# The development and plasticity of alveolar type 1 cells 

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#### Abstract

Alveolar type 1 (AT1) cells cover $>95 \%$ of the gas exchange surface and are extremely thin to facilitate passive gas diffusion. The development of these highly specialized cells and its coordination with the formation of the honeycomb-like alveolar structure are poorly understood. Using new marker-based stereology and single-cell imaging methods, we show that AT1 cells in the mouse lung form expansive thin cellular extensions via a non-proliferative two-step process while retaining cellular plasticity. In the flattening step, AT1 cells undergo molecular specification and remodel cell junctions while remaining connected to their epithelial neighbors. In the folding step, AT1 cells increase in size by more than 10-fold and undergo cellular morphogenesis that matches capillary and secondary septa formation, resulting in a single AT1 cell spanning multiple alveoli. Furthermore, AT1 cells are an unexpected source of VEGFA and their normal development is required for alveolar angiogenesis. Notably, a majority of AT1 cells proliferate upon ectopic SOX2 expression and undergo stage-dependent cell fate reprogramming. These results provide evidence that AT1 cells have both structural and signaling roles in alveolar maturation and can exit their terminally differentiated non-proliferative state. Our findings suggest that AT1 cells might be a new target in the pathogenesis and treatment of lung diseases associated with premature birth.


## KEY WORDS: Lung development, Cell plasticity, Alveolar angiogenesis

## INTRODUCTION

The mammalian lung consists of a tree-like airway compartment and a honeycomb-like gas exchange compartment. The two major epithelial cell types covering the gas exchange compartment are alveolar type 1 (AT1) and type 2 (AT2) cells, which are in close contact with underlying capillaries and fibroblasts (Williams, 2003; Herzog et al., 2008; Weibel, 2015). AT1 cells are flat and cover more than $95 \%$ of the gas exchange surface, whereas AT2 cells are cuboidal and synthesize surfactants to prevent the alveoli from collapsing (Crapo et al., 1982; Williams, 2003). Classical electron microscopy studies show that AT1 cells are extremely thin $(<0.1 \mu \mathrm{~m})$, presumably to facilitate passive gas diffusion, and have a complex morphology that can be traced over multiple alveoli (Weibel, 1971, 2015). Whereas AT2 cells have recently been shown

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to self-renew and give rise to AT1 cells during homeostasis and injury repair (Barkauskas et al., 2013; Desai et al., 2014), AT1 cells are generally considered terminally differentiated in vivo, although they exhibit some plasticity in culture (Danto et al., 1995; Williams, 2003; Gonzalez et al., 2005, 2009). One recent study suggests that, although infrequent, AT1 cells may convert to AT2 cells and proliferate upon pneumonectomy or oncogenic KRAS expression (Jain et al., 2015).
During development, recent studies suggest that a population of bipotential progenitors expressing markers of both AT1 and AT2 cells differentiate into mature AT1 or AT2 cells by upregulating additional markers of the corresponding cell fate and downregulating markers of the alternative cell fate (Desai et al., 2014; Treutlein et al., 2014). However, it is unknown how alveolar cell number, morphology and fate are regulated during subsequent lung maturation. In particular, how do AT1 cells adopt their convoluted morphology in coordination with the formation of the honeycomb-like alveolar structure? When and to what extent are the fates of AT1 and AT2 cells specified?
In this study, we focus on the poorly understood AT1 cells during the perinatal period. We develop a new marker-based stereology method to follow the change in cell number and alveolar surface area, and use single-cell three-dimensional (3D) imaging and three AT1 cell genetic drivers to follow changes in cell morphology and cell fate plasticity. We show that AT1 cells develop via a nonproliferative two-step growth process of cell flattening and cell folding, but retain cellular plasticity. Furthermore, AT1 cells, but not AT2 cells, express Vegfa, and disruption of AT1 cell development leads to reduced alveolar angiogenesis. These findings pave the way for future investigation of the role of AT1 cells in alveolar maturation and of AT1 cell plasticity in vivo.

## RESULTS

## AT1 cell growth fuels postnatal alveolar growth

To understand AT1 cell development, we first set out to determine the number of AT1 cells during postnatal lung growth in mice. AT1 cells have been commonly identified based on morphology using electron microscopy (Stone et al., 1992; Weibel, 2015), which limits the analysis to small regions and makes it technically challenging to obtain the total cell number as it requires the dissector method (Hsia et al., 2010) or an assumption of uniform nuclear shapes (Kauffman et al., 1974; Weibel, 2015). This prompted us to develop a new marker-based stereology method that combines stereological sampling principles with 3D imaging of molecular markers (Fig. S1A). Our method has several advantages. First, we confirmed that, unlike membrane-localized AT1 markers, HOPX stains both the nucleus and cytosol of AT1 cells (Barkauskas et al., 2013), thus allowing nucleus-based cell counting. HOPX expression was AT1 specific throughout postnatal development, as alveolar epithelial cells were marked in a mutually exclusive manner by nuclear HOPX and LAMP3 [an AT2 cell marker (Chang et al., 2013; Desai et al., 2014)] or by nuclear HOPX and cuboidal E-cadherin (E-CAD;
cadherin 1) staining that colocalized with SFTPC (an AT2 cell marker) (Fig. 1A,B, Fig. S1E). Second, we developed a wholemount thick-section staining method that minimized tissue shrinkage as well as dehydration when stained on slides (Fig. S1B,C). Third, we used 3D fluorescence imaging and Imaris software to directly visualize and count cells in large fields to reduce edge effect and bias from field selection (Fig. 1B) (Williams and Rakic, 1988). Results from this direct counting method were comparable to those using the optical dissector method (Hsia et al., 2010) (Fig. S1F). Our measurements of alveolar cell number and surface area in the adult mouse lung were in general agreement with the widely variable measurements in the literature (Fig. S1G).

Using this method, we found that as the lung continued to grow postnatally, there was a parallel increase in the number of AT2 cells, resulting in a nearly 6 -fold increase in AT2 cell number from P0 to P54 (Fig. 1C, Table S1). By contrast, over the same period, the number of AT1 cells only increased $\sim 2$-fold and most of the increase occurred within the first week after birth, possibly owing to the differentiation of remaining embryonic progenitors (Fig. S2; as described below) (Alanis et al., 2014; Yang and Chen, 2014). Such a difference was also reflected in a previously unappreciated decrease in the ratio between the numbers of AT1 and AT2 cells postnatally (Fig. 1C, Table S1). Furthermore, AT2 but not AT1 cells express the proliferative marker KI67 (Fig. S3A). Few
apoptotic AT1 or AT2 cells were observed (Fig. S3B) (Schittny et al., 1998). Despite their smaller number, AT1 cells constituted nearly all the alveolar surface throughout postnatal development, as measured using STEPanizer (Fig. 1C, Table S1). Therefore, AT1 cell growth, but not proliferation, fuels most of the postnatal alveolar growth with a limited contribution from AT2 cells on a structural level.

## AT1 cells flatten in conjunction with molecular specification

Next, we examined how AT1 cells grow and the relationship of this growth to postnatal alveolar maturation. The boundaries of the ultrathin cellular extensions of AT1 cells are only reliably detected by electron microscopy, which limited the analysis to two dimensions and sections capturing only part of an AT1 cell (Weibel, 1971, 2015). As a result, the complete morphology of a single AT1 cell in the developing and mature lung is unknown. To examine whole AT1 cells in 3D, we titrated down the dose of the recombination inducer (tamoxifen) to genetically label isolated cells with a membrane-bound reporter (Muzumdar et al., 2007). We also developed whole-mount multicolor staining and imaging methods to visualize labeled cells without physical sectioning and in conjunction with molecular markers (Figs 2 and 3). Multicolor reporters, such as Rosa Confetti (Snippert et al., 2010), were not used because the soluble reporter proteins do not efficiently mark the



Fig. 2. AT1 cells flatten in conjunction with molecular specification. (A) Confocal images of immunostained strips from E19 Sox $9^{C r e E R /+}$; Rosa ${ }^{m T m G /+}$ lungs with recombination induced at E13 (Tam, tamoxifen). The two leftmost images are maximal projection views of branch tips (dashed outlines). Columnar wedge-shaped epithelial progenitors $(P)$ are found in branch tips. Elongated AT1 cells and cuboidal AT2 cells are found in branch stalks and also shown in section views in subsequent images with the epithelial basement membrane outlined with dashes and the lumen (lu) labeled. Only part of a cell is visible in the section view and therefore may have a different morphology from that in the projection view. Note that AT2 cells, unlike AT1 and progenitor cells, are extruded from the lumen. Elongated AT1 cells have nuclear HOPX expression and have lost progenitor and AT2 cell markers, including SOX9 and SFTPC. (B) Confocal images of E19 lung sections. PODXL-expressing AT1 cells (square bracket) are connected with neighboring AT2 cells through apically restricted tight junctions [ZO1 (TJP1), arrowheads]. E-CAD is also enriched apically at AT1-AT2 and AT1-AT1 junctions, as opposed to accumulating throughout the lateral sides between progenitors (A). Dashed line indicates epithelial basement membrane marked by collagen type IV (COL4). Asterisks mark blood vessels that express PODXL, ZO1 and COL4 but not E-CAD. (C) A model of AT1 cell flattening. As columnar wedge-shaped progenitors flatten to become AT1 cells, apical tight junctions (TJ) are maintained whereas lateral junctions are lost. Cell flattening is accompanied by changes in marker expression including SOX9, SFTPC and HOPX. Scale bars: $10 \mu \mathrm{~m}$.
ultrathin AT1 cell extensions and because the GFP antibody, which provides the necessary signal amplification, does not distinguish GFP variants, including CFP and YFP.

