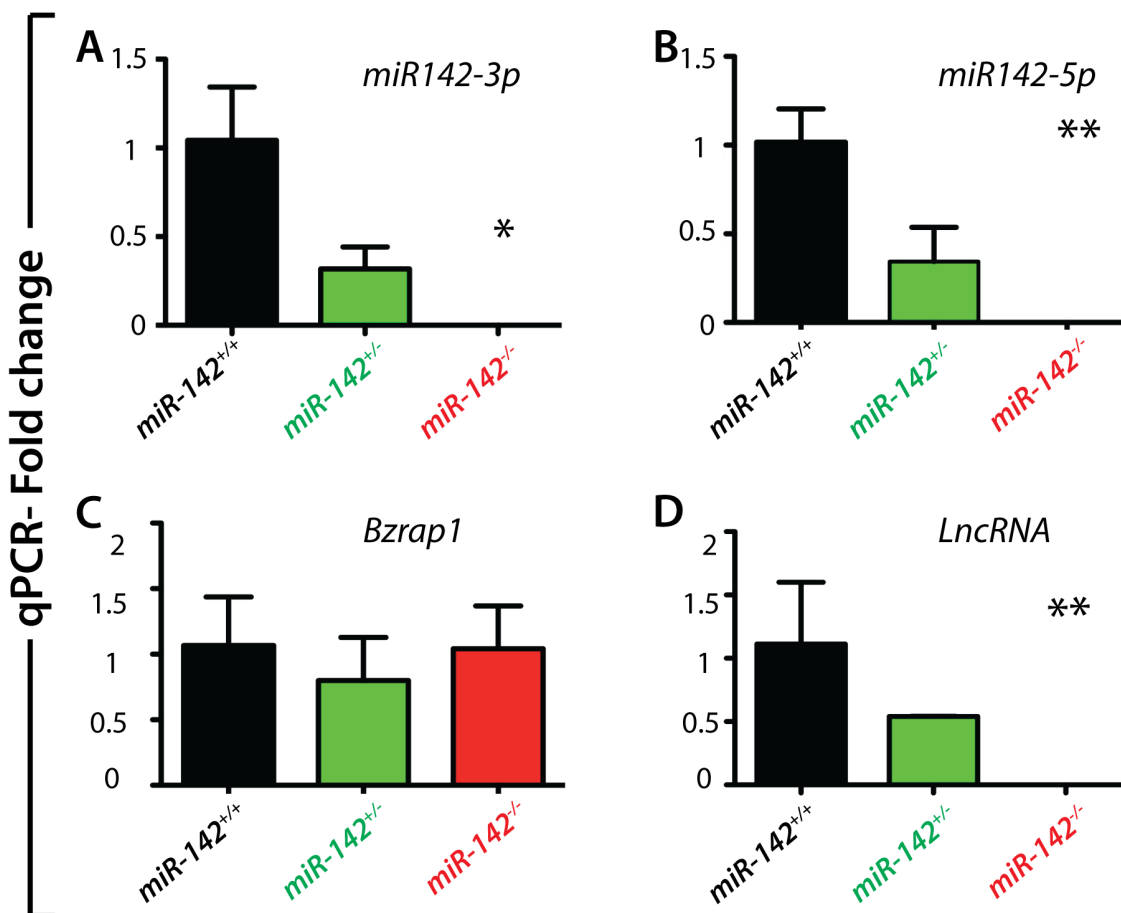


Fig 3. Deletion of *miR-142* successfully abrogates *miR-142-3p* and *miR-142-5p* expression. A-B) qPCR analysis on cDNA obtained from E18.5 embryonic lung sample showing that both *miR-142-3p* and *miR-142-5p* are down-regulated in *miR-142*^{+/-} lungs and completely absent in *miR-142*-null lungs as compared to WT samples. C) The levels of *Bzap1* gene, positioned 3.5kb downstream of the *miR-142* gene, remain unchanged as compared to that of wild type littermates shown. D) Expression of the LncRNA was completely abolished in *miR-142*-null lungs.

doi:10.1371/journal.pone.0136913.g003

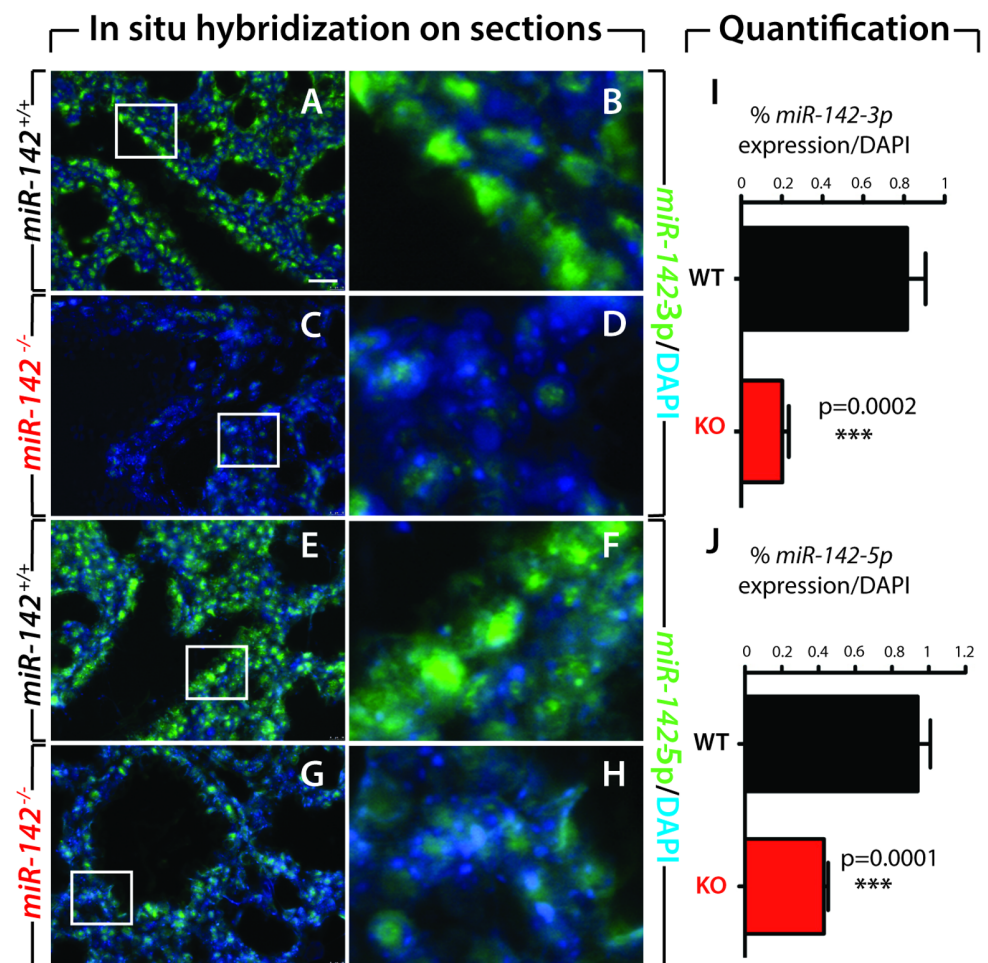


Fig 4. In situ hybridization on E18.5 *miR-142*^{-/-} embryonic lung sections. In situ hybridization for *miR-142-3p* (A-D) and *miR-142-5p* (E-H) showing significant reduction of the corresponding expression levels in *miR-142*^{-/-} compared to WT E18.5 embryonic lung sections. (I-J). Quantification of the in situ hybridization signals indicating a strong reduction of both isoforms. Scale bar: (A,C,E,G): 20 μ m and (B,D,F,H): 4 μ m.

doi:10.1371/journal.pone.0136913.g004

respective increase in *Apc* and *p300* in *miR-142*-null lungs was also validated at the mRNA level (Fig 5N and 5O). *Ctnnb1* mRNA expression is not changed between *miR-142*-null and control lungs (Fig 5M).

miR-142-null mice display a wide range of hematological disorder

miR-142 is highly expressed in hematopoietic cells belonging to both the myeloid and lymphoid lineages. Using an exogenous gene trap technology, Chapnik and colleagues recently reported the phenotypic analysis of *miR-142* knockdown mice. Their mutant mice displayed an array of hematological defects [8]. In order to validate our mouse model, we performed total blood count on 8 week-old *miR-142*^{+/+} and *miR-142*^{-/-} mice ($n = 3$ for each genotype). Our results indicate a significant decrease in the number of white blood cells, lymphocytes, eosinophils, monocytes and platelets in *miR-142*-null vs. wild type animals (Fig 6B–6E and 6G). A significant increase in mean platelet volume was also observed (Fig 6D and 6F).

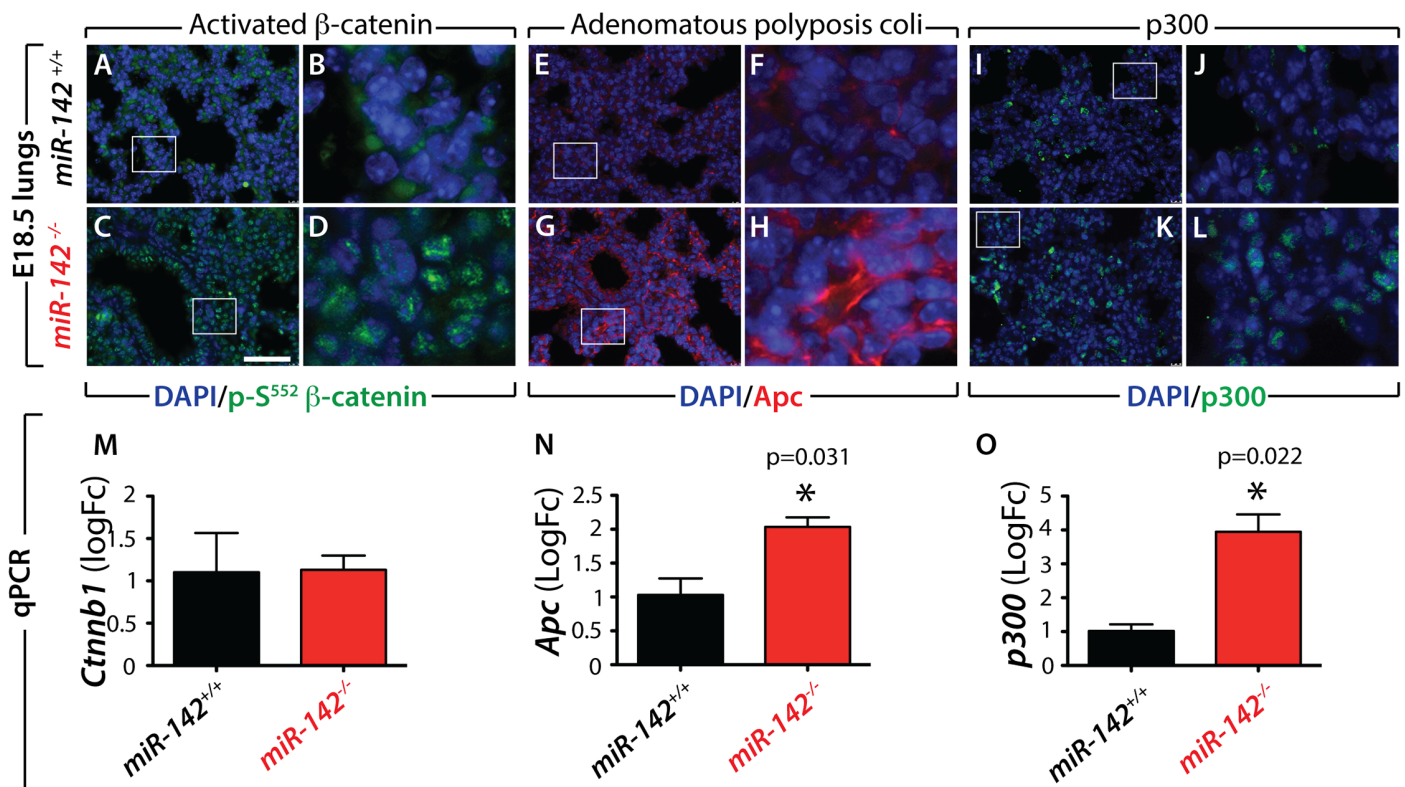


Fig 5. Increased expression of *Apc* and *p300* is associated with increased Wnt signaling in E18.5 *miR-142*-null lungs. (A–L) Immunofluorescence for activated beta-catenin (A–D), Adenomatous polyposis coli (*Apc*) (E–H) and *p300* (I–L) in wild type (A, B, E, F, I, J) and *miR-142* ko (C, D, G, H, K, L) lungs showing increased Wnt signaling, *Apc* and *p300* expression. (M–O) qPCR analysis of beta catenin (*Ctnnb1*), *Apc* and *p300* expression in E18.5 wild type and *miR-142*-null lungs. Scale bar: (A, C, E, G, I, K): 44 μm and (B, D, F, H, J, L): 9 μm .

doi:10.1371/journal.pone.0136913.g005

Discussion

We are the first group to report the generation and validation of classical *miR-142*-null mice. We show that both isoforms of *miR-142*, the 3p and 5p, are no longer expressed in *miR-142*-null mice. The expression of *Bzrap1*, a gene immediately flanking *miR-142*, is not altered while the expression of a lncRNA embedded within the *miR-142* gene is abolished. E18.5 *miR-142*-null lungs display increased Wnt signaling associated with the up-regulation of *Apc* and *p300*, two previously reported targets of *miR-142-3p* and *-5p*, respectively. *miR-142*-null pups are born alive and are normally represented indicating absence of embryonic lethality. Adult *miR-142*-null animals are viable and display impaired hematopoietic lineage formation. This novel, homologous recombination-based, *miR-142*-null mouse line will be useful for the scientific community.

A *miR-142* gene-trap allele was recently created by insertion of an exogenous gene trap sequence 50 bp upstream of the murine pre-*miR-142* [8]. In these mice, the expression of both 3p and 5p was reduced to very low levels but not completely abolished. The authors describe that at E14.5, embryos homozygous for the mutant allele display a normal Mendelian distribution. However, one third of the postnatal homozygous mutant mice died within 3 weeks. Our results support these observations with the exception of the lack of perinatal death. Even though this remains to be shown, perinatal death may be associated with heart failure due to the absence of adaptive growth of the cardiomyocytes [15]. Similarly to the homozygous *miR-*

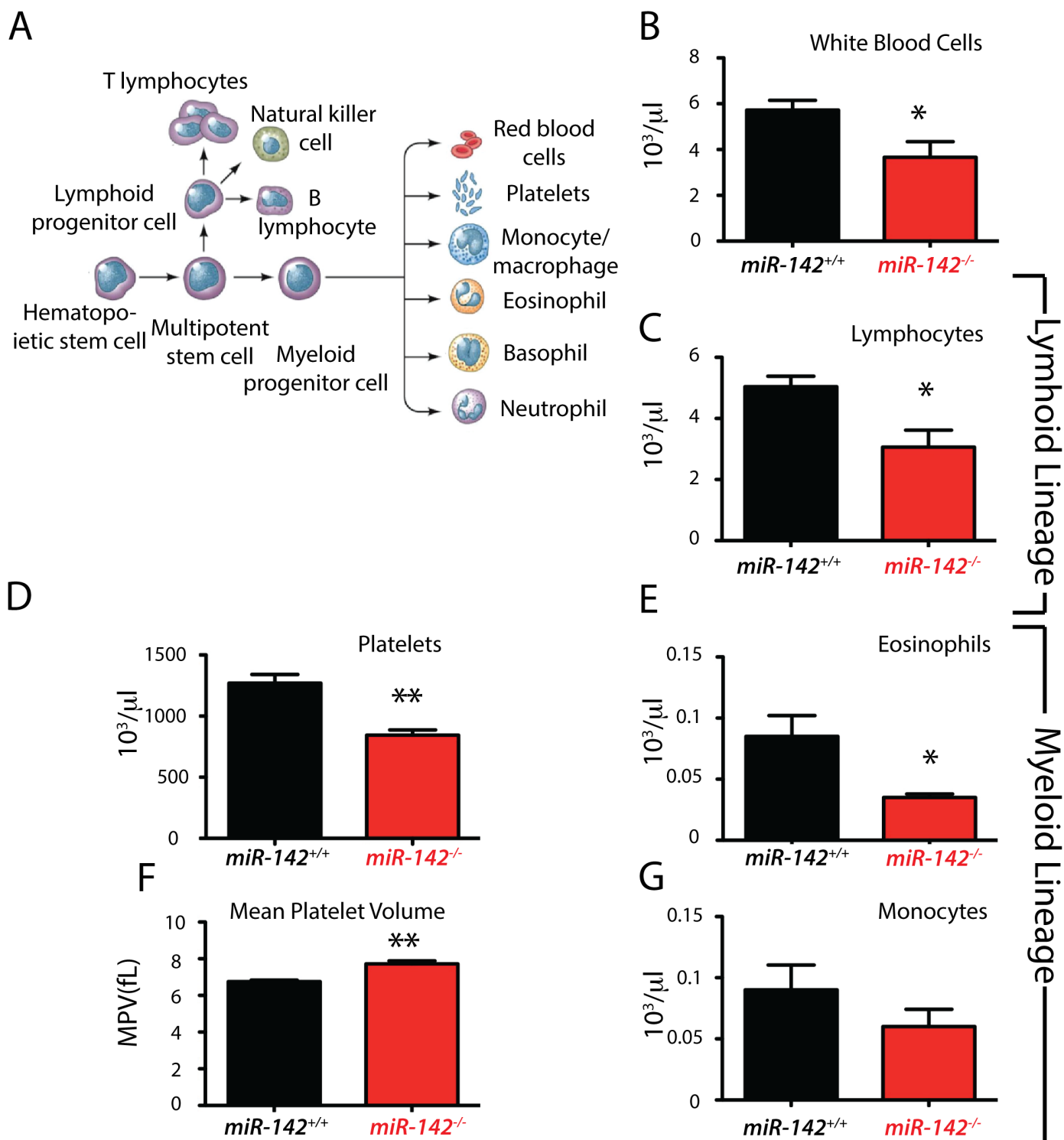


Fig 6. *miR-142* KO mice display hematological abnormalities. (A) Schematic representation of hematopoietic stem cells differentiated into different blood cell types. Peripheral blood cell count in 8 week old *miR-142*^{-/-} mice (n = 3) showing a significant decrease in circulating white blood cells (B), lymphocytes (C), platelets (D), eosinophils (E) and monocytes (G) in addition to an increase in mean platelet volume (F).

doi:10.1371/journal.pone.0136913.g006

142 gene-trap mice, our *miR-142*-null mice also display decreased white blood cells, decreased platelets and increased mean platelet volume. However, we did not observe the decrease in red blood cell numbers as well as the increased level of basophils and reticulocytes (data not shown) initially reported in the homozygous *miR-142* gene-trap mice. The basis for such differences is not known so far and could be linked to the genetic background.

In order to predict the potential defects observed in *miR-142*-null animals, the predicted targets for *miR-142-3p* and *5p* were characterized using Diana-MicroT and Targetscan (Fig 7A). Among the processes and pathways potentially affected, we identified endocytosis, regulation of actin cytoskeleton, pathways in cancer, TGF β signaling and Wnt signaling.

As the *miR-142* conventional KO leads to the deletion of both *miR-142-3p* and *miR-142-5p*, it might be challenging to tease out the activity of each isoform. Others and we have previously reported that *Apc* is a direct target of *miR-142-3p* [3,12]. Fig 7B describes the common targets (in orange) for *miR-142-3p* and *5p* as well as unique targets (yellow) for each isoform in regard to the Wnt pathway. Reduction in *Apc* leads to the activation of canonical Wnt signaling by preventing the recruitment of Ctnnb1 to the degradation complex. Increased *Apc* expression upon *miR-142-3p* silencing causes down-regulation of Wnt signaling [3]. On the other hand Sharma et al. found that *p300* is a target of *miR-142-5p*. Interestingly, this target was not identified as a main putative target through *in-silico* analysis and demonstrates the importance of identifying and confirming interacting partners through pull-down assays followed by gene arrays [3] or next generation sequencing. A mutual inhibitory loop between *p300* and *miR-142-5p* has also been described and under mechanical stress, *p300* is accumulated in the cardiac cells, which results in the down-regulation of *miR-142-5p*. The loss of *miR-142-5p* expression activates genes required for myocyte survival and function [15]. *p300* is a positive regulator of Ctnnb1, which synergistically activates Ctnnb1/TCF transcription [17]. It is therefore not surprising to find that the loss of *miR-142* leads to increased expression of both *Apc* and *p300* (Fig 5). As *Apc* is a negative regulator and *p300* is a positive regulator of Wnt signaling, we expect that upon deletion of *miR-142*, the balance between *Apc* and *p300* will lead to either no change in Wnt signaling, an increase or a decrease in Wnt signaling. In the E18.5 lung, our results indicate that the *Apc/p300* balance favors Wnt signaling. The regulation of the relative expression of both isoforms in specific tissues over time is still unknown. In the embryonic lung at E18.5, both the *-5p* and *-3p* isoforms are normally co-expressed in the epithelium and mesenchyme while *-3p* appears to be more abundantly expressed in the mesenchyme at E12.5 [3]. In the hematopoietic system, *-3p* is the predominant isoform expressed. In the heart, it appears that *-5p* is the major isoform. Our mouse model will allow determining the overall contribution of *-3p* and *-5p* to Wnt signaling in different tissues and at different developmental stages.

Another aspect related to the reported *miR-142*-null mouse is the deletion of the LncRNA together with the *miR-142* gene. LncRNAs contribute also to gene regulation [18] and in the future, the generation of new mouse models allowing the tissue-specific rescue of *miR142-3p/5p* expression in the context of the *miR-142* KO animals will allow determining the role of this LncRNA. Interestingly, the expression of this LncRNA has not been investigated in the *miR-142* gene-trap line. More recently, a conditional KO for *miR-142* was generated by deleting 900 bp of the genomic sequence that encompasses the *miR-142* precursor in the germ line [19]. The authors reported that *miR-142*-null mice are born at the expected Mendelian ratio and appear healthy and fertile with no apparent internal organ defects, similarly to the findings described in this report. They also described a highly penetrant splenomegaly that is also observed in our *miR142*-null mice (data not shown).

