Alveolar cell fate selection and lifelong maintenance of AT2 cells by FGF signaling

The lung’s gas exchange surface is comprised of alveolar AT1 and AT2 cells that are corrupted in several common and deadly diseases. They arise from a bipotent progenitor whose differentiation is thought to be dictated by differential mechanical forces. Here we show the critical determinant is FGF signaling. Fgfr2 is expressed in the developing progenitors in mouse then restricts to nascent AT2 cells and remains on throughout life. Its ligands are expressed in surrounding mesenchyme and can, in the absence of exogenous mechanical cues, induce progenitors to form alveolospheres with intermingled AT2 and AT1 cells. FGF signaling directly and cell autonomously specifies AT2 fate; progenitors lacking Fgfr2 in vitro and in vivo exclusively acquire AT1 fate. Fgfr2 loss in AT2 cells perinatally results in reprogramming to AT1 identity, whereas loss or inhibition later in life triggers AT2 apoptosis and compensatory regeneration. We propose that Fgfr2 signaling selects AT2 fate during development, induces a cell non-autonomous AT1 differentiation signal, then continuously maintains AT2 identity and survival throughout life.

Gas exchange occurs in alveoli, tiny terminal air sacs of the lung lined by two intermingled epithelial cell types: exquisitely thin alveolar type 1 (AT1) cells that provide the gas-exchange surface, and cuboidal AT2 cells that secrete surfactant to prevent alveolar collapse. Alveoli are also the site of some of the most significant but poorly understood and difficult-to-treat human diseases. These include bronchopulmonary dysplasia in infants, COPD/emphysema, pulmonary fibrosis, adenocarcinoma, and infections including severe acute respiratory syndrome (SARS) and COVID-19 in adults. Understanding how these key alveolar cell types arise during development and are maintained throughout life is critical for understanding and treating these diseases, and for guiding developmental, regenerative and tissue engineering approaches to create healthy alveoli and the surfactant that lines them.

Several lines of evidence, including marker expression, lineage tracing, and clonal analysis in mice, indicate that AT1 and AT2 cells arise directly from a common progenitor during embryonic development. This model is supported by single-cell RNA sequencing studies of developmental intermediates that reconstructed the full, bifurcating gene expression program from bipotent progenitors at embryonic day 16.5 (e16.5) to either AT1 or AT2 cells over the next several days of fetal development, although a recent genetic labeling study claims an earlier fate commitment. Multiple developmental signaling pathways and transcription factors can influence alveolar structure, maturation, or the balance of the two cell types, but the key driver of alveolar fate selection and differentiation is thought to be mechanical forces. The build-up and increased movement of luminal fluid in late gestation is proposed to stretch and flatten progenitors into AT1 cells, as stretch does to cultured AT2 cells to cause AT1 transdifferentiation in vitro. Live imaging indicates that alveolar progenitors protected from luminal mechanical forces during budding default to the AT2 cell program.
Here we demonstrate that alveolar cell fate specification is, in fact, dictated by a classic growth factor signal, the same FGF signaling pathway that controls the earlier steps of airway budding and branching\textsuperscript{22-27,30} and also initiates subsequent budding\textsuperscript{31,32}. After branching, the receptor Fgfr2 remains on in alveolar progenitors and dynamically restricts to the AT2 lineage shortly before cellular differentiation, while its ligands Fgf7 and Fgf10 continue to be expressed by surrounding stromal cells. We show by the effects of the ligands on isolated e16.5 progenitors in cultures lacking budding and differential surrounding stromal cells. We show by the effects of the ligands on branching, the receptor Fgfr2 remains on in alveolar progenitors and cells\textsuperscript{31,32}, which, if deprived of the FGF signal early in life, reprogram to where it serves continuously and ubiquitously to maintain healthy AT2 cells\textsuperscript{33,34}, which is known consequence (Fzd8), only adult lung phenotypes (Cd36 and Cd74), or have not yet been examined (Ngfrap1). Fgfr2 is expressed early and throughout the developing lung epithelium and its deletion abrogates airway branching\textsuperscript{22,29,30}, so its later function has been more challenging to investigate\textsuperscript{4,31-33}. The single-cell RNA sequencing (scRNAseq) profiles demonstrated that the iiib isoform of Fgfr2 is expressed in bipotent progenitors and maintained in the AT2 lineage but downregulated in the AT1 lineage (Fig. 1a and Supplementary Fig. 1c). Fgfr2 immunostaining during mouse alveolar development confirmed that Fgfr2 is expressed in bipotent progenitors (Fig. 1c), downregulated in nascent AT1 cells, and maintained in nascent AT2 cells as they bud into the surrounding mesenchyme and become histologically and molecularly distinct from AT1 cells (Fig. 1c and Supplementary Fig. 1a). Indeed, Fgfr2 is among the earliest known markers of AT2 fate selection.

**Results**

Fgfr2 restricts to the AT2 lineage during alveolar differentiation

To identify signaling pathways that might control alveolar cell fate selection, we searched the single-cell transcriptional program of mouse alveolar development\textsuperscript{4} for receptor genes enriched in either the AT1 or AT2 lineages. Of the five receptor genes showing at least twofold enrichment in the AT2 lineage (Fig. 1a; Fgfr2, Fzd8, Cd36, Cd74, and Ngfrap1), only Fgfr2 is known to be required for embryonic lung development\textsuperscript{22,27}. The other four either have no known consequence (Fzd8), only adult lung phenotypes (Cd36 and Cd74), or have not yet been examined (Ngfrap1). Fgfr2 is expressed early and throughout the developing lung epithelium and its deletion abrogates airway branching\textsuperscript{22,29,30}, so its later function has been more challenging to investigate\textsuperscript{4,31-33}. The single-cell RNA sequencing (scRNAseq) profiles demonstrated that the iiib isoform of Fgfr2 is expressed in bipotent progenitors and maintained in the AT2 lineage but downregulated in the AT1 lineage (Fig. 1a and Supplementary Fig. 1c). Fgfr2 immunostaining during mouse alveolar development confirmed that Fgfr2 is expressed in bipotent progenitors (Fig. 1c), downregulated in nascent AT1 cells, and maintained in nascent AT2 cells as they bud into the surrounding mesenchyme and become histologically and molecularly distinct from AT1 cells (Fig. 1c and Supplementary Fig. 1a). Indeed, Fgfr2 is among the earliest known markers of AT2 fate selection.

To identify the relevant Fgfr2 ligands, we performed scRNAseq on the adjacent mesenchymal cells during alveolar differentiation. Two Fgfr2 (isoform iiib) ligands, Fgf7 and Fgf10, were detected in mesenchymal cells (Fig. 1b), predominantly in the previously identified major subpopulation (“matrix fibroblast”) marked by Wnt2 expression (Supplementary Fig. 2a)\textsuperscript{10}. Both Fgf genes are also expressed in lung mesenchyme earlier in development, when Fgf10 serves as the key ligand in Fgfr2-induced branching of the bronchial tree\textsuperscript{22,27}. Visualization of Fgfr2 ligands with the Fgfr2 (isoform iiib) ligand-binding domain fused to the human IgG1 domain showed ligand production in a subset of mesenchymal cells near budding AT2 cells (Fig. 1d, e and Supplementary Fig. 1b).

