Pulmonary alveolar type I cell population consists of two distinct subtypes that differ in cell fate

Yanjie Wang[a,b], Zan Tang[b,c], Huanwei Huang[b], Jiao Li[b,d], Zheng Wang[b,e], Yuanyuan Yu[a,b], Chengwei Zhang[f], Juan Li[b], Huaping Dai[g], Fengchao Wang[b], Tao Cai[b,1], and Nan Tang[b,1]

[a]College of Life Sciences, Tsinghua University, 100084 Beijing, China; [b]National Institute of Biological Sciences, 102206 Beijing, China; [c]College of Life Sciences, Peking University, 100871 Beijing, China; [d]China Agricultural University, 100083 Beijing, China; [e]Graduate School of Peking Union Medical College, 100730 Beijing, China; [f]Beijing Armed Police General Hospital, 100039, China; and [g]China-Japan Friendship Hospital, 100029, China

Edited by Brigid L. M. Hogan, Duke University Medical Center, Durham, NC, and approved January 31, 2018 (received for review November 7, 2017)

Pulmonary alveolar type I (AT1) cells cover more than 95% of alveolar surface and are essential for the air–blood barrier function of lungs. AT1 cells have been shown to retain developmental plasticity during alveolar regeneration. However, the development and heterogeneity of AT1 cells remain largely unknown. Here, we conducted a single-cell RNA-seq analysis to characterize postnatal AT1 cell development and identified insulin-like growth factor-binding protein 2 (Igfbp2) as a genetic marker specifically expressed in postnatal AT1 cells. The portion of AT1 cells expressing Igfbp2 increases during alveologenesis and in post pneumonectomy (PNX) newly formed alveoli. We found that the adult AT1 cell population contains both Hopx$^+$Igfbp2$^+$ and Hopx$^+$Igfbp2$^-$ AT1 cells, which have distinct cell fates during alveolar regeneration. Using an *Igfbp2*-CreER mouse model, we demonstrate that Hopx$^+$Igfbp2$^+$ AT1 cells represent terminally differentiated AT1 cells that are not able to transdifferentiate into AT2 cells during post-PNX alveolar regeneration. Our study provides tools and insights that will guide future investigations into the molecular and cellular mechanism or mechanisms underlying AT1 cell fate during lung development and regeneration.

pulmonary alveolar type I cells | single cell RNA-seq | alveolar development and regeneration | Igfbp2 | lineage tracing

The pulmonary alveolar epithelium is not only essential for lung gas-exchange function but also functions as an important barrier to protect our body from hazards. In response to acute injuries, pulmonary alveoli are able to quickly repair and regenerate new alveolar epithelial cells for restoring an intact epithelial barrier. The pulmonary alveolar epithelium is mainly composed of two types of epithelial cells: alveolar type I (AT1) and type II (AT2) cells. AT2 cells are smaller, cuboidal cells that are best known for their functions in synthesizing and secreting pulmonary surfactant. In addition, AT2 cells serve as alveolar stem cells and can differentiate into AT1 cells during alveolar homeostasis and post injury repair (1–3). AT1 cells are large squamous cells that cover 95% of the alveolar surface area and form the epithelial component of the thin air–blood barrier (4, 5).

At the late embryonic stage, both AT1 and AT2 cells differentiate from alveolar progenitor cells and form distal epithelial saccules (6, 7). After birth, the epithelial saccules are continuously subdivided into numerous smaller mature gas-exchange units called alveoli. This postnatal developmental process is called alveologenesis, which occurs with 90% of human alveoli and all mouse alveoli (8). During alveologenesis, AT1 cells expand their surface area and flatten their cell body to accommodate postnatal lung growth (9). AT1 cells were traditionally considered to be terminally differentiated cells. However, an exciting recent study found that adult AT1 cells retain cellular plasticity and are able to proliferate and give rise to AT2 cells during post-PNX alveolar regeneration (10).

Although a long series of studies has greatly advanced our knowledge of AT1 cells during alveolar development and regeneration (4, 9–18), we still know little about the molecular genetics and fate specification of AT1 cells. Because of the lack of knowledge of the development and heterogeneity of adult AT1 cell population, it is unclear whether all or only a subset of AT1 cells can transdifferentiate into AT2 cells during alveolar regeneration (9, 10). In addition, AT1 cell development during alveologenesis is still poorly characterized at the transcriptome level because of the difficulty of isolating these fragile cells.

Here, we applied a combination of single-cell RNA-seq analysis, mouse genetics, and alveolar-like organoid cultures and characterized AT1 cell development during both postnatal lung development and alveolar regeneration. We identified a genetic marker of postnatal AT1 cells, insulin-like growth factor-binding protein 2 (Igfbp2), and use this marker to demonstrate that the postnatal AT1 cell population contains two unevenly distributed AT1 cell subtypes, Hopx$^+$Igfbp2$^+$ and Hopx$^+$Igfbp2$^-$ AT1 cells. Igfbp2 is heterogeneously expressed in AT1 cells of newborn lungs and newly differentiated AT1 cells (from AT2 cells) that occur during post-PNX alveolar regeneration. Importantly, we demonstrate that Hopx$^+$Igfbp2$^+$ AT1 cells maintain their AT1 cell fate and do not transdifferentiate into AT2 cells in normal or post injury lungs. Therefore, Hopx$^+$Igfbp2$^+$ AT1 cells represent the terminally differentiated population of AT1 cells.

Significance

Pulmonary alveolar type I (AT1) cells are essential for the gas-exchange function of lungs. AT1 cells retain their cellular plasticity during injury-induced alveolar regeneration. However, we know very little about the developmental heterogeneity of the AT1 cell population. Our study identified a robust genetic marker of postnatal AT1 cells, insulin-like growth factor-binding protein 2 (Igfbp2). We use this marker to demonstrate that the postnatal AT1 cell population actually consists of two AT1 cell subtypes (Hopx$^+$Igfbp2$^+$ and Hopx$^+$Igfbp2$^-$ AT1 cells) with distinct cell fates during alveolar regeneration. The large majority of adult AT1 cells expresses Igfbp2 and cannot transdifferentiate into AT2 cells during post pneumonectomy formation of new alveoli. Therefore, Hopx$^+$Igfbp2$^+$ AT1 cells represent the terminally differentiated population of AT1 cells.

Author contributions: Y.W. and N.T. designed research; Y.W., Z.T., Jiao Li, Z.W., Y.Y., C.Z., Juan Li, and F.W. performed research; H.D. contributed new reagents/analytic tools; Y.W., Z.T., H.H., Jiao Li, Z.W., Y.Y., T.C., and N.T. analyzed data; and Y.W., T.C., and N.T. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE106960).

