Identification of dedifferentiation and redevelopment phases during postpneumonectomy lung growth

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1Children’s Hospital Informatics Program, Harvard-MIT Division of Health Sciences and Technology, Boston Children’s Hospital, Boston, Massachusetts; and 2Pulmonary and Respiratory Diseases Division, Boston Children’s Hospital, Boston, Massachusetts

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Kho AT, Liu K, Visner G, Martin T, Boudreault F. Identification of dedifferentiation and redevelopment phases during postpneumonectomy lung growth. Am J Physiol Lung Cell Mol Physiol 305: L542–L554, 2013. First published August 29, 2013; doi:10.1152/ajplung.00403.2012.— Surgical resection of pulmonary tissue exerts a proregenerative stretch stimulus in the remaining lung units. Whether this regeneration process reenacts part or whole of lung morphogenesis developmental program remains unclear. To address this question, we analyzed the stretch-induced regenerating lung transcriptome in mice after left pneumonectomy (PNX) in its developmental context. We created a C57BL/6 mice lung regeneration transcriptome time course at 3, 7, 14, 28, and 56 days post-PNX, profiling the cardiac and medial lobes and whole right lung. Prominent expression at days 3 and 7 of genes related to cell proliferation (Ccnb1, Bub1, and Cdk1), extracellular matrix (Col1a1, Eln, and Tnc), and proteases (Serpib2 and Mmp9) indicated regenerative processes that tapered off after 56 days. We projected the post-PNX transcriptomic time course into the transcriptomic principal component space of the C57BL/6 mouse developing lungs time series from embryonic day 9.5 to postnatal day 56. All post-PNX samples were localized around the late postnatal stage of developing lungs. Shortly after PNX, the temporal trajectory of regenerating lobes and right lung reversed course relative to the developing lungs in a process reminiscent of dedifferentiation. This reversal was limited to the later postnatal stage of lung development. The post-PNX temporal trajectory then moves forward in lung development time close to its pre-PNX state after days 28 to 56 in a process resembling redevelopment. A plausible interpretation is that remaining pulmonary tissue reverts to a more primitive stage of development with higher potential for growth to generate tissue in proportion to the loss.

lungs; regeneration; development

ORGANOGENESIS IS A FINELY choreographed multiscale process that starts with a relatively homogenous cell population and ends with a functioning organ of relatively heterogeneous histomorphology. It is typically recognized to be invoked only once in the lifetime of most mammals. Regeneration is an injury-triggered process that repairs tissue loss with growth of functional tissue. One fundamental question concerning tissue regeneration in a surgically reduced organ of a mature mammal is whether regeneration is achieved by recapitulating parts of the organogenetic machinery or by invoking machinery distinct from organogenesis. In short, does organ regeneration recapitulate organ development? The molecular and histomorphological changes in the kidney and liver during organ regeneration have been described (7, 24). In contrast to true regeneration where tissue regrows at the site of excision, new tissue formation is confined within the remaining organ. Hyperplasia dominates liver tissue growth following hepatectomy (HPX), whereas hypertrophy dominates renal tissue growth postnephrectomy. Human organ surgical reduction is routine and growth is robust in specific cases. For example as much as 50% of surgically resected hepatic tissue can regrow in less than 1 yr (6). Yet the question of whether liver or kidney regeneration recapitulates development is not fully answered (7, 24, 29).

The lungs of small rodents and larger mammals including dogs are also capable of regeneration characterized by expansion of existing airways and formation of new alveoli within the remaining lungs (8, 10, 11). In contrast to the comprehensive work on liver and kidneys, lung growth following pneumonectomy (PNX) has largely remained an experimental curiosity (4) owing to the lack of similar compensatory growth of tissue mass in humans 5 yr of age and older (10, 21). The recent identification of a cohort of novel pulmonary stem cells that might invoke lung growth in adult humans (5, 15, 16, 19) and novel regenerative medicine approaches in development (28, 31) have reignited interest in lung regeneration. Mice models for lung regeneration have lately gained prominence (23, 27, 30, 38). The advantages of the murine model include a vigorous growth post-PNX, the availability of reliable mouse transcriptome profiling assays, and a vast number of genetic tools to engineer their genome. Here, we use the mouse to investigate the relationship between lung regeneration post-PNX and development from a transcriptomic perspective.

The archetypal regeneration model is limb regeneration in adult urodeles, which can be divided into three major phases (2). After completion of wound healing (phase 1), the tissue system enters a dedifferentiation stage (phase 2) whereby cells in the stump near the site of resection undergo a profound genetic reprogramming, and lose their specialized characteristics (dedifferentiate) to create the blastema, a population of undifferentiated cells forming the origin for the redevelopment (phase 3) of the resected limb. This redevelopment phase presents spatial and temporal patterns of gene expression similar to limb development (2).

Lung regeneration is widely perceived as the reinitiation or acceleration of cellular growth (10). Warburton and colleagues (37) have proposed that, similar to limb regeneration, lung development programs are reactivated during regeneration. In short, regeneration in mature lungs would consist of an altered development phase characterized by the reactivation of known lung organogenesis genes such as Shh, Ptc1, Smo, Gli, Fgf, Fgfr, Sprouty, Wnt, Bmp4, Tgf-β, Pdgf, Hoxa5, and Smad (9, 37). However, these classic lung morphogenetic genes and

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associated molecular pathways have not been detected in post-PNX lung growth in rats or dogs (12). Recent studies of mice lung regeneration reported little similarity between the first few days of lung regeneration and early embryonic lung development largely because classic morphogenetic transcription factors of early embryonic lung development (37) were not detected (23, 30, 38). Together these studies suggest the absence of a nonincidental relationship between lung regeneration and embryonic lung development, counter to Warburton’s earlier hypothesis.