**Fig. 1 | Expression of Fgfr2 and its ligands during alveolar differentiation.**

a Expression of Fgfr2 and the four other receptor genes most selectively expressed in alveolar type 2 (AT2) cell lineage (“Receptors”) from single-cell RNA sequencing (scRNAseq) analysis of distal (alveolar) epithelial cells from embryonic day 18.5 (e18.5) mouse lung. Cells (columns) are arranged in developmental pseudotime (BP, bipotent progenitors, center; AT1 lineage to the left; AT2 lineage to the right) determined as described in ref. 11 by expression of alveolar lineage markers like the biological triplicate. pre-AT1, nascent AT1 cells that downregulated Fgfr2. All experiments were repeated at least three times. b Schematic showing inferred signaling from Fgf ligand-expressing mesenchymal cell (fibroblast) to nearby Fgfr2-expressing nascent AT2 cell during alveologenesis. pre-AT1, nascent AT1 cells that downregulated Fgfr2. All experiments were repeated at least three times.
Fgf7 and the Fgfr inhibitor FIIN-1 (bottom panel), cells remained bipotent.

Fig. 2 | Effect of Fgfr2 ligands on purified alveolar epithelial progenitors in culture. Phase images (left panels) and immunostains (right) of epithelial progenitors purified from tips of e16.5 lungs and cultured in Matrigel for the period indicated with media alone (Control, top panels) or media supplemented every two days with Fgf7 (50 ng/ml, middle panels) or Fgf10 (100 ng/ml) and HSPG (100 ng/ml, lower panels). Note an increase in luminal surface area (dashed lines) and basal extrusion (arrowheads) in cultures with Fgf ligands, and differentiation into intermingled cuboidal AT2 (Sftpc+ red) and squamous AT1 cells (RAGE+ green) at day 4 (“alveolospheres”). In control cultures lacking FGFs (top row) or treated both with Fgf7 and the Fgfr inhibitor FIIN-1 (bottom panel), cells remained bipotent progenitors throughout, as shown by co-expression of both markers. nAT1, nascent AT1 cell; nAT2, nascent AT2 cell. Bars, 50 µm (left panels and right +Fgf7 panel), 10 µm (right top and +Fgf10 panels), and 10 µm (Fgf7 + FIIN-1 panel). Co-treatment with both Fgf7 and Fgf10 (with HSPG) gave similar results as each treatment alone. Quantification at right gives the percent of each cell type (BP), AT1, AT2) in the culture at day 4 determined by immunostaining (n = 348 cells scored for control, 843 for Fgf7-treatment, and 1365 for Fgf10-treatment in experimental triplicate). **p = 7.7 x 10^-8 for Fgf7-treatment and 6.4 x 10^-8 for Fgf10-treatment (Student’s two-sided t-test), BP abundance of either Fgf treatment condition vs control.

Fgf2 ligands induce alveolar differentiation in culture

To explore the function of Fgfr2 signaling in alveolar development, we first examined the effect of Fgf7 and Fgf10 on purified epithelial progenitors isolated from the tips of e16.5 lungs, before AT1 and AT2 cells are detected. When the progenitors were cultured for up to 8 days in Matrigel in the absence of exogenous FGFs, they failed to develop into either AT1 or AT2 cells, remaining cuboidal and organizing into multicellular clusters around a lumen (Fig. 2 and Supplementary Fig. 3c). By contrast, in the presence of Fgf7 (50 ng/ml), the progenitors organized into large epithelial spheres containing differentiated cuboidal Sftpc+ RAGE+ AT2 cells intermingled with squamous Sftpc-RAGE- Fdpn- AT1 cells, similar to the structures formed during alveologenesis in vivo beginning at ~e17.5 (Fig. 2 and Supplementary Fig. 3h). The Arp2/3 inhibitor CK666, used to block protrusions and AT2 development in vivo, reduced the transient buds but not Fgf7-induced AT1 and AT2 development in culture (Supplementary Fig. 3i). Thus, Fgfr2 signaling drives differentiation of alveolar progenitors and formation and growth of alveolus-like structures (“alveolospheres”) with intermingled AT1 and AT2 cells, and it does so independent of extrinsic mechanical forces and without the early budding and associated structural features proposed to differentially protect progenitors from such forces. However, it is possible that extrinsic mechanical forces and budding in vivo might regulate some aspect of Fgf signaling or the developmental process that is dispensable in the culture system.

Fgfr2 signaling directly selects AT2 cell fate

The addition of FGF ligands to the cultures triggered the differentiation of progenitors into both AT1 and AT2 cells. To distinguish direct (cell autonomous) effects of FGF from secondary (cell non-autonomous) effects, we devised a strategy to enforce or block Fgfr2 signaling in random subsets of cultured progenitors targeted by a lentiviral vector. The vectors expressed either wild-type Fgfr2 (Lenti-Fgfr2) or a dominant negative form lacking the cytoplasmic domain (Lenti-Fgfr2DN) along with a fluorescent reporter (GFP) to mark infected cells (Fig. 3a). We found that nearly every progenitor cell (95%) infected with virus expressing wild-type Fgfr2 differentiated into an AT2 cell, and conversely infected cells expressing Fgfr2DN almost always (88%) showed no evidence of AT2 cell fate (Supplementary Fig. 3g). The Arp2/3 inhibitor CK666, used to block protrusions and AT2 development in vivo, reduced the transient buds but not Fgf7-induced AT1 and AT2 development in culture (Supplementary Fig. 3i). Thus, Fgfr2 signaling drives differentiation of alveolar progenitors and formation and growth of alveolus-like structures (“alveolospheres”) with intermingled AT1 and AT2 cells, and it does so independent of extrinsic mechanical forces and without the early budding and associated structural features proposed to differentially protect progenitors from such forces. However, it is possible that extrinsic mechanical forces and budding in vivo might regulate some aspect of Fgf signaling or the developmental process that is dispensable in the culture system.
acquired AT1 fate (Fig. 3b, c); the rare cells that did not acquire the predominant fate remained undifferentiated (Fig. 3c and Supplementary Fig. 4a). Experiments with a control virus (AAV-nGFP) that expresses GFP but no Fgfr showed that randomly-infected progenitors have a roughly equal chance of acquiring AT1 or AT2 fate during culture (Supplementary Fig. 4b). These results, together with the above result showing e16.5 distal progenitors cultured for over a week in the absence of FGF fail to differentiate into AT1 or AT2 cells (Fig. 2 and Supplementary Fig. 4c), indicate that the fate of alveolar progenitors is determined by Fgfr2 signaling. The results also imply that the observed induction of AT1 fate by FGF addition to the cultures must be indirect (cell non-autonomous), presumably via a secondary signal produced by maturing AT2 cells and received by neighboring progenitors (see Discussion). AT1 differentiation cannot simply be the default fate because it, too, requires the addition of FGF ligands to the culture.