To ensure unbiased analyses of the earliest stage of AT1 cell growth, we used the Sox $9^{\text {CreER }}$ allele to label lung epithelial progenitors at E13, before any detectable AT1 cell differentiation (Rawlins et al., 2009; Chang et al., 2013; Alanis et al., 2014; Yang and Chen, 2014). As shown in Fig. 2A, labeled E19 lungs contained epithelial cells of three morphological types: columnar wedge-shaped cells in the most distal tube-like structure (branch tips), and a mixture of cuboidal and elongated cells in the near distal sac-like structure (transformed branch stalks, see also Fig. 4A and Fig. S4A). The columnar cells expressed SOX9, a
progenitor marker (Yang and Chen, 2014), together with SFTPC and RAGE (AGER), and were therefore likely to be the bipotential progenitors (Desai et al., 2014). Consistent with this possibility, clusters of SOX9-expressing cells at the distal edge at E19 were lineage traced into both AT1 and AT2 cells in the mature lung (Fig. S2). The elongated cells were variable in morphology, ranging from leaf-shape with irregular smooth protrusions to spindle-shape with stretched sharp tips, and expressed RAGE and nuclear HOPX, consistent with them being developing AT1 cells. Although infrequent, such elongated morphology was detected as early as E17 (Fig. S4A). In comparison, the cuboidal cells expressed SFTPC but not SOX9, consistent with them being developing AT2 cells.


Fig. 3. AT1 cells fold in conjunction with alveolar septation. (A) (Top) Confocal projection images of immunostained strips from a P8 Hopx ${ }^{C r e E R}$; Rosa ${ }^{n T n G /+}$ lung showing infrequent mistargeting of AT2 cells (asterisk) by Hopx ${ }^{C r e E R}$ when induced with 2 mg tamoxifen at E19. Solid arrowhead, AT1 cells; open arrowhead, AT2 cells. To the right is a schematic of a cranial lobe with airways traced according to a real sample stained for SOX2 to illustrate that a strip in the gas exchange compartment is cut along the dashed line and immunostained and the boxed region imaged. The plot shows quantification of AT1 cell total surface area (both the apical and basal surface). Representative examples are shown in the bottom panels. The plot is distributed along the $x$-axis to avoid excessive overlapping. (Bottom) Confocal projection images (top row) of immunostained strips from Hopx ${ }^{C r e E R}$; Rosa ${ }^{m T m G /+}$ lungs at the indicated stages induced with 0.4 mg tamoxifen at E19, with the corresponding surface rendering (bottom row). Arrowheads, 'valleys' of AT1 cells; Asterisk, AT2 cell. (B) Confocal projection images of immunostained lung strips and corresponding surface rendering (beneath each confocal image). At P4 (left column), primary saccules are island-like and associated with deeper and wider grooves (dashed line, see also the PO lung in A) with embedded smooth muscles (open arrowhead). Shallow grooves with smooth muscles begin to form on the surface of primary saccules (solid arrowheads). Vessels are associated with all grooves and only appear double layered (asterisk) between primary saccules. At P19 (right column), SMA-expressing myofibroblasts mostly disappear, whereas vessels persist to mark grooves between mature alveoli. Primary saccules are no longer obvious as secondary septa have deepened. Square brackets indicate pre-capillary arterioles surrounded by smooth muscles. (C) (Left) Projection view (top left), surface rendering (top middle and right) and section views at the indicated depth (bottom row) of confocal images of immunostained strips from a P30 Hopx ${ }^{C r e E R}$; Rosa ${ }^{m T m G /+}$ lung induced with 0.4 mg tamoxifen at E19. A single AT1 cell ( n , nucleus) spans multiple alveoli ( 1 through 5, demarcated by vessels) and the underlying alveolar sac (sac). (Right) Projection view (top) and surface rendering (bottom) of confocal images of immunostained strips from a P24 Hopx ${ }^{C r e E R}$; Rosa ${ }^{\text {tdT/+ }}$ lung induced with 0.4 mg tamoxifen at P23. The soluble reporter (RFP, pseudocolored green) highlights the nucleus of a single AT1 cell (dashed outline). (D) A model of AT1 cell folding. Flattened AT1 cells undergo a>10-fold expansion in surface area and fold where myofibroblasts temporally and vessels permanently reside. As a result, a mature AT1 cell spans multiple alveoli (alv). Scale bars: $10 \mu \mathrm{~m}$.


Fig. 4. Scnn1a-Cre-driven ectopic SOX2 expression reprograms flattened AT1 cells. (A) Confocal projection images of immunostained P0 Scnn1a-Cre; Rosa ${ }^{m T m G /+}$ lung strips. Dashed lines denote a branch-like tube at the lobe edge with distal tips containing progenitors in clusters and proximal stalks containing intermingled flattened AT1 and cuboidal AT2 cells. The boxed region is enlarged to the right. Note that all labeled cells are flattened AT1 cells (demarcated by E-CAD junctions) with nuclear HOPX (arrowhead), rather than cuboidal AT2 cells (asterisk). (B) Confocal projection images of immunostained Scnn1a-Cre; Rosa ${ }^{\text {Sox2/+ }}$ lung strips at the indicated stages showing that isolated flattened mutant AT1 cells (demarcated by E-CAD junctions, dashed lines) form clusters with compact cell arrangement over time. (C-E) Projection (C,D) or section (E) view of confocal images of immunostained strips from littermate control (C) and Scnn1a-Cre; Rosa ${ }^{\text {Sox2/+ }}$ (C,E) or Scnn1a-Cre; Rosa ${ }^{m T m G / S o x 2}$ (D) mutant lungs. (C) Compared with AT1 cells in the control lung or neighboring unrecombined AT1 cells (open arrowhead), mutant AT1 cells (solid arrowhead) lose RAGE expression and have diffuse HOPX expression. Asterisks denote AT2 cells. (D) The Rosa ${ }^{m T m G}$ allele is easier to recombine than the Rosa ${ }^{\text {Sox2 }}$ allele, generating juxtaposed GFP-labeled AT1 cells with and without SOX2 expression. Compared with control AT1 cells (green outline with black dashed nuclei), mutant AT1 cells are smaller and express the proliferation marker KI67 (green outline with red dashed nuclei). Note additional KI67 expression in mesenchymal cells. (E) Mutant AT1 cells express the basal cell marker P63 (arrowhead). (F) Left two panels show confocal images of a control lung section showing that NKX2.1 is expressed by both AT1 (solid arrowheads) and AT2 (open arrowheads, cuboidal E-CAD staining) cells. All epithelial cell nuclei are genetically marked by GFP to allow co-staining with the rabbit anti-NKX2.1 antibody. Right two panels are confocal images of sections from littermate control and Scnn1a-Cre; Rosa ${ }^{\text {Sox2/+ }}$ mutant lungs. Mutant AT1 cells marked by GFP have nuclear (solid arrowhead) or diffuse (arrow) NKX2.1. Inset shows NKX2.1 single-channel image. NKX2.1 expression in AT2 cells (open arrowhead, cuboidal E-CAD staining) of the mutant lung is not affected. (G) Scatterplot of $\log _{2}$ fold change in gene expression between two biological replicates. Compared with littermate control (Scnn1a-Cre; Rosa ${ }^{m T m G /+}$ ) AT1 cells, mutant (Scnn1a-Cre; Rosa ${ }^{\text {Sox2/+ }}$ ) AT1 cells downregulate AT1 markers (green) and upregulate airway markers (red). The number in parenthesis denotes the rank order in fold change among genes that are significantly different between control and mutant. Scale bars: $10 \mu \mathrm{~m}$.

Throughout such changes in cell morphology, the elongated cells remained connected to their cuboidal neighbors via apically restricted tight junctions and apically enriched adherens junctions (Fig. 2B). Interestingly, the basolateral side of the cuboidal cells contained E-CAD and was surrounded by a continuous basement membrane instead of adjacent AT1 cells (Fig. 2B). Such apically restricted contact between AT1 and AT2 cells positioned AT2 cells in the interstitial region rather than the lumenal space (Fig. 2B), an arrangement also observed for AT2 cells labeled individually (Fig. 2A) and at later postnatal stages (Fig. S5). These results suggested that, in the initial step of AT1 cell
growth, columnar wedge-shaped progenitors flatten while maintaining apical tight junctions but losing lateral adherens junctions, a process accompanied by cell type-specific molecular changes (Fig. 2C).