[1]To whom correspondence may be addressed. Email: caitao@nibs.ac.cn or tangnan@nibs.ac.cn.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1719474115/-/DCSupplemental.

Published online February 20, 2018.

Results

Overview of Developmental Changes of Postnatal AT1 Cells at the Single-Cell Level. To gain a comprehensive understanding of postnatal AT1 cell development, we performed single-cell RNA-seq (scRNA-seq) analyses of AT1 cells at postnatal day 3 (P3), P15, and P60. We selected these times because alveologenesis in mice is known to begin at P4, and secondary septation formation is typically completed before P15 (15, 19–21).

We set up a strategy to sort AT1 cells and to avoid the contamination of AT2 cells by generating *Sftpc*-CreER; *Rosa26*-Zsgreen; *Hopx*-tdTomato mice (Fig. 1*A* and *SI Appendix*, Fig. S1 *A–D*). Lungs of TAM-treated *Sftpc*-CreER; *Rosa26*-Zsgreen; *Hopx*-tdTomato mice at P3, P15, and P60 were dissociated in an enzymatic mixture solution (Fig. 1*A*) (10). Single-cell suspensions were sorted by FACS to enrich cells expressing both RFP and Pdpn and to deplete GFP^+ AT2 cells, RFP^+Pdpn^- ciliated cells, and $CD45^+$ cells (Fig. 1*A* and *SI Appendix*, Fig. S1 *E* and *F*). $RFP^+Pdpn^+GFP^-CD45^-$ cells were then analyzed with scRNA-seq. After filtering, normalization, and removal of potential outliers (*SI Appendix*, *SI Materials and Methods*), 3,149 cells from P3 lungs, 2,940 cells from P15 lungs, and 1,337 cells from P60 lungs that had high gene expression signals were used for subsequent analyses (Fig. 1 *B–D* and Dataset S1).

A t-distributed stochastic neighbor embedding (tSNE)-based plot revealed that cells from P3, P15, and P60 lungs can be clustered into four, four, and two main distinct populations, respectively (Fig. 1 *B–D*). Cells of the largest of the populations express high levels of classic AT1 cell markers such as *Hopx*, *Pdpn*, and *Ager*, but have low-level expression of classic AT2 markers such as *Sftpb*, *Sftpc*, and *Sftpd*; ciliated cell marker *Foxj1*; and the endothelial cell marker *Pecam1* (*SI Appendix*, Fig. S2 *A–C*), indicating these cells are AT1 cells. We also used scRNA-seq to identify genes that are specifically expressed in small cell populations. Our results from this analysis revealed several types of expression signatures (Dataset S1) (22), which strongly suggested that there were three distinct cells populations that could be contaminated by AT1 cell debris (*SI Appendix*, Fig. S1*G*): endothelial cells, ciliated cells, and AT2 cells. Thus, we excluded these populations when we performed our hierarchical clustering analysis of gene expression for cells of the largest cell population (AT1 cells).

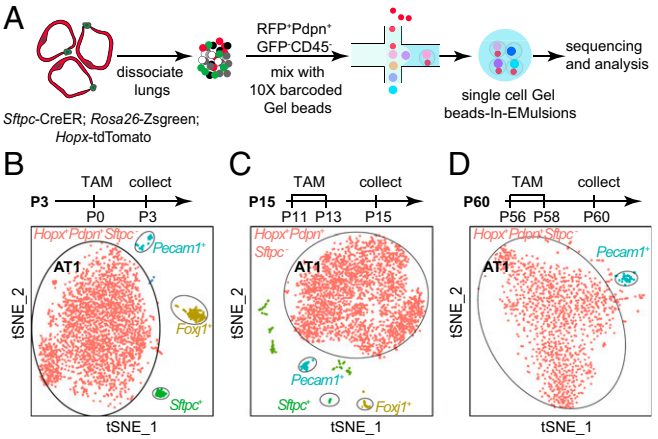

Fig. 1. Analyze the development of postnatal AT1 cells by single-cell RNA-seq (scRNA-seq). (*A*) Schematic illustration of the strategy of lung dissociation, AT1 cell sorting, and single-cell RNA sequencing analysis. (*B–D*) $RFP^+Pdpn^+GFP^-CD45^-$ cells were isolated from TAM-treated *Sftpc*-CreER; *Rosa26*-Zsgreen; *Hopx*-tdTomato mice at P3 (*B*), P15 (*C*), and P60 (*D*). The t-distributed stochastic neighbor embedding plots show cells isolated from P3 (*B*), P15 (*C*), and P60 (*D*) lungs can be clustered into four, four, and two main distinct populations, respectively. AT1 cell population is characterized by expressing *Hopx* and *Pdpn* (AT1 markers), but not *Sftpc* (AT2 marker), *Pecam1* (endothelial cell marker), and *Foxj1* (ciliated cell marker).

Using the clustering analysis, differentially expressed genes were clustered into five groups according to their expression patterns among P3, P15, and P60 AT1 cells (Fig. 2*A* and Dataset S2). The expression levels of genes in group I significantly decreased during postnatal AT1 cell development (Fig. 2*A*). We performed gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses to determine enriched terms of genes in group I. Many genes in the group I are known to regulate pluripotency of stem cells (*Id1*, *Id2*, and *Id3*) (23, 24), cell cycle (*Cdkn1a*) (25), and translation (*Rpl5*) (26) (Fig. 2 *B* and *C* and Dataset S3), indicating that postnatal AT1 cells continue to differentiate during alveologenesis. The expression levels of genes in group II are higher at P15 than at P3 or P60 (Fig. 2*A*). Genes in group II are known to regulate the epidermal growth factor receptor signaling pathway (*Rhbdf1*) and angiogenesis (*Klf2* and *Cdh13*) (Dataset S3). The expression levels of genes in groups III, IV, and V significantly increased during postnatal AT1 cell development (Fig. 2*A*). Genes in these three groups are highly enriched for regulating cell growth (*Igfbp2*) (27), dendrite and neuron projection development (*Bdnf*) (28), exogenous drug catabolic process (*Cyp4b1* and *Cyp2b10*) (29), and angiogenesis (*Ctgf*) (30) (Fig. 2 *B′* and *D* and Dataset S3).