To determine if Fgfr2 signaling plays a similar role in alveolar cell fate selection in vivo, we used an Nkx2.1-Cre transgene with low Cre activity to mosaically delete Fgfr2 in the developing lung epithelium, combined with a fluorescent Cre reporter allele (Rosa26mTmG) to mark the cells in which Cre was active (Fig. 4 and Supplementary Fig. 4c). In control mice carrying a wild-type Fgfr2 allele in trans to the conditional Fgfr2 allele (Tg^[Nkx2.1Cre;Fgfr2^+/Rosa26mTmG]), one-third of distal cells with Cre reporter activity (36%, n = 970 GFP + cells scored in three mice) acquired AT1 fate by the end of fetal life (PN0) and the rest acquired AT2 fate (64%) (Fig. 4a, b). By contrast, in animals with an Fgf2 deletion in trans to the conditional allele (Tg^[Nkx2.1Cre;Fgfr2^+/Rosa26mTmG]), the targeted distal cells almost exclusively (91%, n = 460 GFP + cells scored in three mice) acquired AT1 fate (Fig. 4a, b); the few cells that did not acquire AT1 fate had not yet lost Fgfr2 expression (Fig. 4c). We conclude that Fgfr2 is the critical determinant of AT2 fate and is required cell autonomously for AT2 fate selection in vivo as well as in vitro, and that alveolar progenitors lacking Fgfr2 signaling acquire the alternative, AT1 fate.

Fgfr2 signaling prevents AT2 reprogramming to AT1 fate in juvenile life

Curiously, this developmental signaling pathway remains on later in life, for months or even years, after alveoli have acquired their canonical structure and function. Fgfr2 continues to be selectively expressed in AT2 cells (Fig. 5a, b, d and Supplementary Fig. 5), Fgf7 and Fgf10 continue to be expressed in Wnt2-expressing alveolar fibroblasts and at lower levels in myofibroblasts (Fig. 5e–i and Supplementary Fig. 2b, c), and the Fgfr2 signaling pathway remains active in mature AT2 cells as detected by phosphorylated MAP kinase (Fig. 5c, d) and expression of Fgfr2-induced genes such as Spry2 (Fig. 5a). To investigate the role of this selective and persistent Fgfr2 signaling in AT2 cells, we first used a Cre knock-in allele at the endogenous LysozymeM (LyzM, also known as Lyz2) locus, which turns on in some AT2 cells early in postnatal life...
and is progressively activated later in other AT2 cells (see below), to conditionally delete Fgfr2 in AT2 cells at different ages. There was a dramatic effect of Fgfr2 deletion from AT2 cells during juvenile life and an equally significant but entirely different effect in adults.

LyzM-Cre activity is first detectable in rare AT2 cells at P5, as shown by the Cre reporter (Fig. 5j), and then gradually becomes active in additional AT2 cells (Figs. 5p, 6a (left panel). In juvenile control mice carrying an Fgfr2 conditional allele (Fgfr2<sup>fl/fl</sup>), there was a rapid decline in the proportion of reporter-expressing cells with AT2 identity, from 96% at PNS to 31% at PN17, and a concurrent increase in labeled cells that had their proliferation<sup>32,33</sup>. However, another recent study reported that Fgfr2 signaling is ubiquitously and continuously required for AT2 fate selection in vivo.<sup>36</sup> To more precisely determine the cellular and molecular consequences of acute abrogation of FGF signaling in adult AT2 cells, we tried a tamoxifen-inducible Cre recombinase (Sftpc<sup>CreER</sup>; Fgfr2<sup>del/+</sup>, Rosa26<sup>mTmG</sup>) but found that removal of Fgfr2 occurred but was inefficient under these conditions (Supplementary Fig. 5c). We, therefore, developed three additional approaches, which revealed that Fgfr2 signaling is ubiquitously and continuously required for AT2 cell maintenance throughout adult life, and even brief deprivation of signaling immediately initiates apoptosis.

Two important limitations of the conditional Fgfr2 deletion experiment noted above were that the gene was inefficiently deleted, and even when the gene was deleted, the protein persisted (perdured), limitations that could have contributed to the modest or entire lack of effect observed in other studies<sup>37</sup>. To overcome the former limitation, we designed a combinatorial genetic approach using the MASTR transgene<sup>37</sup> (Fig. 6c), which following Flp-mediated recombination of the transgene provides constitutive and high-level Cre expression to ensure recombination of all Cre-dependent alleles in the cell, such as Fgfr2<sup>δ/δ</sup> and Rosa26<sup>fl/+</sup>. We used the MASTR allele in conjunction with an AAV virus we engineered to express Flp recombinase specifically in AT2 cells using an Sftpc promoter element (Fig. 6c, d); when the virus was instilled intratracheally into adult control mice (Fgfr2<sup>δ/δ</sup>; Rosa26<sup>fl/+;MASTR</sup>), specific expression of Cre-dependent reporter genes was observed in AT2 cells (Fig. 6e; >98% of GFP<sup>+</sup> cells, n = 3 lungs, 168 GFP<sup>+</sup> cells counted per lung at 2 weeks), similar to the AT2 specificity observed in prior studies with this approach<sup>38,40</sup>. When the virus was instead instilled into Fgfr2<sup>δ/δ</sup>; Rosa26<sup>fl/+;MASTR</sup> mice, 60% fewer GFP<sup>+</sup> AT2 cells were observed (Fig. 6e, f); of the GFP<sup>+</sup> AT2 cells that remained, nearly all (96%) were found to have retained Fgfr2 protein due to perdurance (Fig. 6h), and the rare GFP<sup>+</sup> Fgfr2<sup>+</sup> AT2 cells detected (4 of 96 scored GFP<sup>+</sup> AT2 cells from three mice) had been extruded into the alveolar space and had a rounded morphology (Fig. 6g). This result
indicates that adult AT2 cells broadly and uniformly undergo apoptosis following removal of Fgfr2. Apoptosis may even initiate with only partial reduction in Fgfr2 levels because one-third (36%) of the remaining GFP+ AT2 cells in this experiment, nearly all of which expressed detectable levels of Fgfr2 (Fig. 6h), were positive for cleaved Caspase-3 (Fig. 6i, j).