## AT1 cells fold in conjunction with alveolar septation

To specifically label AT1 cells and follow their subsequent growth, we screened seven Cre and CreER alleles of genes that were expected to be active in the lung epithelium and therefore potentially in AT1 cells. We characterized all of the alleles throughout embryonic and postnatal stages using the same Cre reporter

Rosa $a^{m T m G}$ to allow direct comparison. Whereas some alleles had a very low (Krt8-CreER, Krt18-CreER, Krt14-Cre, Cldn6 ${ }^{\text {CreER }}$ ) or non-selective (Nkx2.1 ${ }^{\text {CreER }}$ ) activity in the lung epithelium, Scnn1aCre (described below) and Hopx ${ }^{\text {CreER }}$ were found to be highly selective for AT1 cells (Fig. S6). When recombination was induced before E15, very rare cells were labeled by Hopx ${ }^{\text {CreER }}$ in the lung (Fig. S6F). When induced at E18, Hopx ${ }^{\text {CreER }}$ labeled elongated cells with irregular extensions, similar to the aforementioned developing AT1 cells labeled by Sox9 ${ }^{\text {CreER }}$ (Fig. S4B). Although Hopx ${ }^{\text {CreER }}$ can label AT2 cells (Jain et al., 2015), such leaky targeting was infrequent when induced at E19 using the nuclear reporter Rosa ${ }^{n T n G}$ (Prigge et al., 2013) (7/189 GFP ${ }^{+}$cells from three mice; Fig. 3A), suggesting that Hopx CreER has limited activity in bipotential progenitors. Labeled AT1 cells were readily identified by their characteristic thin cellular extensions that were marked by Rosa ${ }^{m T m G}$ (Fig. 3, Fig. S6F).

Timecourse morphological analysis using Hopx ${ }^{\text {CreER }}$ revealed that AT1 cells underwent a folding process, whereby the initial flattened cells were sculptured into 'mountains' and 'valleys' (Fig. 3A). Although individually variable at a given time point, AT1 cells, as a population, had an increase in total cell surface area of more than 10 -fold and reached a maximum of $\sim 12,000 ~ \mu \mathrm{~m}^{2}$ by P30 (Fig. 3A). The total (apical and basal) surface area of individual cells was comparable to the average apical surface area calculated from the stereology data (Table S1) and the literature (Stone et al., 1992), with the difference attributed to tissue shrinkage (Fig. S1C) and possibly preferential labeling of AT1 cells that are more mature by $H o p x^{C r e E R}$ at a limiting dose of tamoxifen.

Notably, AT1 cell folding became apparent from P5, which coincided with transformation of the smooth-walled primary alveolar saccules into the honeycomb-like mature alveoli (Fig. 3A), a process known as secondary septation (Herzog et al., 2008). To further investigate this, we developed whole-mount staining methods to visualize the septation in 3D. At P4, grooves of various orientation and depth were visible on the alveolar surface subdividing the primary alveolar saccules (Fig. 3A,B). Most grooves contained smooth muscle actin-expressing cells, consistent with them being the myofibroblast-associated secondary septa (Herzog et al., 2008). All the grooves matched a network of vessels, which persisted even after the myofibroblasts disappeared after P19 (Fig. 3B). Therefore, compared with the transient nature of the myofibroblasts, the vessels are a more consistent and permanent marker of secondary septa. The apparent similarity in the size of the alveolar pocket between P4 and P19 is likely to be due to the formation of secondary septation, despite the increase in total alveolar surface area by 3- to 4 -fold (Table S1). Interestingly, unlike the capillary maturation model (Burri, 1984), all vessels were parallel, instead of perpendicular, to the grooves and therefore did not bend to form a hairpin-like double-layered structure inside the grooves. Double-layered vessels were only observed at the interface between adjacent primary alveolar saccules where the vessel networks associated with individual saccules were juxtaposed (Fig. 3B).

Co-staining of individual folded AT1 cells with vessels revealed that the 'valleys' of AT1 cells matched the vessels and thus were considered to result from secondary septation, while the 'mountains' corresponded to alveoli from subdivided primary saccules (Fig. 3). As a result of cell folding, a single AT1 cell could span multiple alveoli and reach the more centrally located alveolar sac (Fig. 3C, see Fig. 3A for additional examples). Similar cell morphology was observed using the soluble reporter Rosa ${ }^{\text {tdT }}$ (Madisen et al., 2010), which also highlighted the nucleus, thus
confirming that our approach labeled individual cells (Fig. 3C). These data suggest that AT1 cells further expand via a folding process that matches the formation of capillaries and secondary septa (Fig. 3D).

## Developing AT1 cells retain cellular plasticity

Having shown that AT1 cells grow via a non-proliferative two-step process, we sought to determine when and to what extent AT1 cells were committed to this growth process. Since little is known about the genes controlling AT1 cell development, we tested whether AT1 cells could change their fate upon ectopic expression of SOX2, a transcription factor that is normally restricted to the airways and promotes airway differentiation (Gontan et al., 2008; Que et al., 2009; Tompkins et al., 2009, 2011; Alanis et al., 2014). We first used the Scnnla-Cre allele identified in our driver screen that targeted AT1 cells in the flattening stage and were thus committed to terminal differentiation. Although SCNN1A was detected in other cell types than AT1 (Borok et al., 2006; Johnson et al., 2002, 2006; Kimura et al., 2011), the Scnn1a-Cre allele was active in AT1 cells after E19, consistent with the perinatal upregulation of Scnn1a in the distal lung epithelium (Fig. S7D) (Chang et al., 2013), and reached a targeting efficiency (defined as the percentage of AT1 cells that were targeted) of $71 \%$ ( $n=153$ cells) and specificity (defined as the percentage of targeted cells that were AT1 cells) of $95 \%$ ( $n=176$ cells from three mice) (Fig. 4A, Fig. S7). As expected from this AT1 cell specificity, SOX2-expressing cells in the Scnn1a-Cre; Rosa ${ }^{\text {Sox2/+ }}$ lung initially had isolated nuclei and the flattened morphology that was demarcated by E-CAD junctions (P2 in Fig. 4B). Strikingly, at P11, mutant cells formed large clusters with a compact cell arrangement reminiscent of that of the airways (Fig. 4B). Mutant cell clusters remained largely monolayered surrounding the airspace and thus appeared less dramatic in section views (e.g. Fig. 4F).

Compared with control lungs or adjacent normal AT1 cells in the same lung, targeted AT1 cells maintained a normal level, albeit diffuse pattern, of HOPX expression, but downregulated other AT1 cell markers including RAGE, upregulated the proliferation marker KI67 ( $26 \%$ of $n=191$ cells at P11; none in control AT1 cells; three mice in each group), and expressed an isoform of P63 (TRP63) specific for basal cells ( $52 \%$ of $n=305$ cells at P11; none in control AT1 cells; three mice in each group) (Fig. 4C-E, Fig. S8A). However, only occasional mutant AT1 cells expressed additional basal or other airway cell markers, including KRT5, KRT14 and CCSP (SCGB1A1) (Fig. S8A), suggesting that the high level of SOX2 expression from the Rosa ${ }^{S o x 2}$ allele did not support physiological airway differentiation. In contrast to prior reports (Ikeda et al., 1995; Zhou et al., 1996), NKX2.1, a lung lineage transcription factor, was expressed by both AT1 and AT2 cells, and frequently lost its nuclear localization in mutant AT1 cells (Fig. 4F). This was associated with upregulation of MUC5AC (Fig. 4G, Fig. S8A), reminiscent of the gene expression changes associated with the formation of mucinous adenocarcinomas upon loss of NKX2.1 (Winslow et al., 2011; Maeda et al., 2012; Snyder et al., 2013). These gene expression changes were confirmed by transcriptome profiling of FACS-purified control and mutant AT1 cells, which identified additional SOX2-suppressed AT1 cell markers (Fig. 4G, Fig. S9, Table S2).

Although Scnn1a-Cre targeted occasional AT2 cells, such leaky targeting (5\%) could not account for the high percentage of mutant AT1 cells expressing KI67 (26\%) and P63 (52\%) and the robust clustering of mutant cells (Fig. 4B). To further substantiate the observed AT1 cell plasticity, we activated Rosa $a^{S o x 2}$ with an independent Cre allele, Aqp $5^{\text {Cre }}$ (Flodby et al., 2010), which


Fig. 5. Aqp $5^{\text {Cre }}$-driven ectopic SOX2 expression reprograms flattened AT1 cells. (A) Confocal projection images of immunostained Aqp5 ${ }^{\text {Cre/+ }} ;$ Rosa $^{\text {Sox2/+ }}$ lung strips at the indicated stages. Some mutant AT1 cells (arrowhead) have retracted their cellular extensions and accumulate a high level of E-CAD at P3. Mutant cell clusters (arrow) are present at P8. (B) Confocal images of sections from littermate control and mutant lungs showing comparable phenotypes of AT1 cells targeted with $A q p 5^{\text {Cre }}$ to those targeted with Scnn1a-Cre (Fig. 4). Mutant AT1 cells (GFP, arrowhead) form clusters, downregulate RAGE (boxed region shown as single-channel images in insets), express KI67 and DNp63 (deltaNp63), and have diffuse (arrow) or nuclear (solid arrowhead) NKX2.1 (single-channel image shown in inset). NKX2.1 expression in AT2 cells (open arrowhead, cuboidal E-CAD staining) of the mutant lung is not affected. KI 67 is expressed in non-AT1 cells in the control lung (Fig. S3A). Scale bars: $10 \mu \mathrm{~m}$.
targeted AT1 cells with an efficiency of $89 \%$ ( $n=329$ cells) and a specificity of $99 \%$ ( $n=317$ cells from three mice) (Fig. S7). Essentially the same phenotypes were observed, including cluster formation over time (Fig. 5A), cell proliferation (KI67, 39\% of $n=161$ cells at P8; none in control AT1 cells; three mice in each group), downregulation of AT1 markers and upregulation of airway markers (P63, $42 \%$ of $n=146$ cells at P8; none in control AT1 cells; three mice in each group) (Fig. 5B, Fig. S8A). SOX2-induced P63 expression was specific to AT1 cells, as Rosa ${ }^{\text {Sox2 }}$ activation in airway cells by Sox2 ${ }^{\text {CreER }}$ (Alanis et al., 2014) or in mesenchymal cells by Tbx4-Cre (Kumar et al., 2014) did not lead to ectopic P63 expression (Fig. S8B,C). Therefore, the overexpression of a single gene is sufficient to reprogram flattened AT1 cells toward the airway cell fate and allow them to proliferate.