In this study, we systematically investigated the transcriptomic relationship between the time course of lung regeneration from before (0) to 56 days following unilateral left PNX, and the entire time series of lung development from embryonic day 9.5 (E9.5) to postnatal day 56 (P56), in the mouse. We uncovered in post-PNX lung regeneration, distinctive transcriptomic signatures analogous to urodeles limb regeneration with alternate phases of dedifferentiation and redevelopment. We observed that post-PNX lung regeneration transcriptomically recapitulates lung development at the later stages when alveoli are forming, rather than the earlier stages of branching and enlargement of primary airways (32). Our analysis suggests that the regenerating lung transcriptome reverses in developmental time (dedifferentiation phase) to a more immature stage in late lung development when cells are likely to have greater potential for growth. This is different from the conceptualization of lung regeneration as reinitiation or acceleration of cellular growth from the early stage of lung development.

MATERIALS AND METHODS

Animals and study design. C57BL/6 pathogen-free male mice (7–9 wk) weighing 22 to 32 g were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were checked for absence of injury and obvious dermatitis. All animal procedures followed NIH Guidelines for the Care and Use of Laboratory Animals and were approved by Animal Resources at Boston Children’s Hospital (Boston, MA). There were two animals per time point: Total RNA from cardiac and medial lobes was isolated from one animal and whole right lung total RNA isolated from another animal. Experimental time points were at baseline (no surgery) on days 0 and 56, and after unilateral left PNX on days 3, 7, 14, 28, and 56. The PNX time course for the cardiac/medial lobes and the whole right lung was derived from 14 distinct mice. Mice were 10–12 wk old at the time of surgery.

Unilateral left PNX. The mice were anesthetized with 2–5% isoflurane in oxygen for 10–15 min and then intubated orotracheally with a 20-gauge Teflon angiocatheter and mechanically ventilated with pure oxygen mixed with 2–3% isoflurane at 120 breaths/min with a tidal volume of 0.3 ml using a rodent ventilator (HSE-HA Minivet, Harvard Apparatus, Holliston, MA). An incision was made through the fourth intercostal to expose the chest cavity. Left lung was gently lifted out of the chest. Complete dissection was performed around the hilar structure. The dissected hilum was tied with a 6–0 silk suture and left lung and hilum were resected. Right lung was slightly overinflated before closing left thoracotomy with 6–0 silk suture and repositioning muscle in their original alignment. Five percent extra oxygen was given during recovery until mice walked and fed properly. Surgery was performed with use of a surgical microscope under sterile conditions. Postoperative pain was managed with a single injection of buprenorphine (0.1 mg/kg sc or ip) at the end of surgery and given every 8 to 12 h for 3 days as needed.

Reverse transcription, real-time PCR, and validation. Messenger RNA was reverse transcribed with TaqMan Gold RT-PCR Kit (Applied Biosystems, Foster City, CA) and Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) following manufacturer’s protocol. Primers for gene of interest were designed with Primer Express 3.0 (Applied Biosystems, Foster City, CA) from publicly available nucleotide sequences (NCBI/NIH, Bethesda, MD): Gapdh, forward: CATGGCTTCCGTGTCCTA, reverse: GCGGCACGT-CAGATCCA and Table 1. Changes in gene level expression were quantified with real-time PCR using SYBR green dye (Applied Biosystems, Foster City, CA) 24 h at 4°C, followed by storage at −80°C until RNA extraction. Total RNA was isolated or RNA concentrations and purity were evaluated by use of a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

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Table 1. Validation of the microarray using qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers Sequence</th>
<th>Correlation qPCR vs. Microarray ($R^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Medial Lobe</td>
</tr>
<tr>
<td>Ccnb1</td>
<td>Forward: AAGGGCGTGACAAAGGCTA</td>
<td>0.673</td>
</tr>
<tr>
<td></td>
<td>Reverse: CATGGTGCGGAATGCATCTG</td>
<td></td>
</tr>
<tr>
<td>Hmmr</td>
<td>Forward: GCCTGAAATCTTCTTGTAGAC</td>
<td>0.771</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCCTGAAATCTTCTTGTAGAC</td>
<td></td>
</tr>
<tr>
<td>Cdk1</td>
<td>Forward: CACCCGGAAGCAACGTGAA</td>
<td>0.904</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGGAGGACAGTGGACGAT</td>
<td></td>
</tr>
<tr>
<td>Cdkn1a</td>
<td>Forward: TGTTGCTCTTCCCTGCTCTTCTT</td>
<td>0.868</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCACTGCTCTTCCCTGCTCTT</td>
<td></td>
</tr>
<tr>
<td>Actb</td>
<td>Forward: AGCTCCCTTCTGCGCGGTT</td>
<td>0.333</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGCCAGGACATATGCATCATC</td>
<td></td>
</tr>
</tbody>
</table>

qPCR, quantitative PCR; Fw, forward; Rv, reverse.
and microarray reported expression per gene for medial, cardiac, and whole right lung over the post-PNX time course.