To overcome the problem of Fgfr2 perdurance in AT2 cells and corroborate these results, we employed two other approaches. In a classical pharmacologic approach (Fig. 6k), we instilled Fgfr inhibitor FIIN-1 into the lungs of adult (P60) Sftpc-CreER;Rosa26- mTmG mouse tamoxifen-induced to label AT2 (GFP) and co-stained for Fgfr2 (isoform iiib) ligand-binding domain to show pathway activity (c). Blue, DAPI counterstain. Bars, 10 µm. d Quantification of b, c. Nearly all labeled cells (GFP+) express AT2 marker (Muc1) and Fgfr2, and show Fgfr2 activity (pMAPK+). n = 300 GFP+ cells scored (three animals). e Fgfr2 ligand expression (scRNAseq) in lung stromal cells. f AT2 in adult Sftpc-CreER/Rosa26-mTmG mouse tamoxifen-induced to label AT2 (GFP+) and co-stained for Fgfr2 (isoform iiib) ligand-binding domain to show Fgfr2 ligands (ligands identified in Supplementary Fig. 2). Blue, DAPI. Bar, 10 µm. g smFISH (Fgf7, Fgf10). Sftpc in adult (P60) lung. Cell neighboring AT2 co-expresses Fgf7 (white dots) and Fgf10 (red dots) (enlarged in inset). Blue, DAPI; arrowhead, AT2. Bar, 5 µm. h, i Quantification of g showing the density of Fgf7 expressing and AT2 cells (h, ***p = 4.8 × 10⁻⁴; Student’s two-sided t-test, data shown as mean ± SD) and distance between them (i, ***p = 2.9 × 10⁻⁵; Mann Whitney, boxplot shows minimum, maximum, first quartile, second quartile (median), and third quartile). n = 182 AT2, 103 Fgf+ cells scored (four animals). (Values indicate Fgf availability and how many AT2 it supports, and how far each Fgfr-expressing cell must extend (or ligand diffuse) for support). j-o Alveolar region of LyzM-CreRosa26-mTmG/Fgf+ AT2 (inset in n) and following Cre activity in AT2 in early postnatal life (p) and identities of GFP+ cells in control (q) and following Fgfr2 loss (r). n = 300 GFP+ cells scored (three animals) per timepoint/condition; *p = 0.01 for (p) and ***p = 0.0002 for (r) (Student’s two-sided t-test, time series end vs. start).

Fig. 5 | Fgfr2 pathway prevents AT2 reprogramming to AT1 after birth.
a scRNAseq of adult AT2 cells. Expression of Fgfr2, downstream transcription factor Ets5, and target genes indicates pathway remains on after birth. AT2, AT2 markers; Ubiq, ubiquitously-expressed genes. b, c AT2 in adult (P60) Sftpc-CreER;Rosa26- mTmG mouse tamoxifen-induced to label AT2 (GFP) and co-stained for Fgfr2 (b) or phosphorylated MAP kinase (pMAPK) to show pathway activity (c). Blue, DAPI counterstain. Bars, 10 µm. d Quantification of b, c. Nearly all labeled cells (GFP+) express AT2 marker (Muc1) and Fgfr2, and show Fgfr2 activity (pMAPK+). n = 300 GFP+ cells scored (three animals). e Fgfr2 ligand expression (scRNAseq) in lung stromal cells. f AT2 in adult Sftpc-CreER/Rosa26-mTmG mouse tamoxifen-induced to label AT2 (GFP+) and co-stained for Fgfr2 (isoform iiib) ligand-binding domain to show Fgfr2 ligands (ligands identified in Supplementary Fig. 2). Blue, DAPI. Bar, 10 µm. g smFISH (Fgf7, Fgf10). Sftpc in adult (P60) lung. Cell neighboring AT2 co-expresses Fgf7 (white dots) and Fgf10 (red dots) (enlarged in inset). Blue, DAPI; arrowhead, AT2. Bar, 5 µm. h, i Quantification of g showing the density of Fgf7 expressing and AT2 cells (h, ***p = 4.8 × 10⁻⁴; Student’s two-sided t-test, data shown as mean ± SD) and distance between them (i, ***p = 2.9 × 10⁻⁵; Mann Whitney, boxplot shows minimum, maximum, first quartile, second quartile (median), and third quartile). n = 182 AT2, 103 Fgf+ cells scored (four animals). (Values indicate Fgf availability and how many AT2 it supports, and how far each Fgfr-expressing cell must extend (or ligand diffuse) for support). j-o Alveolar region of LyzM-CreRosa26-mTmG/Fgf+ AT2 (inset in n) and following Cre activity in AT2 in early postnatal life (p) and identities of GFP+ cells in control (q) and following Fgfr2 loss (r). n = 300 GFP+ cells scored (three animals) per timepoint/condition; *p = 0.01 for (p) and ***p = 0.0002 for (r) (Student’s two-sided t-test, time series end vs. start).
Loss of AT2 cells induces a robust regenerative response

Despite the profound effects of Fgfr2 loss or inhibition on AT2 cells in juvenile and adult life, curiously, alveolar structure and the total number and proportion of AT2 cells were not grossly altered (Fig. 7a, b), obscuring its ubiquitous and constant requirement for AT2 cell maintenance throughout life. This is because the loss of targeted AT2 cells is almost completely compensated by the proliferation of untargeted cells. This was evident in the LyzM-Cre mice ([LyzMcre/+Fgfr2fl/fl;Rosa26mTmG/+]), where the population of untargeted AT2 cells (lacking the Cre-dependent lineage label) in PN60 mice was increased by 52% relative to that in the Fgfr2+ control ([LyzMCre/+;Fgfr2fl/+;Rosa26mTmG/+]), maintaining the normal density of AT2 cells (~5 per 100 um3) and alveolar morphology despite the
after MASTR-mediated Fgf2r8 deletion (e, f) have not fully lost Fgf2r2. Micrograph (g) shows an example of rare GFP-Fgf2r2+ AT2, which were all rounded and extruded into the lumen. Bar, 10 μm. j k Quantification of (j) cCaspase-3 immunostaining (n = 470 GFPpos cells scored/condition, mean ± SD). Note that 36% of GFPpos AT2 cells remaining after MASTR-mediated Fgf2r8 deletion were cCaspase-3+, indicating apoptosis initiated (micrograph, i). Bar, 10 μm.