In conclusion, we report the generation and validation of a novel homologous recombination-based *miR-142* KO mouse model. Interestingly, these mice display differences as compared to the previously described gene-trap model, which will need to be analyzed further to

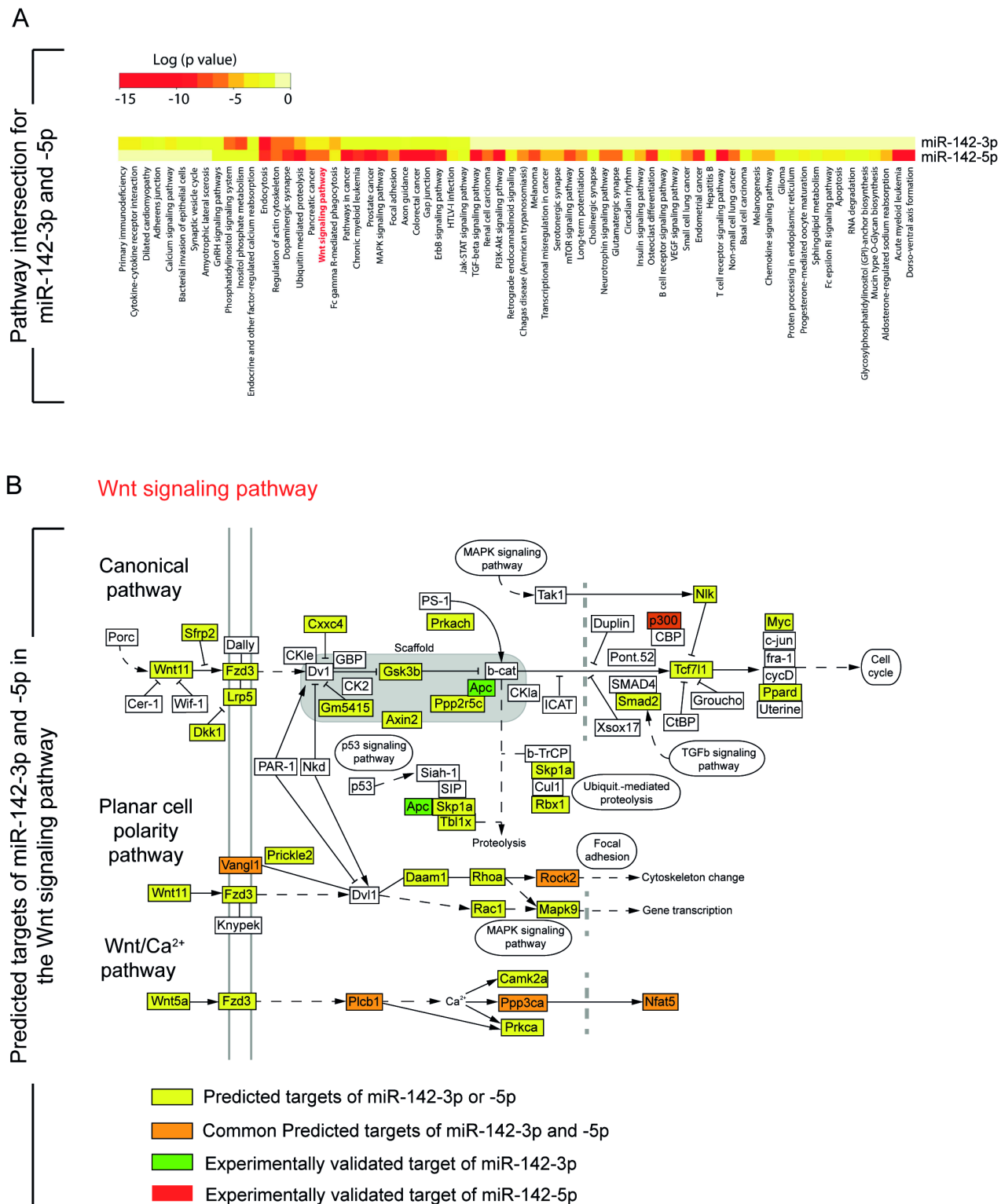


Fig 7. Predicted targets of miR-142-3p and miR-142-5p using Diana-MicroT and Target Scan software prediction tools. (A) Pathway intersection between miR-142-3p and miR-142-5p. **(B)** Predicted targets of miR-142-3p and miR-142-5p in the Wnt signaling pathway. Orange boxes: common predicted targets for both isoforms; yellow boxes: predicted targets for both isoforms; green boxes: experimentally validated target of miR-142-3p; red box: experimentally validated target of miR-142-5p.

doi:10.1371/journal.pone.0136913.g007

validate the proposed function of *miR-142* in hematopoiesis. Together with the recently reported conditional *miR-142* KO, our mouse model will be the first generation of tools designed to decipher the role of *miR-142* in development and disease.

Supporting Information

S1 Fig. Schematic representation of *mir-142* targeting vector. (A) Map of *miR-142* targeting vector plasmid. (B) Deletion of *miR-142* locus in the genome. (TIF)

Acknowledgments

We thank Matt Jones for the genotyping of the animals, Jana Rostkovius for the immunofluorescence experiments and Kerstin Goth for the management of the mouse colony.

Author Contributions

Conceived and designed the experiments: AS GC EEA RM AR GB SB. Performed the experiments: AS RM CMC. Analyzed the data: AS GC CMC RM AR GB SB. Wrote the paper: AS EEA SB.

References

1. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009; 136: 215–233. doi: [10.1016/j.cell.2009.01.002](https://doi.org/10.1016/j.cell.2009.01.002) PMID: [19167326](https://pubmed.ncbi.nlm.nih.gov/19167326/)
2. Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem* 2010; 79: 351–379. doi: [10.1146/annurev-biochem-060308-103103](https://doi.org/10.1146/annurev-biochem-060308-103103) PMID: [20533884](https://pubmed.ncbi.nlm.nih.gov/20533884/)
3. Carraro G, Shrestha A, Rostkovius J, Contreras A, Chao CM, El Agha E, et al. miR-142-3p balances proliferation and differentiation of mesenchymal cells during lung development. *Development* 2014; 141: 1272–1281. doi: [10.1242/dev.105908](https://doi.org/10.1242/dev.105908) PMID: [24553287](https://pubmed.ncbi.nlm.nih.gov/24553287/)
4. Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. *Cell* 2012; 149: 1192–1205. doi: [10.1016/j.cell.2012.05.012](https://doi.org/10.1016/j.cell.2012.05.012) PMID: [22682243](https://pubmed.ncbi.nlm.nih.gov/22682243/)
5. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. *Science* 2004; 303: 83–86. PMID: [14657504](https://pubmed.ncbi.nlm.nih.gov/14657504/)
6. Merkerova M, Belickova M, Bruchova H. Differential expression of microRNAs in hematopoietic cell lineages. *Eur J Haematol* 2008; 81: 304–310. doi: [10.1111/j.1600-0609.2008.01111.x](https://doi.org/10.1111/j.1600-0609.2008.01111.x) PMID: [18573170](https://pubmed.ncbi.nlm.nih.gov/18573170/)
7. Fan HB, Liu YJ, Wang L, Du TT, Dong M, Gao L, et al. miR-142-3p acts as an essential modulator of neutrophil development in zebrafish. *Blood* 2014; 124: 1320–1330. doi: [10.1182/blood-2013-12-545012](https://doi.org/10.1182/blood-2013-12-545012) PMID: [24990885](https://pubmed.ncbi.nlm.nih.gov/24990885/)
8. Chapnik E, Rivkin N, Mildner A, Beck G, Pasvolsky R, Metzl-Raz E, et al. miR-142 orchestrates a network of actin cytoskeleton regulators during megakaryopoiesis. *Elife* 2014; 3: e01964. doi: [10.7554/eLife.01964](https://doi.org/10.7554/eLife.01964) PMID: [24859754](https://pubmed.ncbi.nlm.nih.gov/24859754/)
9. Nimmo R, Ciau-Uitz A, Ruiz-Herguido C, Soneji S, Bigas A, Patient R, et al. MiR-142-3p controls the specification of definitive hemangioblasts during ontogeny. *Dev Cell* 2013; 26: 237–249. doi: [10.1016/j.devcel.2013.06.023](https://doi.org/10.1016/j.devcel.2013.06.023) PMID: [23911199](https://pubmed.ncbi.nlm.nih.gov/23911199/)
10. Kaduthanam S, Gade S, Meister M, Brase JC, Johannes M, Dienemann H, et al. Serum miR-142-3p is associated with early relapse in operable lung adenocarcinoma patients. *Lung Cancer* 2013; 80: 223–227. doi: [10.1016/j.lungcan.2013.01.013](https://doi.org/10.1016/j.lungcan.2013.01.013) PMID: [23410826](https://pubmed.ncbi.nlm.nih.gov/23410826/)
11. Chiou GY, Chien CS, Wang ML, Chen MT, Yang YP, Yu YL, et al. Epigenetic regulation of the miR142-3p/interleukin-6 circuit in glioblastoma. *Mol Cell* 2013; 52: 693–706. doi: [10.1016/j.molcel.2013.11.009](https://doi.org/10.1016/j.molcel.2013.11.009) PMID: [24332177](https://pubmed.ncbi.nlm.nih.gov/24332177/)
12. Isobe T, Hisamori S, Hogan DJ, Zabala M, Hendrickson DG, Dalerba P, et al. miR-142 regulates the tumorigenicity of human breast cancer stem cells through the canonical WNT signaling pathway. *Elife* 2014; 3: 01977

13. Sun Y, Varambally S, Maher CA, Cao Q, Chockley P, Toubai T, et al. Targeting of microRNA-142-3p in dendritic cells regulates endotoxin-induced mortality. *Blood* 2011; 117: 6172–6183. doi: [10.1182/blood-2010-12-325647](https://doi.org/10.1182/blood-2010-12-325647) PMID: [21474672](https://pubmed.ncbi.nlm.nih.gov/21474672/)
14. Trobaugh DW, Gardner CL, Sun C, Haddow AD, Wang E, Chapnik E, et al. RNA viruses can hijack vertebrate microRNAs to suppress innate immunity. *Nature* 2014; 506: 245–248. doi: [10.1038/nature12869](https://doi.org/10.1038/nature12869) PMID: [24352241](https://pubmed.ncbi.nlm.nih.gov/24352241/)
15. Sharma S, Liu J, Wei J, Yuan H, Zhang T, Bishopric N. Repression of miR-142 by p300 and MAPK is required for survival signalling via gp130 during adaptive hypertrophy. *EMBO Mol Med* 2012; 4: 617–632. doi: [10.1002/emmm.201200234](https://doi.org/10.1002/emmm.201200234) PMID: [22367739](https://pubmed.ncbi.nlm.nih.gov/22367739/)
16. Vlachos IS, Kostoulas N, Vergoulis T, Georgakilas G, Reczko M, Maragkakis M, et al. DIANA miRPath v.2.0: investigating the combinatorial effect of microRNAs in pathways. *Nucleic Acids Res* 2012; 40: W498–504. doi: [10.1093/nar/gks494](https://doi.org/10.1093/nar/gks494) PMID: [22649059](https://pubmed.ncbi.nlm.nih.gov/22649059/)
17. Sun Y, Kolligs FT, Hottiger MO, Mosavin R, Fearon ER, Nabel G. Regulation of beta-catenin transformation by the p300 transcriptional coactivator. *Proc Natl Acad Sci U S A* 2000; 97: 12613–12618. PMID: [11050151](https://pubmed.ncbi.nlm.nih.gov/11050151/)
18. Herriges MJ, Swarr DT, Morley MP, Rath KS, Peng T, Stewart KM, et al. Long noncoding RNAs are spatially correlated with transcription factors and regulate lung development. *Genes Dev* 2014; 28: 1363–1379. doi: [10.1101/gad.238782.114](https://doi.org/10.1101/gad.238782.114) PMID: [24939938](https://pubmed.ncbi.nlm.nih.gov/24939938/)
19. Kramer NJ, Wang WL, Reyes EY, Kumar B, Chen CC, Ramakrishna C, et al. Altered lymphopoiesis and immunodeficiency in miR-142 null mice. *Blood* 2015; 125:3420–2730.

ORIGINAL ARTICLE



A critical role for *miR-142* in alveolar epithelial lineage formation in mouse lung development

Amit Shrestha^{1,2} · Gianni Carraro³ · Nicolas Nottet^{4,5} · Ana Ivonne Vazquez-Armendariz² · Susanne Herold² · Julio Cordero⁶ · Indrabahadur Singh⁶ · Jochen Wilhelm² · Guillermo Barreto^{6,7} · Rory Morty⁸ · Elie El Agha^{1,2} · Bernard Mari^{4,5} · Chengshui Chen¹ · Jin-San Zhang^{1,9} · Cho-Ming Chao^{1,2,10} · Saverio Bellusci^{1,2}

Received: 10 January 2019 / Revised: 5 March 2019 / Accepted: 12 March 2019 / Published online: 18 March 2019
© Springer Nature Switzerland AG 2019

Abstract

The respiratory epithelium arises from alveolar epithelial progenitors which differentiate into alveolar epithelial type 1 (AT1) and type 2 (AT2) cells. AT2 cells are stem cells in the lung critical for the repair process after injury. Mechanisms regulating AT1 and AT2 cell maturation are poorly defined. We report that the activation of the glucocorticoid pathway in an in vitro alveolar epithelial lineage differentiation assay led to increased AT2 marker *Sftpc* and decreased *miR-142* expression. Using *miR-142* KO mice, we demonstrate an increase in the AT2/AT1 cell number ratio. Overexpression of *miR-142* in alveolar progenitor cells in vivo led to the opposite effect. Examination of the KO lungs at E18.5 revealed enhanced expression of *miR-142* targets *Apc*, *Ep300* and *Kras* associated with increased β -catenin and p-Erk signaling. Silencing of *miR-142* expression in lung explants grown in vitro triggers enhanced *Sftpc* expression as well as increased AT2/AT1 cell number ratio. Pharmacological inhibition of Ep300- β -catenin but not Erk in vitro prevented the increase in *Sftpc* expression triggered by loss of *miR-142*. These results suggest that the glucocorticoid-*miR-142*-Ep300- β -catenin signaling axis controls pneumocyte maturation.

Keywords Alveolar epithelium · microRNA-142 · Lung · EP300 · Beta-catenin

Amit Shrestha and Gianni Carraro contributed equally.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00018-019-03067-8>) contains supplementary material, which is available to authorized users.

✉ Cho-Ming Chao
Cho-Ming.Chao@paediat.med.uni-giessen.de

✉ Saverio Bellusci
saverio.bellusci@innere.med.uni-giessen.de

¹ Department of Pulmonary and Critical Care Medicine, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China

² Cardio-Pulmonary Institute (CPI), Member of the German Center for Lung Research (DZL), Universities of Giessen and Marburg Lung Center (UGMLC), Justus-Liebig-University Giessen, 35392 Giessen, Germany

³ Department of Medicine, Cedars-Sinai Medical Center, Lung and Regenerative Medicine Institutes, Los Angeles, CA, USA

⁴ Centre National de la Recherche Scientifique, CNRS, UMR 7275, Institut de Pharmacologie Moléculaire et Cellulaire (IPMC), Sophia Antipolis, France

⁵ Université Côte d'Azur, Nice, France

⁶ Lung Cancer Epigenetics, Member of the German Center of Lung Research (Deutsches Zentrum für Lungenforschung, DZL), Max-Planck-Institute for Heart and Lung Research, 61231 Bad Nauheim, Germany

⁷ Institute of Fundamental Medicine and Biology, Kazan (Volga Region) Federal University, 420008 Kazan, Russian Federation

⁸ Department of Lung Development and Remodeling, Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany

⁹ Institute of Life Sciences, Wenzhou University, Wenzhou, Zhejiang, People's Republic of China

¹⁰ Department of General Pediatrics and Neonatology, University Children's Hospital Gießen, Justus-Liebig-University, Giessen, Germany

Introduction

The formation of the alveolar epithelial lineage during lung development is coordinated with the process of branching morphogenesis [1]. Lung development starts at E9.5 with the formation of the laryngotracheal groove on the ventral side of the foregut endoderm. As the trachea separates from the underlying esophagus, the primary lung rudiments appear at the most distal part of the trachea. During the pseudoglandular stage (E9.5–E16.5), the lung epithelium undergoes a coordinated process of branching morphogenesis and differentiation [2]. This allows the formation of a tree-like structure comprised of main branches proximally (the future bronchi) as well as buds. Sox9/Id2-positive cells located at the tip of the epithelial buds during the pseudoglandular stage are considered to be multipotent epithelial progenitor cells [3, 4]. Lineage tracing experiments at E11.5 using *Id2*^{CreERT2} showed that Id2⁺ epithelial cells at the distal tip can give rise to both bronchiolar and alveolar progenitors [3]. Single cell transcriptomic studies of the developing epithelium at E14.5, E16.5 and E18.5 demonstrated the existence of cells expressing both the alveolar epithelial cell type 1 (AT1) marker *Pdpn* and the type 2 (AT2) marker *Sftpc* at E18.5. These cells are called bipotent (BP) progenitor cells [5]. Based on the expression of several AT1 and AT2 markers, it has been suggested that early bipotent progenitors are already present at E16.5 and can give rise progressively to either AT1 or AT2 during the subsequent phases of lung development. AT1 and AT2 cells lining the alveolar sacs are regarded among the most important pulmonary cells allowing the lungs to function properly by mediating gaseous exchange as well as through surfactant production. Supporting BP cells as common progenitors for AT1 and AT2, it was recently reported that alveolar progenitor cells co-expressing *Pdpn* and *Sftpc* at the lung tip are migrating out of the lumen at E16.5–E17.5 and will be destined to become AT2 as they escape the stretching-induced differentiation effect due to the internal pressure. The authors proposed that the cells remaining in the lumen will then differentiate into AT1 cells [6]. It is important to note that the BP model as progenitor for AT1 and AT2 still needs to be validated using lineage tracing approaches. An alternative and non-mutually exclusive model for AT2 formation is that some of the alveolar progenitor cells never transit through a bipotent intermediate but rather give rise to mature AT2 cells through progressive and direct differentiation.

MicroRNAs (miRs) are small regulatory RNA in mammals that account approximately 1% of the genome. They are 22- to 25-nucleotide-long single-stranded RNAs processed from hairpin transcripts, that regulates

post-transcriptional gene regulation in eukaryotes by binding at the 3'-UTR regions of the target mRNA thus leading to mRNA cleavage, degradation or translational repression. The maturation of hairpin transcript give rise to a 3p guide strand and 5p sister passenger strand. In general, only one species remains while the complementary species is degraded but in some cases, both strands can be produced allowing the silencing of specific sets of genes through base pairing to a minimal recognition sequence [7]. In this study, we describe a novel function for *miR-142* in the control of alveolar epithelial lineage formation.

miR-142 is a major regulator of cell fate decision during organogenesis [8]. We previously reported that morpholino-based in vitro knockdown of *miR-142-3p* in the embryonic E11.5 lung led to decreased proliferation and premature differentiation of smooth muscle cell progenitors. Functionally, *miR-142-3p* positively regulates β -catenin signaling via targeting *Adenomatous polyposis coli* (*Apc*), a gene encoding an essential component of the β -catenin (Ctnnb1) degradation complex. Deletion of *Apc* or the ectopic expression of a stable form of β -catenin in the mesenchyme, which led to a rescue of the proliferation and differentiation defects previously observed upon silencing of *miR142-3p*, further validates *Apc* as a functional target downstream of *miR-142-3p* [9]. Interestingly, *miR-142-5p* has been reported to target *Ep300*, a positive regulator of β -catenin signaling [10, 11]. We previously reported the simultaneous upregulation of the negative β -catenin regulator *Apc* and the positive β -catenin regulator *Ep300* in *miR-142* KO E18.5 lungs. This was associated with increased β -catenin signaling in both the epithelium and mesenchyme [12]. Therefore, one reasonable possibility is that, in the context of the *miR-142* KO, *Ep300* is overriding the effect of *Apc* on β -catenin signaling.

We have deployed both in vivo gain and loss of function approaches for *miR-142* to study its role in alveolar epithelial lineage formation. Using FACS-isolated AT2 cells from mutant and control lungs, we carried out gene arrays to determine the corresponding transcriptional changes. An in vitro lung explant culture model allowing alveolar epithelial differentiation was used to monitor the status of *miR-142* expression. In this model, the effect of morpholino-based knockdown of *miR-142* on alveolar lineage formation was also characterized. Pharmacological blockade of *Ep300*/ β -catenin interaction with IQ-1 and Kras/Erk signaling with SCH772984 was carried out. Our results suggest that a glucocorticoid-*miR-142*-*Ep300*- β -catenin signaling axis is in place to control pneumocyte maturation.

Results

Enhancement of alveolar epithelial lineage formation via the activation of the glucocorticoid pathway leads to decreased *miR-142* expression

We previously reported that *miR-142-3p* and *-5p* are expressed in both the epithelium and the mesenchyme at E18.5 [12] suggesting that in addition to its reported role in the mesenchyme [9], *miR-142* could also play a function in the epithelium. We hypothesized that *miR-142* could control alveolar epithelial lineage formation during lung development. First, we took advantage of a previously reported in vitro model where activation of the glucocorticoid receptor pathway with dexamethasone increases alveolar epithelial lineage formation [13]. Treatment of E14.5 embryonic lungs grown in vitro for 4 days with dexamethasone (Fig. 1a) led to an increase in the expression of both AT1 (*Aqp5*, *Pdpn*) and AT2 markers (*Sftpc*) (Fig. 1c). The impact of Dex treatment on *Sftpc* at the protein level was confirmed by IF and western blot (Fig. 1e, f). Next, we investigated the impact of dexamethasone treatment on the expression of the specific *miR-142* isoforms. We observed a strong decrease in *miR-142-3p* and *-5p* expression upon Dex treatment (Fig. 1b) associated with a significant increase in *miR-142* targets *Apc*, *Ep300*, *Il6st* but not *Kras* (Fig. 1d) suggesting that some of the effects of dexamethasone on alveolar epithelial lineage formation could be due to decrease in *miR-142* expression. Downregulation of *miR-142* was already observed as early as after 12 h of culture (data not shown). The expression of the associated lncRNA, which is located in the *miR-142* locus [12], was not affected by dexamethasone.

miR-142-3p and *miR-142-5p* are expressed in both the AT1 and AT2 lineages during lung development

To investigate whether both *miR-142-3p* and *miR-142-5p* are expressed specifically in the AT1 and AT2 cells, we performed fluorescence in situ hybridization specific to *miR-142-3p* and *-5p*, co-stained together with either *Sftpc* (AT2 marker) or *Pdpn* (AT1 marker) antibodies at three different time points (E16.5, E17.5, and E18.5) during alveolar lineage formation (Fig. 2a).