Microarray analyses of post-PNX lung regeneration time course. Total RNA (500 ng per microarray per sample) was reverse transcribed to biotin labeled complementary DNA, and then hybridized onto the Affymetrix GeneChip Mouse Gene 1.0 ST Array (Affymetrix, Santa Clara, CA) which contains 33,297 probes representing 20,818 unique genes following standard Affymetrix protocol. The complete dataset of all samples (cardiac/medial lobes and whole right lung at days 0 and 56 without PNX, and days 3, 7, 14, 28, and 56 post-PNX) was normalized by use of Robust Multichip Averaging (RMA) background correction (1, 14) and the resulting probe signals were represented in logarithmic scale (base 2). The Affymetrix CEL files and RMA normalized data matrix are accessible at the NCBI Gene Expression Omnibus repository (GEO, http://www.ncbi.nlm.nih.gov/geo) series accession number GSE39817. For each probe, we computed the average logarithmic signal fold change in the stretch relative to control samples (denoted Avg.LF2) as the arithmetic average of the stretch sample signals minus the arithmetic average of control sample signals. We say that this fold change is significant if its magnitude exceeds both log2 (1.5-fold) and the maximum logarithmic fold change from signals within the stretch, or within the control sample groups (denoted Nois.LF2) (17). NoisL2F is computed by first sorting the sample signals within a treatment group in ascending order and then calculating the arithmetic average of the upper 50% of sample signals minus the arithmetic average of the lower 50% of sample signals. Gene ontology enrichment analysis was performed by using DAVID 6.7 (http://david.abcc.ncifcrf.gov) (13) for select gene set where the background gene set was all 20,818 unique genes measured by the microarray and the EASE score (modified Fisher exact test probability) for significance was set at <0.05.

Developing mouse lung transcriptome time series. Total RNA was extracted from whole lung of C57BL/6 mice at embryonic days (E) 9.5, 12.5, 14.5, 16.5, 18.5 and postnatal days (P) 0, 2, 4, 7, 11, 13, 18, 24, 30, 56 with biological duplicates for each time point except E18.5 and was profiled by using Affymetrix Mouse GeneChip Mouse Gene 1.0 ST microarray as described in Naxerova et al. (26). This is an expansion of GEO dataset GSE11539 (26) provided by courtesy of Carol Bult (Jackson Laboratory, Bar Harbor, ME). The study protocol was approved by the Jackson Laboratory Animal Care and Use Committee no. 01011.

Principal component analysis of samples in gene expression space. Principal component analysis (PCA) was used to identify the dominant directions of sample variation in transcriptome space for the developing lungs time series, and then the PNX lung regeneration time course samples were projected into this space. This method has been described (17, 18). Briefly, the developing lung transcriptome time series is represented as a matrix D of N genes x M0 samples whose columns are rank normalized and standardized to average 0 and variance 1. We apply PCA of samples in gene space on D to obtain the N-vector principal components (PCs) that we write as column vectors of the matrix P of N genes x E principal components. The 4th column of P is the 4th principal component (PC4). In this study, we use E = 3. The developing lung transcriptome is visualized in the PC1–3 space as YD = P0·D, where columns of YD correspond to different sample time points and rows of YD are coordinates in PC1–3. The PNX lung regeneration time course is represented by the matrix R of N genes x M56 samples whose columns are rank normalized and standardized similar to D. We project the lung regeneration data into lung development PC space by YR = P1·R, where columns of YR correspond to different PNX lung regeneration samples and rows of YR are coordinates in PC1–3 of lung development. We postulated that our analysis would not be limited by the fact that whole lungs rather than individual lobes were used to generate the developing lungs time series. Even though the morphology of individual lobes is not identical, functionally they can be conceptualized as independent breathing units that possess similar key anatomical features (airways, vasculature, and alveoli) and populated largely by cells of similar phenotypes, and hence their transcriptome profiles ought to be largely similar at each developmental stage.

Two-sided t-test with unequal variance was used to assess the difference in PC1 coordinates of PNX sample time points projected into mouse lung development PCA space, with a significance threshold of P < 0.05.

RESULTS

Interlobar growth variation and their gene expression kinetics. Here we investigate the transcriptomic time course of the
whole right lung and its specific lobes after unilateral left lung PNX in C57BL/6 mice at 3, 7, 14, 28, and 56 days postsurgery. Interlobar growth variations following unilateral left PNX have been reported in mice (35). Because of spatioanatomical factors, the cardiac and medial lobes experience greater growth post-PNX than the cranial and caudal lobes. We confirmed this with measurements of total DNA content (Fig. 1A). Despite differences in interlobar growth, the remaining right lung grows to almost the size of an intact pair of lungs with complete total DNA restoration 14–21 days post-PNX (35).

**Table 2. Statistical test of post-PNX time samples in mouse lung dev PCA space**

<table>
<thead>
<tr>
<th>Day 0 vs. Day...</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>28</th>
<th>56</th>
<th>56 ctl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two-Sided t-Test Unequal Variance of PC1 Coordinates</td>
<td>0.04377*</td>
<td>0.01926*</td>
<td>0.29900</td>
<td>0.72669</td>
<td>0.28782</td>
<td>0.51548</td>
</tr>
</tbody>
</table>

PNX, pneumonectomy; PCA, principal component analysis; PC1, principal component 1; ctl, control. *P < 0.05.
Table 3. Post-PNX differential gene expression in medial lobe, cardiac lobe, and whole right lung

<table>
<thead>
<tr>
<th>Dedifferentiation (0.56 CTL→3–7 days)</th>
<th>Number of Genes</th>
<th>Medial Lobe</th>
<th>Cardiac Lobe</th>
<th>Whole Right</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up</td>
<td>74</td>
<td>300</td>
<td>195</td>
<td></td>
</tr>
<tr>
<td>Down</td>
<td>6</td>
<td>108</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Redevelopment (3–7→28–56 days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Up</td>
<td>29</td>
<td>80</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Down</td>
<td>87</td>
<td>304</td>
<td>163</td>
<td></td>
</tr>
</tbody>
</table>

CTL, control.