## AT1 cells are the source of Vegfa

Interestingly, the angiogenic factor Vegfa was downregulated by 16-fold in SOX2-expressing mutant AT1 cells (Fig. 4G, Table S2), an unexpected change given previous evidence for AT2 cells being the source of Vegfa (Bhatt et al., 2001; Ng et al., 2001). To pinpoint the cellular source of Vegfa, we developed a combined fluorescence in situ hybridization and immunostaining protocol and found that the perinuclear localization of the Vegfa mRNA allowed assignment of its expression to AT1 cells, but not AT2 cells (Fig. 6A), consistent with a recent single-cell transcriptome analysis (Treutlein et al., 2014). Vegfa mRNA was present in the alveolar epithelium at E18 and at a lower level in the distal progenitors (Fig. 6A). Notably, Vegfa expression was lost in SOX2-expressing mutant AT1 cells, whereas the AT2 cell marker Sftpc was not affected (Fig. 6B,C). This was accompanied by a simplified vascular network, as determined using the endothelial marker ICAM2, which labels both cell junctions and cell surface (Halai et al., 2014), and a lower, albeit variable, density of endothelial cells as determined using the endothelial nuclear marker ERG (Birdsey et al., 2015) (three mice in each group;

Fig. 6C). These results suggested that normal AT1 cell development is required for alveolar angiogenesis.

## Mature AT1 cells retain cellular plasticity

Lastly, we examined the plasticity of mature AT1 cells by activating Rosa ${ }^{\text {Sox } 2}$ with the inducible $H_{o p x}{ }^{C r e E R}$ allele. Hopx ${ }^{C r e E R}$-induced recombination was almost exclusive to AT1 cells after 5 weeks, with an efficiency of $21 \%$ and specificity of $98 \%$ ( $n=248$ cells from three mice) using Rosa ${ }^{m T m G}$ (Fig. 7A). When Ros $a^{\text {Sox2 }}$ was activated at least 5 weeks after birth in AT1 cells that had completed the folding process, mutant AT1 cells fully retracted their cellular extensions within 21 days and therefore were much smaller than their normal counterparts (Fig. 7A). This difference was better demonstrated when comparing control and mutant AT1 cells in the same lung, taking advantage of the lower recombination efficiency of the Rosa ${ }^{\text {Sox2 }}$ allele compared with the Rosa ${ }^{m T m G}$ allele (Fig. S10A).

Although a subset of mutant AT1 cells $(11 \%, n=72$ cells from three mice) expressed KI67 at a given time (Fig. S10B), over time the majority of targeted AT1 cells formed doublets (33\%) or clusters ( $53 \% ; n=73$ from three mice) (Fig. 7B). Owing to the low efficiency of Hopx $x^{\text {CreER }}$ in recombining the Rosa ${ }^{S o x 2}$ allele, individual mutant doublets or clusters were isolated from each other (Fig. 7B,C) and thus were considered to originate from a single targeted AT1 cell. All mutant cells, including singlets, accumulated a high level of ECAD (Fig. 7B). Most mutant AT1 cells, including singlets (81\%, $n=43$ cells from three mice), activated the cell cycle gene Ccndl, a putative direct target of SOX2 (Chen et al., 2008) (Fig. 7C, Fig. S10C). Such activation occurred 9 days after recombination, when mutant cells still had a single nucleus and elaborate cellular extensions and began to accumulate excessive E-CAD (Fig. 7C). Although NKX2.1 became diffuse, AT1 cell markers were maintained and no airway markers, such as P63, CCSP, FOXJ1 and MUC5AC, were observed in mutant AT1 cells (Fig. S10A,D). Therefore, compared with those in the flattening stage, AT1 cells


Fig．6．AT1 cells are the source of Vegfa．（A）Combined fluorescence in situ hybridization and immunostaining of wild－type lung sections．（Top）Vegfa RNA localizes to oval－shaped AT1 cell bodies that are positive for NKX2．1 and HOPX（solid arrowheads），but not to LAMP3－positive cuboidal AT2 cells （open arrowhead）．（Bottom）The left panel shows，as a technical control，that Sttpc RNA localizes to cuboidal AT2 cells that are positive for SFTPC and LAMP3．Boxed regions are enlarged in subsequent panels．To the right is a colorimetric in situ hybridization of an E18 lung section near the tissue edge showing Vegfa RNA in the alveolar epithelium（arrow，bronchoalveolar duct junction）and，at a lower level，the distal progenitors（open arrow）．The epithelium is traced with a dashed line． （B）Section colorimetric in situ hybridization（left two columns）and confocal projection images of immunostained strips（right two columns）from littermate control and Scnn1a－Cre；Rosa ${ }^{\text {Sox2／＋}}$ lungs at the indicated stages．Vegfa expression （solid arrowheads）is lost in mutant AT1 cells，whereas Sftpc expression（open arrowheads）is not affected．Asterisks indicate regions with fewer vessels in the mutant．（C）Section colorimetric in situ hybridization（left）and confocal projection images of immunostained strips（right two columns）from littermate control and Aqp5 ${ }^{\text {Cre／＋}}$ ；Rosa ${ }^{\text {Sox2／＋}}$ lungs at the indicated stages．The numbers indicate the vessel areas（ $P=0.02$ ， Student＇s $t$－test）and the number of endothelial cells（ $P=0.1$ ，Student＇s $t$－test）for three image fields of $318 \times 318 \times 20 \mu \mathrm{~m}$ ．Scale bars： $10 \mu \mathrm{~m}$ ．


Fig. 7. Mature AT1 cells retain cellular plasticity. (A) Stereomicroscopy images of lung strips 21 days after recombination induced at P56 showing a robust response of individual AT1 cells (arrowheads) to ectopic SOX2 expression by retracting their cellular extensions. Different doses of tamoxifen were used to achieve comparable recombination efficiency of the Rosa ${ }^{m T m G}(0.4 \mathrm{mg})$ and Rosa ${ }^{\text {Sox2 }}$ ( 4 mg daily for three consecutive days) alleles. (B) Confocal projection images of immunostained lung strips at the indicated days after recombination induced at P35. Boxed regions are enlarged in subsequent panels showing that mutant AT1 cells lose extended morphology (green outline at 4 days; arrowhead indicates another mutant cell) and form clusters, doublets or singlets (21 days). The total numbers of each type examined are shown in the bar chart. All singlets examined at 4 days after recombination have extended morphology, indicating their AT1 cell identity. Mutant AT1 cells including singlets have retracted their cellular extensions (surface rendering shown) and accumulate a high level of E-CAD.
(C) Confocal images of immunostained lung sections at 9 or 21 days after recombination induced at P56. Boxed regions are shown as single-channel images. Whereas control AT1 cells do not express CCND1 (Fig. S10C), mutant AT1 cells activate CCND1 when they are still singlets (solid arrowhead), retracting their cellular extensions and starting to accumulate E-CAD (punctate staining in inset). Open arrowhead, CCND1expressing AT2 cells. Scale bars: $100 \mu \mathrm{~m}$ in A; $10 \mu \mathrm{~m}$ in B,C.
after the folding process are less plastic in cell fate, but a majority of them ( $86 \%$, doublets and clusters) retain some degree of proliferative potential.

## DISCUSSION

Our results support a model of AT1 cell development whereby AT1 cells normally undergo terminal differentiation via a nonproliferative two-step process-cell flattening and cell folding. However, during and even after this process, AT1 cells maintain proliferative potential and can undergo developmental stagedependent cell reprogramming (Fig. S11). These results demonstrate in vivo the plasticity of a majority of AT1 cells that had previously been suggested in isolated cells in vitro (Williams, 2003; Gonzalez et al., 2009). Such plasticity is consistent with a
recent study showing that, at a low frequency, AT1 cells may convert to AT2 cells and proliferate upon pneumonectomy or oncogenic Kras expression (Jain et al., 2015). Additional work is necessary to understand whether and how AT1 cells retract their cellular extensions when converting to AT2 cells. In addition, our study has identified a list of genes suppressed by SOX2 expression (Table S2) that may control the convoluted morphology of AT1 cells or serve as signaling molecules (e.g. VEGFA) underlying the physical association between AT1 cells and endothelial cells and myofibroblasts.