In our experimental conditions, *Pdpn* expression was not detectable by IF at E16.5 in contrast with the robust *Sftpc* expression at that stage. At E17.5 and E18.5, both *Sftpc* and *Pdpn* were detected. *miR-142-3p* and *5p* were expressed in *Sftpc*-positive cells at E16.5 (the alveolar progenitors) as well as in progressively differentiated AT2

cells at E17.5–E18.5. Interestingly, *miR-142* expression is mostly detected in the nuclear compartment rather than in the cytoplasm. This result was confirmed by qPCR for the two *miR-142* isoforms from the enriched cytoplasmic or nuclear fractions using adult mouse lungs (Fig. S1A, B). Finally, at E17.5 and E18.5, we clearly observed *Pdpn*-positive AT1 cells co-expressing *miR-142-3p* or *5p*. We further confirmed this observation using antibodies against *Hopx*, a well-defined AT1 marker (Fig. S1C). In addition, we noticed cells negative for *miR-142-3p* or *miR-142-5p* but positive for *Sftpc* or *Pdpn* and vice versa. This result supports previous observations made concerning the heterogeneity of the alveolar epithelial lineage in the adult lung [5, 14]. These results indicate that both miRs are expressed in the alveolar epithelium during development. Using FACS-based approach, with a combination of antibodies against *Cd45* and *Cd31* to exclude the hematopoietic and endothelial cells as well as *Epcam* antibodies for capturing epithelial (*Epcam*⁺) or mesenchymal (*Epcam*⁻) cells, we isolated resident epithelial and mesenchymal lung cells at E14.5, E16.5 and E18.5 and examined miR expression by qPCR (Fig. 2b). The validation of this approach was carried out for known epithelial (*Cdh1*, *Epcam*, *Fgfr2b*) or mesenchymal (*Fgf10*, *Acta2*, *Vimentin*) markers (Fig. S1D, E). The expression of *miR-142-3p* is higher in the mesenchyme than in the epithelium at E16.5 and E18.5 but not at E14.5. Interestingly, between E14.5 and E18.5, *miR-142-3p* expression in the epithelium progressively decreases while the expression of its bona fide targets *Apc*, *Ep300*, *Il6st*, and *Kras* was increased (Fig. 2e). Consistent with our previous observations, *miR-142-5p* was expressed at much lower level than *miR-142-3p* [9].

We also investigated the expression of these two microRNAs in E16.5, E17.5 and E18.5 FACS-isolated AT1 and AT2 cells (Fig. 2c). Alveolar epithelial cells were identified as *CD45*^{-ve}/*CD31*^{-ve}/*CD49f*^{low}/*Epcam*^{int} and AT2 cells were identified as *CD45*^{-ve}/*CD31*^{-ve}/*CD49f*^{low}/*Epcam*^{int} and *Pdpn*^{-ve} while AT1 cells were identified as *CD45*^{-ve}/*CD31*^{-ve}/*CD49f*^{low}/*Epcam*^{int} and *Pdpn*⁺.

At E16.5, the captured cells for the AT1 lineage (*Pdpn*⁺) are likely to contain, provided these cells have a biological significance, the putative bipotent progenitors [5, 15]. In addition, the captured cells for the AT2 lineage (*Pdpn*^{-ve}) are mostly made of alveolar progenitors. For the AT1 lineage, *miR-142-3p* was expressed at similar level between E16.5 and E18.5. On the contrary, the expression of *miR-142-5p* increased as the formation of the AT1 lineage progressed. Similar expression patterns for *miR-142-3p* and *5p* were observed in the formation of the AT2 lineage. Interestingly, we noted the higher enrichment of *miR-142-5p* in AT1 compared to AT2 cells at E18.5. Furthermore, data mining analysis on the expression of *miR-142* targets using the single cell AT1 and AT2 transcriptomic data at E18.5

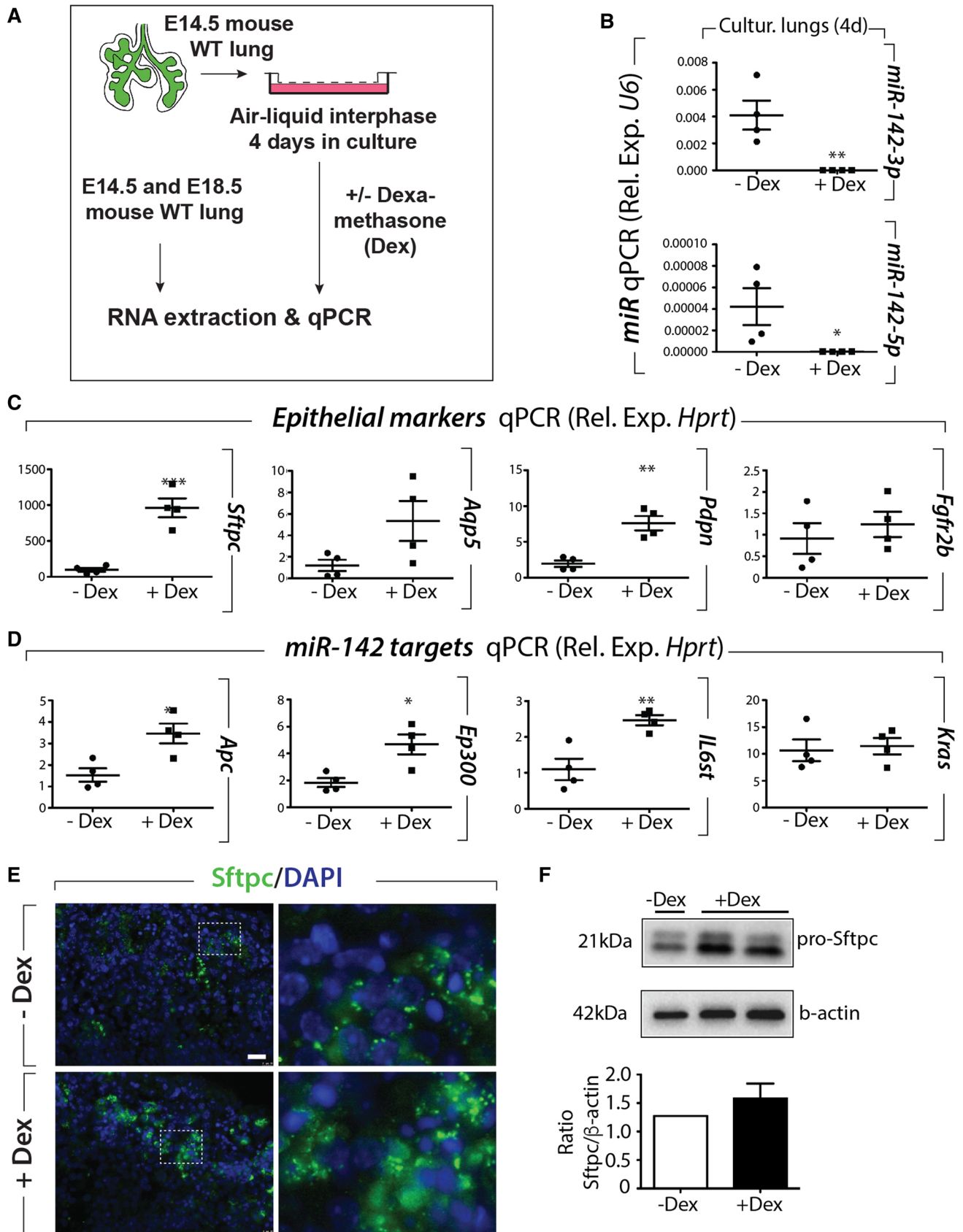


Fig. 1 Effect of dexamethasone on E14.5 embryonic lung cultured in vitro for 4 days. **a** Experimental design ($n=4$ for each $\pm$ dexamethasone). **b** qPCR analysis showing the expression level of *miR-142-3p* and *miR-142-5p* as well as the expression of (**c**) *Sftpc*, *Aqp5*, *Pdpn* and *Fgfr2b*. **d** *Apc*, *Ep300*, *Il6st* and *Kras* on E14.5 lung explants grown for 4 days in presence of dexamethasone (100 nM). **e** IF staining for Sftpc on E14.5 lung explants cultured with and without dexamethasone (100 nM) for 4 days. **f** Representative western blot validating the increase in Sftpc expression in dexamethasone-treated lung explants. Scale bar in (**e**) low magnification: 16 μ m, high magnification: 4 μ m

previously published [5] was carried out (Fig. 2d). We have determined for each of the individual cells in the AT1 and AT2 pools, whether or not they express the different *miR-142* targets (*Apc*, *Ep300*). We have defined these cells as positive or negative for the expression of the target genes. For sake of simplification, we have not made a distinction in the “positive” cells between cells expressing high or low level of the target genes. We noticed that a higher number of individual cells in the AT2 pool expressed *Apc* and *Ep300* (7 out of 12 cells total analyzed and 5 out of 12 cells total analyzed, for *Apc* and *Ep300*, respectively) compared to AT1 (12 out of 41 cells total analyzed and 10 out of 41 cells total analyzed, for *Apc* and *Ep300*, respectively) (Fig. 2d). This supports our result that *miR-142* expression is low in AT2 compared to AT1 cells. Interestingly, a higher number of cells in the AT1 compared to AT2 pools expressed *Kras* (19/41 and 2/12, for AT1 and AT2, respectively) suggesting that *Kras* expression level is not regulated by *miR-142* in AT1 cells. *Il6st* was expressed by a low number of cells in the AT1 and AT2 pools (3/41 and 1/12, for AT1 and AT2, respectively). Altogether, the increase in these *miR-142* targets appears to occur more in AT2 than AT1, confirming the observed differential expression of *miR-142* in these cells. Therefore, it is logic to expect that the formation of the AT1 cells will be disrupted upon loss of *miR-142*.

At the pseudoglandular stage of lung development, *miR-142* KO lungs display alteration in the morphology of the epithelial cells located at the tip but without major branching defects

To further explore the function of *miR-142* during lung development, we generated *miR-142* KO mice (Fig. 3a) leading to the inactivation of the -3p and -5p isoforms simultaneously [12]. No obvious macroscopic abnormalities in terms of branching were observed at E12.5 (Fig. 3b). This result was quite surprising as we previously reported that the knockdown of *miR-142-3p* using morpholino in E11.5 lungs grown in vitro led to impaired branching and loss of β -catenin signaling in the mesenchyme. The latter was associated with increased Adenomatous polyposis coli (*Apc*) expression and arrested proliferation [9]. The lack of obvious

branching phenotype in *miR-142* KO E12.5 lungs suggested that *miR-142-5p* could also play a functional role. *Apc* and *Ep300*, known targets for *miR-142-3p* and *miR-142-5p*, respectively, appeared to be upregulated at the protein level in KO lungs (Fig. 3c, d) while no significant change at the transcriptional level was observed (Fig. 3e). This could be explained by the fact that microRNAs do not act only at the level of the transcripts stability of the target genes but also at the translational level. In addition, in spite of elevated levels of β -catenin (*Ctnnb1*) mRNA (Fig. 3e), no significant changes of activated β -catenin (p^{S552} Ctnnb1) level in E12.5 *miR-142* KO compared to wild-type littermate controls was observed. Several possibilities for this discrepancy can be proposed. For example β -catenin protein degradation could be drastically increased at that stage in *miR-142* KO lungs. In addition, a disconnect between the levels of *Ctnnb1* at the transcriptional levels and at the protein level could also explain this lack of impact on β -catenin signaling at this early stage.

Further, in vitro knockdown experiments using morpholinos against *miR-142-3p* (*mo-3p*), -5p (*mo-5p*) or (*3p+5p*) (*mo-(3p+5p)*) indicated that simultaneous inactivation of both isoforms leads to the rescue of the branching defects reported upon *miR-142-3p* knockdown alone (Fig. S2 and [9]).

Careful examination of E12.5 *miR-142* KO lung epithelium, by immunofluorescence for E-cadherin (*Cdh1*), suggested a disruption in the epithelial cuboidal cell morphology, and acquisition of a round shape with the overall disorganization of the epithelial layer (Fig. 3b). Global transcriptomic analysis using gene arrays with RNA originating from the whole control and KO E12.5 lungs was also carried out. The heat map (Fig. 3f) shows sets of up- or down-regulated genes selected based on their p value in control and KO lungs ($n=3$). KEGG gene set analysis indicated perturbations in pathways involved in regulation of actin cytoskeleton, focal adhesion and endocytosis as well as ECM receptor interaction. All these pathways, which have been shown to modulate epithelial integrity and maturation, may be involved in the observed phenotype. Interestingly, PI3K-Akt, Mapk, Ras, Hippo and Wnt signaling were also perturbed (Fig. 3f).

The alveolar epithelial lineage is perturbed in E18.5 *miR-142* KO lungs

Macroscopic analysis of the E18.5 KO lungs indicated no obvious changes in terms of size or shape, when compared to wild-type littermates. Analysis of the microscopic phenotype by H&E staining ($n=3$) also revealed no major structural abnormalities (Fig. 4a). Transcriptomic analysis between KO and control lungs at E18.5 indicated perturbation in the extracellular matrix-receptor interaction, focal

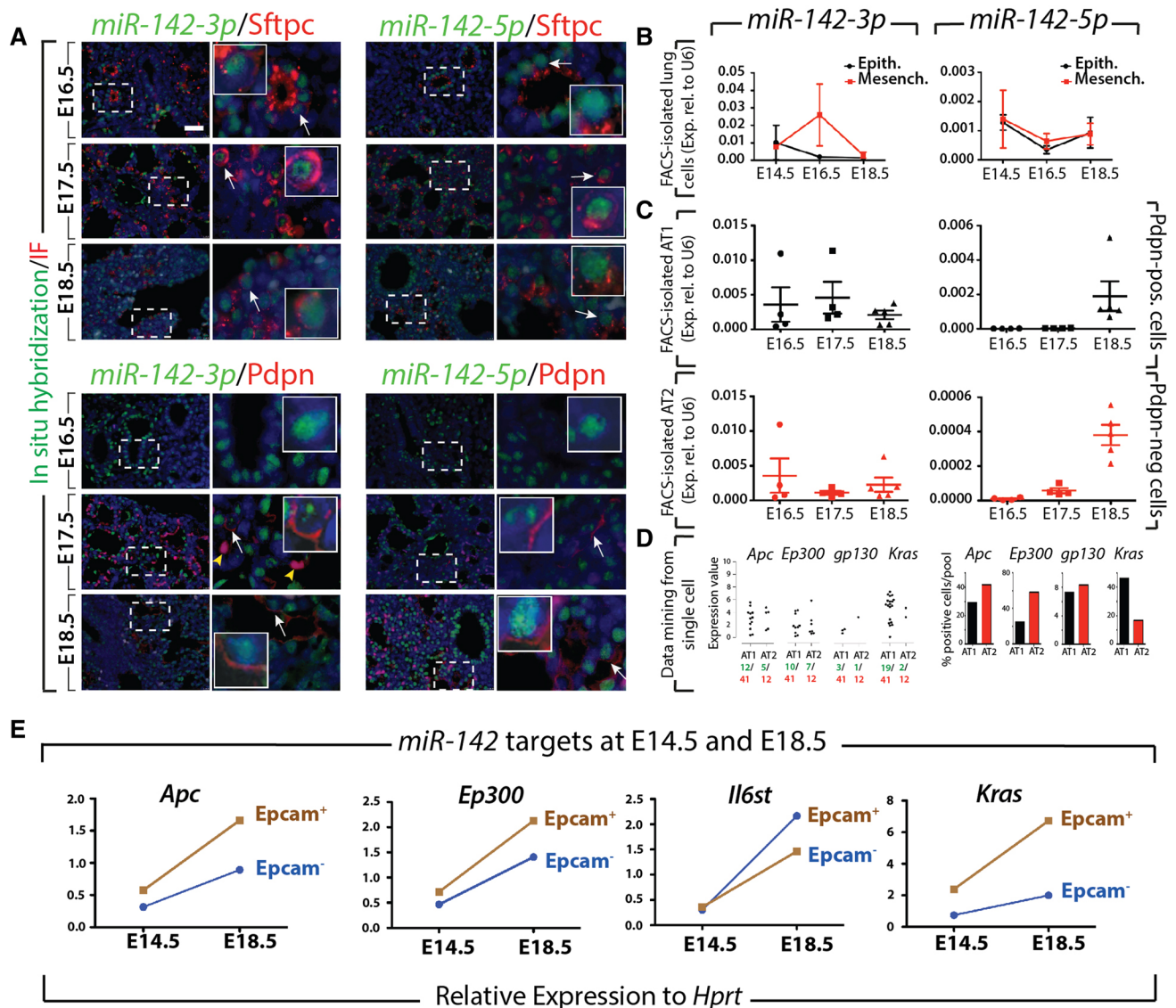


Fig. 2 Gene expression analysis during lung development of *miR-142-3p* vs. *miR-142-5p*. **a** In situ hybridization of *miR-142-3p* or *5p* and co-immunofluorescence staining for either Sftpc (AT2 marker) or Pdpn (AT1 marker). High magnification insets for Sftpc/*miR-142-3p* and -5p-positive and Pdpn/*miR-142-3p* and -5p-positive cells in both WT embryos at 16.5, E17.5 and E18.5 are shown ($n=3$ for each time point). **b** qPCR on FACS-isolated lung epithelium and mesenchyme at E14.5, E16.5 and E18.5 ($n=4$ for each time point).

c qPCR on FACS-isolated AT1 and AT2 cells at E16.5, E17.5 and E18.5 ($n=4$ for each time point). **d** Mining of single cell transcriptomic data for AT1 and AT2 cells at E18.5 (using data from [5]). **e** Expression analysis of *miR-142* targets *Apc*, *Ep300*, *Il6st* and *Kras* in the mesenchyme as well as in the epithelium ($n=3$). *Hprt* is used as a house-keeping gene. Scale bar in **a** low magnification: 30 μ m, high magnification: 8 μ m. White arrows indicate cells with positive signals whereas yellow arrowheads show background signals

adhesion, hematopoietic cell lineage and Wnt signaling (Fig. S3). Further validating our previous findings [12], we observed a significant increase in the level of expression of *Apc*, *Ep300*, and *Il6st* in E18.5 KO lungs in mRNA level by qPCR (Fig. 4b). We further confirmed at the protein level by immunofluorescence staining the upregulation of Apc (Fig. 4c) and Ep300 (Fig. 4d) with an increased level of activated β -catenin (p^{S52} Ctnnb1) compared to the control lungs, suggesting increased Wnt signaling (Fig. 4e). However, the expression of Wnt components *Lef1* and

Ctnnb1 were not significantly affected (Fig. 4b). Finally, we observed an increased p-Erk expression (Fig. 4f), correlating with increased *Kras* mRNA levels (Fig. 4b). Gene expression analysis by qPCR on the whole lung indicated that the AT2 marker, *Surfactant protein C* (*Sftpc*) was upregulated in KO vs. control lungs ($p=0.0074$, $n=3$) (Fig. S4C). Both FACS and immunofluorescence confirmed an increase in the expression level of Sftpc in the KO (Fig. 4g, h). The expression of the AT1 marker Podoplanin (*Pdpn*) ($p=0.06$, $n=3$), the Club cell marker *Scgblal* and the

basal cell marker *p63* were not significantly changed (Fig. S4C). Flow cytometry analysis using Epcam, CD49f, Pdpn antibodies showed an increase in the percentage of AT2 cells ($84.9\% \pm 0.2\%$ vs. $79.8\% \pm 1.04\%$ in KO vs. control, respectively, $p = 0.002$, $n = 3$), and a decrease in the percentage of AT1 cells ($9.05\% \pm 0.68\%$ vs. $12.3\% \pm 0.66\%$ in KO vs. control, respectively, $p = 0.004$, $n = 3$) (Fig. 4g). The increase in Sftpc-positive cells (AT2) and decrease in Hopx-positive cells (AT1) in KO vs. WT lungs were confirmed by IF (Fig. 4h, $n = 3$). Analysis of Ki67 immunoreactivity, in the alveolar epithelial layer of control and KO lungs at E18.5, indicated no changes in epithelial proliferation (Fig. S4D). Furthermore, by flow cytometry analysis no changes in the percentage of Epcam-positive cells (Epcam⁺) were observed (Fig. S4A), suggesting that the loss of *miR-142* is not affecting overall, epithelial proliferation. We also carried out using gene arrays, the transcriptomic analysis of isolated AT2 cells between KO and control lungs (Fig. 4i). Our KEGG analysis indicated that the metabolic pathways, endocytosis, focal adhesion, actin cytoskeleton regulation, peroxisome and Hippo signaling were affected.

Next, we explored the status of Fibroblast growth factor 10 (*Fgf10*) signaling, a key pathway reported to influence alveolar epithelial lineage formation [16, 17]. We found that the expression of *Fgf10* was not changed but a very significant increase in the expression of its receptor, *Fgfr2b*, in KO vs. control lungs ($p = 0.0018$) was observed (Fig. S5A). The protein expression levels were in line with the gene expression levels (Fig. S5B). Analysis of *Sprouty2* and *Etv5*, two previously reported *Fgf10* downstream targets [18, 19], showed no significant difference between control and KO lungs (Fig. S5A), suggesting that *Fgf10* signaling per se was not affected.

In conclusion, we demonstrate increased AT2/AT1 cell number ratio in KO vs. control lung.

Impact of cell autonomous overexpression of *miR-142* in alveolar progenitors

The expression of *miR-142* in the epithelium as well as the epithelial alveolar lineage phenotype upon global loss of function of *miR-142* suggested that *miR-142* could play a cell autonomous role in the epithelium. To test this possibility, we generated a knock in of the *LoxP-Stop-LoxP-miR142* cassette in the *Rosa26* locus (*Rosa26*^{*miR-142/miR-142*}). We crossed the *Rosa26*^{*miR-142/miR-142*} mice with *Sftpc*^{*CreERT2/+*}; *Tomato*^{*flox/flox*} mice, to generate control *Sftpc*^{*+/+*}; *Rosa26*^{*miR-142/+*}; *Tomato*^{*flox/+*} and gain of function (GOF) *Sftpc*^{*CreERT2/+*}; *Rosa26*^{*miR-142/+*}; *Tomato*^{*flox/+*} embryos. This Cre-based recombination, allows an irreversible activation of *miR-142* expression in the epithelium. The expression of *miR-142* was induced with tamoxifen IP injection at E14.5 and E15.5. The resulting lung phenotype was analyzed at

E18.5 (Fig. 5a). Experimental lungs were detected via the expression of red fluorescent protein (RFP) in the epithelium and confirmed by genotyping. Validating our approach, we found increased *miR-142-3p* ($p = 0.0024$) and *-5p* ($p = 0.0004$) expression in GOF vs. control lungs (Fig. 5b). FACS analysis of CD49f^{low}/Epcam^{int} alveolar epithelial cells as well as AT1 (Pdpn⁺) and AT2 (Pdpn⁻) cells indicated no change in the percentage of alveolar epithelial cells (over total cell population). However, a significant decrease in the percentage of AT2 cells ($82.4\% \pm 1.24\%$ vs. $86.6\% \pm 0.99\%$ in GOF vs. control, $p = 0.004$, $n = 4$) and an increase in the percentage of AT1 cells ($10.6 \pm 0.88\%$ vs. $8.7 \pm 0.38\%$ in GOF vs. control, $p = 0.001$, $n = 4$) was observed (Fig. 5c). Immunofluorescence staining for Sftpc and Hopx indicated a decrease in AT2 and increase in AT1 cell number in E18.5 GOF embryos compared to control (Fig. 5d). The general pattern and level of expression of Pdpn was also increased in GOF vs. control lungs supporting the increase in AT1 cell number in GOF lungs by FACS (data not shown). Quantification of the percentage of [Sftpc⁺; Pdpn⁺] cells over total number of cells (Fig. 5d) also indicates a trend towards an increase in GOF vs. control lungs ($3.18\% \pm 0.74\%$ vs. $1.16\% \pm 0.08\%$, $p = 0.06$).