**Fig. 3.** PCA representation of post-PNX dedifferentiation and redevelopment genes in time points space of the developing mouse lung. Each gray dot marks a gene. A gene located in the left hemisphere tends to be more highly expressed earlier than later during lung development. A gene located in the right hemisphere tends to be more highly expressed later than earlier during lung development. The triangles mark genes that are significantly differentially expressed (down or up relative to post-PNX days 3–7) in either the redevelopment or dedifferentiation phases of the post-PNX time course.
Although negligible for end point biological markers, differences in interlobar growth might impact the dynamics and synchronicity of mRNA transcription. For example, cell proliferation is increased post-PNX as indicated by the overexpression of cell cycle related genes such as Cnnb1, Hmmr, and Cdk1 (30). We observed by real-time PCR that expression of Cnnb1, Hmmr, and Cdk1 genes in the medial lobe peaked a few days later and had a lesser amplitude than in the cardiac lobe post-PNX (Fig. 1B). In theory, expression of genes in the whole right lung would be the composite average across all four lobes (Fig. 1B). Thus lumping together the different lobes for transcriptome profiling could muddle the dynamics of lobe specific gene expression and their individual regenerative response. Together these findings motivated us to isolate, in addition to whole right lung, the cardiac and medial lobes for a more complete analysis of lung regeneration biology.

Post-PNX lung regeneration recapitulates later stages of lung development at the transcriptomic scale. Here we investigate the post-PNX regenerating lungs time course relative to the developing whole lungs time series at the transcriptomic scale. First, we verified the correlation between real-time PCR-quantified and microarray-reported expression profiles for a select gene set across the post-PNX time course (Table 1); there was good correlation. Our C57BL/6 mouse developing lung transcriptome time series span the time interval from E9.5 to P56 (see MATERIALS AND METHODS). PCA of samples (developmental time points) in gene space was applied on the developing lung data to identify and visualize the dominant sample variations or PCs in transcriptome space that forms a trajectory or chreod (36) in PC1–3 (Fig. 2, A and B, green-colored points and trajectory). The five classical stages of lung development are indicated: embryonic, pseudoglandular, canalicular, saccular, and alveolar.

The sample transcriptome profiles of the post-PNX regenerating lungs time course were then projected into this developing lung transcriptome PC space (Fig. 2A) to visualize regeneration from a lung development perspective on a transcriptomic scale. Note that the 70-day-old pre-PNX (day 0) and 126-day-old non-PNX control (day 56.c) right lungs localized around the late section of the developing lung trajectory (around P56); this is a qualitative reality check of lung maturity (see Fig. 2A, bottom right and inset). In fact, the post-PNX sample transcriptome profiles largely localized around the late section of the developing lung trajectory.

Of the three regeneration time courses projected into the developing lung transcriptome PC space, the cardiac lobe has...
the greatest transcriptome scale variation as indicated by the greater displacement of its sample time points (Fig. 2, C and D). Although we do not have similar data from the cranial and caudal lobes, we assume that the whole right lung transcriptomic variation is a composite average of its four component lobes (Fig. 2E). Cardiac lobe growth post-PNX appears to be completed in a short time interval of developmental changes: moving backward (days 0 to 3) and forward (days 7 to 28) along the later section of the developing lung trajectory. There appears to be a common transcriptome dynamics trend in all regenerating lung lobes (Fig. 2B) whereby shortly after PNX the transcriptome profile moves backward and then forward along the developing lung trajectory, before returning close to its pre-PNX transcriptomic state 28–56 days later. The entire regeneration process appears to be largely complete by day 28. The backward displacements of the post-PNX transcriptomes to days 3 and 7, relative to day 0, were significant (Table 2). We will call this backward and forward movement of post-PNX regenerating lung transcriptome profiles along the lung development trajectory the “redevelopment” and “dedifferentiation” phases, respectively.

As in silico validation that the movement of the post-PNX regenerating lung transcriptome along the lung development trajectory is not particular to our data, we projected whole lung transcriptome data from a cognate study (30) into the developing lung transcriptome PC space as above. These data (GEO accession GSE15999) consist of pooled whole right lung (n = 4) of C57BL/6 mice (female, 10–12 wk old) at 6 h; 1, 3, and 7 days post-PNX; and post-sham thoracotomy. Figure 7A shows the backward movement from 6 h to 7 days post-PNX that is not observed for corresponding post-sham thoracotomy time points.

Together these observations suggest that, from a transcriptomic scale, post-PNX lung regeneration recapitulates lung development at the later alveolar stage rather than the earlier stages that include the primordial lung bud and branching morphogenesis. Indeed, post-PNX lung regeneration is largely comprised of the creation and enlargement of alveoli, rather than de novo branching of existing airways (10). From this lung developmental perspective, whole lung regeneration would require that the regenerating tissue system move backward along the developing lung trajectory in proportion to the type of growth required to compensate for specialized tissue loss. For example, if new airways were required, then the regenerating system would have to move backward transcriptomically to the developmental stage when airway branching morphogenesis occurs.

**Lung regeneration genes: developmental correlates and ontological enrichments.** Motivated by the observation of dedifferentiation (days 0 to 3 post-PNX) and redevelopment (days 7 to 28 post-PNX) phases in the post-PNX lung regeneration time course at the transcriptomic scale, we investigate the genes that are differentially expressed in these two phases. For the dedifferentiation phase, we identified genes differentially expressed between days 0 (pre-PNX) and 56.c (non-PNX), and days 3–7 post-PNX. Here we reasoned that lung transcriptome at days 0 and 56.c in mature mice should essentially be similar. For the redevelopment phase, we identified genes differentially expressed between days 3–7 post-PNX and days 28–56 post-PNX. Next, we investigated the expression profiles of these genes in the developing mouse lung and their ontological enrichments.