**n** Acute inhibition of Fgf2r2 signaling by FIIN-1. k Scheme for co-instillation of FIIN-1 and fluorescent marker WGA-405 into lungs of adult SftpcCreER+/Rosa26mTmG/+ mice tamoxifen (Tam)-treated to label AT2 then analyzed over 2 weeks. Bar, 200 μm. l I Lung regions exposed to FIIN-1 (WGA-labeled, left) and shown merged (right) with mTmG fluorescence (AT2 lineage GFP-labeled, other cells tdTomato (m)-labeled). Bar, 250 μm. m WGA-labeled alveolar regions exposed 2 h to the vehicle (top) or FIIN-1 (bottom). FIIN-1-exposed AT2 cells (GFPpos) undergo apoptosis (cCaspase-3+, arrowheads). Bar, 20 μm. n Kinetics of AT2 apoptosis in (m). In vehicle control, <2.5% of AT2 were cCaspase-3+ (n = 650 cells scored/condition, three experiments, mean ± SD). No conversion of labeled AT2 to AT1 was detected during this period (n = 390 cells scored/condition, three experiments) (see also Suppl. Fig. 7). o q Effect of acute Fgf2r2 inhibition in AT2 cells by Fgf2r2ib-Fc protein. o Scheme. After Tam-induction of adult SftpcCreER+/Rosa26mTmG/+ mice to Fgf2r2iiib-Fc (vehicle) and WGA-405 were co-instilled and lungs subsequently stained for cCaspase-3. p WGA-labeled alveolar regions from vehicle control (top) and Fgf2r2ib-Fc (bottom) instilled lungs. Bar, 25 μm. q Quantification of p, n, cells scored, six experiments (mean ± SD). ***p = 3.4 × 10^-3 for (f), **p = 3.4 × 10^-3 for (j), and ***p = 2.5 × 10^-3 for (q) (Student’s two-sided t-test) (panels f, j, q).

**Discussion**

The results identify FGF signaling as the critical factor that induces and controls alveolar epithelial fate selection. Fgf2r2 is expressed in bipotent alveolar progenitors but rapidly restricts to nascent AT2 cells and remains exclusively in the AT2 lineage, while its ligands Fgf7 and Fgf10 are expressed in surrounding mesenchyme. The addition of either ligand to cultured progenitors induced the formation of alveolus-like structures with intermingled AT2 and AT1 cells, in the absence of any extrinsic forces or cell budding that might protect some progenitors from such forces. In mosaic cultures, randomly selected progenitors with constitutive Fgf2r2 expression exclusively acquired AT2 fate, whereas those with the Fgf2r2 pathway inhibited exclusively acquired AT1 fate, and a similar result was obtained for Fgf2r2 progenitors in mosaic alveoli in vivo. The results demonstrate that Fgf2r2 signaling serves as a developmental switch in alveolar development, inducing bipotent progenitors to AT2 fate (Fig. 7j); progenitors with no or low Fgf2r2 signaling acquire AT1 fate through a secondary, cell non-autonomous signal provided by nascent AT2 cells. Recently, we identified a secondary signal (Notch pathway) required for AT1 fate selection (Gilllich et al., in revision); we suspect this secondary signal could be enhanced by (or synergize with) some subsequent local mechanical change, which would integrate our findings with the longstanding models implicating mechanical forces in alveolar fate acquisition.2,35,48

The FGF developmental pathway remains on in AT2 cells throughout life. Loss of Fgf2r2 signaling during the juvenile period results in AT2 reprogramming to AT1 fate, perhaps by direct reprogramming without proliferation41 or by transient reversion to bipotent progenitor identity (Supplementary Fig. 3d, e). Loss or reduction of Fgf2r2 signaling during adult life, even briefly at any time or position in the lung, triggers rapid AT2 apoptosis, followed by a robust regenerative response (Fig. 7i). The anti-apoptotic effect of Fgf2r2 signaling is transduced through Akt. Thus, the Fgf2r2 pathway directly selects AT2 cells during development and then ubiquitously and continuously maintains them throughout life.

The Fgf2r2 pathway also plays a critical role in the first six days of lung development, inducing and patterning airway branching.26,27,29,30,48 The signaling pathway is thus reused throughout the life of the animal but with changing roles and cellular consequences at each stage. As the lung forms in the embryo (e10–e16), Fgf2r2 signaling provides mitogenic and motogenic functions to pattern airway epithelium budding and branching. During late fetal life (e16–e19), its budding function may be sufficient to continue branching, whereas for the rest of life, it thereby consolidating the AT2 fate decision, whereas for the rest of life, it serves as a survival signal, continuously suppressing AT2 apoptosis likely through Akt. From an AT2 fate, thereby consolidating the AT2 fate decision, whereas for the rest of life, it serves as a survival signal, continuously suppressing AT2 apoptosis likely through Akt. AT2 cells also require Fgf2r2 during alveolar repair32,36. Although there has long been evidence of Fgf2r2’s importance beyond airway branching2,10,21,25,46, its crucial functions in AT2 selection and lifelong maintenance have been obscured by the pathway’s earlier embryonic roles, by the difficulty in rapidly and completely deleting Fgf2r2 and depleting Fgf2r2 protein, and by the robust regenerative response to alveolar cell loss by wild-type AT2 cells.

An important future goal will be to molecularly elucidate how the FGF signaling pathway selects and maintains AT2 cells, and how it results in distinct cellular outcomes at different stages in lung development. The same ligand (Fgf7, Fgf10) and receptor (Fgf2r2) genes are deployed throughout life, so presumably, changes in the downstream
signal transduction and effector pathway or in parallel pathways account for the stage-specific effects of Fgfr signaling. Such a model is operative in Drosophila respiratory system development where the initial round of Fgfr signaling induces changes in the expression of downstream effectors that alter the response in the next signaling round. Appealing candidates for the differential apoptotic response of juvenile vs adult AT2 cells are anti-apoptotic BH3 proteins regulated by Akt activity, which could buffer them against apoptosis. Other possible stage-specific effectors include ETV4 and ETV5, related transcription factors induced by Fgf7 and Fgf10 at bud tips during airway branching that regulate bud size, growth, and number but whose expression restricts such that ETV5 becomes specifically expressed in the AT2 lineage, where it is required to maintain expression of AT2-selective genes in cultured cells as well as the adult mouse lung.

The Fgfr2 pathway remains active in AT2 cells and sustains them throughout life (Fig. 7j). Why would a developmental signaling pathway remain on for months or even years after its developmental role has been completed, simply to keep the selected cell alive? The considerable energy expenditure involved in lifelong signaling suggests some substantial benefit of active AT2 maintenance. Perhaps it allows rapid conversion to a progenitor state and other fates following alveolar injury to quickly restore gas-exchange function, akin to the rapid transdifferentiation of club into ciliated airway cells when Notch signaling is blocked. Or perhaps it prevents initiation of lung damage associated with AT2 overgrowth.
adenocarcinoma, the leading cancer killer, when an AT2 cell divides and a daughter loses contact with the underlying stromal cells that express the Fgf7 and Fgf10 survival signals (Fig. 7k). Whatever the reason, the pathway plays an important role in human lung health because genetic studies identify Fgf7 as a susceptibility locus for the common and devastating disease COPD (chronic obstructive lung disease)\(^{30}\).

Our results show that loss or inhibition of Fgf2 signaling has dire consequences for the alveolus and gas exchange, though they may not become apparent immediately because of the robust regenerative response activated by AT2 cell death. However, widespread targeting of FGF signaling remains active in adults, maintaining AT2 (middle). Loss of FGF in the stressed alveolus (right) triggers AT2 apoptosis and rapid replacement. FGF signaling in adults might prevent AT2 overgrowth/tumors by depriving daughters (dashed) moving away from the FGF source of survival signal. All experiments were repeated at least three times.