Next, we carried out gene arrays from E18.5 control and GOF FACS-isolated AT2 cells ($n = 3$, Fig. 5e). KEGG analysis indicates that G-coupled protein-mediated olfactory transduction, metabolic pathways, and RNA degradation were affected.

In conclusion, gain of *miR-142* expression in alveolar epithelial cells led to a decreased AT2/AT1 cell ratio.

Loss of *miR-142* expression in vitro starting at E14.5 is sufficient to increase the AT2/AT1 cell ratio

As the *miR-142* KO lung phenotype (where both isoforms are deleted) is different from the phenotype observed upon silencing of *miR-142-3p* in vitro [9], it was important to validate our morpholino approach against *miR-142*. We carried out the simultaneous silencing of both isoforms (*miR-142-3p* and *miR-142-5p*) at E12.5 in vitro. The silencing of *miR-142-3p* was used as a positive control for our experiments. Our data indicate that the silencing of both isoforms has no impact on lung branching, a phenotype observed in vivo in the *miR-142* KO (Fig. S2A). Silencing only *miR-142-3p* leads to the impaired branching phenotype as previously reported [9]. qPCR data on the in vitro-cultured lungs indicated that silencing both isoforms led to the loss of expression of both *miR-142-3p* and *-5p*. Silencing only *miR-142-3p* led to the loss of *miR-142-3p* but not *-5p* (Fig. S2B).

To evaluate the impact of *miR-142* loss of function at the time in which AT2 (*Sftpc*) and AT1 (*Pdpn*) cell markers arise, we performed in vitro treatment with morpholino specific for *miR-142-3p* and *-5p* on E14.5 lung explants and

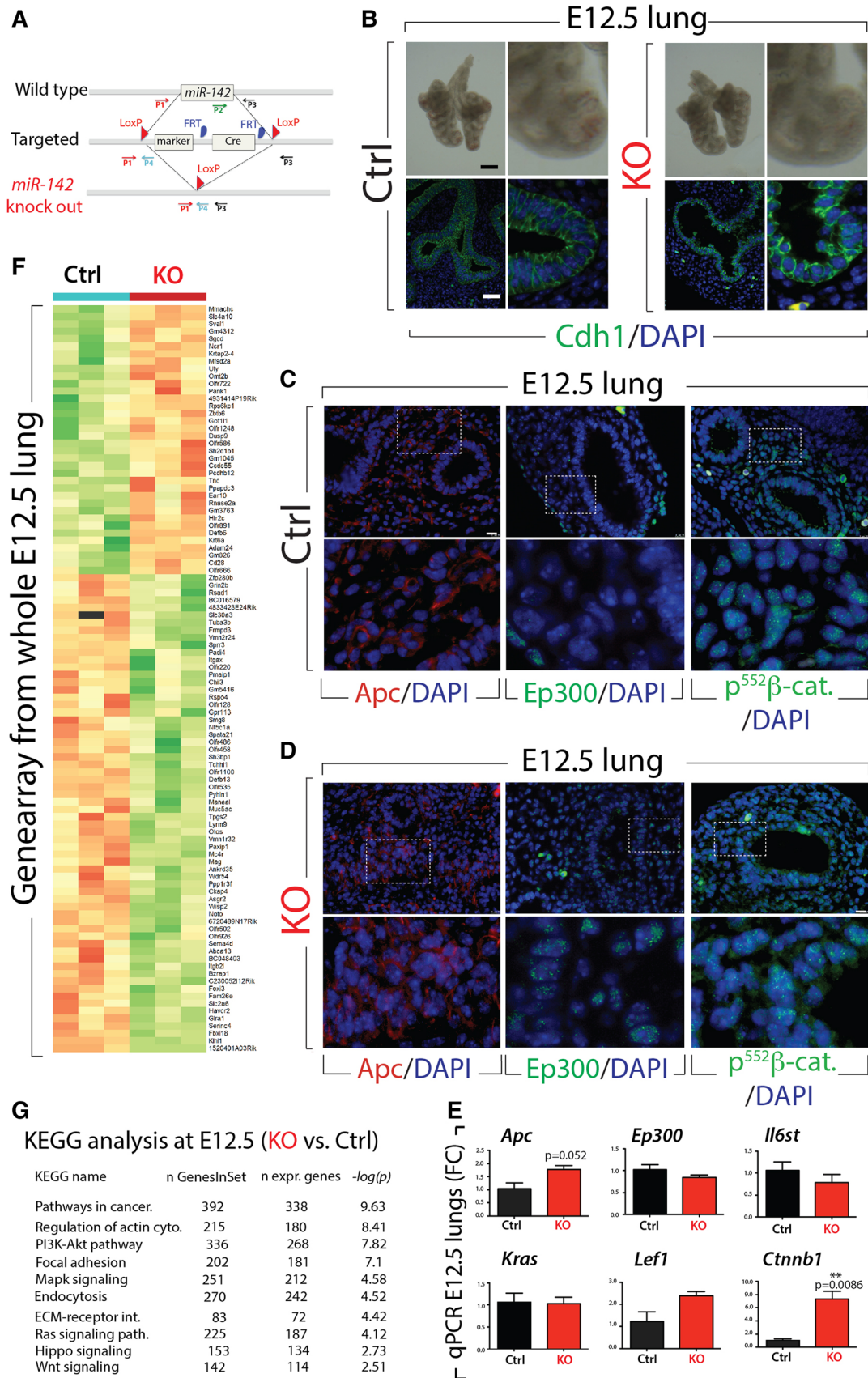


Fig. 3 Analysis of the *miR-142* KO at E12.5. **a** Generation of the *miR-142* KO by homologous recombination. **b** Bright field picture and Cdh1 staining of control (WT) littermate and *miR-142* KO lungs at E12.5 (minimum $n=3$). **c**, **d** Immunofluorescence staining for Apc, Ep300 and activated β -catenin (p^{S52} Ctnnb1) ($n=3$) and **e** gene expression analysis of *miR-142* target genes by qPCR in E12.5 controls and *miR-142* KO lungs ($n=3$). **f** Heat map of the most differentially expressed genes (according to their p values) between KO and control lungs at E12.5 ($n=3$). **g** Corresponding KEGG pathway analysis. Scale bar for bright field: low mag: 400 μ m, high mag: 100 μ m. Scale bar for immunofluorescence: low mag: 40 μ m, high mag: 10 μ m

cultured them for 4 days (Fig. 6a). This approach allowed analyzing the effect of *miR-142* LOF at a time when early alveolar progenitors have yet to differentiate into AT1 and AT2 cells [5]. We observed a significant decrease in the expression of *miR-142-3p* and *-5p*, respectively (Fig. 6b). Treatment with both morpholinos together (*mo*-(*3p+5p*)) led to the simultaneous knockdown of both *miR-142* isoforms (Fig. 6b). The decrease in the expression of these *miRNAs* was further confirmed by upregulation of their respective target genes such as *Apc*, *Ep300* and *Kras* (Fig. 6c). Interestingly, the attenuation of *miR-142-3p* and/or *-5p* led to the increase in *Sftpc* expression while decreasing the expression of *Pdpn* (Fig. 6d). In accordance with our in vivo data, FACS analysis of AT2 and AT1 cells in these lungs grown in vitro demonstrated an increase in the percentage of AT2 ($14.8\% \pm 1.33\%$ vs. $7.9\% \pm 4.8\%$ in *mo*-(*3p+5p*) vs. *Scramble*, respectively, $p=0.049$, $n=4$) and a decrease in AT1 ($0.86\% \pm 0.2\%$ vs. $2.8\% \pm 1.1\%$ in *mo*-(*3p+5p*) vs. *Scramble*, respectively, $p=0.01$, $n=4$) (Fig. S6). The phenotype resulting from the knockdown of *miR-142* in vitro using morpholinos was validated in *miR-142* KO and WT lungs grown in vitro (Fig. S7). In particular, we found that *miR-142* KO lungs grown in vitro display increased *Sftpc* expression compared to the corresponding control. Therefore, our results demonstrate that in vitro knockdown of *miR-142* recapitulates the in vivo phenotype in terms of AT2/AT1 ratio. This result reinforces the concept that the observed phenotype is due to a direct effect of perturbing *miR-142* signaling, rather than to secondary effects due to prolonged absence of *miR-142*.

Blockade of Ep300/ β -catenin (Ctnnb1) in vitro using the pharmacological inhibitor IQ-1 prevents *miR-142*-LOF-induced increase in *Sftpc*

To identify the molecular mechanism responsible for the perturbation of alveolar lineage formation by silencing *miR-142*, the activity of its downstream targets were modulated. We employed the pharmacological inhibitor IQ-1 (blocks Ep300/ β -catenin interaction) and SCH772984, a specific inhibitor of Erk1/2 (inhibits Kras/Erk signaling). Treatment with IQ-1 (10 μ m) and SCH772984 (10 μ m) on E14.5 lung

explants for 4 days showed that both inhibitors were efficient in reducing *Sftpc* expression (Fig. 6e, h, j) but did not alter the level of *Pdpn* expression (data not shown). It is not clear whether this effect is *miR-142* dependent or independent. Given the fact that *miR-142* silencing enhances *Sftpc* expression compared to scramble, we investigated the relevance of the Erk and Ep300/ β -catenin signaling downstream of *miR-142* using these inhibitors in our lung explant model (Fig. 6f).

In presence of IQ-1, silencing *miR-142* in E14.5 lung explants were not able to rescue the increased expression of *Sftpc* phenotype being observed in *miR-142* knockdown alone (Fig. 6g, h). On the other hand, silencing *miR-142* in SCH772984 (Erk inhibitor) treated E14.5 lung explants led to a moderate but significant increase in the expression of *Sftpc* compared to the control scramble treated with Erk inhibitor (Fig. 6i, j). These results suggest that Ep300/ β -catenin rather than Kras/Erk signaling is downstream of *miR-142* to control *Sftpc* expression.

Validating our in vitro approach using morpholinos, KO lungs grown in vitro showed increased *Sftpc* expression compared to the corresponding control wild-type lung (Fig. S7A) confirming the results obtained in vivo. In addition, KO lungs ($n=3$) grown in vitro in presence of Erk inhibitor displayed increased *Sftpc* expression compared to the corresponding control wild-type lungs (also grown with Erk inhibitor) ($n=3$) (Fig. S7B). Further supporting our in vitro morpholino-based approach, KO lungs ($n=4$) grown in vitro in presence of IQ1 displayed no change in *Sftpc* expression compared to the corresponding control wild-type lungs (also grown with IQ1) ($n=3$) (Fig. S7C).

Discussion

The mechanisms regulating alveolar epithelial cells proliferation and differentiation as well as the advancement of distinct lung progenitor cells towards given mature alveolar cell types are poorly understood [20–22]. Recently, studies based on the use of cell-specific markers as well as single cell transcriptomic of the epithelium during lung development suggested that AT1 and AT2 cells derive from common bipotent progenitor cells [5, 15]. The biological relevance of the BP model as progenitor for AT1 and AT2 is still unclear. Alternatively, *Sftpc*-positive alveolar progenitors could progressively differentiate into either AT1 or AT2. The differentiation towards the AT1 lineage would require losing *Sftpc* expression/AT2 markers and acquiring AT1 markers.

Interestingly, at E16.5, alveolar progenitors (Epcam⁺ve Cd49f⁺ve) can be subdivided by FACS into two populations based on the expression of *Pdpn*. We propose that *Pdpn*⁺ve cells are alveolar progenitor cells differentiating towards the AT1 lineage and expressing, therefore, *Pdpn*; these cells can

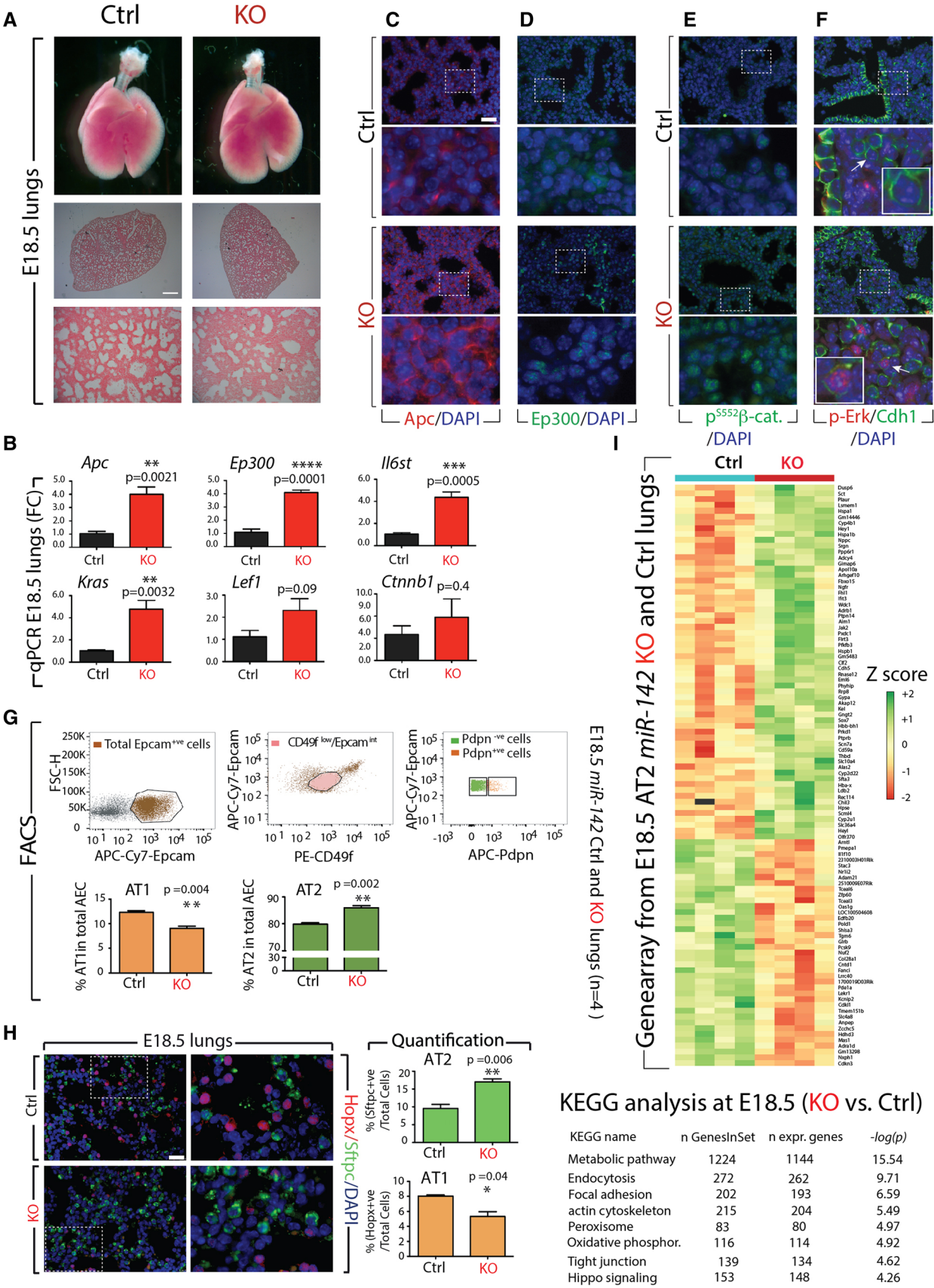


Fig. 4 Analysis of the alveolar epithelial lineage phenotype of the *miR-142* control and KO lungs at E18.5. **a** Bright field pictures, H&E staining of control and *miR-142* KO lungs at E18.5 ($n=3$). **b** Gene expression analysis of *miR-142* target genes by qPCR in E18.5 controls and *miR-142* KO lungs ($n=3$). Immunofluorescence staining for **c** Apc, **d** Ep300, **e** activated β -catenin (p^{S552} Ctnnb1) and **f** p-Erk/Cdh1 ($n=3$ each). **g** FACS of control and *miR-142* KO lungs ($n=3$) using Epcam, Cd49f and Pdpn antibodies. Quantification of AT1 (Cd49^{low}; Epcam^{int}, Pdpn^{+ve}) and AT2 (Cd49^{low}; Epcam^{int}, Pdpn^{-ve}) cells. **h** Hopx/Sftpc/DAPI IF staining in control and KO E18.5 lungs and the corresponding quantification of AT1 and AT2 cells ($n=3$). **i** Heatmap of the most differentially expressed genes (according to their p values) between AT2-isolated cell from KO and control lungs at E18.5 ($n=4$) and corresponding KEGG pathway analysis. Scale bar **a** low mag: 125 μ m, high mag: 31 μ m; scale bar **c-f**: low mag: 25 μ m, high mag: 6 μ m. Scale bar **h**: low mag: 20 μ m, high mag: 5 μ m

either be BP cells (Sftpc^{+ve} and Pdpn^{+ve}) or simply alveolar progenitor cells which have acquired Pdpn and also lost Sftpc. On the other hand, we expect the Pdpn^{-ve} cells to be the alveolar progenitor cells differentiating towards the AT2 lineage.

The fact that we can detect two populations (Pdpn^{+ve} and Pdpn^{-ve}) at E16.5 indicates that the bipotent mode is not widely applicable (otherwise we should only detect the Pdpn^{+ve} cells). It is likely that only a small proportion of the alveolar progenitors (Sftpc^{+ve}) acquire Pdpn expression at E16.5 to become bipotent progenitor cells. The overall majority of the alveolar progenitors express only Sftpc. Again, in the future, lineage tracing experiments for the BP should be carried out to elucidate their exact contribution to the AT1 and AT2 lineages.

Using a constitutive KO mouse model, we report that in the absence of *miR-142* (abolishing both the -3p and -5p strands), there is an relative increase of AT2 and a decrease in AT1 cell number (leading to an increase in the AT2/AT1 cell number ratio). Examination of the KO versus control lungs at E18.5, revealed enhanced expression of the *miR-142* targets *Apc* and *Ep300*, associated with increased β -catenin and p-Erk signaling. Supporting a cell autonomous function for *miR-142* in the epithelium, increased *miR-142* from E14.5 to E18.5 in alveolar progenitor cells led to the opposite effect. Interestingly, no changes in the number of Epcam or Ki-67-positive cells were observed suggesting a direct impact of *miR-142* in epithelial cell differentiation. Activation of the glucocorticoid pathway in an in vitro alveolar epithelial lineage differentiation assay was sufficient to achieve decreased *miR-142* expression and enhanced *Sftpc* expression. Morpholino-based knockdown of *miR-142* was sufficient to induce *Sftpc*, decrease *Pdpn* expression and increase AT2/AT1 cell number ratio. In addition, *Apc*, *Ep300* and *Kras* expression were up-regulated. Pharmacological inhibition of Ep300/ β -catenin but not the Kras/Erk signaling completely prevented *miR-142* morpholino-based increase in *Sftpc* expression. These results suggest that a

glucocorticoid-*miR-142*-Ep300 signaling axis controls the differentiation of alveolar progenitors and maintains the balance between AT1 and AT2 cells.

Recently, we showed that Fgf10 might represent a crucial molecule, controlling the differentiation of alveolar epithelial progenitor cells towards the AT2 lineage [16]. Using *Fgf10* heterozygous (*Fgf10*^{+/-}) lungs, we demonstrated a decrease in the AT2/AT1 cell ratio as well as surfactant production impairment in newborn pups. These changes are associated with a fully penetrant lethal phenotype after hyperoxia injury. Furthermore, a defect in epithelial differentiation and proliferation was observed in *Fgf10* hypomorphic lungs showing impairment in AT2 lineages [17]. While we cannot exclude changes in components of Fgf10 signaling in the *miR-142* KO, *Fgf10* as well as its downstream targets *Etv5* and *Sprouty2* were unchanged in our model. Furthermore, the fact that pharmacological inhibition of Ep300/ β -catenin but not the Kras/Erk signaling completely prevented *miR-142* morpholino-based increase in *Sftpc* expression, suggests a prominent role for Wnt signaling downstream of *miR-142*.

Recently, a role for *histone deacetylase 3* (*Hdac3*) in the spreading of AT1 cells and lung sacculization was reported. It was shown that *Hdac3* expressed in alveolar progenitors represses the expression of *miR-17-92* [23]. *miR-142* does not appear to impact the spreading of AT1 cells as no such defects were detected at E18.5 or postnatally (data not shown). In addition, the adult KO lungs are functional and appear histologically normal ([12] data not shown). There is no evidence so far of an organized cross-talk between *miR-142* and *miR-17-92* during the late phase of lung development. During early development *miR-17-92* was shown to modulate Fgf10-Fgfr2b signaling by specifically targeting *Stat3* and *Mapk14*, hence regulating Cdh1 expression. Cdh1 expression level in turn fine-tunes Ctnnb1 signaling in the epithelium, which is critical for epithelial bud morphogenesis triggered by Fgf10 [24]. Interestingly, mutant lungs with specific deletion of *Hdac3* in the mesenchyme also display impairment of AT1 differentiation, correlating with decreased Ctnnb1 signaling in the epithelium. Rescue of Ctnnb1 signaling in the mutant lung partially rescues AT1 cell differentiation defects [25]. Again, as Ctnnb1 signaling is increased in the *miR-142* KO epithelium, it is very unlikely that this leads to the perturbation of AT1 differentiation, conclusion that is supported by our analysis.