Our single-cell analysis offers unprecedentedly high resolution to define the specificity and temporal expression patterns of genes expressed in postnatal AT1 cells. This allows us to identify biomarkers that are specifically expressed in adult AT1 cells. We compared the scRNA-seq profiles between AT1 and AT2 cells of P60 lungs (*SI Appendix*, Fig. S3). AT2 cells (GFP^+CD45^-) were isolated from the same lungs of *Sftpc*-CreER; *Rosa26*-Zsgreen; *Hopx*-tdTomato mice that we used for P60 AT1 cell scRNA-seq analysis (*SI Appendix*, Fig. S3*A*). After removing the potential outliers, 2,093 cells with high gene expression levels of *Sftpb*, *Sftpc*, *Sftpd*, and *Cxcl15* were extracted for the AT2 cell scRNA-seq analysis (*SI Appendix*, Fig. S3 *B* and *C* and Dataset S1). The GO and KEGG pathway enrichment analyses show that genes upregulated in AT1 cells mainly function in regulating cell shape, cell adhesion, cytoskeleton, and angiogenesis (*SI Appendix*, Fig. S3*D* and Dataset S4). By comparing the gene expression between AT1 and AT2 cells by both scRNA-seq analysis and quantitative real-time PCR analysis, we identified many specific biomarkers of adult AT1 and AT2 cells that have not been previously described (*SI Appendix*, Fig. S4 and Dataset S5).

The Portion of AT1 Cells Expressing Igfbp2 Gradually Increases During Postnatal Lung Development. Among the previously characterized and newly identified AT1 cell markers, we found that *Igfbp2* is one of the most specific biomarker genes of adult AT1 cells (*SI Appendix*, Fig. S4 *A* and *C*). Interestingly, the portion of cells expressing *Igfbp2* increases during postnatal AT1 cell development (Fig. 2*D* and *SI Appendix*, Fig. S2*D*), whereas classic AT1 biomarker genes such as *Pdpn*, *Hopx*, *Aqp5*, and *Ager* are invariantly expressed in almost all AT1 cells during postnatal AT1 cell development (*SI Appendix*, Fig. S2*D*). We performed immunostaining experiments using an anti-Igfbp2 antibody to validate the expression of Igfbp2 in prenatal and postnatal AT1 cells (Fig. 3 *A* and *B* and *SI Appendix*, Fig. S5 *A* and *B*). Unlike classic AT1 cell markers such as Pdpn, Hopx, Aqp5, and Ager that are expressed in AT1 cells starting from embryonic (E) 16.5 (6, 10, 22), Igfbp2 is not expressed in AT1 cells until P1. At P1, fewer than 20% of $Hopx^+$ AT1 cells are positive for Igfbp2 expression. Within 15 d after birth, however, the percentage of $Hopx^+Igfbp2^+$ AT1 cells increases to 85%. By P60, 95% of the $Hopx^+$ AT1 cells are positive for Igfbp2 expression. Importantly, there is a small subset of $Hopx^+$ AT1 cells (less than 5%) that does not express Igfbp2 at P60 (*SI Appendix*, Fig. S5*B*; n = 5 mice; total, 1,958 cells). This immunostaining result is consistent with our scRNA-seq result for P60 lungs, which showed that 5% $Hopx^+$ AT1 cells do not express Igfbp2 (*SI Appendix*, Fig. S2*D*). Note that no Igfbp2 protein expression was detected

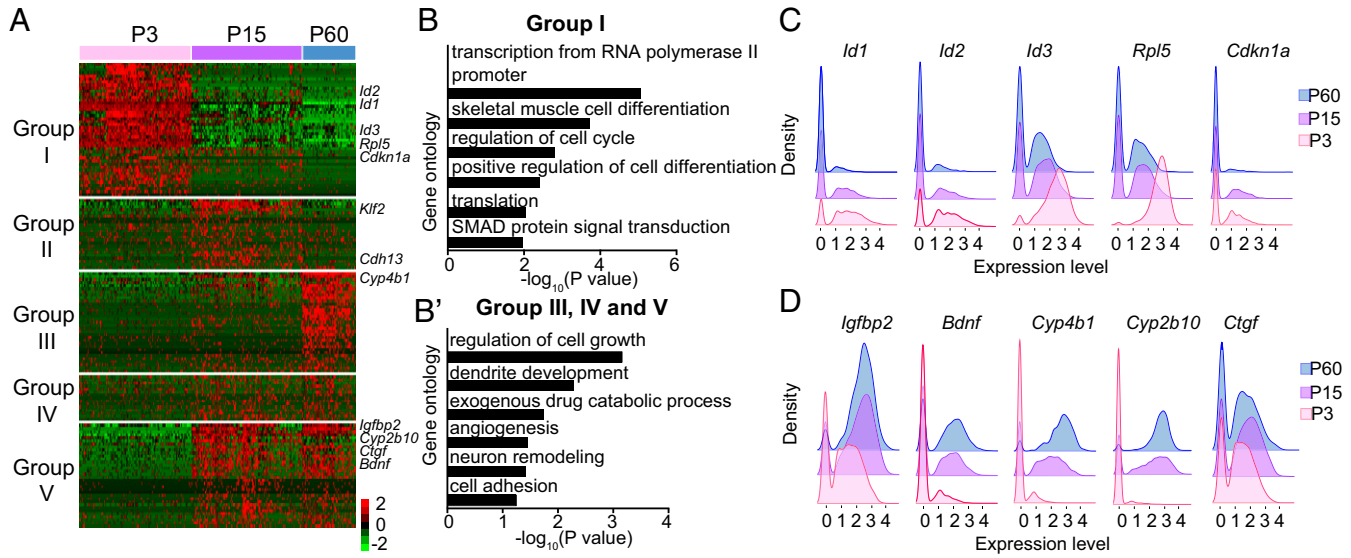

Fig. 2. scRNA-seq analysis shows that postnatal AT1 cells continue to differentiate from P3 to P60. (*A*) The clustering of genes from randomly picked 1,000 AT1 cells of P3, P15, and P60 lungs. Differentially expressed genes (Dataset S2) can be clustered into five groups according to their expression patterns from P3 to P60. (*B* and *B'*) Selected GO terms of genes in group I genes (*B*) and genes in groups III, IV and V (*B'*). (*C*) Density plots of selected genes in group I. In the density plot, *x*-axis represents the gene expression level, and *y* axis represents the density of numbers of cells. (*D*) Density plots of selected genes in groups III, IV and V.

in any of the P60 AT2 cells (*SI Appendix*, Fig. S5 *C* and *D*; n = 5 mice; total, 3,377 cells) and club cells (*SI Appendix*, Fig. S5 *E* and *F*; n = 5 mice; total, 2,691 cells). We also investigate whether IGFBP2 is expressed in adult human AT1 cells. We found that IGFBP2 is also specifically expressed in adult human AT1 cells (*SI Appendix*, Fig. S5*G*). Therefore, IGFBP2 is a specific AT1 cell marker in both human AT1 cells and mouse AT1 cells.