This study provides insights into the cell biology of perinatal lung cell maturation, a process that is disrupted in premature birth, as well as new imaging and genetic tools for the further study of this process. Given that AT1 cells constitute $>95 \%$ of the alveolar
surface area, the cellular changes of AT1 cells are expected to reflect how the tubular epithelium generated by branching morphogenesis is transformed into a honeycomb-like alveolar epithelium during the saccular and alveolar stages of lung development (Yang and Chen, 2014). According to our singlecell morphology analyses, the first flattening step increases epithelial surface to facilitate saccule formation while maintaining an interconnected epithelial sheet. Flattening of AT1 cells initiates behind the SOX9-positive branch tips, at a location where saccules first appear (Chang et al., 2013; Desai et al., 2014). After flattening, individual AT1 cells increase surface area by more than 10 -fold and undergo a folding step that matches with the formation of the honeycomb-like mature alveoli, resulting in a single AT1 cell spanning multiple alveoli.

Our multicolor 3D imaging of intact alveolar tissues suggests that, besides myofibroblasts and vessels, AT1 cells are an integral component and a potential regulator of secondary septation. On sections, secondary septation appears as club-like inward growth of the saccule wall, which in 3D corresponds to grooves running in different directions along the saccule surface made of a single folded AT1 cell. On sections, the septal tips often contain smooth muscle actin-expressing myofibroblasts, which in 3D correspond to fibrous smooth muscle bundles embedded in the aforementioned grooves. Conceivably, these smooth muscle bundles might function as a 'belt' to limit structural changes locally while the rest of the saccule wall expands as the lung grows in volume. If true, the septa might not in fact grow inward but only appear to do so as the result of the relative outgrowth of the non-septa regions. Vessels are associated with the grooves as early as myofibroblasts and remain associated after the myofibroblasts disappear. This raises the intriguing possibility that septa formation might be driven and/or stabilized by vessels, while myofibroblasts might be required to overcome greater tissue mechanical resistance during septa initiation. Notably, as judged by 3D analysis, all vessels run in parallel, instead of perpendicular, to the grooves. We do not observe the double capillaries that have previously been suggested to form via hairpinlike bending of single capillaries into the septa (Burri, 1984). Further studies are necessary to test whether the previously reported double capillaries are juxtaposed vessels that are associated with two primary saccules (Fig. 3B). Given the coordination among AT1 cells, myofibroblasts and vessels, it is tempting to speculate that AT1 cells might play a signaling role in addition to their structural role during secondary septation. Indeed, our results show that AT1 cells, instead of AT2 cells, are the source of the key alveolar angiogenic factor VEGFA (Kasahara et al., 2000; Stenmark and Abman, 2005). Future genetic experiments should reveal whether AT1 cells signal to the endothelial cells by secreting angiogenic factors, such as VEGFA, and/or by forming permissive matrix substrates.

Although AT1 cells are traditionally viewed as terminally differentiated (Williams, 2003), our study shows that, at a high frequency, flattened AT1 cells undergoing terminal differentiation can be reprogrammed toward the airway fate and proliferate, and that fully differentiated AT1 cells can retract their elaborate cellular extensions and proliferate. As AT1 cells require the overexpression of a single gene to activate proliferation, it is tempting to speculate that AT1 cells might also serve a stem cell function or as the cell-oforigin for a subset of lung cancers, both of which have been shown for AT2 cells (Barkauskas et al., 2013; Desai et al., 2014). Given the substantial difference between AT1 and AT2 cells, the corresponding tumors may have distinct molecular signatures and genetic alterations. A deeper understanding of the tumorigenic potential of AT1 cells, as well as of the mechanisms normally
restricting their proliferation, might implicate AT1 cells as a new cellular source for lung repair and lung cancer.

## MATERIALS AND METHODS

Mice
The following mouse (Mus musculus) strains were used: RosamTmG (Muzumdar et al., 2007), Rosa ${ }^{R G}$ (Shioi et al., 2011), Rosa ${ }^{n T n G}$ (Prigge et al., 2013), Rosa ${ }^{\text {Sox2 }}$ (Lu et al., 2010), Rosa ${ }^{\text {tdT }}$ (Madisen et al., 2010), Shh ${ }^{\text {Cre }}$ (Harfe et al., 2004), Hopx ${ }^{\text {CreER }}$ (Takeda et al., 2011), Sox2 ${ }^{\text {CreER }}$ (Arnold et al., 2011), Sox $9^{\text {CreER }}$ (Soeda et al., 2010), Scnnla-Cre (JAX stock number 009613), Tbx4-Cre (Kumar et al., 2014), Aqp $5{ }^{\text {Cre }}$ (Flodby et al., 2010), Krt8CreER (Van Keymeulen et al., 2011), Krt18-CreER (Van Keymeulen et al., 2009), Krt14-Cre (Dassule et al., 2000), Cldn $6^{\text {CreER }}$ (Anderson et al., 2008) and $N k x 2.1^{\text {CreER }}$ (Taniguchi et al., 2011). The amount of tamoxifen to achieve the desired level of recombination was determined empirically, as detailed in the supplementary Materials and Methods.

## Marker-based stereology

Lungs were inflation fixed at $25 \mathrm{~cm} \mathrm{H}_{2} \mathrm{O}$ pressure with $0.5 \%$ paraformaldehyde (P6148, Sigma) in phosphate-buffered saline (PBS, pH 7.4). The left lobes were cryosectioned exhaustively at $60 \mu \mathrm{~m}$ with one in every 15 sections collected for whole-mount immunostaining. STEPanizer (Tschanz et al., 2011) was used to obtain lung volume and alveolar surface area. The number of AT1 and AT2 cells in the entire stack $\left(N_{v}\right)$ was directly counted using Imaris (Bitplane). See the supplementary Materials and Methods for details.

## Whole-mount and section immunostaining

Immunostaining was performed following published protocols (Chang et al., 2013; Alanis et al., 2014). Cell and tissue surface rendering was generated in Imaris with default settings: smoothing with surface area detail level at $0.621 \mu \mathrm{~m}$ and thresholding by local contrast at $2.33 \mu \mathrm{~m}$. For further details, including the antibodies used, see the supplementary Materials and Methods.

## Fluorescence and colorimetric in situ hybridization

Colorimetric section in situ hybridization was carried out following published protocols (Chang et al., 2013; Alanis et al., 2014). For fluorescence in situ hybridization, sections were incubated with $0.05 \mu \mathrm{~g} / \mathrm{ml}$ riboprobes and a fluorescein tyramide signal amplification system (PerkinElmer, NEL741001KT) was used to detect the hybridized riboprobes. See the supplementary Materials and Methods for details.

## Transcriptome profiling of FACS-purified AT1 cells

Cell dissociation and purification were performed based on a previous protocol with modifications as detailed in the supplementary Materials and Methods (Chang et al., 2013). RNA was extracted from at least $10^{5}$ purified cells using Trizol reagents (Invitrogen, 15596018) and an RNeasy Micro Kit (Qiagen, 74004). RNAseq libraries were prepared using an mRNA isolation kit (New England BioLabs, E7490) and a NEBNext Ultra RNA Library Prep Kit (New England BioLabs, E7530S) and were sequenced on an Illumina HiSeq2000. Approximately $35-80$ million 76 nt pair-end reads were generated for each sample and analyzed using standard tophat2, bowtie2 and cufflinks modules in R. The raw data were deposited in GEO under accession number GSE73861.

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## Competing interests

The authors declare no competing or financial interests.

## Author contributions

J.Y., B.J.H., D.M.A. and J.C. designed research; J.Y., B.J.H., D.M.A., O.N., L.V.-E. and E.J.O. performed research; E.J.O. analyzed the transcriptome data; H.A. provided the Sox $9^{\text {CreER }}$ mice; S.E.E. provided mice; J.C. and E.J.O. wrote the paper; all authors read and approved the paper.

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## Supplementary information

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## Supplementary Materials and Methods

## Mice (Mus musculus)

The day on which a vaginal plug was observed was designated as E1. The day on which mice were born was designated as P0. For the CreER driver screen, 2 mg tamoxifen (T5648, Sigma) dissolved in corn oil (C8267, Sigma) was injected intraperitoneally 2 days before harvest except that 60 ug tamoxifen was injected in P2 Nkx2.1 ${ }^{\text {CreER }}$ and Hopx ${ }^{\text {CreER }}$ mice. For sparse cell labeling experiments, the tamoxifen dosage was determined empirically for each time point. For Sox $9^{\text {CreER }}, 250$ ug tamoxifen was injected intraperitoneally at E13 for E19 harvest; 0.4 mg (low dose) and 3.5 mg (high dose) tamoxifen was given via oral gavage at E19 for P20 harvest (Fig. S2B). For Hopx ${ }^{C r e E R}, 0.4 \mathrm{mg}$ tamoxifen was given via oral gavage at E18 and E19 for E19 and postnatal harvest, respectively; postnatally, $7.5 \mathrm{ug}, 10 \mathrm{ug}, 25 \mathrm{ug}, 100 \mathrm{ug}$ and 400 ug tamoxifen injected intraperitoneally was determined to label isolated AT1 cells at P1, P3, P7, P14 and P24, respectively. Although at the same genomic locus, the Rosa ${ }^{S o x 2}$ allele was activated much less efficiently than the Rosa ${ }^{m T m G}$ allele by $H o p x^{C r e E R}$ and required daily gavage of 4 mg tamoxifen for three days after P35. For Sox2 ${ }^{\text {CreER }}, 2 \mathrm{mg}$ tamoxifen was injected intraperitoneally at P28 for P49 harvest. All animal experiments were approved by the institutional animal care and use committee at Texas A\&M Health Science Center Institute of Biosciences and Technology (protocol number 2014-0001-IBT and 2014-0002-IBT).

