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

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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 used this marker to demonstrate that the postnatal AT1 cell population contains two unevenly distributed AT1 cell subtypes, Hopx\textsuperscript{+}Igfbp2\textsuperscript{+} and Hopx\textsuperscript{+}Igfbp2\textsuperscript{−} 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\textsuperscript{+}Igfbp2\textsuperscript{+} AT1 cells maintain their AT1 cell fate and do not transdifferentiate into AT2 cells in normal or post injury lungs. Therefore, Hopx\textsuperscript{+}Igfbp2\textsuperscript{+} 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\textsuperscript{+}Igfbp2\textsuperscript{+} and Hopx\textsuperscript{+}Igfbp2\textsuperscript{−} 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 pneumocyte formation of new alveoli. Therefore, Hopx\textsuperscript{+}Igfbp2\textsuperscript{+} AT1 cells represent the terminally differentiated population of AT1 cells.

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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).

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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-tTomato mice (Fig. 1A and SI Appendix). Lungs of TAM-treated Sftpc-CreER; Rosa26-Zsgreen; Hopx-tTomato mice at P3, P15, and P60 were dissociated in an enzymatic mixture solution (Fig. 1A) (10). Single-cell suspensions were sorted by FACS to enrich cells expressing both RFP and Pdpm and to deplete GFP+ AT2 cells, RFP+ Pdpm− ciliated cells, and CD45+ cells (Fig. 1A and SI Appendix, Fig. S1 A–D). Lungs 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 cell clusters from P15 lungs, and 1,337 cells from P60 lungs that had high gene expression signals were used for subsequent analyses (Fig. 1B–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. 1B–D). Cells of the largest of the populations express high levels of classic AT1 cell markers such as Hopx, Pdpm, 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 cell populations that could be contaminated by AT1 cell debris (SI Appendix, Fig. S1G): 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).

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. 2A and Dataset S2). The expression levels of genes in group I significantly decreased during postnatal AT1 cell development (Fig. 2A). 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 (Rps6fb) (26) (Fig. 2B 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. 2A). Genes in group II are known to regulate the epidermal growth factor receptor signaling pathway (Rhhedj1) 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. 2A). Genes in these three groups are highly enriched for regulating cell growth (Igfbp2) (27), dendrite and neuron projection development (Bdnf) (28), exogenous drug catalytic process (Cyp4b1 and Cyp2b10) (29), and angiogenesis (Ctgf) (30) (Fig. 2B’ and D and Dataset S3).

Our single-cell analysis offers unprecedented 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-tTomato mice that we used for P60 AT1 cell scRNA-seq analysis (SI Appendix, Fig. S3A). 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 showed that genes up-regulated in AT1 cells mainly function in regulating cell shape, cell adhesion, cytokine, and angiogenesis (SI Appendix, Fig. S3D 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. 2D and SI Appendix, Fig. S2D), whereas classic AT1 biomarker genes such as Pdpm, Hopx, Aqp5, and Ager are invariably expressed in almost all AT1 cells during postnatal AT1 cell development (SI Appendix, Fig. S2D). 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 Pdpm, 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. S5B; 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. S2D). Note that no Igfbp2 protein expression was detected.
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. S5G). 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 transcriptions 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. 3D 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. 3I) (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. 3J, K, and N). By day 14 post plating of the culture, 30% of the Hopx+ AT1 cells were expressing Igfbp2 (Fig. 3L–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. 3N). 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 Igfbp2-CreER knock-in allele (SI Appendix, Fig. S6A). We first performed a short-term lineage tracing experiment to validate the expression of Igfbp2 in adult lungs (Fig. 4A). 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 Segblal1+ 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. 4F and SI Appendix, Fig. S6D). 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 D) 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–I) 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–M) Organoids grown from lineage-labeled AT1 cells (l) 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.)

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 regeneration 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 bidirectional 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. 5A). 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. 5B). Among all the lungs examined, no GFP lineage-labeled cells expressed Prosc or Sgrh1b1 (Fig. 5B and SI Appendix, Fig. S7A). 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 × 10^5 Hopx+Pdpn+ AT1 cells (RFP+Pdpn+GFP+CD45+) were isolated by FACS and plated in a 3D organoid culture system (Fig. 5C). Consistent with a previous finding (10), many GFP+ organoids that contain both Hopx+ AT1 cells and Prosc+ AT2 cells had formed by day 14 (Fig. 5D and E and SI Appendix, Fig. S7B). Many flattened Hopx+ cells in the interior luminal side of the organoids expressed Igfbp2 (Fig. 5E). Recall that we showed that AT1 cells express Hopx, but not Igfbp2, at post plating day 9 (Fig. 3K). 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. 5F 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...
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AT1 cells in adult
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SI Appendix
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AT1
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AT1 cells. Consideration of
organoid formation by day 14 of culture (n
AT1 cells contain both
AT1 cells in normal adult lungs seem to represent an
organoid formation by day 14 represents the 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
AT1 cells can transdifferentiate

contained both Hopx
Igfbp2
AT1 cells and Prospc
AT2 cells (Fig. 5H and SI Appendix, Fig. S7C). 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 × 10^5
Igfbp2
AT1 cells (RFP
Pdpn
CD45
) were isolated by FACS
and plated in a 3D organoid culture system (Fig. S1). We saw no RFP
organoid formation by day 14 of culture (n = 6 experiments) (Fig. 5J and K). To monitor the conditions of our organoid culture
system, we isolated AT2 cells (GFP
CD45
) from TAM-treated
Stiff-CreER; Rosa26-mTmG mice and Igfbp2
AT1 cells (RFP
Pdpn
CD45
) from TAM-treated Igfbp2
CreER; Rosa26-tdTomato mice (SI Appendix, Fig. S7D). We then mixed 5,000
AT2 cells (GFP
CD45
) with 5 × 10^5 Igfbp2
AT1 cells in the same Transwell cell culture insert (SI Appendix, Fig. S7D). Many organoids had formed by day 14. All the clones
grew GFP, but not RFP (SI Appendix, Fig. S7E–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.
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 (Igfbp2−/−) 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 biochemical 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 this study is provided in SI Appendix, SI Materials and Methods. Multiple mouse lines were used. Primary AT1 cells were isolated, 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).

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