Our observation of the differential expression of Igfbp2 prompted us to examine our scRNA-seq data set to identify other differences in the transcriptomes between Igfbp2$^+$ and Igfbp2$^-$ AT1 cells during alveologenesis. Specifically, at the individual cell level, *Igfbp2* can be detected in 62% of *Hopx*$^+$ AT1 cells at P3, 85% of *Hopx*$^+$ AT1 cells at P15, and 94% *Hopx*$^+$ AT1 cells at P60 (Fig. 3 *B* and *C* and *SI Appendix*, Fig. S2 *D–F*). A GO analysis of the 32 genes that are consistently up-regulated in Igfbp2$^+$ AT1 cells among P3, P15, and P60 lungs revealed strong enrichment for the following terms: regulation of cell growth, angiogenesis, extracellular matrix organization, positive regulation of cell migration, and patterning of blood vessels (Fig. 3*D* and Dataset S6). Moreover, GO analysis of the 31 genes that are consistently up-regulated in Igfbp2$^-$ AT1 cells revealed strong enrichment for the following terms: translation, regulation of cell cycle, and epithelial cell differentiation (Dataset S7). In addition, the expression level of *Sftpd* is significantly increased in Igfbp2$^-$ AT1 cells compared with Igfbp2$^+$ AT1 cells. These results support our findings that the expression of Igfbp2 is positively associated with AT1 cell development during alveologenesis.

Igfbp2 Is a Late AT1 Cell Marker During Post Injury Alveolar Regeneration. Our result that the expression of Igfbp2 is associated with AT1 cell development prompted us to investigate the expression of Igfbp2 in newly differentiated AT1 cells that occur during alveolar regeneration. We therefore investigate the expression of Igfbp2 in newly regenerated alveoli, using a PNX-induced alveolar regeneration mouse model (10, 31).

We used *Sftpc*-CreER mice and *Rosa26*-mTmG reporter alleles for lineage labeling AT2 cells to track the differentiation of AT2 cells into AT1 cells. We performed a PNX treatment on *Sftpc*-CreER; *Rosa26*-mTmG mice and collected lungs for analysis at post-PNX day 14, 21, and 45 (Fig. 3 *E–H*). By post-PNX day 14, many lineage-labeled AT2 cells had differentiated into AT1 cells that express Hopx, Ager, and Aqp5 (*SI Appendix*, Fig. S5 *H* and *I*); notably, these newly differentiated cells did not express Igfbp2 (Fig. 3 *F* and *H*). The percentage of Hopx$^+$ AT1 cells expressing Igfbp2 increases over time. By post-PNX day 45, most of the post-PNX differentiated Hopx$^+$ AT1 cells were expressing Igfbp2 (Fig. 3 *G* and *H*).

We also monitored Igfbp2 expression in experiments with 3D alveolar-like organoids (1). Lineage-labeled AT2 cells from TAM-treated *Sftpc*-CreER; *Rosa26*-Zsgreen mice were isolated by FACS and plated in culture medium with 50% Matrigel in Transwell cell culture inserts, as previously described (Fig. 3*I*) (1). At day 9 post plating of the culture, we started to observe some flattened cells in the interior luminal side of the organoids that expressed Hopx, but did not express Igfbp2 (Fig. 3 *J*, *K*, and *N*). By day 14 post plating of the culture, 30% of the Hopx$^+$ AT1 cells were expressing Igfbp2 (Fig. 3 *L–N*). Similar to what we observed in our analysis of post-PNX lungs, the ratio of Hopx$^+$Igfbp2$^+$ cells to Hopx$^+$ cells increases over time (Fig. 3*N*). Collectively, our extensive in vivo and in vitro organoid results support that the expression of Igfbp2 is later than the expression of Hopx for newly differentiated AT1 cells during alveolar regeneration.

Igfbp2$^+$ AT1 Cells Maintain Their AT1 Cell Fate During Alveologenesis. We showed two Igfbp2 expression patterns in postnatal and post-PNX AT1 cells (Fig. 3 *A*, *B*, and *F–H*). Previous studies have established that Hopx$^+$ alveolar epithelial cells are able to become both AT1 and AT2 cells during the first month of postnatal life (10), immediately raising a question about the fate of Igfbp2$^+$ cells during alveologenesis. To specifically label Igfbp2$^+$ AT1 cells, we generated an *Igfbp2*-CreER knock-in allele (*SI Appendix*, Fig. S6*A*). We first performed a short-term lineage tracing experiment to validate the expression of Igfbp2 in adult lungs (Fig. 4*A*). About 84 ~ 90% of the Hopx$^+$ AT1 cells (n = 5 mice; total, 3,158 cells) were lineage labeled (Fig. 4 *B* and *C*), and none of Prospc$^+$ cells (n = 5 mice; total, 5,127 cells) or Scgb1a1$^+$ cells (n = 5 mice; total, 3,565 cells) expressed any GFP (Fig. 4 *D* and *E* and *SI Appendix*, Fig. S6 *B* and *C*).

Igfbp2-CreER, *Rosa26*-tdTomato pups were given one TAM injection at P5, and lungs were collected for analysis 24 h later, at P30, or at P336 (Fig. 4*F* and *SI Appendix*, Fig. S6*D*). At P6, about 65% Hopx$^+$ of the AT1 cells were lineage-labeled (*SI Appendix*,