## Marker-based stereology

Inbred FVB/NJ (Stock 001800, Jackson Laboratory) mice of mixed gender were anaesthetized with Avertin (T48402, Sigma) and placed in the supine position. The trachea was cannulated with blunt needles of the appropriate size: 25 G (B25-50, SAI) for P0-P6, 23 G (B23-50, SAI) for P7-P14, 20 G (B20-50, SAI) for >P14. The descending aorta was cut and the rib cage partially removed. After perfusion through the right ventricle with phosphate-buffered saline (PBS, pH 7.4), the lung was inflated with $0.5 \%$ paraformaldehyde (PFA; P6148, Sigma) in PBS at 25 cm $\mathrm{H}_{2} \mathrm{O}$ pressure. After 5 min , the trachea was tied to maintain the pressure and the lung was carefully dissected and submersion fixed in $0.5 \%$ PFA in PBS on a rocker at room temperature for $4 \sim 6 \mathrm{hr}$. The lung was subsequently washed in PBS at $4^{\circ} \mathrm{C}$ overnight with the trachea cut to facilitate diffusion. This fixation protocol was compatible with fluorescence immunostaining for most antibodies and achieved fixation stability because the lung volume measured by the fluid displacement method (Scherle, 1970) was largely unchanged between immediately after fixation and after overnight PBS wash (Fig. S1B).

The left lobe, constituting $\sim 1 / 3$ of the total lung at all postnatal stages (Fig. S1D), was trimmed free of extra-pulmonary tissue and cryoprotected in 20\% sucrose in PBS and 33\% optimal cutting temperature compound (OCT; 4583, Tissue-Tek) at $4^{\circ} \mathrm{C}$ overnight and then embedded in OCT with its long axis perpendicular to the section plane. As illustrated in Fig. S1A, the left lobe was exhaustively sectioned at 60 um on a calibrated cryomicrotome (CM1850, Leica), with one in every 15 sections collected, resulting in a section interval of 900 um. To avoid dehydration and therefore vertical tissue shrinkage from section immunostaining, the sections were transferred to PBS to dissolve OCT and the tissue was processed for wholemount immunostaining as described below.

After staining, the tissue was fixed with 2\% PFA in PBS at room temperature for 2 hr and mounted under 22 mm square cover slips (48366067, VWR) that were spaced from the slide with tapes. Care was taken to avoid tissue folding during mounting. Confocal imaging verified that the mounted tissue maintained its original 60 um thickness. The tissue was first imaged at a low magnification with a stereomicroscope (M205C, Leica). The total area and airway/major vessel area were measured using STEPanizer (Tschanz et al., 2011) with at least 150 points counted for each lobe and the volume calculated using Cavalieri's principle. STEPanizer was used to systematic uniform randomly select 5~7 fields from all tissue sections of each lobe. The selected fields were located with a 10x objective and then imaged with a 40x oil objective with a field size of 318 um x 318 um x 30 um and a pixel dimension of $512 \times 512 \times 30$ on a confocal microscope (A1plus, Nikon). Fields containing airways or major vessels were not imaged. To avoid surface artifacts from physical sectioning, images were collected several microns below the surface.

The resulting image stack was visualized using Imaris (Bitplane) and the number of AT1 and AT2 cells in the entire stack ( $N_{v}$ ) was directly counted instead of using a dissector method (Williams and Rakic, 1988; Hsia et al., 2010). To account for edge effects, only cells with at least half of their nuclei visible were counted. Section view was used to resolve ambiguity in cell identification. Results from repeated counting vary by less than 5\%. To measure alveolar surface area, the $10^{\text {th }}$ section in each confocal stack was quantified using the line probes in STEPanizer with the intercepts assigned to AT1 or AT2 cells based on HOPX and E-Cadherin staining.

## Whole mount and section immunostaining

Immunostaining were performed following published protocols with minor modifications (Chang et al., 2013; Alanis et al., 2014). For section immunostaining, OCT sections at 10 um thickness were blocked in PBS with $0.3 \%$ Triton X-100 and 5\% normal donkey serum (017-000-121, Jackson ImmunoResearch) and then incubated with primary antibodies diluted in PBS with $0.3 \%$ Triton X-100 in a humidified chamber at $4{ }^{\circ} \mathrm{C}$ overnight. The sections were washed with PBS in a coplin jar for 1.5 hr and incubated with secondary antibodies and 4',6-diamidino-2phenylindole (DAPI) diluted in PBS with $0.3 \%$ Triton X-100 at room temperature for 2 hr . After another 1.5 hr wash with PBS, the sections were mounted with Aquamount (18606, Polysciences).

For whole mount immunostaining, 60 um frozen sections prepared as described above or 1~2 mm wide strips cut from around the edge of cranial and left lobes (Fig. 7A) were blocked in 2 mL Eppendorf tubes containing PBS with $0.3 \%$ Triton X-100 and 5\% normal donkey serum and then incubated with primary antibodies diluted in PBS with $0.3 \%$ Triton X-100 on a rocker at $4^{\circ} \mathrm{C}$ overnight. The following day, the tissues were washed in PBS with $1 \%$ Triton X-100 and $1 \%$ Tween-20 for 3~6 hr and incubated with secondary antibodies diluted in PBS with $0.3 \%$ Triton X-100 on a rocker at $4^{\circ} \mathrm{C}$ overnight. The third day, the tissues were washed as before and fixed with $2 \%$ PFA in PBS for 2~3 hr. For tissues expressing green or red fluorescent protein, the native fluorescence but not that from secondary antibodies was quenched after immunostaining by incubation with methanol with 6\% hydrogen peroxide (H1009, Sigma) at $4^{\circ} \mathrm{C}$ overnight. Lobe strips were used to preserve the alveolar structure and whole AT1 cells and were thin enough for high resolution imaging with a 40x oil objective. Cell and tissue surface rendering was generated in Imaris with default settings: smoothing with surface area detail level at 0.621 um and thresholding by local contrast at 2.33 um . Vessel areas were measured using a threshold of 60 and a volume cut-off of 50 for both control and mutant lungs.

## Antibodies

The following antibodies were used: chicken anti-green fluorescent protein (GFP, 1:1,000, AB13970, Abcam), rabbit anti-HOPX (1:500, sc-30216, Santa Cruz Biotechnology), rat anti-ECadherin (ECAD, 1:2000, 131900, Invitrogen), rabbit anti-Ki67 (1:1000, RM9106S0, Thermofisher), rat anti-Ki67 (1:1000, 14-5698-80, eBioscience), rabbit anti-Krt5 (1:1000, RM2106S0, Thermofisher), rabbit anti-Krt14 (1:1000, RB9020P0, Thermofisher), goat antiCCSP (1:2500, a gift from Dr. Barry Stripp), rabbit anti-CCND1 (1:1000, RM9104S0,

Thermofisher), rabbit anti-FOXJ1 (1:1000, HPA005714, Sigma), chicken anti-MUC5AC (1:1000, a gift from Dr. Samuel Ho), goat anti-SRY-box-containing gene 9 (SOX9, 1:1,000, AF3075, R\&D Systems), rabbit anti-prosurfactant protein C (SFTPC, 1:1,000, AB3768, Millipore), rat antiRAGE (1:1,000, MAB1179, R\&D Systems), goat anti-Podocalyxin-like (PODXL, 1:250, AF1556, R\&D Systems), rabbit anti-ZO1 (1:50, 187430, Invitrogen), rabbit anti-collagen type IV (COL4, 1:5000, LSL-LB-1403, CosmoBioUSA), Cy3-conjugated mouse anti-smooth muscle actin (SMA, 1:1000, C6198, Sigma), rabbit anti-Aquaporin 5 (AQP5, 1:2500, ab78486, Abcam; non-specific staining was observed in embryonic lungs), rat anti-Intercellular adhesion molecule 2 (ICAM2, 1:2500, 14-1021-80, eBioscience), rabbit anti-Avian erythroblastosis virus E-26 (v-ets) oncogene related (ERG, 1:5000, ab92513, Abcam), rat anti-Lysosomal-associated membrane protein 3 (LAMP3, 1:1000, DDX0192, Imgenex), goat anti-SRY-box-containing gene 2 (SOX2, 1:250, sc-17320, Santa Cruz Biotechnology), rabbit anti-deltaNp63 (DNp63, 1:1000, 619001, Biolegend) and rabbit anti-p63 (1:250, sc-8343, Santa Cruz Biotechnology). Fluorescent secondary antibodies were obtained from Invitrogen or Jackson ImmunoResearch and used at 1:1000. Apoptotic cells were detected with a TUNEL (terminal deoxynucleotidyl transferasemediated dUTP nick end labeling) kit (11684795910, Roche) following the manufacturer's protocol.