To identify the molecular mechanism involved in the regulation of alveolar epithelial phenotype by *miR-142*, we employed a well-established model to activate glucocorticoid signaling using dexamethasone to stimulate the maturation of alveolar cells into functional AT1 and AT2 cells [13, 26]. Interestingly, we noted a reduced expression of *miR-142-3p* and *miR-142-5p* in lung explants treated with glucocorticoid agonists, suggesting reduced level of *miR-142*

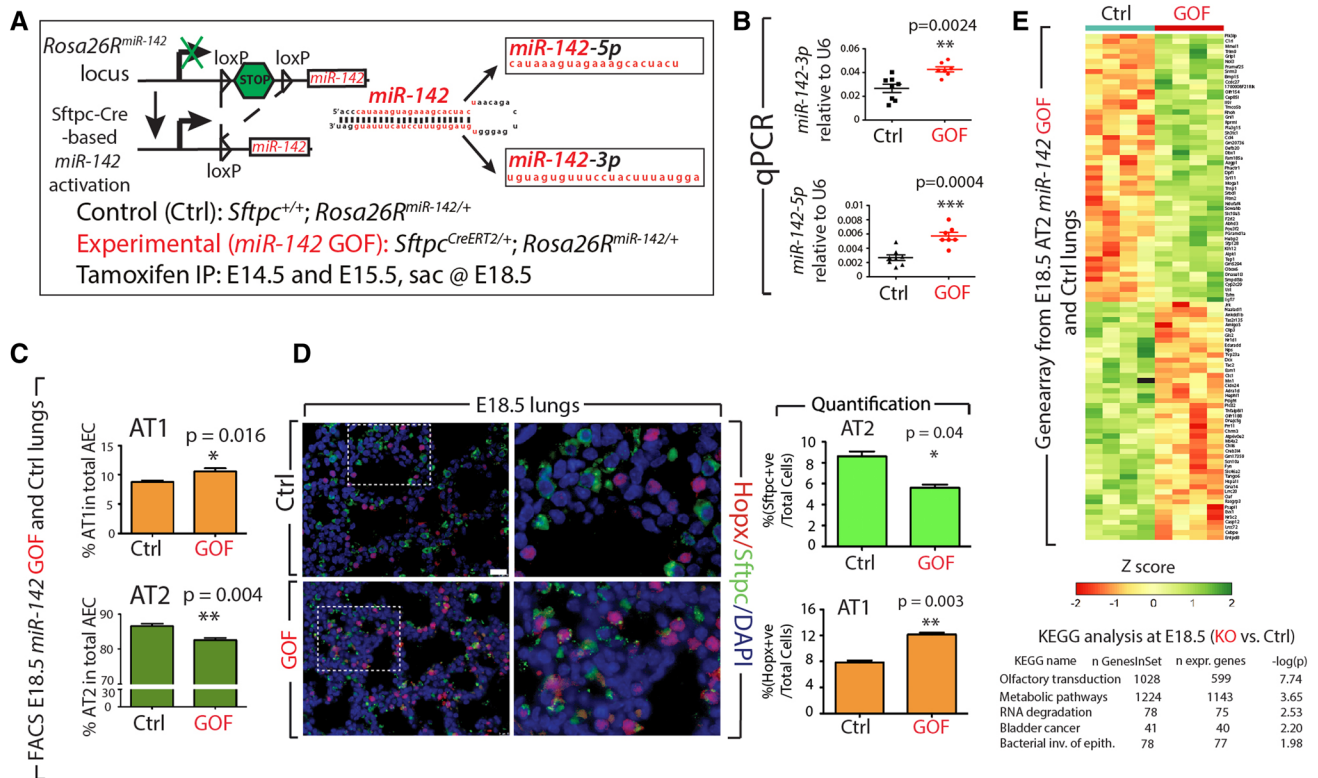


Fig. 5 Analysis of the alveolar epithelial lineage phenotype of the *miR-142* gain of function (GOF) and littermate control lungs at E18.5. **a** *LoxP-Stop-LoxP-miR-142* knock in mice (in the *Rosa26* locus) are crossed with *Sftpc^{CreERT2}* mice. Cre activation in the alveolar epithelial progenitor cells following tamoxifen IP injection to pregnant females at E14.5 and E15.5 leads to *miR-142* overexpression in these cells. Lungs are then analyzed at E18.5. **b** Validation of *miR-142-3p* and *-5p* overexpression in E18.5 GOF and control litter-

mate lungs. **c** FACS analysis of control and GOF E18.5 lungs ($n=4$) for total Epcam, AT1 and AT2 cells. **d** Hopx/Sftpc/DAPI IF staining in E18.5 GOF and control littermate lungs and the corresponding quantification. **e** Heatmap of the most differentially expressed genes (according to their p values) between AT2-isolated cell from GOF and control lungs at E18.5 ($n=4$) and corresponding KEGG pathway analysis. Scale bar **d**: low mag: 16 μ m, high mag: 4 μ m

is required for the differential of alveolar epithelial cells. Furthermore, we demonstrated two equally important pathways downstream of *miR-142* playing an important role in the formation of the alveolar lineage. Ep300/ β -catenin interaction has been shown to be one of the pathways involved in the differentiation of adult epithelial progenitors [27] as well as differentiation of embryonic stem cells and regulation of proximal–distal axis during lung development [28]. *Apc* and *Ep300*, two critical targets of *miR-142*, can control the β -catenin pathway. *Apc* is part of the degradation complex for β -catenin and is, therefore, a negative regulator of Ctnnb1 signaling. Conversely, Ep300 binds to β -catenin and acts as a co-transcriptional activator. In vitro blockade of Ep300/ β -catenin interaction with the use of IQ-1 showed complete downregulation of *Sftpc* expression, suggesting impairment in the alveolar epithelial lineage, while silencing *miR-142* in IQ-1-treated lung explants were unable to rescue the expression of the AT2 marker (Fig. 6g, h). The other pathway controlled by *miR-142* is the Kras/Erk pathway. A recent report indicated that *miR-142* is highly expressed in

undifferentiated mouse embryonic stem cells (mESCs) and downregulated in differentiated cells. It was also reported that overexpression of *miR-142* interrupted mESC differentiation. A double-negative feedback loop between Kras/Erk and *miR-142* levels has been suggested. Low level of *miR-142* triggers Kras and Erk phosphorylation, which in turn induces mESC differentiation and vice versa [29]. However, evidence suggests that Kras represses the formation of the alveolar lineage as forced activation of Kras in the distal lung epithelium in vivo suppresses the alveolar differentiation program [30]. In our in vitro model, lung explants treated with Erk inhibitor alone showed reduced *Sftpc* expression whereas Erk inhibitor treatment in combination with morpholino specific to *miR-142* showed a moderate increase in the *Sftpc* expression indicating a mild rescue in the alveolar lineage phenotype (Fig. 6i, j).

In conclusion, we report for the first time, an important role played by both isoforms of *miR-142* in alveolar epithelial lineage formation. We show that *miR-142* governs the formation of AT1 progenitors thereby controlling the AT2/AT1

cell number ratio. We propose that a glucocorticoid-*miR-142*-p300- β -catenin signaling axis controls alveolar epithelial lineage formation (Fig. 6k).

Materials and methods

Mice

The *miR-142* KO mice on a pure C57BL6 background were previously generated [12]. *miR-142* heterozygous males and females were crossed to generate KO and WT littermate embryos at different stages. We also generated a knock in of *miR-142* in the *Rosa26R* locus. *Rosa26-LoxP-STOP-loxP-miR-142* (also known as *Rosa26R^{miR-142/miR-142}*) mice were crossed with *Sftpc^{CreERT2/+}* mice (kind gift from Dr. Chapman) to generate control (*Sftpc^{+/+}; Rosa26^{miR-142/+}*) and experimental (*Sftpc^{CreERT2/+}; Rosa26R^{miR-142/+}*) embryos. Pregnant females were injected with tamoxifen IP (0.1 mg/g of mouse) at E14.5 and E15.5 and collected at E18.5. Animal experiments were approved by the Federal Authorities of Animal Research of the Regierungspräsidium Giessen, Hessen, Germany with approved protocol numbers 405_M, 423_M, G 47/2017 and G54/2017. Animal protocols for the use of genetically modified animals were approved by Wenzhou University/Wenzhou Medical University animal care and use committee.

Quantitative real-time PCR analysis

Briefly, total RNA from embryonic lungs were extracted and used for cDNA synthesis. RT-PCR for mRNA was carried out using Quantitative Reverse Transcription kit (205314; Qiagen) and Taqman MicroRNA Reverse Transcription kit (4366597; Applied Biosystem) was used in RT-PCR for miRNA. In both cases, reactions were assembled following the manufacturer's recommendations. qPCR was performed on a Light Cycler 480 system (Roche Applied Science). The TaqMan microRNA assay (Applied Biosystem) was used for screening the differential expression of miRNAs whereas SYBR Green (Platinum SYBR Green qPCR SuperMix-UDG Invitrogen) was used for the analysis of mRNA expression. *U6* and *Hprt* (Hypoxanthine phosphoribosyl transferase 1) were used as a reference for normalization of miRNA and mRNA, respectively. Results were collected from at least three lung samples and each reaction was run in triplicate. Primers for *miR142-3p*, *miR142-5p* and *U6* were obtained from Applied Biosystems.

In situ hybridization and immunofluorescence staining

Freshly isolated lungs were washed in PBS, then fixed in 4% PFA, gradually dehydrated in ethanol, impregnated with

xylene, embedded in paraffin and sectioned into 5 μ m slices on poly-L-lysine-coated slides. Antigen retrieval was performed by treating the sample with Proteinase K for 1 min at 37 °C. The slides were blocked 2 times 5 min with Dako (DAB Emission + Dual Linksystem HRP, Life Technologies) and then incubated with digoxigenin-labeled LNA probes (Exiqon, miRCURY LNA Detection probe, Vedback, Denmark) specific for *miR142-3p* and *miR142-5p* and Anti-Digoxigenin-POD, Fab fragments (11207733910; Roche Diagnostics) were used to detect the signal by in situ hybridization.

For immunofluorescence staining, the slides were deparaffinized, blocked with 3% bovine serum albumin (BSA) and 0.4% Triton X-100 in Tris-buffered saline (TBS) at room temperature (RT) for 1 h and then incubated with primary antibodies against Apc (ab15270, Abcam; 1:250), phospho-S⁵⁵²- β -catenin (9566, Cell Signaling; 1:250), Ep300 (sc-585, Santa Cruz; 1:250) and p-Erk (4376, Cell Signaling; 1:250), Sftpc (AB3786, Millipore; 1:500), Cdh1 (610181, BD Trans. Lab; 1:250), Fgfr2-BEK (sc-122, Santa Cruz; 1:250), Pdpn (8.1.1, DSHB; 1:250), Hopx (HPA030180, Atlas antibodies, 1:300) at 4 °C overnight. After incubation with primary antibodies, slides were washed three times in TBST (Tris buffer saline + 0.1% Tween 20) for 5 min, incubated with secondary antibodies at RT and washed three times with TBST before being mounted with Prolong Diamond Anti-fade Mountant with DAPI (4',6-diamidino-2-phenylindole; Invitrogen). Photomicrographs of immunofluorescence staining were taken using a Leica DMRA fluorescence microscope with a Leica DFC360 FX camera (Leica, Wetzlar, Germany). Figures were assembled using Adobe Illustrator. The data are representative of at least three lungs from independent experiments.

FACS analysis and cell sorting

Lungs from E18.5 embryonic mice were dissected and processed for flow cytometry analysis using BD LSR FORTRESSA™ (BD Bioscience). Isolation of epithelium and mesenchyme as well as isolation of AT2 cells was performed using the FACSARIA™ III (BD Bioscience) cell sorter. Following antibodies were used for analysis of AT1 and AT2 cell number [102513, 488-CD31 (1:50), Biolegend; 103108, FITC-CD45 (1:50); 118217, Apc Cy7 EpCam (1:50), or 47-5791-80, Apc-eFluor-780-EpCam (1:50), eBioscience; AB3786, proSPC (1:500), Millipore; 127409, Apc-Podoplanin (1:20), Biolegend; 402012, Apc Isotype Ctrl (1:20), Biolegend]. Fc block (Gamunex10%—1:10) was used for the blocking the non-specific binding and Saponin (558255, Cabiochem) was used for the permeabilization step. For the fluorescence-activated cell sorting of epithelium and mesenchyme, the cells were subsequently labeled with following antibodies [488-CD31 (1:50); FITC-CD45 (1:50); Apc

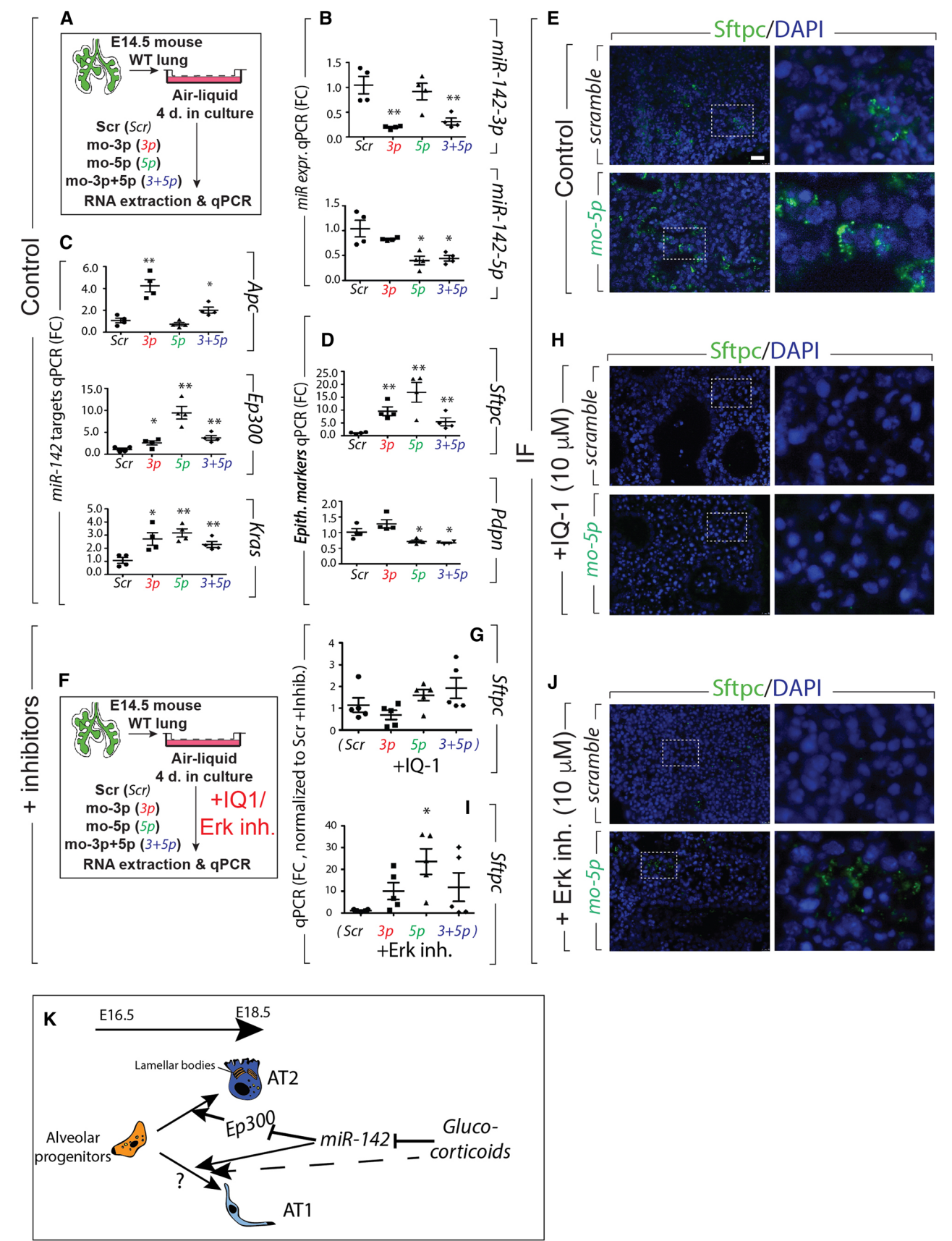


Fig. 6 In vitro differentiation of the alveolar epithelial progenitors. **a** Schematic showing the in vitro treatment of E14.5 WT lung explants with morpholinos specific to *miR-142-3p* and *miR-142-5p* for 4 successive days ($n=4$ for each condition). Impact of morpholino (*mo-3p*, *mo-5p*, *mo-(3p+5p)*) treatment on **b** *miR-142-3p* and *-5p* expression. **c** *Apc*, *Ep300* and *Kras* expression **d** *Sftpc*, *Pdpn* expression, **e** IF staining of *Sftpc* on E14.5 lung explants cultures treated with *Scramble* or *mo-5p* for 4 days. **f** Schematic showing the in vitro culture of E14.5 WT lung explants with *Scramble* or morpholinos against *miR-142-3p* or *-5p* for 4 successive days in presence of either IQ-1 (10 μ M) or SCH772984 (10 μ M) (a specific inhibitor of ERK1/2). qPCR analysis (**g–i**, $n=5$) and IF staining (**h–j**) showing the level of expression of *Sftpc* on E14.5 lung explants cultures in presence of either IQ-1 or SCH772984 treated with morpholino specific to *miR-142-5p*. **k** Glucocorticoids (GC) are enhancing AT1 and AT2 formation. Inhibition of *miR-142* is sufficient to increase the AT2 to AT1 cell number ratio but does not perturb the flattening of the AT1 cells. It is still unclear whether *miR-142* acts directly or indirectly on the differentiation of the alveolar progenitors towards the AT1 lineage. Blockade of Ep300/ β -catenin with IQ1 suppresses the increase in *Sftpc* expression triggered by the loss of *miR-142*. We propose that *miR-142* is a negative regulator of alveolar progenitor differentiation towards AT2 cells. In addition, GC can act in a *miR-142*-independent fashion to increase the differentiation of alveolar progenitors towards AT1 cells. Scale bar **e**, **h**, **j**: low mag: 16 μ m, high mag: 4 μ m

Cy7 EpCam (1:50)] whereas [488-CD31 (1:50); FITC-CD45 (1:50); Apc Cy7 EpCam (1:50), Apc-Podoplanin (Pdpn) (1:20); Apc Isotype Ctrl (1:20)] antibodies were used for isolation of AT1 and AT2 cells. Alveolar epithelial cells were identified as CD45^{ve}/CD31^{ve}/CD49f^{low}/Epcam^{int} and AT2 cells were identified as CD45^{ve}/CD31^{ve}/CD49f^{low}/Epcam^{int} and Pdpn^{ve} while AT1 cells were identified as CD45^{ve}/CD31^{ve}/CD49f^{low}/Epcam^{int} and Pdpn^{ve}. Cells were sorted through a flow chamber with a 100- μ m nozzle tip under 25 psi sheath fluid pressure. Isolated cells were used for RNA isolation. As a main criterion for gating, we used the settings allowing capturing 98% of the cells in the isotype control and then we applied these gating conditions to the stained cells.

Microarray experiment

RNA was isolated using the RNeasy Mini Kit (217004; Qia-gen). Purified total RNA was amplified using the Ovation PicoSL WTA System V2 kit (NuGEN). Per sample, 2 μ g amplified cDNA was Cy5-labeled using the SureTag DNA labeling kit (Agilent). 2 μ g of the labeled cDNA was hybridized on Agilent-074809 SurePrint G3 Mouse GE v2 8 $\times$ 60 K Microarrays for 22 h at 65 °C in Agilent hybridization chambers. The cDNA was not fragmented before hybridization. Dried slides were scanned at 2 μ m/pixel resolution using the InnoScan 900 (Innopsys). The analysis was performed with R and the limma package. Gene set analyses were done using the Wilcoxon tests of the t statistics. The data are deposited in GEO and are available through the accession number GSE106411.

Embryonic lung explant cultures

Timed pregnant wild-type mice were killed on E14.5, the embryonic lungs were harvested and placed on 8 μ m Nucleopore Track-Etch membranes (110414; Whatman). Vivo-morpholinos specific for *miR-142-3p* and/or *miR-142-5p* (Gene Tools LLC) were added at 5 μ M to E14.5 lung explants. Lungs were grown for 96 h at 37 °C with 5% CO₂ prior to analysis. In a second set of experiments, 100 nM dexamethasone (Dex, D1756; Sigma Aldrich), 10 μ M IQ-1 (Ep300/ β -catenin (Ctnnb1) inhibitor, S8248; Selleckchem), 10 μ M SCH772984 (Erk inhibitor, 19166; CayMan Chemical) either alone or in combination with morpholinos were added to the E14.5 lung explants.

Western blotting

Immunoblotting was performed using antibodies against pro-Sftpc (AB3786, Millipore; 1:1000), Lamin B1 (B-10) (sc-374015 from SantaCruz; 1:200) and beta-actin (ab8227, Abcam; 1:50000).

Statistical analysis

Data were assembled using Graph Pad Prism Software (Graph Pad software, USA) and presented as average values $\pm$ S.E.M. Statistical analyses were performed using Student's *t* test. Data were considered significant if $p < 0.05$. Figures were assembled using Adobe Photoshop CS6 and Adobe Illustrator CS6.

Acknowledgements We acknowledge the precious help provided by Kerstin Goth and Jana Rostkovius in managing the mouse colonies, and Shirisha Bagari for the genotyping of the mice. C.M.C. and E.E.A. were funded by start-up grants from the Cardio Pulmonary Institute (CPI) (previously known as Excellence Cluster Cardio-Pulmonary System (ECCPS)). C.M.C. also acknowledges the support of the University Hospital Giessen and Marburg (UKGM). E.E.A. acknowledges the support of the University Hospital Giessen and Marburg (UKGM) and the German Center for Lung Research (DZL). S.B. was supported by grants from the Deutsche Forschungsgemeinschaft (DFG; BE4443/1-1, BE4443/4-1, BE4443/6-1, BE4443/14-1, KFO309 P7 and SFB1213-projects A02 and A04), UKGM, Universities of Giessen and Marburg Lung Center (UGMLC) and the Deutsche Zentrum für Lungenforschung (DZL). J.S.Z. was funded through a start-up package from Wenzhou Medical University and the National Natural Science Foundation of China (Grant Number 81472601). S.H. was supported by University Hospital Giessen and Marburg (FOKOOPV), the German Center for Lung Research (DZL), and Grants from the DFG (KFO309 P2/8; SFB1021 C05, SFB TR84 B2). CC was supported by the Interventional Pulmonary Key Laboratory of Zhejiang Province, the Interventional Pulmonology Key Laboratory of Wenzhou City, the Interventional Pulmonology Innovation Subject of Zhejiang Province, the National Nature Science Foundation of China (81570075, 81770074), Zhejiang Provincial Natural Science Foundation (LZ15H010001), Zhejiang Provincial Science Technology

Department Foundation (2015103253) as well as the National Key Research and Development Program of China (2016YFC1304000). B.M. acknowledges the ANR-18-CE92-0009-01.