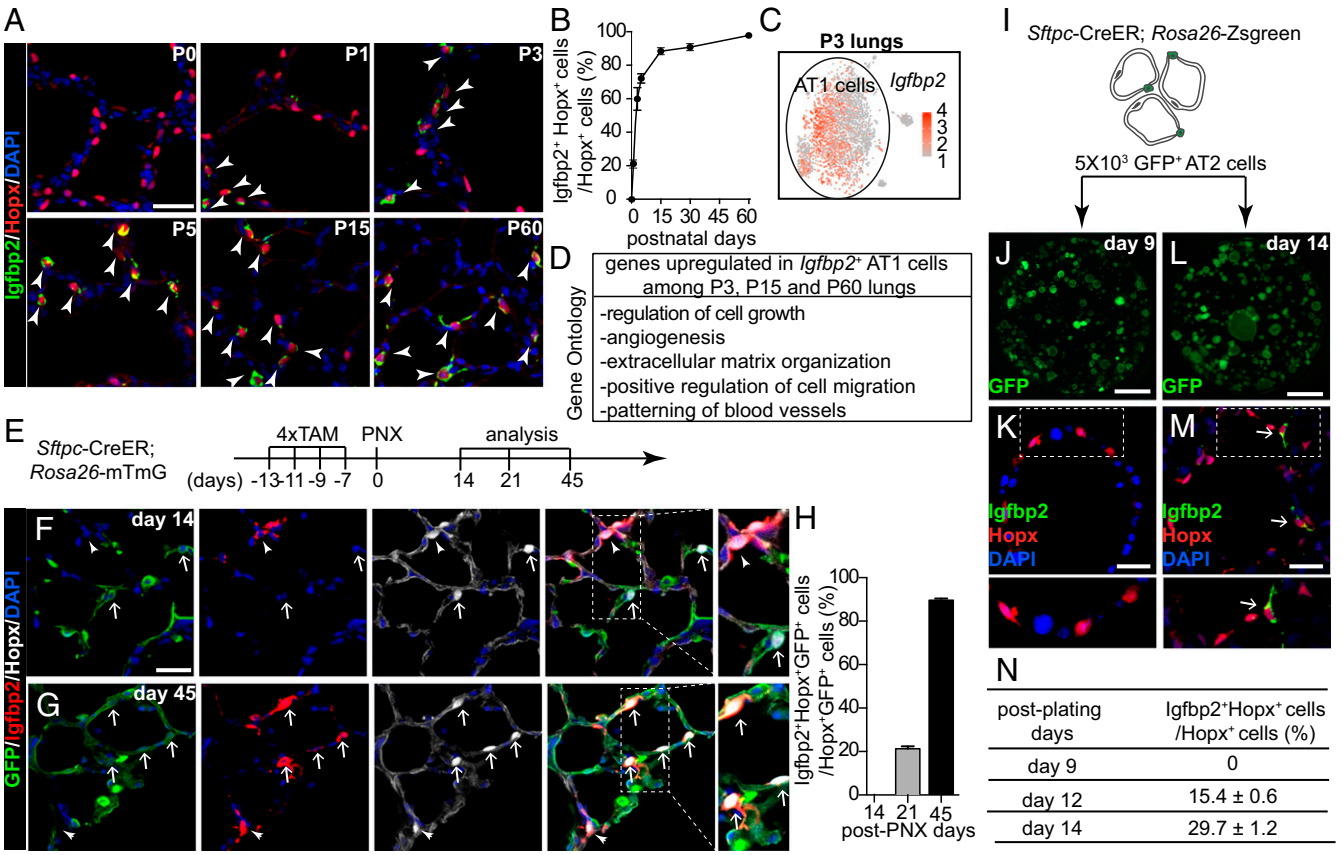

Fig. 3. The expression of Igfbp2 is associated with alveologenesis and AT1 cell differentiation during alveolar regeneration. (*A* and *B*) The percentages of Igfbp2 expressing AT1 cells were quantified (mean ± SEM; $n = 3$) by immunostaining with anti-Igfbp2 and anti-Hopx antibodies. Arrowheads indicate AT1 cells that express Igfbp2. (*C*) The t-distributed stochastic neighbor embedding plot of Igfbp2 expression in P3 AT1 cells. (*D*) The GO analysis of up-regulated genes in Igfbp2$^+$ AT1 cells among P3, P15, and P60 lungs. (*E–H*) Lungs of TAM-treated *Sftpc*-CreER; *Rosa26*-mTmG mice (*E*) at post-PNX day 14 (*F*), 21, and 45 (*G*) were analyzed by antibodies against GFP, Igfbp2, and Hopx. Arrowheads indicate the original AT1 cells. Arrows indicate newly differentiated AT1 cells. The percentages (mean ± SEM, $n = 3$) of newly differentiated AT1 cells expressing Igfbp2 in all newly differentiated AT1 cells are quantified (*H*). (*I–N*) Organoids grown from lineage-labeled AT2 cells (*I*) were collected at post plating day 9 (*J*) and day 14 (*L*) and stained with antibodies against Igfbp2 and Hopx (*K* and *M*). The proportion (mean ± SEM, $n = 3$) of Hopx$^+$Igfbp2$^+$ cells among the Hopx$^+$ cells was quantified. (Scale bars: *A*, *F*, *G*, *K*, and *M*, 25 μm; *J* and *L*, 1 mm.)

Fig. S6 *E* and *F*; n = 3 mice), all of which were Pdpn$^+$ AT1 cells (Fig. 4*G*). There were still 65–70% lineage-labeled Hopx$^+$ AT1 cells present in the lungs at P30 and at P336 (*SI Appendix*, Fig. S6 *E* and *F*). We also did not detect any AT2 cells or club cells expressing RFP at any of the sampled times from P6 to P336 (Fig. 4 *G* and *H* and *SI Appendix*, Fig. S6*G*). In addition, we found that these lineage-labeled AT1 cells were still expressing Igfbp2 in lungs at P336 post-TAM injection (Fig. 4*I*), revealing that these Igfbp2 lineage-labeled AT1 cells maintain their Igfbp2 expression, and suggesting that the turnover of the Igfbp2$^+$ AT1 cells is slow in the steady-state condition.

Igfbp2$^+$ AT1 Cells Are Terminally Differentiated AT1 Cells During PNX-Induced Alveolar Regeneration. It was shown that adult Hopx$^+$ AT1 cells are able to transdifferentiate into AT2 cells, and thereby contribute to lung regrowth during post-PNX alveolar regeneration (10, 31). Our characterization of the two distinct AT1 cell subtypes in Hopx$^+$ AT1 cells motivated us to investigate whether a bi-directional lineage relationship exists between Igfbp2$^+$ AT1 cells and AT2 cells during PNX-induced alveolar regeneration.

We gave *Igfbp2*-CreER; *Rosa26*-Zsgreen mice four doses of TAM to label Igfbp2$^+$ AT1 cells, and performed a left lung resection (Fig. 5*A*). At 26 d after PNX treatment, we collected lungs from PNX-treated mice ($n = 5$ mice each group). In PNX-treated lungs, more than 85% of Hopx$^+$ AT1 cells were lineage labeled, and all the GFP$^+$ cells were Hopx$^+$ AT1 cells (Fig. 5*B*). Among all the lungs examined, no GFP lineage-labeled cells expressed Prospc or Scgb1a1 (Fig. 5*B* and *SI Appendix*, Fig. S7*A*). These results indicate that Igfbp2$^+$ AT1 cells cannot transdifferentiate into AT2 cells or club cells after PNX treatment. Furthermore, we found that no Igfbp2$^+$ AT1 cells expressed Ki67 during alveolar development or during PNX-induced alveolar regeneration (*SI Appendix*, Table S1), indicating that Igfbp2$^+$ AT1 cells cannot proliferate in vivo.