Table 3 shows the count of genes that were identified to be differentially expressed in the two phases. In both the differentiation and redevelopment phases, the cardiac lobe has the largest number of differentially expressed genes, followed by the whole right lung and the medial lobe. In each phase, there is only one gene with opposite differential expression profiles in different lobes: During the dedifferentiation phase, Nppa increases with time (higher at days 3–7 than days 0 and 56.c) in the cardiac lobe and decreases with time in the whole right lung; during the redevelopment phase, Igkv4–91 increases with time (higher at days 28–56 than 3–7 post-PNX) in the medial lobe and decreases with time in whole right lung.

Table 4. *Computational enrichment analysis of dedifferentiation and redevelopment phases (selected terms)*

<table>
<thead>
<tr>
<th>Biological Terms</th>
<th>P Value</th>
<th>Fold Enrichment</th>
<th>Gene Count</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dedifferentiation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Up</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cell cycle</td>
<td>7 × 10⁻³²</td>
<td>5.49</td>
<td>69 (20%)</td>
</tr>
<tr>
<td>Extracellular matrix</td>
<td>6 × 10⁻¹⁷</td>
<td>5.94</td>
<td>35 (10%)</td>
</tr>
<tr>
<td>Response to wounding</td>
<td>1 × 10⁻¹³</td>
<td>4.80</td>
<td>34 (9.9%)</td>
</tr>
<tr>
<td>Chemotaxis</td>
<td>1 × 10⁻⁶</td>
<td>9.17</td>
<td>10 (2.9%)</td>
</tr>
<tr>
<td>Metalloprotease</td>
<td>5 × 10⁻³</td>
<td>3.46</td>
<td>9 (2.6%)</td>
</tr>
<tr>
<td>Down</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidoreductase</td>
<td>1 × 10⁻⁵</td>
<td>3.93</td>
<td>16 (11%)</td>
</tr>
<tr>
<td>PPAR signaling</td>
<td>4 × 10⁻⁵</td>
<td>10.47</td>
<td>7 (4.9%)</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>5 × 10⁻⁴</td>
<td>8.86</td>
<td>6 (4.2%)</td>
</tr>
<tr>
<td>Immunoglobulin</td>
<td>4 × 10⁻³</td>
<td>29.33</td>
<td>3 (2.1%)</td>
</tr>
<tr>
<td><strong>Redevelopment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Up</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Immunoglobulin</td>
<td>6 × 10⁻⁹</td>
<td>82.39</td>
<td>6 (5.5%)</td>
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<tr>
<td>Carboxylesterase activity</td>
<td>1 × 10⁻⁴</td>
<td>12.16</td>
<td>6 (5.5%)</td>
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<tr>
<td>Oxidation reduction</td>
<td>4 × 10⁻³</td>
<td>3.14</td>
<td>10 (9.1%)</td>
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<tr>
<td>PPAR signaling</td>
<td>1 × 10⁻²</td>
<td>8.14</td>
<td>4 (3.6%)</td>
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<td>Down</td>
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<tr>
<td>Cell cycle</td>
<td>2 × 10⁻¹⁴</td>
<td>6.15</td>
<td>81 (23%)</td>
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<tr>
<td>Extracellular matrix</td>
<td>4 × 10⁻¹⁷</td>
<td>5.83</td>
<td>36 (10%)</td>
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<tr>
<td>Response to wounding</td>
<td>3 × 10⁻¹²</td>
<td>4.44</td>
<td>33 (9.3%)</td>
</tr>
<tr>
<td>Chemotaxis</td>
<td>9 × 10⁻⁹</td>
<td>6.90</td>
<td>16 (4.5%)</td>
</tr>
<tr>
<td>Metalloprotease</td>
<td>4 × 10⁻⁴</td>
<td>4.09</td>
<td>11 (3.1%)</td>
</tr>
</tbody>
</table>

Parenthetical percentages under Gene Count represent % gene in sample.
We investigated the expression profile of the differentially expressed genes from the two phases in the developing mouse lung transcriptome from E9.5 to P56 (Fig. 3). Here PCA of genes in sample (developmental time point) space was used to visualize the expression profile of genes during lung development, where each gene’s expression profile has been standardized to average 0 and variance 1 from E9.5 to P56. The resulting PC representation resembles a disk where each dot marks a gene. A gene on the left hemisphere (9 o’clock) generally has an expression profile that decreases with developmental time (e.g., cyclins), whereas a gene on the right hemisphere (3 o’clock) generally has an expression profile that increases with developmental time (e.g., surfactants); see Fig. 4. Among the differentially expressed genes during the dedifferentiation phase, the ones that were increasing with time (De-diff. Up) tend to have higher expression earlier (59 – 64%) rather than later in the developing lung, whereas the genes that were decreasing with time (De-diff. Down) tend to have higher expression later (82–100%) rather than earlier in the developing lung. Among the differentially expressed genes during the redevelopment phase, the ones that were increasing with time (Re-dev. Up) tend to higher expression later (82–100%) rather than earlier in the developing lung, whereas the genes that were decreasing with time (Re-dev. Down) tend to have higher expression earlier (61 – 79%) rather than later in the developing lung. These developmental trends were observed in individual lobes as well as whole right lung.