## Fluorescence and colorimetric in situ hybridization

Postnatal lungs were harvested and processed as described for section immunostaining except $0.5 \%$ PFA was included for the sucrose/OCT overnight incubation to minimize RNA degradation. Colorimetric section in situ hybridization was carried out following published protocols (Chang et al., 2013; Alanis et al., 2014). For fluorescence in situ hybridization, sections were incubated with $1 / 20$ of the amount of riboprobes ( $0.05 \mathrm{ug} / \mathrm{mL}$ ). After washing with 0.2 X SSC (sodium chloride and sodium citrate), sections were incubated with PBS with $3 \%$ hydrogen peroxide for 1 hr at room temperature to quench the endogenous peroxidase activity. An anti-digoxigenin POD Fab fragment (1:2000, Roche 11207733910) and a fluorescein tyramide signal amplification system (NEL741001KT, Perkin Elmer) were used to detect the hybridized riboprobes. The biotin tyramide signal amplification system (NEL749A001KT, Perkin Elmer) was not used due to the presence of endogenous biotin in AT2 and club cells (Kuhn, 1988). Subsequently, sections were immunostained for SFTPC, LAMP3, HOPX or NKX2.1 antibodies as described above. The Vegfa riboprobe spanned exon 2 to 6 and detected all Vegfa isoforms (Ng et al., 2001). The peri-nuclear localization of the Vegfa mRNA allowed unequivocal
identification of its cellular source. Control and mutant samples were processed on the same section to minimize technical variations.

## Transcriptome profiling of FACS purified AT1 cells

Cell dissociation and purification was performed based on a previous protocol with modifications (Chang et al., 2013). Briefly, postnatal day 3 or 4 Scnn1a-Cre; Rosa ${ }^{m T m G /+}$ and Scnn1a-Cre; Rosa ${ }^{\text {Sox2/t }}$ littermate lungs were dissected in PBS. Around 3 mm wide strips were cut along the lobe edge, minced into pieces with forceps and digested in PBS with $2 \mathrm{mg} / \mathrm{mL}$ collagenase type I (Worthington, CLS-1) and $0.2 \mathrm{mg} / \mathrm{mL}$ DNase I (Worthington, D) for 40 min at $37^{\circ} \mathrm{C}$. An equal volume of $0.25 \%$ Trypsin/EDTA (Invitrogen, 25200-056) was added and incubated for another 10 min at $37^{\circ} \mathrm{C}$. Fetal bovine serum (FBS, Invitrogen, 10082-139) was added to a final concentration of $5 \%$ and the tissue was triturated for $40 \sim 50$ times. Dissociated cells were spun down at 6000 rpm for 1 min , resuspended in red blood cell lysis buffer ( $15 \mathrm{mM} \mathrm{NH} 4 \mathrm{Cl}, 12 \mathrm{mM}$ $\mathrm{NaHCO}_{3}, 0.1 \mathrm{mM}$ EDTA, pH 8.0) for 3 min , washed with PBS with $1 \%$ FBS, SYTOX-red (Invitrogen, S34859) added to exclude dead cells and sorted on a BD Biosciences Influx sorter.

RNA was extracted from at least $10^{5}$ purified cells using Trizol reagents (Invitrogen, 15596018) and an RNeasy Micro kit (Qiagen, 74004). 100~200 ng total RNA was used to prepare an RNAseq library using an mRNA isolation kit (New England BioLabs, E7490) and a NEBNext Ultra RNA library prep kit (New England BioLabs, E7530S) with a final double ( 0.65 x $-1 \times$ bead volume) size selection step using a SPRIselect reagent kit (Beckman Coulter, B23318). The libraries were bar-coded (New England BioLabs, E7335S) and sequenced on an Illumina HiSeq2000. 35~80 million 76 nucleotide pair-end reads were generated for each sample and aligned to the UCSC mm10 reference genome using tophat2 and bowtie2 in R (Langmead and Salzberg, 2012; Kim et al., 2013). Transcript abundance, differential expression, and isoform quantitation were calculated using the cufflinks suite in $R$ from each pair of littermate samples or pooled control and mutant samples (Roberts et al., 2011a; Roberts et al., 2011b; Trapnell et al., 2012; Trapnell et al., 2013).





G

| Year | Author | $\mathrm{V}\left(\mathrm{cm}^{3}\right)$ | $\mathrm{S}\left(\mathrm{cm}^{2}\right)$ | $\mathrm{AT} 1\left(10^{6}\right)$ | $\mathrm{AT} 2\left(10^{6}\right)$ |  |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| 1992 | Stone | 0.65 | 500 | 11.6 | 14.8 |  |  |  |  |  |  |
| 2004 | Ochs | 1.21 | 1001 |  | 13.0 |  |  |  |  |  |  |
| 2005 | Jung |  |  |  | 15.3 |  |  |  |  |  |  |
| 2007 | Knudsen | 1 | 597.7 |  | 12.6 |  |  |  |  |  |  |
| 2009 | Knudsen | 0.68 | 575.3 |  | 7.7 |  |  |  |  |  |  |
| 2012 | Jung | 0.71 | 547 |  | 5.3 |  |  |  |  |  |  |
| 2014 | Knudsen | 1.17 | 734.7 |  | 9.7 |  |  |  |  |  |  |
| This study |  |  |  |  |  |  | Yang | 0.39 | 336 | 7.2 | 18.9 |

Fig. S1. Marker-based stereology. (A) Schematics of the marker-based stereology method. After volume measurement using the fluid displacement method, systematic uniform random sampling (SURS) is applied to the left lung by exhaustive sectioning at 60 um. One in every 15 sections is collected and whole mount (WM) immunostained to avoid dehydration. Tissue volume ( $V$ ) is obtained using STEPanizer software and Cavalieri's principle. AT1 and AT2 cell densities ( $N v$ ) are obtained by counting HOPX and ECAD expressing cells in confocal Z-stacks using Imaris software. (B) Lung volumes measured by the fluid displacement method change minimally immediately or 24 hr after fixation indicating fixation stability. The intercept is set as 0 in the linear regression analysis and R denotes the correlation coefficient (same for (C) and (D)). (C) Left lung volumes measured by the fluid displacement method correlate linearly with those measured by Cavalieri's principle using STEPanizer. The volumes shrink by ~23\% during embedding, sectioning and immunostaining. (D) Right lung volumes are twice left lung volumes as measured
by the fluid displacement method. (E) Confocal projection images of immunostained P17 lung strips showing that cuboidal ECAD-expressing cells (open arrowhead) express SFTPC. The boxed region is enlarged. Scale: 10 um . (F) Cell counting results are comparable using marker-based stereology or optical dissector method (Hsia et al., 2010). AT1 cells in six 30-um image stacks from P54 lungs are counted using the optical dissector method at an interval of 3, 6 or 9 um. The results are plotted against the numbers calculated as a fraction of the total cell number using markerbased stereology (Table S1). The diagonal line is drawn. (G) Comparison of results from our method and those in PubMed searched using "mouse lung stereology" as keywords (Stone et al., 1992; Ochs et al., 2004; Jung et al., 2005; Knudsen et al., 2007; Knudsen et al., 2009; Jung et al., 2012; Knudsen et al., 2014). Cell numbers were counted on single plane images and corrected by a shape factor by Stone et al. Measurements of P54 left lobes (Table S1) are multiplied by 3 (Fig. S1D) to represent those of the entire lung. V: lung parenchyma volume; S: total alveolar surface area.


Fig. S2. AT1 cells are descendants of SOX9 progenitors. (A) Confocal projection images of immunostained lung strips at indicated embryonic and postnatal stages showing that SOX9-expressing progenitors are distal to HOPXexpressing AT1 cells and are present at P7. Scale: 10 um. (B) Confocal images of immunostained P20 lung strips that have been lineage-labeled at E19 (Tam, tamoxifen). Most alveolar cells near the lobe edge are labeled at a high dose (left), whereas a cluster of 1 AT1 cell and 8 AT2 cells are labeled at a low dose (right, nuclei rendered with Imaris). Scale: 10 um.


|  | HOPX $^{+}$KI67 $^{+} /$HOPX $^{+}$ | SFTPC $^{+}$KI67 ${ }^{+} /$SFTPC $^{+}$ |
| :---: | :---: | :---: |
| P4 | $0 / 70(0 \%)$ | $35 / 99(35 \%)$ |
| P8 | $0 / 32(0 \%)$ | $37 / 149(25 \%)$ |
| P14 | $0 / 46(0 \%)$ | $17 / 165(10 \%)$ |
| P21 | $0 / 21(0 \%)$ | $0 / 120(0 \%)$ |



|  | HOPX $^{+}$TUNEL $+/$HOPX $^{+}$ | ECAD $^{+}$TUNEL + /ECAD |
| :---: | :---: | :---: |
| P4 | $0 / 49(0 \%)$ | $0 / 107(0 \%)$ |
| P8 | $0 / 42(0 \%)$ | $0 / 133(0 \%)$ |
| P14 | $0 / 35(0 \%)$ | $1 / 118(0.8 \%)$ |
| P21 | $0 / 42(0 \%)$ | $0 / 175(0 \%)$ |

Fig. S3. Epithelial cell proliferation and apoptosis. (A) Confocal images of lung sections showing KI67 positive AT2 cells (filled arrowhead) and KI67 negative AT1 and AT2 cells (open arrowhead). AT2 cells are labeled with the rabbit anti-SFTPC antibody instead of the rat anti-ECAD antibody so that the same rat anti-KI67 antibody can be used for both AT1 and AT2 cells to allow direct comparison. Scale: 10 um. The number and percentage of KI67 positive AT1 and AT2 cells at indicated postnatal days are tabulated. (B) Confocal images of lung sections containing occasional TUNEL positive cells (arrowhead), which do not co-localize with HOPX or ECAD. Scale: 10 um. The number of percentage of TUNEL positive AT1 and AT2 cells at indicated postnatal days are tabulated.