We further investigated the clonal formation capacity of Hopx$^+$ AT1 cells and Igfbp2$^+$ AT1 cells in a 3D organoid culture system. *Sftpc*-CreER; *Rosa26*-Zsgreen; *Hopx*-tdTomato mice were treated with four doses of TAM to label AT2 cells. Three days after the last dose of TAM treatment, 5 × 10^4 Hopx$^+$Pdpn$^+$ AT1 cells (RFP$^+$Pdpn$^+$GFP$^-$CD45$^-$) were isolated by FACS and plated in a 3D organoid culture system (Fig. 5*C*). Consistent with a previous finding (10), many GFP$^-$ organoids that contain both Hopx$^+$ AT1 cells and Prospc$^+$ AT2 cells had formed by day 14 (Fig. 5 *D* and *E* and *SI Appendix*, Fig. S7*B*). Many flattened Hopx$^+$ cells in the interior luminal side of the organoids expressed Igfbp2 (Fig. 5*E*). Recall that we showed that AT1 cells express Hopx, but not Igfbp2, at post plating day 9 (Fig. 3*K*). Therefore, we first cultured lineage-labeled GFP$^+$ AT2 cells isolated from *Sftpc*-CreER; *Rosa26*-Zsgreen; *Hopx*-tdTomato mice. At post plating day 9, we isolated GFP$^+$Hopx$^+$Pdpn$^+$ AT1 cells (Fig. 5 *F* and *G*) and cultured these GFP$^+$Hopx$^+$Pdpn$^+$ AT1 cells in a 3D organoid culture system. We found that by post plating day 14, these organoids

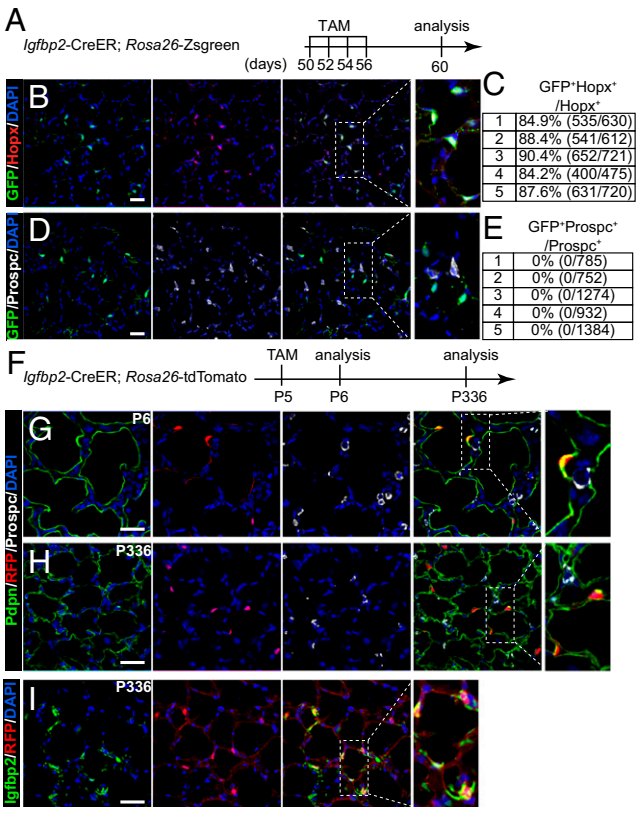

Fig. 4. Igfbp2$^+$ AT1 cells maintain their AT1 cell fate during homeostasis. (*A*–*C*) Lungs ($n = 5$) of TAM-treated *Igfbp2*-CreER; *Rosa26*-Zsgreen mice were analyzed with antibodies against GFP and Hopx (*B* and *C*) or antibodies against GFP and Prospc (*D* and *E*). (*F*–*I*) Lungs of *Igfbp2*-CreER; *Rosa26*-tdTomato mice (TAM-treatment at P5) (*F*) were analyzed using antibodies against Pdpn, RFP, and Prospc at P6 (*G*) and P336 (*H*). Lungs from P336 old mice (*H*) were analyzed using antibodies against Igfbp2 and RFP (*I*). (Scale bars: *B*, *D*, and *G*–*I*, 25 μm.)

contained both Hopx$^+$Igfbp2$^+$ AT1 cells and Prospc$^+$ AT2 cells (Fig. 5*H* and *SI Appendix*, Fig. S7*C*). Beyond highlighting the cellular plasticity of these Hopx$^+$Igfbp2$^-$ AT1 cells, these results show that Igfbp2$^+$ AT1 cells are derived from Hopx$^+$Igfbp2$^-$ AT1 cells.

In a parallel experimental approach, we labeled Igfbp2$^+$ AT1 cells by injecting four doses of TAM to *Igfbp2*-CreER; *Rosa26*-tdTomato mice. Three days after the last dose of TAM treatment, 5×10^4 Igfbp2$^+$ AT1 cells (RFP$^+$Pdpn$^+$CD45$^-$) were isolated by FACS and plated in a 3D organoid culture system (Fig. 5*I*). We saw no RFP$^+$ organoid formation by day 14 of culture (n = 6 experiments) (Fig. 5 *J* and *K*). To monitor the conditions of our organoid culture system, we isolated AT2 cells (GFP$^+$CD45$^-$) from TAM-treated *Sftpc*-CreER; *Rosa26*-mTmG mice and Igfbp2$^+$ AT1 cells (RFP$^+$Pdpn$^+$CD45$^-$) from TAM-treated *Igfbp2*-CreER; *Rosa26*-tdTomato mice (*SI Appendix*, Fig. S7*D*). We then mixed 5,000 AT2 cells (GFP$^+$CD45$^-$) with 5×10^4 Igfbp2$^+$ AT1 cells (RFP$^+$Pdpn$^+$CD45$^-$) in the same Transwell cell culture insert (*SI Appendix*, Fig. S7*D*). Many organoids had formed by day 14. All the clones expressed GFP, but not RFP (*SI Appendix*, Fig. S7 *E*–*G*), indicating that these clones were generated from AT2 cells but not Igfbp2$^+$ AT1 cells. Both our in vivo and in vitro results demonstrated that Hopx$^+$Igfbp2$^+$ AT1 cells are terminally differentiated AT1 cells that cannot proliferate and transdifferentiate into AT2 cells.