Next, we performed ontology enrichment analyses on the above four gene sets from the two phases as reported on Table 4: (De-diff. Up) union of genes that increased with time during the dedifferentiation phase (higher at days 3 – 7 than days 0 and 56.c) in all three samples (cardiac, medial, and whole right lung); (De-diff. Down) union of genes that decreased with time during the dedifferentiation phase in all three samples; (Re-dev. Up) union of genes that increased with time during the redevelopment phase (higher at days 28 – 56 than 3 – 7 post-PNX) in all three samples; (Re-dev. Down) union of genes that decreased with time during the redevelopment phase in all

### Table 5. Complete list of genes for gene ontology analysis of dedifferentiation and redevelopment phases as tallied in Table 4

<table>
<thead>
<tr>
<th>Biological Terms</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dedifferentiation</td>
<td></td>
</tr>
<tr>
<td><strong>Up</strong></td>
<td></td>
</tr>
<tr>
<td>Cell cycle</td>
<td>Exo1, Spag5, Mad21I, Oip5, Ncap2, Sgo2, Sgo1, Anln, Spc24, E2f8, Ccn2a, Ccn1, Dlgap5, Tacc3, Mki67, Mis18B1, Racgap1, Nuf2, Hells, Rbpb8, Tpx2, Ncap2, Chaf1a, Zwilch, Cks2, Spx25, Rhou, Cda3a, Smpd3, Cdc20, Mapk13, Chkl2, Cda8, Pnm1, Fam83d, Cenpf, Prc1, Ccnb1, H2afx, Cep55, Bub1b, Nld1, Cda2, Tripl3, Fxox5, Bub1, Esco2, Mcm7, Birc5, Cnek2, Cnek1, Cnb2, Smc2, Cks1b, Ubrf1, Cnfn, Ncapb, Cda3a, Aurka, Aurkb, Lgll1, Cenef, Genas23, Pkl, Aspm, 463243411R, Kf, F630043A04R, Cdk1</td>
</tr>
<tr>
<td>Extracellular matrix</td>
<td>Col5a1, Ch1, Col5a2, Vit, Cpx, Col6a2, Nid2, Fbn2, Clip, Adam12, Fbn1, Vcan, Mmp14, Mmp19, Adamts9, Eln, Mmp12, Col1a2, Col1a1, Adamts7, Ofm12M, Mmp5, Tcc, Cde80, Lox11, Col3a1, Lox, Elmilin2, Timp1, Elmilin1, Lumi, Wnt4, Muc5ac, Adamts12, Fn1</td>
</tr>
<tr>
<td>Response to wounding</td>
<td>C1q, Crl2, Crl1, Ccl7, C16H3, Chl34, Selp, Ccl8, F7, Reg3 g, Ccl3, C1 s, Cth, F10, Ptg3er, Arg1, Ccr2, Ccl2, C1, Orl1, Serpina3n, Gtn, Cxcl2, Thbs1, Col1, F13a1, Cnp, Ifla1, C1qb, Cxcl13, Saa3, F1n, I6, C1qc</td>
</tr>
<tr>
<td>Chemotaxis</td>
<td>Cxcl2, Ccl7, C3ar1, Ccl11, C9, Ccl8, Ccl2, Cxcl13, Ccl2, Ccl3</td>
</tr>
<tr>
<td>Metalloprotease</td>
<td>Cpxn1, Adam12, Pappa, Adamts7, Mmp14, Cpz, Mmp19, Mmp12, Cilp12</td>
</tr>
<tr>
<td><strong>Down</strong></td>
<td></td>
</tr>
<tr>
<td>Oxidoreductase</td>
<td>Cyp4b1, Fno5, Fno1, Maob, Adhn3a1, Cxoxb, Acox1, Cyp2a4, Cyp3a13, Cyp2a5, Cyp2b10, Noxa, Fno2, Cyp2c1, Cpl1a1, Cyp4a12b</td>
</tr>
<tr>
<td>PPAR signaling</td>
<td>Fabp3, Plin1, Pclk1, Fabp1, Aup7, Adipoq, Cyp4a12b</td>
</tr>
<tr>
<td>Myofibril</td>
<td>Csrp3, Tm1od1, Myh6, Tnn3, Tnnt2, Myoz2</td>
</tr>
<tr>
<td>Immunoglobulin</td>
<td>Igk-V28, Igk-v9-120, Igk-v6-14</td>
</tr>
<tr>
<td>Redevelopment</td>
<td></td>
</tr>
<tr>
<td><strong>Up</strong></td>
<td></td>
</tr>
<tr>
<td>Immunoglobulin</td>
<td>Igk-V28, Igk-V3558, Igk-v4-70, Igk-lv1, Igkm, Igk-v6-14</td>
</tr>
<tr>
<td>Carboxylesterase activity</td>
<td>Ces1e, Ces1d, Pon1, Lipf, Ces1j, Ces1g</td>
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<tr>
<td>Oxidation reduction</td>
<td>Fno3, Akr1c19, ATP6, Cyp3a13, Cyp2b10, Maob, Agmo, Abp1, Fno2, Cyp2c1</td>
</tr>
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</tr>
<tr>
<td>Down</td>
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<tr>
<td>Cell cycle</td>
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</tr>
</tbody>
</table>
three samples. The complete list of genes can be found on Supplementary Tables S1 (Dedifferentiation) and S2 (Redevelopment) (supplemental material for this article is available online at the Journal website).

As in silico qualitative validation of the whole right lung dedifferentiation phase (days 0 and 56.6 relative to days 3–7 post PNX) genes above, we investigated their concordance with genes twofold differentially expressed at 7 days post-sham thoracotomy relative to 7 days post-PNX in the cognate study GSE15999 (30) mentioned in the previous result. Note that their 7 days post-sham thoracotomy condition is the closest to our non-PNX controls (days 0 and 56.6) as an unperturbed reference whole lung transcriptome. Figure 7B shows strong and significant agreement in direction of expression change between these datasets: odds ratio 40.1 and $\frac{P}{H_{11005}} \times \frac{1}{H_{11003}} \times \frac{71}{H_{11002}}$ (2-sided Fisher’s exact test).