Methods

Mouse strains

All mouse experiments followed applicable regulations and guidelines and were approved by the Institutional Animal Care and Use Committee at Stanford University (Protocol 9780). Mice were housed under a 14 h light/10 h dark cycle or 12 light/12 dark cycle, and lights were not used during the dark cycle. Mouse housing temperature was kept at 65–75 °F (~18–23 °C) and humidity at 40%. Timed-pregnant C57BL/6 J females (abbreviated B6; Jackson Laboratories) were used for postnatal time points, mice were euthanized by carbon dioxide inhalation and dissected to both expose the lungs and allow for exsanguination via the abdominal aorta. Phosphate buffered saline (PBS; Ca\(^{2+}\)- and Mg\(^{2+}\)-free, pH 7.4) was gently perfused into the right ventricle of the heart by syringe with a 21 gauge needle until the lungs appeared white. For postnatal time points at or after 8 days, the trachea was then cannulated with a blunt needle and the lungs were gently inflated to full capacity with molten low melting point agarose (Sigma, 2% in PBS).

For all time points, lungs were then separated by lobe and containing, a vibrating microtome (Leica) was used to generate embryonic lungs (see Fig. 6k -n) and subsequent staining (AT2 lineage mGFP, cCasp3, Ki67, Pdpn). mGFP\(^{fl/+}\) (AT2 lineage) cells were scored in instilled (WGA\(^{+}\)) regions: uninstilled regions and vehicle-instilled lungs showed no changes. n = 300 mGFP\(^{fl/+}\) cells scored per stain/timepoint (21 animals, mean ± SD). **H: Vehicle and Fltn1-1-treated alveolar regions 2 weeks after instillation. Note clusters of lineage-labeled AT2 (asterisks) and conversion to AT1 (arrowheads) in Fltn1-1 lungs and, overall restoration of the alveolar structure. Bar, 100 μm. Stain repeated in biological triplicate. 1 Sequential roles of Fgf/Fgf2 pathway in alveolus: AT2 selection and 2 induction of AT1 (embryo), AT2 fate consolidation and prevention of reprogramming to AT1 (juvenile), and AT2 survival (adult, mediated by Akt). AT2 loss triggers regeneration by AT2\(^{-}\)J Source and dynamics of FGF signaling. After AT2 selection in development (left), a 2\(^{+}\) signal induces other progenitors to AT1 fate. FGF signaling remains active in adults, maintaining AT2 (middle). Loss of FGF in the stressed alveolus (right) triggers AT2 apoptosis and rapid replacement. \& FGF signaling in adults might prevent AT2 overgrowth/tumors by depriving daughters (dashed) moving away from the FGF source of survival signal. All experiments were repeated at least three times.

Immunostaining

Immunohistochemistry was performed as previously described in ref. 59 using primary antibodies against the following epitopes (used at 1:500 dilution unless otherwise noted): pro-StfPC (rabbit, Chemicon AB3786), RAGE (rat, R&D MAB179), E-cadherin (rat, Life Technologies ECD-2), Podoplanin (hamster, DSHB 8.1.1), McIun (hamster, Thermo Scientific HM1630 and rabbit, Novus NB20-15481), Ki67 (rat, DAKO M7249), Fgf2 (rabbit, SCBT SC-722), cleaved Caspase-3 (rabbit, Novus NB00-56708), GFP (chicken, Abcam ab3970), Fgf2iiib-Fc (human, R&D Systems) and Phospho-44/42 MAPK (rabbit, CST D13.14.4E). Briefly, fixed lung slices were permeabilized and blocked overnight in 5% goat serum/PBS/0.5% Triton X-100 at 4 °C. Antibody incubation steps were conducted in a blocking buffer overnight and washing steps were performed in three rounds (15 min/round) using PBST (0.1% Tween-20/PBS). Primary antibodies were subsequently detected using Alexa Fluor-conjugated secondary antibodies (Life Technologies), including Goat anti-rabbit IgG Alexa 568 conjugated (Invitrogen, A1036), Goat anti-rat IgG Alexa 488 conjugated (Invitrogen, A1006), Goat anti-hamster Alexa 633 (Invitrogen, A-21113), and Goat anti-Human (Invitrogen, A-11014) unless noted otherwise and incubated in Vectashield with DAPI (5 μg/ml, Vector labs). EdU was detected using the standard Click-IT reaction (Thermo Fisher). Images were acquired using a laser-scanning confocal microscope (Zeiss LSM 780) and subsequently processed using Zen and ImageJ.

Lung isolation and processing

For prenatal time points, individual embryos were staged by fetal crown–rump length before sacrifice and lungs removed en bloc. For postnatal time points, mice were euthanized by carbon dioxide inhalation and dissected to both expose the lungs and allow for exsanguination via the abdominal aorta. Phosphate buffered saline (PBS; Ca\(^{2+}\)- and Mg\(^{2+}\)-free, pH 7.4) was gently perfused into the right ventricle of the heart by syringe with a 21 gauge needle until the lungs appeared white. For postnatal time points at or after 8 days, the trachea was then cannulated with a blunt needle and the lungs were gently inflated to full capacity with molten low melting point agarose (Sigma, 2% in PBS).

For all time points, lungs were then separated by lobe and fixed in either Zinc Formalin, 4% PFA, or Dent’s fixative at 4 °C. For immunostaining, a vibrating microtome (Leica) was used to generate embryonic (200 μm) or adult (450 μm) tissue sections of uniform thickness. For in situ hybridization, lobes were submerged in OCT (Tissue Tek) in an embedding mold and frozen on dry ice. Tissue sections (10 μm) were obtained using a Leica CM3050S cryostat, collected on glass slides, and subsequently processed.
In situ hybridization

Two methods of multiplexed single-molecule fluorescence in situ hybridization (smFISH) of mRNAs was performed. To simultaneously detect Sftpc, Fgf7, and Fgf10 RNAs, RNAscope multiplex assay V1 kit (ACD) was used as described. To visualize Sftpc and Fgfr2 RNAs, proximity ligation in situ hybridization (PLISH) was used. Briefly, 20-μm thick sections were cut from OCT-embedded, cryopreserved tissue and hybridized with multiple anti-sense probe pairs that hybridize to the target transcript. Probe targets that targeted each gene share a common barcode that is unique and complementary to circle and bridge constructs. The circle and bridge constructs undergo proximity ligation to form a closed circle that undergoes rolling circle amplification. Detection oligonucleotides conjugated to fluorescent probes anneal to the rolling circle amplification product, generating discrete puncta for each transcript. The following sets of primer pairs were used to detect transcripts of the indicated genes:

**Sftpc**
- 5'TCGTACGCTCTAACCTAGCTGTTGAGCTGAGTGCG3′
- 5'GGTCTTTCTGGCCTTACTAGTCGAGTGTTTATGTGTGG3′
- 5'GGTGCTTTCTGGCCTTACTAGTCGAGTGTTTATGTGTGG3′
- 5'TGTTTCTCAATTTACGTCGTTATGTTTATTTTGTGATAG3′
- 5'TGGTTTTCACATCAGGCTGCTTATACGTCGAGTTGAGAACAA CCTG3′

**Fgfr2**
- 5'TGATACCCACGCCAACCTAGTCGTTATGTTTATTTTGTGATAG3′
- 5'TGTTTTCACATCAGGCTGCTTATACGTCGAGTTGAGACAA CCTG3′
- 5'TGTTTTCACATCAGGCTGCTTATACGTCGAGTTGAGACAA CCTG3′
- 5'TGTTTTCACATCAGGCTGCTTATACGTCGAGTTGAGACAA CCTG3′
- 5'TGTTTTCACATCAGGCTGCTTATACGTCGAGTTGAGACAA CCTG3′

**Fgf7**
- 5'TGTTTTCACATCAGGCTGCTTATACGTCGAGTTGAGACAA CCTG3′
- 5'TGTTTTCACATCAGGCTGCTTATACGTCGAGTTGAGACAA CCTG3′

**Fgf10**
- 5'TGTTTTCACATCAGGCTGCTTATACGTCGAGTTGAGACAA CCTG3′

Expression was detected by confocal fluorescence microscopy as -0.5 μm fluorescent puncta.

Cell isolation and culture

Adult AT2 cells and embryonic (e16.5) bipotent alveolar epithelial progenitors were purified as previously described in ref. 11. Adult 2-month-old mice were euthanized by administration of CO2. For e16.5, embryos were removed from the mother and their lungs were isolated en bloc without perfusion and pooled by litter (five to seven embryos) for further processing. Lungs were microdissected to remove the proximal lung tissue, leaving only the distal (alveolar region) tissue. Distal e16.5 lung cells were dissociated in dispase (BD Biosciences) and triturated with glass Pasteur pipettes until a single-cell suspension was attained. For adult lungs, the vasculature was perfused through the right ventricle with 37 °C media (DMEM/F12, Life Technologies). The lungs were flushed with digestion buffer containing DNase I (0.33 U/ml; Roche), incubated with proteoglycans (HSPG, 100 ng/ml, Sigma) to the indicated concentration, stained with Rock inhibitor Y27632 (20 μM, R&D Systems), and heparan sulfate proteoglycans (HS, 100 ng/ml, Sigma) for 10 min, and then centrifuged at 400×g for 10 min. Pelleted cells were resuspended in red blood cell lysing solution (BD Biosciences), incubated for 2 min, passed through a 40 μm mesh filter (Fisher), centrifuged at 400×g for 10 min, and then resuspended in MACS buffer (2 mM EDTA, 0.5% BSA in PBS, filtered and degassed) for perfusion.

Live imaging of alveolar progenitors in culture

Time-lapse microscopy of cultured alveolospheres was conducted using an inverted Zeiss LSM 580 confocal microscope equipped with environmental control to maintain 37 °C and 5% CO2. Isolated bipotent progenitors were plated and cultured for -24 h on coverslips (LabTek) coated with growth factor reduced Matrigel (BD, Biosciences) for 30 min at 37 °C. Cells were supplemented with Fgf7 (50 ng/ml, R&D Systems), Fgf10 (100 ng/ml, R&D Systems), FGF11 (20 μM, Tocris), Fgf21–fib-Fc (2 μg/ml, R&D Systems), Arp2/3 inhibitor CK666 (40 μM, R&D Systems), ROCK inhibitor Y27632 (20 μM, R&D Systems), and heparan sulfate proteoglycans (HS, 100 ng/ml, Sigma) for 10 min, and then centrifuged at 400×g for 10 min, and then stained with Rock inhibitor Y27632 (20 μM, R&D Systems), and heparan sulfate proteoglycans (HS, 100 ng/ml, Sigma) for 10 min, and then added to the lysis reaction and processed in parallel (Internal RNA Controls Consortium) RNAspike-in Mix (Ambion, Life Technologies) was added to the lysis reaction and processed in parallel (Internal RNA Controls Consortium) RNAspike-in Mix (Ambion, Life Technologies) was added to the lysis reaction and processed in parallel (Internal RNA Controls Consortium) RNAspike-in Mix (Ambion, Life Technologies) was added to the lysis reaction and processed in parallel (Illumina Nextera XT DNA Sample Preparation kit), and single-cell RNA sequencing libraries were constructed (Fluidigm C1 system) in experimental duplicate). To enrich for adult fibroblasts, MACS depletion was conducted as above using antibodies against CD31, CD45, and EpCAM.

Single-cell RNA sequencing

Processing, scRNAseq, and analysis of e18.5 and adult mouse lung cells was conducted as previously described with minor alterations to enrich the respective populations. Briefly, single-cell suspensions sorted by MACS as CD45−CD31+ EpCAM (for mesenchymal cells) or CD45−CD31− EpCAM (for endothelial cells) were subsequently incubated with a viability stain (Sytox Blue, Life Technologies) for 15 min and loaded on a medium-sized (10–17 μm cell diameter) microfluidic RNA-seq chip (Fluidigm) for 15 min and loaded on a medium-sized (10–17 μm cell diameter) microfluidic RNA-seq chip (Fluidigm) using the Fluidigm C1 system. Cells were loaded at a concentration of 300–500 cells/ml and imaged by phase-contrast and fluorescence microscopy to assess the number and viability of cells per capture site. Only single, live cells were included in the analysis. For scRNAseq experiments, cDNAs were prepared on a chip using the SMARTer Ultra Low RNA kit for Illumina (Clontech). ERRCC (External RNA Controls Consortium) RNAspike-in Mix (Ambion, Life Technologies) was added to the lysis reaction and processed in parallel to cellular messenger RNA. Sequencing libraries were constructed (Illumina Nextera XT DNA Sample Preparation kit), and single-cell libraries pooled and sequenced 100 base pairs (bp) paired-end to a
depth of 2 to 6 × 10^4 reads. Sequencing data were processed as described in ref. 11, and transcript levels quantified as fragments per kilobase of transcript per million mapped reads (FPKM) generated by TopHat/Cufflinks. Cells not expressing (FPKM <1) three of four housekeeping genes (Actb, Gapdh, Ubc, Ppia), or expressing them less than three standard deviations below the mean, were removed from the analysis. Recombinoblasts were identified as cells expressing canonical fibroblast markers Mgp, Coll1a1, and Coll1a2 that also lacked expression of canonical markers of the major airway or alveolar epithelial cell types. Subsequent analysis, including hierarchical clustering, was performed as described in ref. 11. To determine isoform expression of Fgfr2, STAR was used to align and count reads specific to either exon Iib or Ilc. To determine receptor genes restrictively expressed along either AT1 or AT2 lineage, receptor genes were first screened for ones detectably expressed in at least six cells, then Welch's t-test was used to identify receptor genes with at least twofold higher expression in each respective lineage (p ≤ 0.01).