Discussion

In this study, we characterized the developmental changes of AT1 cells during alveologenesis and alveolar regeneration. Our study revealed that the adult AT1 cell population contains two distinct types of cells: Hopx$^+$Igfbp2$^+$ and Hopx$^+$Igfbp2$^-$ AT1 cells. Adult Hopx$^+$Igfbp2$^+$ AT1 cells represent the large majority of Hopx$^+$ AT1 cells and are terminally differentiated; that is, they cannot transdifferentiate into AT2 cells and cannot proliferate during alveolar regeneration. Hopx$^+$Igfbp2$^-$ AT1 cells, which account for fewer than 5% of the Hopx$^+$ AT1 cells in adult lungs, contain cell populations that maintain cellular plasticity and can generate alveolar-like organoids. Both a post-PNX alveolar regeneration mouse model and in vitro organoid culture results show that Igfbp2 expression occurs later than Hopx expression in AT1 cells that have differentiated from AT2 cells. Notably, organoids generated from Hopx$^+$ AT1 cells contain both Hopx$^+$Igfbp2$^+$ and Hopx$^+$Igfbp2$^-$ AT1 cells. Consideration of these multiple lines of evidence lead us to speculate that Hopx$^+$ Igfbp2$^-$ AT1 cells in normal adult lungs seem to represent an intermediate stage of AT1 cell differentiation during the ongoing physiological renewal of the alveolar epithelium. Some of these newly differentiated Hopx$^+$Igfbp2$^-$ AT1 cells likely to maintain developmental plasticity in the period before these cells begin to express Igfbp2; these Hopx$^+$Igfbp2$^-$ cells can transdifferentiate

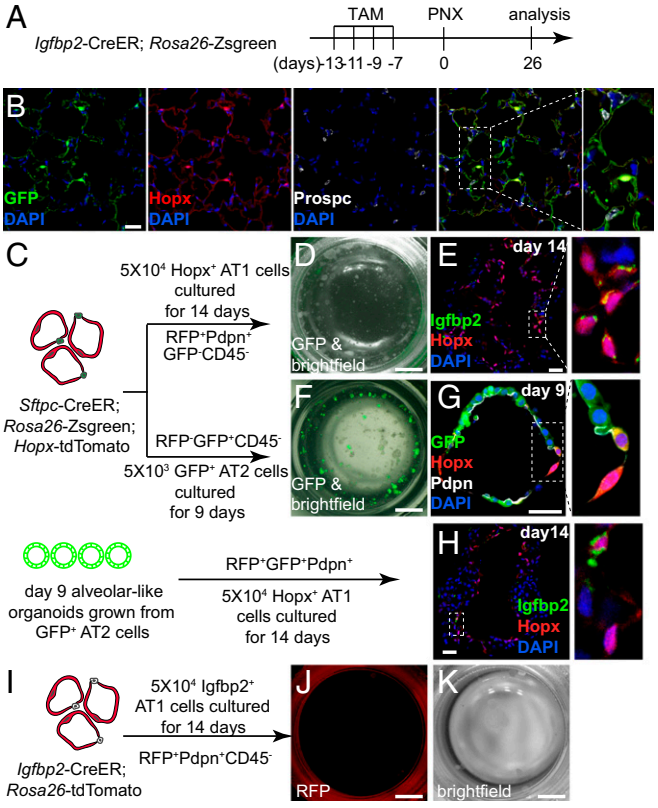

Fig. 5. Igfbp2$^+$ AT1 cells are terminal differentiated AT1 cells and can differentiated from Hopx$^+$ AT1 cells. (*A*–*C*) Lungs of PNX-treated *Igfbp2*-CreER; *Rosa26*-Zsgreen mice (*A*) were analyzed with antibodies against GFP, Hopx, and Prospc (*B*). (*C*) Organoids were grown from either Hopx$^+$ AT1 cells or GFP$^+$ lineage-labeled AT2 cells. (*D* and *E*) At post plating day 14, organoids growth Hopx$^+$ AT1 cells (*D*) were analyzed with antibodies against Igfbp2 and Hopx (*E*). (*F*–*H*) At post plating day 9, some flatten cells at the luminal side of organoids formed from GFP$^+$ AT2 cells (*F*) expressed both Hopx and Pdpn (*G*). Hopx$^+$ cells were then isolated from these day 9 organoids and cultured in a 3D organoid culture system. At post plating day 14, some Hopx$^+$ AT1 cells expressed Igfbp2 (*H*). (*I*–*K*) Lineage-labeled Igfbp2$^+$ AT1 cells were plated for the alveolar organoid culture (*I*). No RFP$^+$ organoids formed by day 14 of culture (*J* and *K*). (Scale bars: *B*, *E*, *G*, and *H*, 25 μm; *D*, *F*, *J*, and *K*, 1 mm.)

into AT2 cells, and thus help maintain tissue integrity in the context of repair during injury-induced alveolar regeneration.

Our scRNA-seq analysis shows that postnatal AT1 cells continue to differentiate and enhance their functions in air–blood barrier and alveolar angiogenesis during postnatal lung development. Compared with AT1 cells in neonatal mice, adult AT1 cells are much larger, squamous cells with thin cytoplasmic extensions. Our scRNA-seq analysis shows that genes regulating cell growth, extracellular matrix organization, and angiogenesis are up-regulated in $Hopx^{+}Igfbp2^{+}$ AT1 cells compared with $Hopx^{+}Igfbp2^{-}$ AT1 cells among P3, P15, and P60 lungs. In addition, $Hopx^{+}Igfbp2^{+}$ AT1 cells do not proliferate and do not transdifferentiate into AT2 cells during alveologenesis. All these results support our conclusion that Igfbp2 is an informative and highly specific genetic marker for the terminal differentiation of AT1 cells.

Igfbp2's function in AT1 development remains to be elucidated. It belongs to an evolutionarily conserved IGFBP superfamily. Many studies support that IGFBPs promote growth through both IGF-dependent and IGF-independent mechanisms (32, 33). We generated *Igfbp2* null ($Igfbp^{-/-}$) mice and conducted exploratory functional analysis of Igfbp2 in AT1 cells. We found that $Igfbp2^{-/-}$ mice are healthy and fertile, and H&E staining showed no obvious differences between the lungs of $Igfbp2^{-/-}$ and littermate control mice at 12 mo of age (*SI Appendix*, Fig. S8). One explanation for this lack of any obvious phenotype is that multiple IGFBP family members expressed in AT1 cells may play redundant functions in regulating AT1 cell functions. It is notable that the cellular localization of IGFBP2 is different between human AT1 cells and mouse AT1 cells. Both mouse *Igfbp2* and human *IGFBP2* contain a nuclear localization signal. However, we found that only human IGFBP2 is localized in the nucleus of AT1 cells. Thus, it is clear that future investigations will need to precisely define the biomolecular function or functions of IGFBP2 and other IGFBP family members in AT1 cells.

More and more evidence is suggesting that adult AT1 cells have a variety of other functions beyond their known roles in the formation of the air–blood barrier and in transporting ions and water (14, 34, 35). Our study establishes that the AT1 cell population is actually composed of two distinct AT1 cell types during alveolar development and during alveolar regeneration. This knowledge and our demonstration of Igfbp2 as a robust and reliable marker of the terminal differentiation of AT1 cells will enable future investigation of the genetic and cellular mechanisms that control the function of AT1 cells in development, diseases, and lung regeneration.

Materials and Methods

A detailed description of the materials and methods used in the study is provided in *SI Appendix, SI Materials and Methods*. Multiple mouse lines were used. Primary AT1 and AT2 cells isolation, single-cell RNA-seq analysis, immunostaining, pneumonectomy, lineage-tracing experiments, alveolar-like organoid culture, and quantitative RT-PCR were performed. The data reported in this paper have been deposited in the NCBI GEO database, https://www.ncbi.nlm.nih.gov/geo/ (accession no. GSE106960).