Gene ontology analysis revealed a dedifferentiation response characterized by prominent expression of genes associated with cell proliferation (e.g., Ccnb1, Bub1, Cdc2a, and Hmmr), extracellular matrix (e.g., Col1a1, Eln, and Tnc), proteases (e.g., Serpinb2, Mmp9) and chemotaxis, all hallmarks of tissue growth and remodeling process. The redevelopment response strikingly counterbalanced this rise with a profound attenuation of those gene categories (Table 4), indicating that the lung went back to an earlier pre-PNX steady phase of growth. This indicated that the transient phase of tissue growth peaked around 3–7 days post-PNX and tapered off after 28–56 days. The downregulated genes during dedifferentiation showed similar transient changes, albeit for a smaller number of genes and for fewer categories. Noteworthy among these is the peroxisome proliferator-activated receptors (PPAR) pathway, suggesting a transient decrease in lipid-associated processes during the peak of regeneration at 3–7 days consistent with an intense phase of tissue growth. The complete list of genes for each selected ontology category is listed on Table 5.

Specific gene expression profiles post-PNX and during development. The expression profiles for specific genes (Bub1, Ccnb1, Col1a1, Eln, Serpinb2, Cxcl2, Adipoq, and Cyp2e1) with representative expression changes during the post-PNX regeneration time course are illustrated on Fig. 5A. Although the kinetics and amplitude differ between the analyzed lobes and the whole right lung, as indicated earlier, a common profile is observable with changes peaking around 3 to 7 days. The pattern of gene expression changes in developing whole lungs time series for each of those genes are plotted on Fig. 5B.

Previous studies have suggested the role of stem cells in lung tissue regeneration and a literature survey returned Scgb1a1, Itgb4, and c-kit (in human lungs) as lung stem cell marker genes (5, 16, 19). We examined the RMA level profile of these three genes in the lung regeneration time course and their expression level remained unchanged or trending slightly downward for c-kit and Itgb4 (Fig. 6A). During lung development, only Scgb1a1 presents a notable change with a marked

![Fig. 5. Expression profile of select genes related to cell proliferation (Ccnb1, Bub1), extracellular matrix (Col1a1, Eln), metalloproteases (Serpinb2), chemotaxis (Cxcl1), and PPAR signaling (Adipoq) and Cyp2e1) with representative expression changes during the post-PNX regeneration time course are illustrated on Fig. 5A. Although the kinetics and amplitude differ between the analyzed lobes and the whole right lung, as indicated earlier, a common profile is observable with changes peaking around 3 to 7 days. The pattern of gene expression changes in developing whole lungs time series for each of those genes are plotted on Fig. 5B.](http://ajplung.physiology.org/)

Values for 56-day control (ctl) added as single point where indicated on graph.
increase at E14.5 consistent with increase in surfactant production later in lung development (Fig. 6B). Note that the whole lobe/organ profiling used here could very well dilute the expression of cell type-specific marker genes.

A dedifferentiation phase during post-HPX liver regeneration at the transcriptomic scale. To investigate whether developmental processes are recapitulated during regeneration in another organ, we analyzed the post-HPX liver regenerating liver time course relative to the developing whole liver time series at the transcriptomic scale, as we have done for the lung above. Otu et al. (29) investigated the first 3 days of post-HPX liver regeneration in 10-wk-old mice vis-à-vis the developing mouse liver from E10.5 to E16.5 to find little similarity between the two phenomena from a transcriptomic perspective, aside from cell proliferation-associated genes and ontologies (GSE6998/Ref. 29). Using the similar PCA projection method above, we projected their post-HPX time course into a developing mouse liver time series from E11.5 to mature adult/P21 (GSE13149/Ref. 22), Fig. 7C. Here the HPX regenerating liver sample profiles localized near the later section of the developing liver trajectory. Upon visually zooming in on the HPX regenerating liver profiles, we observed the beginnings of a reversal of development toward an earlier stage (Fig. 7C), akin to the dedifferentiation phase described in the post-PNX regenerating lung above. We conjecture that the short time span of the study (3 days post-HPX) is not sufficient to observe the later redevelopment phase. Note the marked resemblance of post-HPX regenerating liver trajectory against regenerating lung shown in Fig. 7A (inset) including the transient deviation at 6 h post-HPX (or post-PNX) within the postsurgical stress period.

DISCUSSION

Knowledge of the innate capacity of organs to regenerate dates more than a century ago (9), but recent progress on stem cells research and highly awaited regenerative medicine applications ignited renewed enthusiasm for whole organ regeneration studies. Post-PNX lung regeneration in particular has recently been the subject of comprehensive research work leveraging the murine model and its superior genomic and transcriptomic capacities to address the hypothesis that lung regeneration recapitulates development (30, 38). In general, their conclusions agree with our findings: lung regeneration does not recapitulate lung development at the embryonic stage or shortly after birth. Our findings suggest instead that lung growth post-PNX recapitulates only late stages of development via a mechanism similar to limb regeneration in amphibians with a short dedifferentiation phase followed by a redevelopment phase.

Our findings point toward the existence of developmental plasticity for postnatal lung that ought to be reflected at the anatomical level. Accordingly, several morphological features observed within regenerating lungs of several species, including thickening of septal wall characterized by a central sheet of interstitial tissue, strong expression of type I procollagen and tropoelastin in alveolar walls of rat lungs in a pattern of expression reminiscent of the one observed during alveolarization in humans, and prevalence of double capillary layer (11, 12, 20, 35), are all evidence that regenerating lungs have indeed reverted to a more immature stage of development. The observation that lungs can go back in their developmental history
has been reported in other context. Lungs of newborn rats treated briefly (4 days) with glucocorticoids exhibit a regression to an earlier stage of maturity with increased number of double capillary-layered septa followed by a second round of alveolarization when glucocorticoids are withheld (33).