References

- McCulley D, Wienhold M, Sun X (2015) The pulmonary mesenchyme directs lung development. *Curr Opin Genet Dev* 32:98–105
- El Agha E, Bellusci S (2014) Walking along the fibroblast growth factor 10 route: a key pathway to understand the control and regulation of epithelial and mesenchymal cell-lineage formation during lung development and repair after injury. *Scientifica* 2014:538379
- Rawlins EL, Clark CP, Xue Y, Hogan BL (2009) The Id2+ distal tip lung epithelium contains individual multipotent embryonic progenitor cells. *Development* 136:3741–3745
- Rockich BE, Hrycaj SM, Shih HP, Nagy MS, Ferguson MA, Kopp JL, Sander M, Wellik DM, Spence JR (2013) Sox9 plays multiple roles in the lung epithelium during branching morphogenesis. *Proc Natl Acad Sci USA* 110:E4456–E4464
- Treutlein B, Brownfield DG, Wu AR, Neff NF, Mantalas GL, Espinoza FH, Desai TJ, Krasnow MA, Quake SR (2014) Reconstructing lineage hierarchies of the distal lung epithelium using single-cell RNA-seq. *Nature* 509:371–375
- Li J, Wang Z, Chu Q, Jiang K, Li J, Tang N (2018) The strength of mechanical forces determines the differentiation of alveolar epithelial cells. *Dev Cell* 44:297–312 e295
- Jonas S, Izaurralde E (2015) Towards a molecular understanding of microRNA-mediated gene silencing. *Nat Rev Genet* 16:421–433
- Shrestha A, Mukhametshina RT, Taghizadeh S, Vasquez-Pacheco E, Cabrera-Fuentes H, Rizvanov A, Mari B, Carraro G, Bellusci S (2017) MicroRNA-142 is a multifaceted regulator in organogenesis, homeostasis, and disease. *Dev Dyn* 246:285–290
- Carraro G, Shrestha A, Rostkovius J, Contreras A, Chao CM, El Agha E, Mackenzie B, Dilai S, Guidolin D, Taketo MM, Gunther A, Kumar ME, Seeger W, De Langhe S, Barreto G, Bellusci S (2014) miR-142-3p balances proliferation and differentiation of mesenchymal cells during lung development. *Development* 141:1272–1281
- Sun Y, Kolligs FT, Hottiger MO, Mosavin R, Fearon ER, Nabel GJ (2000) Regulation of beta-catenin transformation by the p300 transcriptional coactivator. *Proc Natl Acad Sci USA* 97:12613–12618
- Sharma S, Liu J, Wei J, Yuan H, Zhang T, Bishopric NH (2012) Repression of miR-142 by p300 and MAPK is required for survival signalling via gp130 during adaptive hypertrophy. *EMBO Mol Med* 4:617–632
- Shrestha A, Carraro G, El Agha E, Mukhametshina R, Chao CM, Rizvanov A, Barreto G, Bellusci S (2015) Generation and validation of miR-142 knock out mice. *PLoS One* 10:e0136913
- Laresgoiti U, Nikolic MZ, Rao C, Brady JL, Richardson RV, Batchen EJ, Chapman KE, Rawlins EL (2016) Lung epithelial tip progenitors integrate glucocorticoid- and STAT3-mediated signals to control progeny fate. *Development* 143:3686–3699
- Kotton DN, Morrissey EE (2014) Lung regeneration: mechanisms, applications and emerging stem cell populations. *Nat Med* 20:822–832
- Desai TJ, Brownfield DG, Krasnow MA (2014) Alveolar progenitor and stem cells in lung development, renewal and cancer. *Nature* 507:190–194
- Chao CM, Yahya F, Moiseenko A, Tiozzo C, Shrestha A, Ahmadvand N, El Agha E, Quantius J, Dilai S, Kheirollahi V, Jones M, Wilhem J, Carraro G, Ehrhardt H, Zimmer KP, Barreto G, Ahlbrecht K, Morty RE, Herold S, Abellar RG, Seeger W, Schermuly R, Zhang JS, Minoo P, Bellusci S (2017) Fgf10 deficiency is causative for lethality in a mouse model of bronchopulmonary dysplasia. *J Pathol* 241:91–103
- Ramasamy SK, Mailleux AA, Gupte VV, Mata F, Sala FG, Veltmaat JM, Del Moral PM, De Langhe S, Parsa S, Kelly LK, Kelly R, Shia W, Keshet E, Minoo P, Warburton D, Bellusci S (2007) Fgf10 dosage is critical for the amplification of epithelial cell progenitors and for the formation of multiple mesenchymal lineages during lung development. *Dev Biol* 307:237–247
- Herriges JC, Verheyden JM, Zhang Z, Sui P, Zhang Y, Anderson MJ, Swing DA, Zhang Y, Lewandoski M, Sun X (2015) FGF-regulated ETV transcription factors control FGF-SHH feedback loop in lung branching. *Dev Cell* 35:322–332
- Mailleux AA, Tefft D, Ndiaye D, Itoh N, Thiery JP, Warburton D, Bellusci S (2001) Evidence that SPROUTY2 functions as an inhibitor of mouse embryonic lung growth and morphogenesis. *Mech Dev* 102:81–94
- McQualter JL, Yuen K, Williams B, Bertonecello I (2010) Evidence of an epithelial stem/progenitor cell hierarchy in the adult mouse lung. *Proc Natl Acad Sci USA* 107:1414–1419
- Weiss DJ, Kolls JK, Ortiz LA, Panoskaltis-Mortari A, Prockop DJ (2008) Stem cells and cell therapies in lung biology and lung diseases. *Proc Am Thorac Soc* 5:637–667
- Zemke AC, Teisanu RM, Giangreco A, Drake JA, Brockway BL, Reynolds SD, Stripp BR (2009) beta-Catenin is not necessary for maintenance or repair of the bronchiolar epithelium. *Am J Respir Cell Mol Biol* 41:535–543
- Wang Y, Frank DB, Morley MP, Zhou S, Wang X, Lu MM, Lazar MA, Morrissey EE (2016) HDAC3-dependent epigenetic pathway controls lung alveolar epithelial cell remodeling and spreading via miR-17-92 and TGF-beta signaling regulation. *Dev Cell* 36:303–315
- Carraro G, El-Hashash A, Guidolin D, Tiozzo C, Turcatel G, Young BM, De Langhe SP, Bellusci S, Shi W, Parnigotto PP, Warburton D (2009) miR-17 family of microRNAs controls FGF10-mediated embryonic lung epithelial branching morphogenesis through MAPK14 and STAT3 regulation of E-Cadherin distribution. *Dev Biol* 333:238–250
- Wang X, Wang Y, Snitow ME, Stewart KM, Li S, Lu M, Morrissey EE (2016) Expression of histone deacetylase 3 instructs alveolar type I cell differentiation by regulating a Wnt signaling niche in the lung. *Dev Biol* 414:161–169
- Alanis DM, Chang DR, Akiyama H, Krasnow MA, Chen J (2014) Two nested developmental waves demarcate a compartment boundary in the mouse lung. *Nat Commun* 5:3923
- Rieger ME, Zhou B, Solomon N, Sunohara M, Li C, Nguyen C, Liu Y, Pan JH, Minoo P, Crandall ED, Brody SL, Kahn M, Borok Z (2016) p300/beta-catenin interactions regulate adult progenitor cell differentiation downstream of WNT5a/protein kinase C (PKC). *J Biol Chem* 291:6569–6582
- Sasaki T, Kahn M (2014) Inhibition of beta-catenin/p300 interaction proximalizes mouse embryonic lung epithelium. *Transl Respir Med* 2:8
- Sladitschek HL, Neveu PA (2015) The bimodally expressed microRNA miR-142 gates exit from pluripotency. *Mol Syst Biol* 11:850
- Chang DR, Martinez Alanis D, Miller RK, Ji H, Akiyama H, McCrea PD, Chen J (2013) Lung epithelial branching program antagonizes alveolar differentiation. *Proc Natl Acad Sci USA* 110:18042–18051

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

RESEARCH

Open Access



Neonatal exposure to hyperoxia leads to persistent disturbances in pulmonary histone signatures associated with NOS3 and STAT3 in a mouse model

Cho-Ming Chao^{1,2}, Rhea van den Bruck³, Samantha Lork³, Janica Merkle³, Laura Krampen³, Patrick P Weil³, Malik Aydin³, Saverio Bellusci¹, Andreas C. Jenke^{3,4*}  and Jan Postberg^{3†}

Abstract

Background: Early pulmonary oxygen exposure is one of the most important factors implicated in the development of bronchopulmonary dysplasia (BPD).

Methods: Here, we analyzed short- and long-term effects of neonatal hyperoxia on NOS3 and STAT3 expression and corresponding epigenetic signatures using a hyperoxia-based mouse model of BPD.

Results: Early hyperoxia exposure led to a significant increase in NOS3 (median fold change $\times$ 2.37, IQR 1.54–3.68) and STAT3 (median fold change $\times$ 2.83, IQR 2.21–3.88) mRNA levels in pulmonary endothelial cells with corresponding changes in histone modification patterns such as H2aZac and H3K9ac hyperacetylation at the respective gene loci. No complete restoration in histone signatures at these loci was observed, and responsiveness to later hyperoxia was altered in mouse lungs. In vitro, histone signatures in human aortic endothelial cells (HAEC) remained altered for several weeks after an initial long-term exposure to trichostatin A. This was associated with a substantial increase in baseline eNOS (median 27.2, IQR 22.3–35.6) and STAT3 α (median 5.8, IQR 4.8–7.3) mRNA levels with a subsequent significant reduction in eNOS expression upon exposure to hypoxia.

Conclusions: Early hyperoxia induced permanent changes in histones signatures at the NOS3 and STAT3 gene locus might partly explain the altered vascular response patterns in children with BPD.

Background

Bronchopulmonary dysplasia (BPD) is a chronic lung disease of prematurely born infants and remains a leading cause of morbidity and mortality. Currently, there is no curative therapy available. Based on the severity-based definition of BPD (inclusion of infants with mild BPD), 68% of premature infants born with a gestational age (GA) $\leq$ 28 weeks develop BPD [1–3]. The risk of developing BPD correlates inversely with the gestational

age (GA) and birth weight (BW) [4]. Since premature infants are born with a lung which is in the canalicular or saccular stages of development, the lung structure is therefore not adequate to provide sufficient ventilation and gas exchange. Thus, mechanical ventilation and high oxygen concentration are often necessary at birth. Barotrauma induced by mechanical ventilation as well as oxygen toxicity and inflammation are major contributing factors responsible for the pulmonary damages in the immature lung. In addition, some studies have suggested a strong genetic component in BPD [5]. In fact, clinical studies suggest that decisions during the first minutes of life [6] or even events before the delivery might be crucial for the later development of BPD. In line with these clinical data are observations that hyperoxia in rat pups leads to increased endothelial nitric oxide synthase (eNOS)

* Correspondence: Andreas.jenke@uni-wh.de

†Equal contributors

³Department of Pediatrics, HELIOS Medical Center Wuppertal, Center for Clinical & Translational Research (CCTR), Center for Biomedical Education & Research (ZBAF), Witten/Herdecke University, Wuppertal, Germany

⁴EKO Children's Hospital, Oberhausen, Witten/Herdecke University, Alfred-Herrhausen Str. 40, Witten, Germany

Full list of author information is available at the end of the article



© The Author(s). 2018 **Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated.

levels, nitric oxide (NO) activity, hyperemia [7, 8], and possibly eNOS uncoupling eventually leading to BPD.

Importantly, BPD does not only affect neonatal mortality but also leads to long-term morbidity, e.g., increased susceptibility to upper respiratory infections during the first year of life [9] and pulmonary hypertension [10]. Again, neonatal oxygen seems to be an important pathological factor since it increases for example the sensitivity to influenza A virus infection by suppressing epithelial expression of Ear1 [11]. In fact, extremely and moderately preterm infants face a 3.6 times increased risk of being hospitalized due to respiratory infection in the first year of life [12].

Whereas it seems clear from twin studies that genetic susceptibility plays an important role for disease development [13], the exact mechanism remains unclear but epigenetic mechanisms seem to be part of it since several recent publications have reported abnormalities of histone acetylase activity and the chromatin remodeling pathway in BPD patients [14, 15]. Thus, persistent changes in epigenetic signatures might be also at least partly responsible for the later development of pulmonary hypertension and the increased susceptibility to respiratory tract infections. Since we have recently shown that epigenetic signatures at NOS3 encoding eNOS in human umbilical artery endothelial cells are substantially shaped by prenatal events such as placental insufficiency and that there exists an interdependency between NOS3 and the gene activity of signal transducer and activator of transcription 3 alpha (STAT3 α) as well as Stat3-transcription factor-binding in the NOS 3 promoter. Moreover, in human, a 27-nt non-coding RNA becomes co-expressed with NOS3 and post-transcriptionally regulates STAT3 expression [16]. We thus aimed to utilize the same gene loci to analyze the effect of early neonatal

oxygen exposure on the pulmonary vascular endothelial epigenetic landscape and the associated consequences for oxygen exposure later in life.

Therefore, we analyzed NOS3 gene expression in response to early neonatal hyperoxia at first and then studied the respective changes in epigenetic signatures as consequences of this first early oxygen exposure on later events of hyperoxia and hypoxia later in life.

Methods

Mice

Wildtype mice (males and females, CD1 background) were crossed to generate time-pregnant females. All mice received food and water ad libitum.

Hyperoxia injury (BPD mouse model)

Newborn pups were exposed to either normoxia (NOX) or hyperoxia (HOX) within 24 h after birth (P0) (Fig. 1). In experimental group 1, dams and pups were kept in NOX from P0 to P16. In experimental group 2, dams and pups were kept in NOX from P0 to P15. From P15 to P16, the dams and the pups were exposed to HOX (85% O₂). In experimental group 3, newborn pups were subjected to HOX (85% O₂) injury from P0 to P8 in a chamber (Proox Model 110, Biospherix). To minimize oxygen toxicity and bias, nursing dams were rotated every 24 h between NOX and HOX. Afterwards, nursing dams and pups were exposed to NOX (21% O₂) from P8 to P16. From P15 to P16, the dams and the pups were re-exposed to HOX (85% O₂). In experimental groups 1 and 3, lungs were harvested at P8. In all groups, lungs were harvested at P16. All dams and pups received food and water ad libitum.

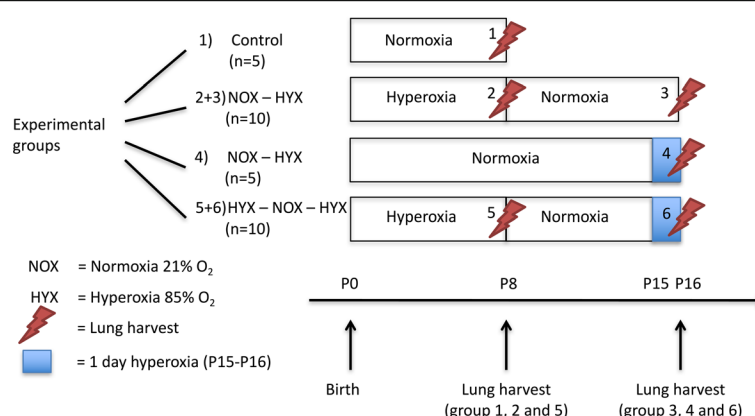


Fig. 1 Experimental setting. The experimental setting consists of six groups. Each group consists of five animals. The experiments were performed in two steps. The first comprised a control group at P8 in normoxia (group 1), a group after neonatal hyperoxia from P0 to P8 (group 2), and a group exposed to neonatal hyperoxia from P0 to P8 with eight additional days of normoxia until P16 (group 3). The second comprised a control group in normoxia at P16 (group 4), a group after neonatal hyperoxia from P0 to P8 (group 5), and a group exposed to neonatal hyperoxia (P0 to P8) followed by normoxia (P8 to P15), and a second exposure to hyperoxia for 1 day before analysis at P16 (group 6). P postnatal

Lung perfusion, isolation, tissue processing, and histology

Mice were euthanized by intraperitoneal injection of a solution made of ketamin, dormitor, heparin, and saline. After sternotomy, the lung was perfused transcatheterially (through the right ventricle) by using PBS (1×), then isolated and incubated for 30 min at 4 °C in COLD medium, stored at −20 °C overnight, and finally stored at −80 °C till further tissue processing.

For histology, the lung was flushed from the right ventricle to remove blood cells then perfused through the trachea with a pressure of 20 cm H₂O with 5 ml 4% PFA. The trachea was tied off with a string, and the lung was removed and placed in 4% PFA for max. 24 h at 4 °C. Lungs were then progressively dehydrated (30, 50, 70, and 99% ethanol, 3 h each), incubated in xylene, then in paraffin overnight and finally embedded with a Leica embedding machine (EG 1150C). Paraffin blocks were kept cold, and 5 µm sections were cut. Hematoxylin and eosin staining was performed according to protocols previously published.

Positive selection for vascular endothelial cells

Positive selection for vascular endothelial cells was performed by magnetic separation with human CD146 Microbeads (Miltenyi®, Bergisch-Gladbach, Germany) following manufacturers' instructions with minor modifications as described previously [17].

RNA isolation

Total RNA was isolated using Trizol (Sigma-Aldrich) and isopropanol precipitation and further purification on columns. Next, RNA integrity was assayed using the Agilent Bioanalyzer 2000. Only samples with non-fragmented RNA were included.

Gene expression analyses

Gene expression analyses were performed using quantitative real-time PCR (qPCR) analyses on a Rotor-Gene 6000 (Qiagen). For PCR reactions, QuantiTect SYBR Green qPCR Master Mix (Qiagen) containing Hot Start Taq DNA polymerase and SYBR Green was used. Primers were used as described earlier [18]. The expression of gene of interest was normalized using three housekeeping genes (BACT, GAPDH, PECAM1). PCR conditions were as follows: 95 °C for 15 min, 40× [95 °C for 15 s, 60 °C for 30 s]. Melting of PCR product was done using a gradient from 55 to 95 °C rising in 0.5 °C increments.

Antibodies

Primary antibodies used in this study were (1) Rabbit anti-H2A.Zac (Diagenode pAb-173-050), (2) Rabbit anti-H3K9ac (Active Motif pAb#39137), and (3) Rabbit anti-H3K4me3 (Diagenode pAbCSP-030-050).

Chromatin purification and ChIP assays

Chromatin purification and ChIP assays were performed as described previously [15]. Quantitative PCR analyses were performed using a Rotorgene 6000 (Qiagen). The relative amounts of specifically immunoprecipitated DNA were estimated as “percent of input” and quantified using individual standard curves for each amplicon. Primer pairs were used as described earlier [18].

Pyrosequencing

DNA methylation signatures of promoter segments of NANOG, NFE2L2, and STAT3 were analyzed using a Qiagen Pyromark Q24 sequencer as previously described (Plos One Jenke et al.). Briefly, after standard sodium bisulfite conversion using the EZ DNA Methylation-Gold Kit (Zymo Research, USA) pyrosequencing methylation analysis was conducted using the PyroMark Q24 (Qiagen, Germany) according to the manufacturer's protocol. Therefore, we designed and made use of the following oligonucleotides: 1. for NFE2L2—primer F1 (5'-gga gtt aga ggg gat agt ggt t-3'), 5'-biotinylated primer (5'-acc cca cca aat caa aac ttc ct-3'), and sequencing primer S1 (5'-agg gta aag gag gat g-3'); 2. for STAT3—primer F1 (5'-ggg gta ggg tgg ggt tat t-3'), 5'-biotinylated primer (5'-acc cta tat atc tcc tcc tat cct-3'), and sequencing primer S1 (5'-ggg tgg ggt tat ttt t-3'); 3. for NANOG (DNA methylation positive control)—primer F1 (5'-gta gga tag gaa tgg ggg ttg-3'), 5'-biotinylated primer (5'-acc tta aat tta ccc caa att cta c-3'), and sequencing primer S1 (5'-aat ggg ggt tgg gga-3'). No reliable NOS3 DNA methylation assays meeting our quality standards could be designed. The level of methylation was analyzed using PyroMark Q24 2.0.6 Software (Qiagen). Non-CpG cytosine residues and a standard fully methylated DNA (Zymo Research, USA) were used as controls to verify bisulfite conversion.

Small RNA-seq and analyses pipeline

Total RNA was purified as described above. For multiplexing, we made use of different multiplex sequencing barcodes for sequencing in a single lane as described [19]. Briefly, total RNA was separated by polyacrylamide gel electrophoresis. Gel fragments corresponding to 15 to 35 nt RNA molecules were cut, and RNA was eluted. These small RNAs were directly used for the construction of sequencing libraries in four steps: step 1: ligation of DNA oligos to the 3'-end of the RNA; step 2: ligation of RNA or, respectively, chimeric RNA/DNA oligos to the 5'-end of RNAs; step 3: cDNA library synthesis by reverse transcriptase; and step 4: amplification of the cDNA library. Subsequently, after final quality checks by microcapillary electrophoresis and qPCR, the libraries were sequenced on an Illumina HiSeq 2000 platform.

(single end, 50 bp). This work has benefited from the facilities and expertise of the high-throughput sequencing core facility of IMAGIF Gif-sur-Yvette (Centre de Recherche de Gif—www.imagif.cnrs.fr). The initial data analysis pipeline was as follows: CASAVA-1.8.2 was used for demultiplexing, Fastqc 0.10.1 for read quality assessment and Cutadapt-1.3 for adaptor trimming, resulting in an average sequence number for each developmental time point sample of 7.58 Mbp. File conversions, filtering, and sorting as well as mapping (Bowtie2) were done using “Galaxy” [20–22], a platform for data intensive biomedical research (<https://usegalaxy.org/>), and “Chimera” [23], respectively.