ACKNOWLEDGMENTS. We thank Dr. Brigid Hogan for providing us the *Sftpc*-CreER mice. We thank Dr. Jason Rock for his suggestions on setting up the alveolar-like organoid culture system. This study was funded by Beijing Major Science and Technology Projects, Z171100000417003 (to N.T.) and the National Key Research and Development Program of China (2017YFA0103501).

1. Barkauskas CE, et al. (2013) Type 2 alveolar cells are stem cells in adult lung. *J Clin Invest* 123:3025–3036.
2. Desai TJ, Brownfield DG, Krasnow MA (2014) Alveolar progenitor and stem cells in lung development, renewal and cancer. *Nature* 507:190–194.
3. Rock JR, et al. (2011) Multiple stromal populations contribute to pulmonary fibrosis without evidence for epithelial to mesenchymal transition. *Proc Natl Acad Sci USA* 108:E1475–E1483.
4. Haies DM, Gil J, Weibel ER (1981) Morphometric study of rat lung cells. I. Numerical and dimensional characteristics of parenchymal cell population. *Am Rev Respir Dis* 123:533–541.
5. Stone KC, Mercer RR, Gehr P, Stockstill B, Crapo JD (1992) Allometric relationships of cell numbers and size in the mammalian lung. *Am J Respir Cell Mol Biol* 6:235–243.
6. Rawlins EL, Clark CP, Xue Y, Hogan BL (2009) The Id2+ distal tip lung epithelium contains individual multipotent embryonic progenitor cells. *Development* 136: 3741–3745.
7. Nikolić MZ, et al. (2017) Human embryonic lung epithelial tips are multipotent progenitors that can be expanded in vitro as long-term self-renewing organoids. *eLife* 6: e26575.
8. Churg A, Wright JL (2005) Proteases and emphysema. *Curr Opin Pulm Med* 11: 153–159.
9. Yang J, et al. (2016) The development and plasticity of alveolar type 1 cells. *Development* 143:54–65.
10. Jain R, et al. (2015) Plasticity of Hopx(+) type I alveolar cells to regenerate type II cells in the lung. *Nat Commun* 6:6727.
11. Amy RW, Bowes D, Burri PH, Haines J, Thurlbeck WM (1977) Postnatal growth of the mouse lung. *J Anat* 124:131–151.
12. Weibel ER (2009) What makes a good lung? *Swiss Med Wkly* 139:375–386.
13. Massaro D, Teich N, Maxwell S, Massaro GD, Whitney P (1985) Postnatal development of alveoli. Regulation and evidence for a critical period in rats. *J Clin Invest* 76: 1297–1305.
14. Dobbs LG, Johnson MD, Vanderbilt J, Allen L, Gonzalez R (2010) The great big alveolar TI cell: Evolving concepts and paradigms. *Cell Physiol Biochem* 25:55–62.
15. Mund SI, Stampanoni M, Schittny JC (2008) Developmental alveolarization of the mouse lung. *Dev Dyn* 237:2108–2116.
16. Bourbon J, Boucherat O, Chailley-Heu B, Delacourt C (2005) Control mechanisms of lung alveolar development and their disorders in bronchopulmonary dysplasia. *Pediatr Res* 57:38R–46R.
17. Borok Z, et al. (2002) Na transport proteins are expressed by rat alveolar epithelial type I cells. *Am J Physiol Lung Cell Mol Physiol* 282:L599–L608.
18. Williams MC (2003) Alveolar type I cells: Molecular phenotype and development. *Annu Rev Physiol* 65:669–695.
19. Branchfield K, et al. (2016) A three-dimensional study of alveologenesis in mouse lung. *Dev Biol* 409:429–441.
20. Herring MJ, Putney LF, Wyatt G, Finkbeiner WE, Hyde DM (2014) Growth of alveoli during postnatal development in humans based on stereological estimation. *Am J Physiol Lung Cell Mol Physiol* 307:L338–L344.
21. Schittny JC, Mund SI, Stampanoni M (2008) Evidence and structural mechanism for late lung alveolarization. *Am J Physiol Lung Cell Mol Physiol* 294:L246–L254.
22. Treutlein B, et al. (2014) Reconstructing lineage hierarchies of the distal lung epithelium using single-cell RNA-seq. *Nature* 509:371–375.
23. Niola F, et al. (2012) Id proteins synchronize stemness and anchorage to the niche of neural stem cells. *Nat Cell Biol* 14:477–487.
24. Lasorella A, Benezra R, Iavarone A (2014) The ID proteins: Master regulators of cancer stem cells and tumour aggressiveness. *Nat Rev Cancer* 14:77–91.
25. Deng C, Zhang P, Harper JW, Elledge SJ, Leder P (1995) Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* 82:675–684.
26. Singh SA, et al. (2014) p53-Independent cell cycle and erythroid differentiation defects in murine embryonic stem cells haploinsufficient for Diamond Blackfan anemia-proteins: RPS19 versus RPL5. *PLoS One* 9:e89098.
27. Wood TL, Rogler LE, Czick ME, Schuller AG, Pintar JE (2000) Selective alterations in organ sizes in mice with a targeted disruption of the insulin-like growth factor binding protein-2 gene. *Mol Endocrinol* 14:1472–1482.
28. Conover JC, et al. (1995) Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4. *Nature* 375:235–238.
29. Cui JY, Renaud HJ, Klaassen CD (2012) Ontogeny of novel cytochrome P450 gene isoforms during postnatal liver maturation in mice. *Drug Metab Dispos* 40:1226–1237.
30. Brigstock DR (2002) Regulation of angiogenesis and endothelial cell function by connective tissue growth factor (CTGF) and cysteine-rich 61 (CYR61). *Angiogenesis* 5: 153–165.
31. Liu Z, et al. (2016) MAPK-mediated YAP activation controls mechanical-tension-induced pulmonary alveolar regeneration. *Cell Rep* 16:1810–1819.
32. Hwa V, Oh Y, Rosenfeld RG (1999) The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocr Rev* 20:761–787.
33. Rosenfeld RG, et al. (1999) The insulin-like growth factor binding protein superfamily: New perspectives. *Pediatrics* 104:1018–1021.
34. Qiao R, et al. (2003) Identification of three genes of known function expressed by alveolar epithelial type I cells. *Am J Respir Cell Mol Biol* 29:98–105.
35. McElroy MC, Kasper M (2004) The use of alveolar epithelial type I cell-selective markers to investigate lung injury and repair. *Eur Respir J* 24:664–673.