For the cell type-specific gene expression analysis in published scRNAseq datasets, the processed mRNA counts for each cell were used from developing (GSE196874) and adult mouse lung (GSE109774) datasets. Expression matrices were analyzed via R using the Seurat package. Clusters of cells with similar expression profiles were identified via shared nearest neighbor analysis using the Louvain algorithm. Clusters were visualized in UMAP plots, and expression of specific genes was represented in heatmaps, violin plots, and dot plots generated in Seurat.

**Mosaic and inducible deletion of Fgfr2**

To mosaically modulate Fgfr2 activity in culture, a lentiviral approach was developed using the Lenti-X Tet-Off 3 G Inducible Expression System (Clontech). The ORF encoding Fgfr2iiib (Origene; MC221076), the IRES sequence from pLVX-IRES-Puro (Clontech; 632186), and the sfGFP (superfolder GFP) coding sequence from pBAD-sfGFP (Addgene; 54319) were PCR-amplified with adapter sequences (IDT) and assembled (NEB; E5520) into the lentiviral backbone pTetOne (Takara; 63018S) linearized by EcoRI/BamHI digestion (NEB) to generate pTetOne-Fgfr2-IRES-sfGFP (Fig. 3a). To generate a dominant negative form of Fgfr2iiib, a truncated version of Fgfr2iiib lacking the tyrosine kinase domain was also PCR-amplified with adapter sequences (as above) to assemble pTetOne-Fgfr2DN-IRES-sfGFP. Lentiviruses (“Lenti-Fgfr2,” “Lenti-Fgfr2DN”) were produced from these plasmids following the manufacturer's instructions for transfection, lentiviral concentration, and titer estimation (Lenti-X Expression System, Clontech). An AAV virus that constitutively expresses eGFP from CMV promoter (Addgene, 103543-AAV9) was used as a cell labeling control (Supplementary Fig. 4b). Purified bipotent progenitors plated on Matrigel in a standard medium as above were treated with cationic polymer polybrene (8 ng/ml, EMD Millipore, to enhance viral infection) 10 min before viral addition. After 24 h of infection at 37°C in media containing doxycycline (100 ng/ml, Sigma) to induce expression of the Fgfr2iiib gene, media was replaced with media containing doxycycline and Fgf7 (50 ng/ml). Four days later, cultured cells were fixed and analyzed by immunostaining.

For in vivo mosaic labeling and deletion of the floxed Fgfr2 allele in bipotent progenitors, Tg^NesCre/Etv2, Rosa26^tm1(CAG/2)Mmiş/Mmiş mice were used. For analogous experiments in AT2 cells, LyzM^mTmG, Rosa26^tm1(CAG/2)Mmiş/Mmiş and Sftpc^CreERT2-rtTA-rtT, Rosa26^tm1(CAG/2)Mmissão/Mmissão were bred and induced by intraperitoneal injection of tamoxifen. At time points and conditions indicated, lineage-labeled (mGFP+) and unlabeled (mGFP−) cell types (BP, AT2, or AT1, as indicated) identified by marker expression were counted from large alveolar fields (25x objective, ~500 μm × 500 μm × 100 μm).

To mosaically but efficiently delete Fgfr2 in adult AT2 cells, we used a recombinant AAV described below to infect and express Flp recombinase mosaically in AT2 cells, and the Flp-dependent Rosa26-MSTR allele to constitutively express Cre recombinase to delete the conditional Fgfr2 allele. The pAAV-EF1α-Flpo plasmid was modified using the assembly scheme described above (NEB; E5520) to replace the EF1α promoter (removed by MluI/BamHI restriction digestion) with a previously defined 320 bp minimal promoter element of Sftpc was that chemically synthesized with added adapter sequences (IDT) (Fig. 6c). Recombinant AAV-Sftpc-Flpo virus was prepared by the HHMI Janene Farnen Viral Tools facility (2 × 10^9 GC (genome copies)/ml, serotype 2/9) and endotracheally instilled (1 μl of viral solution diluted in 50 μl PBS) into the lungs of mice harboring Rosa26^MSTR/mTmG and either heterozygous or homozygous for the floxed Fgfr2 allele. The efficiency of Fgfr2 deletion was assessed by immunostaining for Flp. For adult time points, the density of AT2 cells was calculated and expressed as the number of AT2 cells per 100 μm × 100 μm × 100 μm region of alveolar tissue. To determine AT2 proliferation, EdU (1 mg/ml, Cedar Lane) was administered in the drinking water for the indicated period.

**Pharmacologic inhibition of Fgfr2 and activation of Akt**

To rapidly and persistently inhibit Fgfr2 signaling, the irreversible Fgfr2 inhibitor FIN1 (14.75 mg/kg, Tocris) or recombinant Fgfr2iiib-Fc (100 μg/30 g mouse, R&D Systems) was used. Mice at least 2 months (and up to over 1 year) of age carrying the Sftpc^CreERT2-rtTA-rtT, Rosa26^tm1(CAG/2)Mmissão alleles were administered tamoxifen (3 mg/30 g mouse, Sigma) via intraperitoneal injection to label >95% of AT2 cells. After 1 week, the mice were anesthetized with isoflurane and administered by endotracheal instillation either vehicle alone (PBS with biotinylated WGA), or vehicle with the inhibitor. To test the effect of Akt signaling, the small molecule Akt activator SC79 (Tocris) was administered (0.04 mg drug per g mouse) via intraperitoneal injection 30 min prior to Fgfr2 inhibition. Lungs were isolated at the indicated time points and areas of high exposure to inhibitors were identified by WGA immunostaining.

**Statistical analysis**

Data analysis and statistical tests were performed with R or GraphPad Prism software. Replicate experiments were all biological replicates with different animals, and quantitative values are presented as mean ± SD unless indicated otherwise. Student's t-tests were two-sided. No statistical method was used to predetermine sample size, and data distribution was tested for normality prior to statistical analysis and plotting. Both male and female animals were used in experiments, and subjects were age- and gender-matched in biological replicates and in comparisons of different groups. Post hoc power analysis was done (Supplemental Table 1).

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The scRNAseq datasets are available in the GEO repository under the accession numbers "GSE109774," "GSE32583" for E18 distal lung epithelial cells, "GSE196874" for E18 distal lung mesenchymal and endothelial cells, "GSE109444" for adult lung mesenchymal cells, and "GSE119228" for E16 lung cells. Source data are provided with this paper.

**Code availability**

Code to reproduce the analyses described in this manuscript can be accessed at https://github.com/dbrownfield/Brownfield_et_al, 451970378.

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