Immunofluorescence staining for eNOS

Paraffin sections were deparaffinized. Antigen retrieval was performed for 15 min in citrate buffer using a rice cooker, then slides were cooled down on ice for 20 min. After washing slides three times in TBST (TBS buffer + 0.1% Tween 20) for 5 min, slides were blocked with 3% bovine serum albumin (BSA) and 0.4% Triton X-100 [in Tris-buffered saline (TBS)] at room temperature (RT) for 1 h and then incubated with primary antibody against eNOS (ThermoFisher Scientific, PA1-037; 1:100) at 4 °C overnight. Afterwards, slides were again washed three times in TBST for 5 min, incubated with secondary antibody (Goat Anti-Rabbit Alexa 555, ThermoFisher Scientific, A-21429; 1:500) in room temperature for 1 h, and then washed three times in TBST before being mounted with Prolong Gold Anti-fade Reagent with DAPI (4,6-diamidino-2-phenylindole; ThermoFisher Scientific, P36931). Fluorescent images were acquired using Leica DM5500 B fluorescence microscope connected to Leica DFC360 FX camera.

Statistical analysis

Data were compared using Mann-Whitney *U* test according to normality assumptions on univariate analysis followed by Bonferroni correction for multivariate analysis. Categorical variables were compared using the Fisher exact test. Statistical analyses were performed with GraphPad Prism 5.0.

Cell culture

Human arterial endothelial cells (HAEC) were bought from PromoCell (Heidelberg, Germany) and cultivated upon manufacturer's recommendations. For cDNA synthesis, we used 500 ng RNA per sample using the QuantiTect Reverse Transcription kit (Qiagen). DNA was isolated by phenol:chloroform:isoamyl alcohol extraction followed by precipitation with isopropanol. For *in vitro* experiments, HAEC (PromoCell) were cultivated upon manufacturer's recommendations. For HDAC inhibition, HAEC were treated with 1 μ M trichostatin A

(TSA) for either 72 h or twice for 6 h with an 60-h interval in between. Hypoxia experiments were performed using a hypoxia incubator chamber (STEMCELL Technologies, Grenoble, France) exposing cells to a ppO_2 of 0.12 bar corresponding to an oxygen fraction of 12% for 24 h. Control experiments were performed under normoxic conditions (ppO_2 = 0.21 bar).

Results

Neonatal hyperoxia from P0 to P8 leads to increased NOS3 expression—in both whole lung tissue and CD146-positive cells

For our study, we used a well-established mouse model for bronchopulmonary dysplasia. Newborn pups were exposed to 85% oxygen from P0 to P8 then stayed in normoxia from P8 to P16. Lungs were analyzed at P8 and P16. In neonatal mouse lungs, exposure to hyperoxia (85% oxygen) from P0 to P8 led to a significant increase in NOS3 (median fold change $\times$ 2.37, IQR 1.54–3.68, p = 0.003), GPX1 (median fold change $\times$ 1.73, IQR 1.28–2.08, p = 0.001), and STAT3 α and β (median fold change $\times$ 2.83, IQR 2.21–3.88, p = 0.001) mRNA levels at P8. Other genes, such as HIF1A for example, were not differentially expressed on the mRNA level (Fig. 2a). At P16, 8 days after hyperoxia, mRNA levels of GPX1 and STAT3 dropped to levels comparable to the normoxia group at P8. NOS3 mRNA levels also dropped but tended to be slightly above the level of the normoxia group at P8—even though this difference did not reach statistical significance except for primer pair matching to exon10/11 (Fig. 2a). In addition, we aimed to determine whether expression profiles upon oxygen exposure differ between whole lung tissue and pulmonary endothelial cells. Therefore, we isolated vascular endothelial cells from whole lung tissue using magnetic separation with human CD146 microbeads. Results obtained from CD146-positive cell population are shown in Fig. 2b. For all analyzed genes of interest, we did not see any differences in expression profiles. After hyperoxia, histology showed simplification of alveoli with increased airspace and dilated alveoli (Fig. 3c, d) compared to the normoxia group (Fig. 3a, b) indicating hypoalveologenesis due to disturbed secondary septa formation. This could be associated with the vascular malformation seen in children with BPD [24, 25],

Responsivity to later hyperoxia at P15 is altered in mouse lungs that have been exposed to hyperoxia in the neonatal period

Exposure to hyperoxia later in life at P15 led to substantial differences in mRNA levels compared to mice previously exposed to hyperoxia during the neonatal period and those without. More specifically, there was less

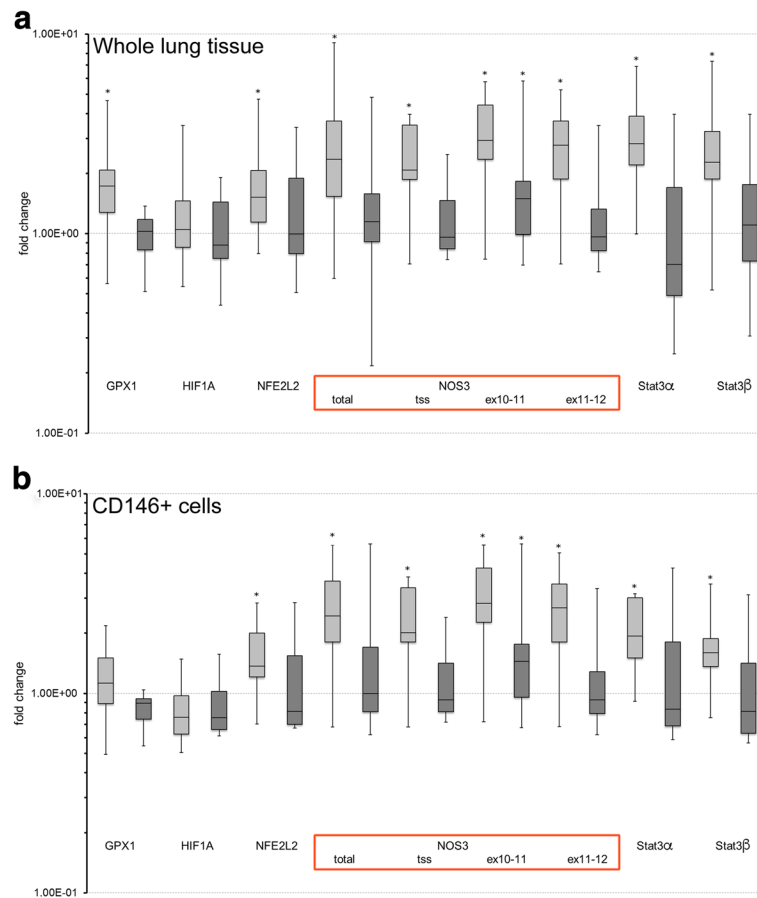


Fig. 2 Expression profile of whole lung tissue versus endothelial cells (CD 146+). The upper part shows expression patterns in whole lung tissue (a), the lower in CD146-positive endothelial cells (b). Light gray bars indicate mice immediately after hyperoxia (group 2), dark gray 8 days of normoxia after hyperoxia from P0 to P8 (group 3). Expression data is normalized to the control group 1. Every group contained five animals. Error bars indicated 95% CI. Asterisks indicate significant difference to the control group ($p < 0.05$)

increase in NOS3 mRNA levels (median fold change $\times 1.90$, IQR 0.92–2.72) in mice that were previously exposed to hyperoxia compared to mice exposed to normoxia (median fold change $\times 3.93$, IQR 2.50–8.51). Interestingly, the increase in STAT3 mRNA was similar in both groups—even though it tended to be higher in the hyperoxia (median 3.73, IQR 1.38–7.29) compared to the normoxia group (median 2.30, IQR 0.74–4.53)—when compared to the median fold change ($\times 2.83$, IQR 2.21–3.88) after the first exposure. With respect to the other genes investigated, such as NFE2L2 (Fig. 4c), GPX1, and HIF1A (data not shown), we found no significant differences between mice with and without previous hyperoxia exposure (Fig. 4). Since in human, a small 27 nt-RNA is apparently involved in the negative regulation of NOS3 mRNA via STAT3 mRNA targeting, we performed holistic analyses of microRNA (miR) profiles in purified lung epithelial cell obtained from experiments under normoxic (group 1), hyperoxic (groups 2/5), and after repetitive hyperoxic treatment

(group 6). Notably, an ortholog of human 27 nt-RNA could not be identified in the mouse genome. Therefore, we aimed to identify differentially expressed murine candidate miRs, which potentially could target STAT3 mRNA in a way reminiscent of human 27 nt-RNA and human STAT3 mRNA. In total, we identified 301 different miRs from which 101 were non-marginally expressed and thus were considered for differential analyses. Figure 4d shows the relative average enrichment of these 101 miRs and below the group-specific quantitative profiles as a heat map. Interestingly, almost general drop of miR abundance was observed after hyperoxic treatment (groups 2/5) when compared with normoxia (group 1), whereas the abundance of many miRs increases markedly, when a repetitive oxygen treatment was applied (group 6). Notably, we identified several miRs differentially overexpressed under these conditions, where strong evidence exists for STAT3 mRNA targeting in mice (Fig. 4e), i.e., mmu-let-7b-5p, mmu-miR-181a-5p, mmu-miR-93-5p, mmu-miR-17-5p, and mmu-miR125a-5p.

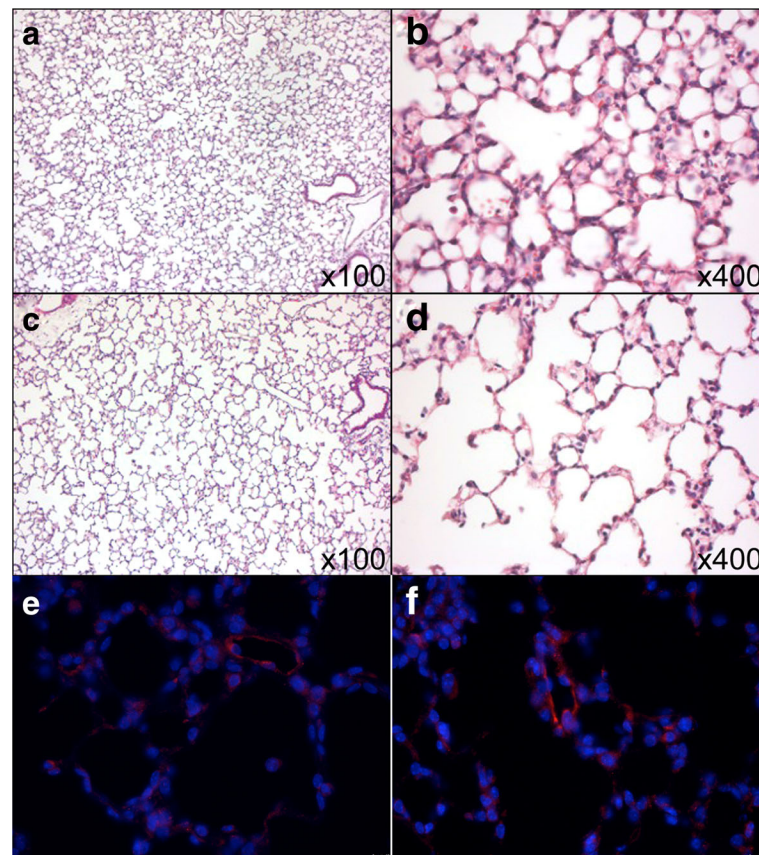


Fig. 3 Neonatal hyperoxia leads to hypoalveogenesis at P8. **a, b** The lungs of the normoxia group at P8 (group 1) show normal developed alveoli. **c, d** The lungs from the hyperoxia group (group 2) at P8 after exposure to 85% oxygen from P0 to P8 reveal a significant hypoalveogenesis with dilated alveoli and increased airspace macroscopically due to disturbed secondary septa formation. H&E. **e, f** Immunofluorescence staining on P8 comparing normoxia (**e**, group 1) versus hyperoxia (**f**, group 2) show higher expression after hyperoxia. **a, c** $\times 100$; **b-f** $\times 400$

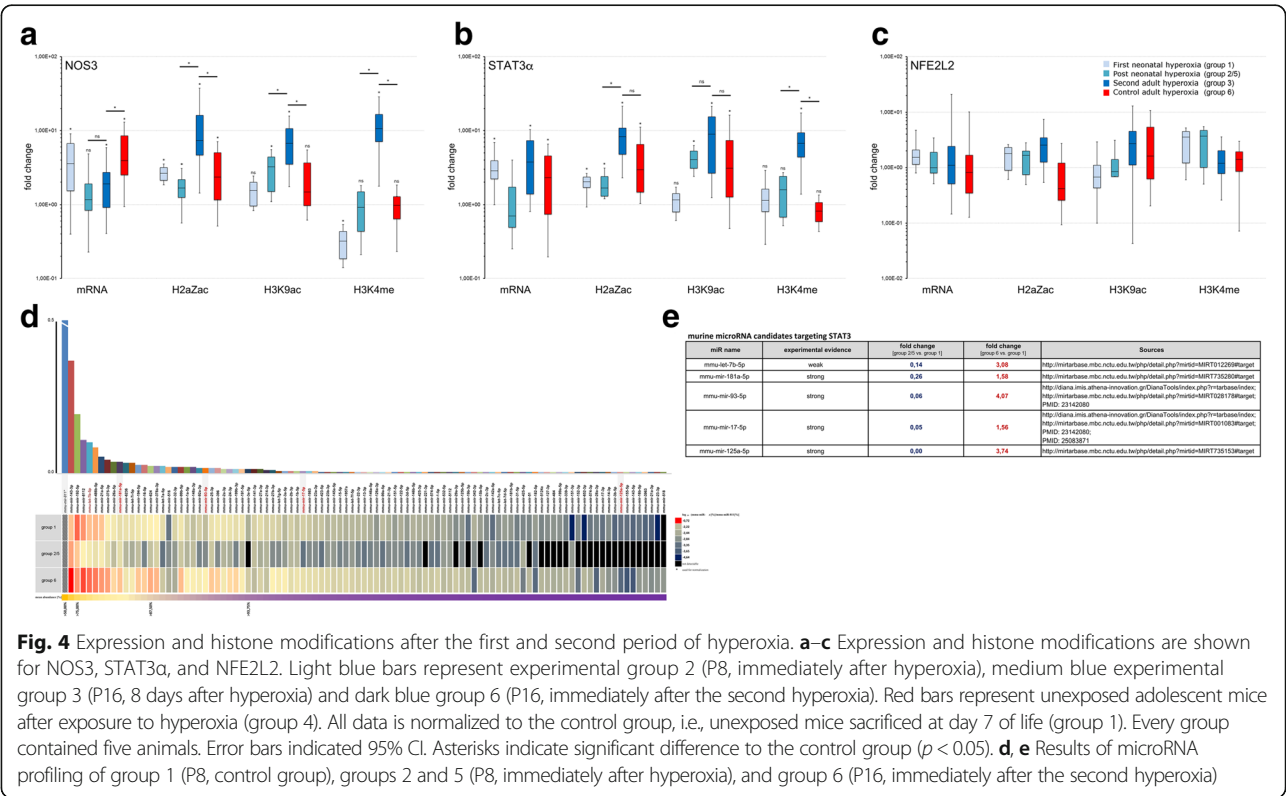
Modifications in histone signatures at the NOS3 and STAT3 gene locus in response to hyperoxia exposure are not restored

We then further analyzed in pulmonary vascular endothelial cells whether changes in histone and/or CpG signatures were associated with the observed expression patterns. Whereas we did not see any significant differences in CpG methylation (Table 1), histone modifications patterns at the NOS3 and STAT3 gene locus changed substantially. Upon initial exposure to hyperoxia, we noted an increase in H2aZac at both loci and an additional decrease in H3K4me3 at the NOS3 locus. No significant alterations were observed for H3K9ac. Importantly, levels for H2aZac did not return to a baseline level but remained elevated and levels for H3K9ac increased significantly after 8 days of normoxia at both loci (Fig. 4a, b). Interestingly, upon second exposure to hyperoxia, we observed an even further increase in H2aZac, H3K4me, and H3K9ac in mice with previous excessive oxygen exposure when compared to mice

raised in a normoxic environment, which showed an acetylation/methylation pattern very similar to the initial pattern observed upon the primary excessive oxygen exposure (Fig. 4a, b). At the NFE2L2 locus, no changes were noted (Fig. 4c).

Long-lasting disturbances in histone modification at the NOS3 gene locus lead to disruption of the regulation of eNOS expression upon physiological stimuli later on

To further analyze whether persistent histone modifications are primarily responsible for the altered eNOS response pattern of vascular endothelial cells upon physiological stimuli later on, we used human aortic endothelial cells (HAEC) which were exposed to trichostatin A (TSA). To mimic persistent environmental stimulation, we incubated HAEC with TSA for 72 h and compared these to unexposed HAEC and HAEC that were exposed twice for 6 h with an interval of 60 h in between to simulate a more physiological situation. Whereas persistent stimulation led to a change in



histone acetylation as for example at H3K9, this effect was much weaker in HAEC subjected to repetitive stimulation (Fig. 5). Importantly, we also noticed significant differences between both groups in eNOS and STAT3α expression. Whereas after repetitive stimulation eNOS (median 0.52, 0.4–0.67) and STAT3α (median 0.23, 0.2–0.29) dropped to levels below baseline measurement levels after 4 weeks (Fig. 6a, c), we noticed a substantial increase in baseline eNOS (median 27.2, 22.3–35.6) and STAT3α (median 5.8, 4.8–7.3) expression in HAEC initially subjected to long-term TSA treatment (Fig. 6b, d). Interestingly, the drop to baseline expression of eNOS and STAT3α in the group with repetitive

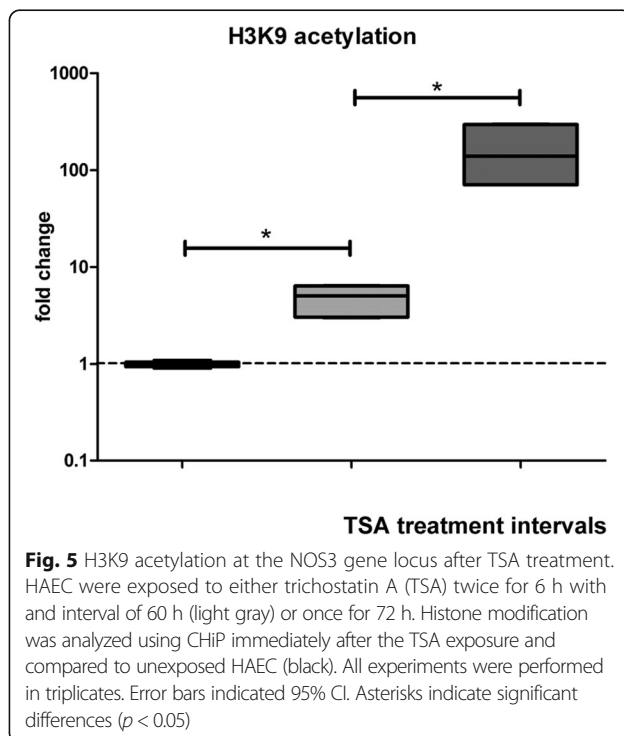
stimulation took significantly more time compared with the first stimulus.

In the last part of this study, we analyzed the response of the TSA-exposed HAEC to a physiological stimulus. Since in contrast to pulmonary vascular endothelial cells, eNOS expression in peripheral arterial endothelial cells is stimulated by hypoxia, HAEC—either exposed to two 6-h treatments with TSA or one 72-h TSA treatment—were exposed to hypoxia after cultivation for 3 weeks under normal growth conditions. As can be seen in Fig. 7, HAEC with a previous long-term exposure to TSA did show a pathological response to hypoxia with a significant reduction in eNOS expression whereas cells

Table 1 CpG methylation

	NANOG				NFE2L2						STAT3					
	CpG 1	CpG 2	CpG 3	CpG 4	CpG 1	CpG 2	CpG 3	CpG 4	CpG 5	CpG 6	CpG 1	CpG 2	CpG 3	CpG 4	CpG 5	CpG 6
Ctrl	5.00	10.00	11.50	13.00	3.50	1.00	1.50	3.00	1.50	1.50	2.50	2.00	5.50	1.50	1.50	0.50
Hyperoxia	5.00	11.00	12.00	12.00	3.00	1.00	1.00	2.00	1.00	1.00	3.00	1.00	3.00	1.00	1.00	1.00
P[Ctrl/hyperoxia]	0.43	0.47	0.44	0.43	0.14	0.68	0.43	0.09	0.29	0.05	0.48	0.12	0.35	0.86	0.93	0.53
Hyperoxia/5d release	8.00	13.00	12.00	16.00	3.00	1.00	1.00	3.00	1.00	1.00	3.00	2.00	5.00	1.00	1.00	2.00
P[Ctrl/hyperoxia-release]	0.80	0.56	0.48	0.71	0.39	1.00	0.79	0.44	0.93	0.28	0.75	0.11	0.89	0.64	0.93	0.18

The mean percentage of methylation at the respective CpGs located in the different promoter regions is shown



with a previous short-term repetitive exposure to TSA showed a response pattern very similar to control HAEC. This pathological response was accompanied by altered histone modifications at the NOS3 gene locus (Fig. 7d, e). Importantly, as previously mentioned, HAEC with previous long-term exposure to TSA showed persistence of H3K9ac at the NOS3 gene locus whereas in cells with short-term repetitive TSA treatment histone marks at the NOS3 gene locus were restored. Interestingly, we also noticed high levels of the repressive histone mark H3K27me3 which persisted during hypoxia.