An obvious consequence of a regenerative mechanism mostly recapitulating late-stage development process is dysanaptic growth. This pathological post-PNX lung growth refers to the observed imbalance, despite normalization of the lung mechanics and total volume, between conducting airways and blood vessels with respect of acinar tissue (10). Anatomic studies have shown that total secondary pulmonary lobule volume in parenchyma, making the bulk of lung volume, increases more than volume and cross-sectional area of conducting airways after PNX. Since recapitulating late development stage means recapitulating mostly late alveolarization (3, 32), this excludes early fetal lung development processes such as branching and enlargement of conducting airways and is a plausible explanation for the observed dysanapsis.

Our interpretation of the step backward in the lung developmental scale as dedifferentiation is largely inspired from the limb regeneration. In contrast to the well-characterized limb redevelopment phase, much less is known, however, about the molecular events leading to the organization of the blastema and the dedifferentiation phase preceding and participating to its formation (2). Although blastema cells clearly bear undifferentiated phenotype, the origin of those cells has never been entirely resolved and the extent of dedifferentiation that occurs in those cells prior to migration and formation of the blastema remains unclear. As mentioned earlier, several lines of anatomical and biochemical evidences in post-PNX regenerative lung support our interpretation that a dedifferentiation process is taking place and is rightly reflected in our PC analysis.

The contribution of stem cells to post-PNX lung regeneration is poorly defined, and current observations tend to limit their role in this process. In the mouse, bronchioalveolar stem cells contribute modestly to formation of new lung tissue post-PNX with the suggestion that “the observed AECII density was sufficient to account for all or a large part of alveolar regrowth” (27). In addition, circulating vascular progenitor cells were not recruited following PNX (34). Furthermore, markers of cell proliferation in post-PNX murine lungs have been detected by coimmunostaining in several populations of fully differentiated cells that are otherwise largely quiescent in control lungs (30, 35). In our analysis, we did not observe prominent enrichment in markers of lung stem cells consistent with the notion that mature cells are moving back into development. A caveat here is that our experiment design, temporal and spatial (whole lobe), was not designed to capture a cell...

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**Fig. 7. In silico validation.** A: PCA of the developing mouse lungs time series in light green time points annotated and connected with a green line. Data from Paxson et al. (30) are projected therein with green X marking Control and magenta X marking post-PNX sample time points 6 h and 1, 3, and 7 days (magnified in figure inset). B: contingency table and 2-sided Fisher’s exact test of all genes 2-fold differentially expressed at 7 days post-PNX relative to 7-day control in Paxson et al. against our list of significant genes during dedifferentiation in whole right lung. Sig., significantly; Not-sig., not significantly. C: PCA of sample time points in the transcriptomic space of the developing whole lung series (GSE13149/Ref. 22), and the projection of post-HPX liver regeneration (GSE6998/Ref. 29) time course transcriptome profiles into the transcriptomic PC space of the former. Green: developing liver time series. Magenta: post-HPX liver regeneration time course. HPX, hepatectomy; dev., developing.
type-specific profile of gene that could be diluted in a hetero-
genous organ complex.

How does our model relate to current paradigms in kidney and liver regeneration? Cell division is prominent in a regener-
ergating liver and has historically been the key index for
assessing the level of regenerative activity in an organ. Thus
kidney regeneration with its limited postsection mitotic ac-
tivity has not been considered a true regenerative process (24).
This interpretation could change if regeneration is understood
relative to the later stage of an organ’s developmental history.
Interestingly, late postnatal stage growth in the liver lobule is
mainly characterized by extensive hepatocytes proliferation
(hyperplasia) whereas nephrons at this cognate stage of de-
velopment grow largely by increasing cell size (hypertrophy) (7,
25). Furthermore, a common trait emerges when one considers
regeneration of lungs, liver, and kidney together: the bulk of
the volume expansion and growth is not achieved through de
 novo creation of the functional unit (FU) (secondary pulmo-
nary lobule, hepatic lobule, or nephron) but through enlarge-
ment of preexisting FU. In these organs, the morphological
transformations that establish the number of FU occur early in
development whereas organ growth during the late postnatal
stage occurs mostly through FU enlargement by a hypertropic
(kidney) or hyperplastic (liver/lungs) mechanism.

Although important questions remain regarding the physio-
logical mechanisms (and their precise triggers) that underwrite
lung regeneration, it might be productive to focus on dissecting
the molecular pathways that drive mature lung cells toward
dedifferentiaiton in the quest to invoke growth in mature
human lungs. The role of mechanical stretch, which has been
found to be the chief stimulus driving lung regeneration post-
PNX, needs to be further clarified to identify specific mecha-
nosensitive pathways that could reactivate lung growth in
humans.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
A.T.K., K.L., T.M., and F.B. conceived and designed the research; A.T.K. and
F.B. analyzed data; A.T.K., K.L., T.M., and F.B. interpreted the results of experi-
ments; A.T.K., K.L., and F.B. drafted the manuscript; A.T.K., K.L., and F.B.
analyzed data; A.T.K., K.L., G.V., T.M., and F.B. performed experiments; A.T.K.,
K.L., G.V., T.M., and F.B. edited and revised the manuscript; A.T.K., K.L.,
G.V., T.M., and F.B. prepared figures; A.T.K. and F.B. approved the final version
of the manuscript; K.L. and F.B. performed the experiments.

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