Fig. S4. AT1 cell morphology during embryonic development. (A) Confocal projection images of immunostained strips from E17 Sox9CreER/+; Rosa ${ }^{m T m G /+}$ lungs with recombination induced at E 13 (Tam, tamoxifen). The boxed region is enlarged on the right. Occasional elongated presumable AT1 cells are found in branch stalks and outlined with green lines and their nuclei marked with black dashed lines. Scale: 10 um . (B) Confocal projection images of immunostained strips from E19 Hopx ${ }^{C r e E R /+}$; Rosa ${ }^{m T m G /+}$ lungs with recombination induced at E 18 (Tam, tamoxifen). Boxed regions are enlarged on the right. HOPX-expressing elongated AT1 cells are indicated with arrowheads or outlined with green lines and the nucleus marked with red dashed lines. They have a similar morphology to those labeled with SoxgCreER (Fig. 2A). Infrequent cuboidal SFTPC-expressing AT2 cells are also labeled (right panels) possibly due to the low diffuse expression of HOPX in the progenitors. Scale: 10 um.


Fig. S5. Interstitial positioning of AT2 cells. Confocal images of immunostained lung sections at indicated postnatal days. AT2 cells (open arrowhead, cuboidal ECAD staining) are in the interstitial regions instead of protruding into the air lumen (L). Scale: 10 um.


Fig. S6. Cre driver screen. Confocal images of immunostained sections from $\operatorname{Krt8-CreER(A),Krt18-CreER~(B),~}$
 induced at indicated stages (Tam, tamoxifen). Occasional epithelial cells are labeled with Krt8-CreER, Krt18-CreER
and $C l d n 6^{C r e E R}$ (filled arrowhead) at all examined stages. No Cldn6 ${ }^{C r e E R}$ activity is observed postnatally. Krt18CreER also labels mesenchymal cells (open arrowhead). Krt14-Cre does not label KRT5-expressing basal cells (open arrowhead), but instead occasional vasculature-associated cells (filled arrowhead). Nkx2.1 ${ }^{\text {CreER }}$ labels most epithelial cells (arrowhead, AT1 cells) at all stages examined. HopxCreER recombination is rare before E15 (filled arrowhead) and is observed in occasional AT2 cells at P2 (open arrowhead). Dashed lines denote conducting airways. Scale: 100 um.


Fig. S7. Scnn1a-Cre and Aqp5 ${ }^{\text {Cre }}$ activity in the lung. Confocal images of immunostained lung sections (A) and strips ( $\mathbf{B}, \mathbf{C}$; projection view). Scnn1a-Cre recombination is rare before E19 (filled arrowhead) and is observed in occasional AT2 cells at P4 (open arrowhead). The nuclear reporter Rosa ${ }^{R G}$ and $R_{\text {os }}{ }^{n T n G}$ allows unambiguous identification of recombined AT2 cells that are surrounded by AT1 cells. Note that HOPX is expressed at a variable level in AT1 cells and therefore gives different shades of yellow when overlapping with GFP. Asterisks denote unrecombined AT1 cells. Dashed lines denote conducting airways. Scale: 100 um (A) and 10 um (B, C). (D) Average fold changes of gene expression showing upregulation of Scnn1a and Aqp5 in FACS-isolated distal epithelial cells at indicated embryonic days. The original data and experimental details are previously reported (Chang et al., 2013).


Fig. S8. Marker analyses of Scnn1a-Cre, Aqp5 ${ }^{\text {Cre }}$, Sox2 ${ }^{\text {CreER }}$ and Tbx4-Cre targeted cells. (A, B) Confocal images of immunostained sections from littermate control and mutant lungs. Mutant AT1 cells (GFP, arrowhead) activate an isoform of P63 (DNp63) that is specific for basal cells. Occasional mutant AT1 cells express additional basal cell markers (KRT5, KRT14) and a club cell marker (CCSP). A ciliated cell marker (FOXJ1) is observed only in Aqp5 ${ }^{\text {Cre }}$ targeted AT1 cells but aberrantly co-expressed with CCSP (inset showing single channel images). A low but detectable level of a goblet cell marker (MUC5AC) is present in mutant AT1 cells, consistent with the RNA-seq data (Fig. 4G and Table S2). Scale: 10 um. (C) A confocal image of immunostained section from the Sox2 ${ }^{\text {CreER/+; }}$ Rosa ${ }^{\text {Sox2/+ lung induced with } 2 \mathrm{mg} \text { tamoxifen (Tam) at P28. No DNp63 cells are observed. Airways are outlined with }}$ dashes. Scale: 10 um. (D) Projection view of a confocal stack of an immunostained Tbx4-Cre; Rosa ${ }^{\text {Sox2/+ lung strip. }}$ No DNp63 cells are observed. Scale: 10 um.


Fig. S9. FACS purification of AT1 cells. Representative FACS plots of dissociated live AT1 cells from P3 littermate Scnn1a-Cre; Rosa ${ }^{m T m G /+}$ (A, control) and Scnn1a-Cre; Rosa ${ }^{S o x 2 /+}$ (B, mutant) lungs. GFP positive cells represent 2~3\% of total cells. Note that GFP expression from Rosa ${ }^{\text {Sox2/+ }}$ is lower than that from Rosa ${ }^{m T m G /+}$ and Tomato expression perdures after recombination.


Fig. S10. Marker analyses of targeted mature AT1 cells. (A) Projection (left) and sectional (right) views of confocal images of immunostained strips from Hopx ${ }^{C r e E R /+}$; Rosa $a^{m T m G / S o x 2}$ lungs at 21 days after recombination induced at P35 (Tam, tamoxifen). Rosa ${ }^{m T m G}$ is easier to recombine than Rosa ${ }^{\text {Sox2; }}$ therefore GFP marks both control (open arrowhead) and SOX2-expressing mutant (filled arrowhead) AT1 cells. No P63 staining is observed. Scale: 10 um. (B) Confocal projection images of immunostained strips at 21 days after recombination induced at P56. The boxed region is shown as single channel images. While absent in control AT1 cells (Fig. S3A), KI67 is activated in a subset of mutant AT1 cells ( $11 \%, n=72$ cells). (C) Confocal images of immunostained sections showing that in a control lung CCND1 is expressed in AT2 cells (open arrowhead, cuboidal ECAD staining), but not AT1 cells (arrowhead). All epithelial cell nuclei are genetically marked by GFP to allow co-staining with the rabbit anti-CCND1 antibody. (D) Confocal images of immunostained strips (top row) and sections (bottom row) at 21 days after recombination induced at P35 (Tam, tamoxifen). AQP5, RAGE and HOPX are mostly unaffected. NKX2.1 is diffuse in mutant AT1 cells (arrow, marked by GFP) as compared to non-targeted AT1 cells (arrowhead) and AT2 cells (open arrowhead). No MUC5AC, CCSP and FOXJ1 are detected in mutant AT1 cells. Scale: 10 um.


Fig. S11. A model summarizing AT1 cell development and plasticity combining Fig. 2C, 3D and the ectopic SOX2 expression experiments. AT1 cells have a potential signaling role by expressing Vegfa.

## Table S1: Quantification of AT1 and AT2 cells at postnatal stages.

The lung volume and total AT1 and AT2 alveolar surface are obtained using STEPanizer. Major vessels are defined as those that parallel airways; therefore the parenchyma volumes include small vessels in the gas exchange region. AT1 and AT2 cell numbers are averaged over image fields and corresponding standard errors calculated. The ratio between AT1 and AT2 cell numbers is calculated for each image field and then averaged. The total cell number in the left lung is calculated by multiplying average cell number per image field ( $0.003037537 \mathrm{uL}, 318.2 \mathrm{um} \times 318.2 \mathrm{um}$ $x 30 \mathrm{um}$ ) by the parenchyma volume. The average AT1 apical surface area is calculated by dividing the total AT1 apical surface area by the total AT1 cell number.

## Click here to Download Table S1

Table S2: Transcriptome comparison of FACS purified littermate control and SOX2-expressing mutant AT1 cells from two biological replicates. Analyses are performed for individual pairs and pooled samples. Significance is defined as false discovery rate by Benjamini-Hochberg procedure (q) $<0.05$.

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