Discussion

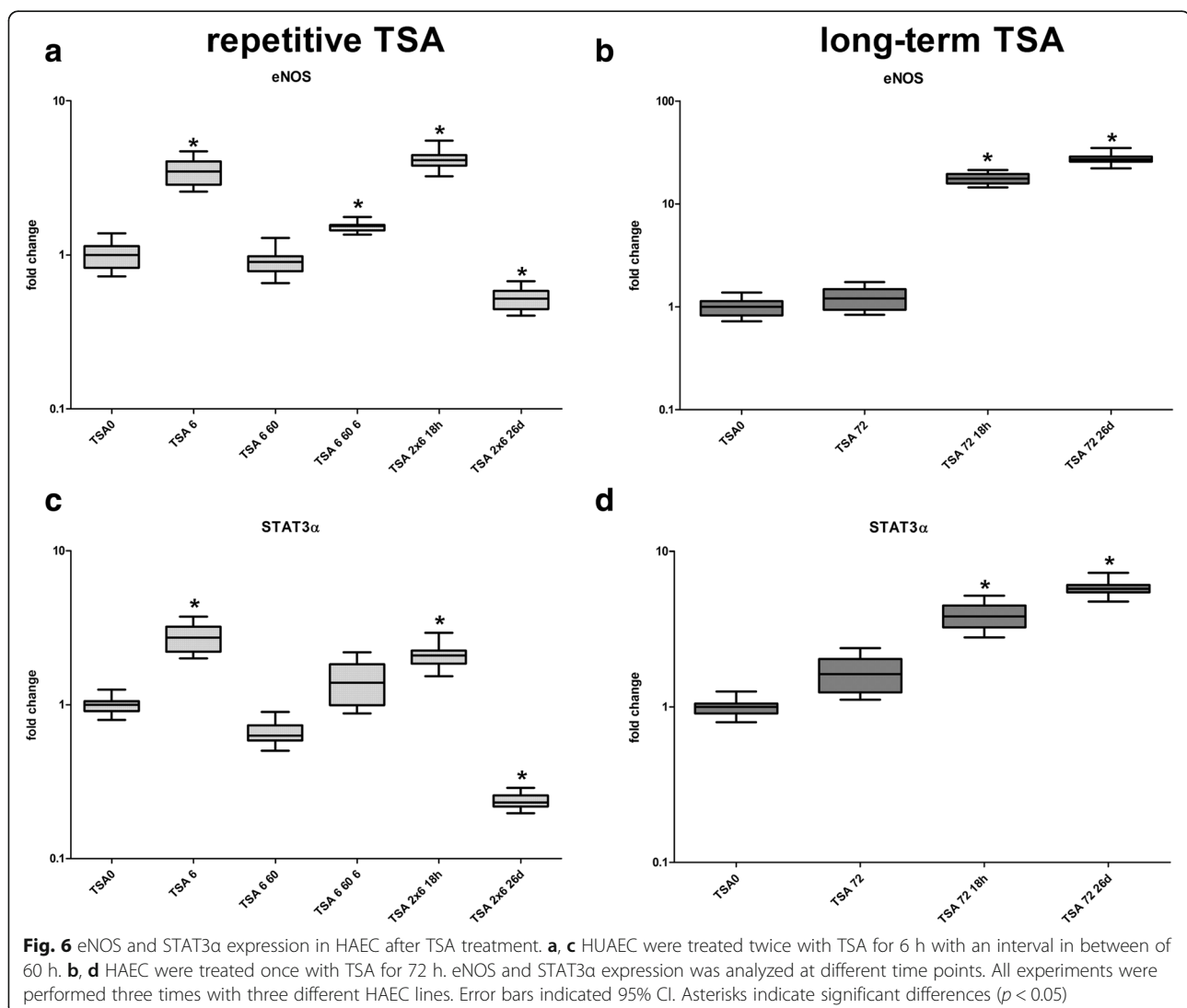
Bronchopulmonary dysplasia (BPD) remains one of the main problems of prematurity even in the post-surfactant area. Importantly, BPD is also the main reason for pulmonary hypertension in the pediatric population. Early oxygen exposure has been identified as one of the most important risk factors for the development of BPD. Even though the exact mechanism remains unclear, it seems to involve high oxygen exposure possibly by inducing excessive pulmonary vasodilatation and production of oxygen radicals followed by tissue damage. However, to date, no pathophysiological link has been identified between early oxygen exposure and later pathological response patterns of the pulmonary vasculature in pulmonary hypertension.

Here, we provide the first evidence that excessive oxygen exposure early in life leads to permanent changes in

the epigenetic signatures at the NOS3 locus in pulmonary endothelial artery cells in a mouse model. These changes seem to be associated with a non-physiological expression pattern of eNOS upon oxygen exposure later in life. Moreover, non-physiological alterations of epigenetic signatures at the NOS3 gene locus induce similar effects in human aortic endothelial cells (HAEC) in vitro.

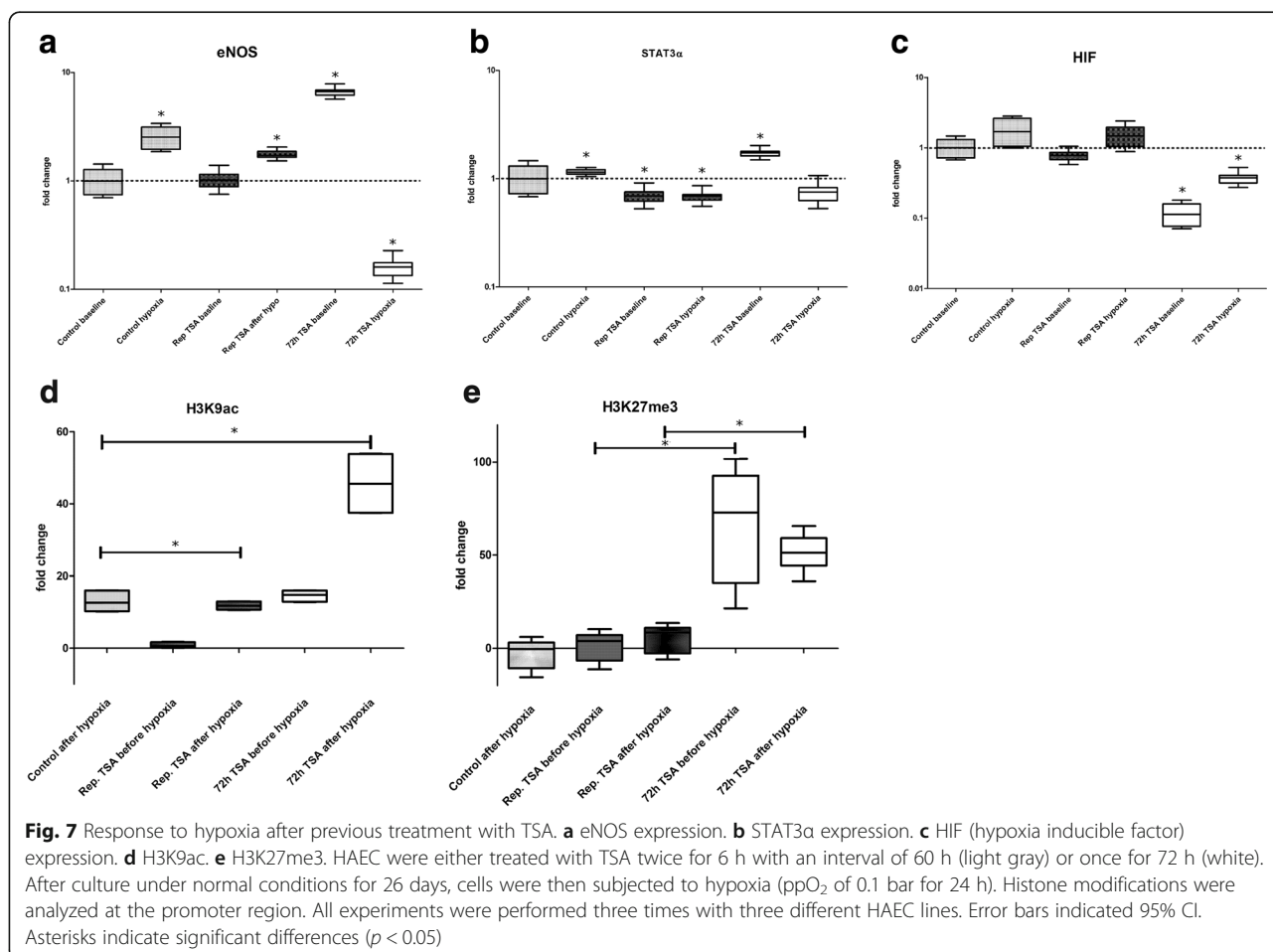
Specifically, in the first part of the study, we demonstrated that NOS3 mRNA levels increased in pulmonary artery cells upon neonatal oxygen exposure in newborn mouse. This was paralleled by hyperemia and later disruption of the alveolar structure as shown by histology. This is in line with previous reports demonstrating increased protein concentrations [7] and increased NOS3 mRNA levels upon early excessive oxygen exposure in various models [26] and human tissue samples [27]. Interestingly, we did not observe any changes in RNA expression of NFE2L2, a transcription factor that regulates the expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation. This fits to the previous observation that SOD3, an Nrf2-independent antioxidant, was not found to be upregulated as one might expect in the O₂-exposed neonatal mice compared with room air [28]. In fact, this possibly reflects the inefficiency of counteractive antioxidant mechanisms in the neonatal lung.

The observed increase of NOS3 mRNA levels was paralleled by an increase in H3K9 and H2Az acetylation very similar to our previous observations in HUAEC [18]. Importantly, we did not observe a restitution of acetylation patterns at the NOS3 gene locus after 5 days of normoxia. Moreover, upon a second exposure to hyperoxia later in life, we noticed a dramatic increase in H3K9 and H2Az acetylation and also H3K4 methylation in mice that have been exposed to neonatal hyperoxia. However, NOS3 mRNA levels did not increase but were even found to be substantial lower in these mice when compared to healthy controls. Obviously, the substantial increase in activating histone marks at the NOS3 gene locus did not lead to an increase in transcription in these mice. This is in line with a previous report demonstrating a suppressive effect on NOS3 mRNA synthesis after 12 h incubation with TSA [29]. As we have previously shown the most reasonable explanation for this phenomenon is an increased activity of counteracting processes mainly due to a negative feedback loop, which in humans presumably involves a co-processed 27 nt-ncRNA encoded in NOS3 intron 5 [16, 30], which targets and suppresses STAT3 mRNA [31]. Our analyses allow us to speculate that a negative regulatory function in mice, where a STAT3-targeting 27 nt-RNA was not identified, could be fulfilled by one or several of the differentially expressed miRs, which we identified by microRNA expression profiling.



Moreover, we also observed increased levels of H3K27me3—a repressive histone mark—which might function as an additional counteracting process responsible for the absent increase in NOS3 mRNA levels. Considering a recent report demonstrating eNOS uncoupling and decreased NO production with an enhanced superoxide production in adult rats after neonatal hyperoxia [32], the observed downregulation of NOS3 mRNA production in our study might be a protective mechanism downregulating eNOS expression upon oxygen exposure to prevent further superoxide production and tissue damage. Overall, the observed changes in epigenetic signatures are in line with recent reports such as the study by Zhu et al. who found that H3K27 trimethylation is present in a BPD mouse model and involved in RUNX3 downregulation, a gene associated with pulmonary development [33].

In the second part of the study, we provide further evidence for the relevance of the observed histone modifications for the regulation of NOS3 expression using an in vitro cell culture model. For correct interpretation of the data, it is important to mention that the physiological response pattern of human peripheral arterial endothelial cells is opposite to the response pattern of pulmonary endothelial artery cells, i.e., vasodilatation and NOS3 upregulation upon hypoxia and not hyperoxia. To induce a response pattern comparable to the mouse model upon hyperoxia, we therefore subjected HAEC to hypoxia after exposure to TSA. Similar to the mouse model, we observed long persistence of altered acetylation patterns after long-term exposure to TSA whereas histone signatures were restored after repetitive short-term exposure. In HAEC with long-term exposure to TSA, NOS3 mRNA levels remained high corresponding to an increase H3K9ac at the NOS3 gene locus.



However, upon exposure to hypoxia, we observed a substantial decrease in NOS3 mRNA levels in these cells even though levels of H3K9ac further increased. Currently, similar to the constant increase in H3K27me3 at the NOS3 gene locus in these cells, we have no good explanation for this phenomenon. Possibly, this just reflects a complete disturbance and malfunction of the epigenetic code, but this needs to be further elucidated.

One major limitation of our study is the lack of functional studies such as measurement of NO. Since many previous studies have already demonstrated that upregulation of NOS3 corresponds very well with increased NO production in the hyperoxia mouse model [7], we believe that mRNA levels are sufficient physiological to characterize the cellular response patterns. In addition, even if that is not the case, it does not change the disturbances observed on the level of mRNA expression and in epigenetic signatures. The second major limitation of this study is the lack of further functional analysis on the role of miRNA on the development of BPD. However, since this seems to be rather complex [34] and considering the focused rather than generalistic approach of

this study, this would have been beyond the primary scope of this study.

Conclusions

This study provides further evidence for the long-term relevance of early life events on changes in epigenetic signatures using a newborn mouse model of hyperoxia-induced BPD. It demonstrates the occurrence and relevance of persisting epigenetic marks for regulation of vascular endothelial response patterns upon physiological stimuli later in life. Even though currently very theoretical, this suggests that treatment of BPD and its sequelae by modification of epigenetic mechanisms might at least be an option.

Abbreviations

BPD: Bronchopulmonary dysplasia; BW: Birth weight; eNOS: Endothelial nitric oxide synthase; GA: Gestational age; HAEC: Human arterial endothelial cell; HDAC: Histone deacetylase; HOX: Hyperoxia; NO: Nitric oxide; NOX: Normoxia; PCR: Polymerase chain reaction; TSA: Trichostatin A

Acknowledgements

We would like to sincerely thank Claudia Förster and Prof. Klaus-Peter Zimmer for their constant support.

Funding

Rhea van den Bruck received an internal research grant from the University of Witten/Herdecke.

Availability of data and materials

Not applicable.

Authors' contributions

AJ, CC, SM, JM, LK, PW, MA, and RB have performed data acquisition and analyzed the data. AJ and CC wrote the manuscript. AJ, CC, and JP took part in the main study design and supervision of the study. SB took part in the supervision of the study. SB and JP critically reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval

All animal experiments were performed in accordance with the National Institutes of Health Guidelines for the Use of Laboratory Animals. Animal experiments were approved by the Federal Authorities for Animal Research of the Regierungspräsidium Giessen, Hessen, Germany, protocol 105/2011. This study was conducted with the approval of the Witten/Herdecke University Ethics board.

Competing interests

All authors declare that they have no competing interests.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details

¹Excellence Cluster Cardio-Pulmonary System (ECCPS), Member of the German Center for Lung Research (DZL), Department of Internal Medicine II, Universities of Giessen and Marburg Lung Center (UGMLC), Aulweg 130, 35392 Giessen, Germany. ²University Children's Hospital Gießen, Division of General Pediatrics and Neonatology, Justus-Liebig-University, Gießen, Germany. ³Department of Pediatrics, HELIOS Medical Center Wuppertal, Center for Clinical & Translational Research (CCTR), Center for Biomedical Education & Research (ZBAF), Witten/Herdecke University, Wuppertal, Germany. ⁴EKO Children's Hospital, Oberhausen, Witten/Herdecke University, Alfred-Herrhausen Str. 40, Witten, Germany.

Received: 31 July 2017 Accepted: 9 March 2018

Published online: 20 March 2018

References

- Northway WH Jr, Rosan RC, Porter DY. Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. *N Engl J Med*. 1967;276(7):357–68.
- Stoll BJ, Hansen NI, Bell EF, Shankaran S, Laptook AR, Walsh MC, Hale EC, Newman NS, Schibler K, Carlo WA, et al. Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. *Pediatrics*. 2010;126(3):443–56.
- Baraldi E, Filippone M. Chronic lung disease after premature birth. *N Engl J Med*. 2007;357(19):1946–55.
- Bhandari A, Bhandari V. Pitfalls, problems, and progress in bronchopulmonary dysplasia. *Pediatrics*. 2009;123(6):1562–73.
- Shaw GM, O'Brodovich HM. Progress in understanding the genetics of bronchopulmonary dysplasia. *Semin Perinatol*. 2013;37(2):85–93.
- Vento M, Moro M, Escrig R, Arruza L, Villar G, Izquierdo I, Roberts LJ 2nd, Arduini A, Escobar JJ, Sastre J, et al. Preterm resuscitation with low oxygen causes less oxidative stress, inflammation, and chronic lung disease. *Pediatrics*. 2009;124(3):e439–49.
- Potter CF, Kuo NT, Farver CF, McMahon JT, Chang CH, Agani FH, Haxhiu MA, Martin RJ. Effects of hyperoxia on nitric oxide synthase expression, nitric oxide activity, and lung injury in rat pups. *Pediatr Res*. 1999;45(1):8–13.
- Kondrikov D, Elms S, Fulton D, Su Y. eNOS-beta-actin interaction contributes to increased peroxynitrite formation during hyperoxia in pulmonary artery endothelial cells and mouse lungs. *J Biol Chem*. 2010;285(46):35479–87.
- Simpson SJ, Hall GL, Wilson AC. Lung function following very preterm birth in the era of 'new' bronchopulmonary dysplasia. *Respirology*. 2015;20(4):535–40.
- Mourani PM, Sontag MK, Younoszai A, Miller JL, Kinsella JP, Baker CD, Poindexter BB, Ingram DA, Abman SH. Early pulmonary vascular disease in preterm infants at risk for bronchopulmonary dysplasia. *Am J Respir Crit Care Med*. 2015;191(1):87–95.
- O'Reilly MA, Yee M, Buczynski BW, Vitiello PF, Keng PC, Welle SL, Finkelstein JN, Dean DA, Lawrence BP. Neonatal oxygen increases sensitivity to influenza A virus infection in adult mice by suppressing epithelial expression of Ear1. *Am J Pathol*. 2012;181(2):441–51.
- Dommm W, Misra RS, O'Reilly MA. Affect of early life oxygen exposure on proper lung development and response to respiratory viral infections. *Front Med*. 2015;2:55.
- Bhandari V, Bizzarro MJ, Shetty A, Zhong X, Page GP, Zhang H, Ment LR, Gruen JR, Neonatal Genetics Study G. Familial and genetic susceptibility to major neonatal morbidities in preterm twins. *Pediatrics*. 2006;117(6):1901–6.
- Londhe VA, Sundar IK, Lopez B, Maisonet TM, Yu Y, Aghai ZH, Rahman I. Hyperoxia impairs alveolar formation and induces senescence through decreased histone deacetylase activity and up-regulation of p21 in neonatal mouse lung. *Pediatr Res*. 2011;69(5 Pt 1):371–7.
- Ni W, Lin N, He H, Zhu J, Zhang Y. Lipopolysaccharide induces up-regulation of TGF-alpha through HDAC2 in a rat model of bronchopulmonary dysplasia. *PLoS One*. 2014;9(3):e91083.
- Postberg J, Kanders M, Forcob S, Willems R, Orth V, Hensel KO, Weil PP, Wirth S, Jenke AC. CpG signalling, H2A.Z/H3 acetylation and microRNA-mediated deferred self-attenuation orchestrate foetal NOS3 expression. *Clin Epigenetics*. 2015;7(1):9.
- Jenke AC, Postberg J, Raine T, Nayak KM, Molitor M, Wirth S, Kaser A, Parkes M, Heuschkel RB, Orth V, et al. DNA methylation analysis in the intestinal epithelium-effect of cell separation on gene expression and methylation profile. *PLoS One*. 2013;8(2):e55636.
- Postberg J, Kanders M, Forcob S, Willems R, Orth V, Hensel KO, Weil PP, Wirth S, Jenke AC. CpG signalling, H2A.Z/H3 acetylation and microRNA-mediated deferred self-attenuation orchestrate foetal NOS3 expression. *Clin Epigenetics*. 2015;7:9.
- Weil PP, Jaszczyszyn Y, Baroin-Tourancheau A, Postberg J, Amar L. Holistic and affordable analyses of MicroRNA expression profiles using tagged cDNA libraries and a multiplex sequencing strategy. *Methods Mol Biol*. 2017;1654:179–96.
- Blankenberg D, Von Kuster G, Coraor N, Ananda G, Lazarus R, Mangan M, Nekrutenko A, Taylor J. Galaxy: a web-based genome analysis tool for experimentalists. *Curr Protoc Mol Biol*. 2010;Chapter 19(Unit 19 10):11–21.
- Giardine B, Riemer C, Hardison RC, Burhans R, Eltnitski L, Shah P, Zhang Y, Blankenberg D, Albert I, Taylor J, et al. Galaxy: a platform for interactive large-scale genome analysis. *Genome Res*. 2005;15(10):1451–5.
- Goecks J, Nekrutenko A, Taylor J, Galaxy T. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biol*. 2010;11(8):R86.
- Vitsios DM, Enright AJ. Chimira: analysis of small RNA sequencing data and microRNA modifications. *Bioinformatics*. 2015;31(20):3365–7.
- Thebaud B. Angiogenesis in lung development, injury and repair: implications for chronic lung disease of prematurity. *Neonatology*. 2007;91(4):291–7.
- Thebaud B, Abman SH. Bronchopulmonary dysplasia: where have all the vessels gone? Roles of angiogenic growth factors in chronic lung disease. *Am J Respir Crit Care Med*. 2007;175(10):978–85.
- Feng X, Guo Z, Nourbakhsh M, Hauser H, Ganster R, Shao L, Geller DA. Identification of a negative response element in the human inducible nitric-oxide synthase (hNOS) promoter: the role of NF-kappa B-repressing factor (NRF) in basal repression of the hNOS gene. *Proc Natl Acad Sci U S A*. 2002;99(22):14212–7.
- Davis CW, Gonzales LW, Ballard RA, Ballard PL, Guo C, Gow AJ. Expression of nitric oxide synthases and endogenous NO metabolism in bronchopulmonary dysplasia. *Pediatr Pulmonol*. 2008;43(7):703–9.
- Poonyagariyagorn HK, Metzger S, Dikeman D, Mercado AL, Malinina A, Calvi C, McGrath-Morrow S, Neptune ER. Superoxide dismutase 3 dysregulation in a murine model of neonatal lung injury. *Am J Respir Cell Mol Biol*. 2014;51(3):380–90.
- Rossig L, Li H, Fisslthaler B, Urbich C, Fleming I, Forstermann U, Zeiher AM, Dimmeler S. Inhibitors of histone deacetylation downregulate the expression of endothelial nitric oxide synthase and compromise endothelial cell function in vasorelaxation and angiogenesis. *Circ Res*. 2002;91(9):837–44.
- Zhang MX, Ou H, Shen YH, Wang J, Wang J, Coselli J, Wang XL. Regulation of endothelial nitric oxide synthase by small RNA. *Proc Natl Acad Sci U S A*. 2005;102(47):16967–72.
- Yan L, Hao H, Elton TS, Liu Z, Ou H. Intronic microRNA suppresses endothelial nitric oxide synthase expression and endothelial cell proliferation via inhibition of STAT3 signaling. *Mol Cell Biochem*. 2011;357(1–2):9–19.

32. Zyzorczyk C, Comte B, Huyard F, Cloutier A, Germain N, Bertagnolli M, Nuyt AM. Developmental programming of eNOS uncoupling and enhanced vascular oxidative stress in adult rats after transient neonatal oxygen exposure. *J Cardiovasc Pharmacol*. 2013;61(1):8–16.
33. Zhu Y, Fu J, Yang H, Pan Y, Yao L, Xue X. Hyperoxia-induced methylation decreases RUNX3 in a newborn rat model of bronchopulmonary dysplasia. *Respir Res*. 2015;16:75.
34. Nardiello C, Morty RE. MicroRNA in late lung development and bronchopulmonary dysplasia: the need to demonstrate causality. *Mol Cell pediatr*. 2016;3(1):19.

Submit your next manuscript to BioMed Central and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at
www.biomedcentral.com/submit

