Generation of functional lungs via conditional blastocyst complementation using pluripotent stem cells

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Millions of people worldwide with incurable end-stage lung disease die because of inadequate treatment options and limited availability of donor organs for lung transplantation1. Current bioengineering strategies to regenerate the lung have not been able to replicate its extraordinary cellular diversity and complex three-dimensional arrangement, which is indispensable for life-sustaining gas exchange2,3. Here we report the successful generation of functional lungs in mice through a conditional blastocyst complementation (CBC) approach that vacates a specific niche in chimeric hosts and allows for initiation of organogenesis by donor mouse pluripotent stem cells (PSCs). We show that wild-type donor PSCs rescued lung formation in genetically defective recipient mice (PSCs). We show that wild-type donor PSCs rescued lung formation in genetically defective recipient mouse embryos unable to specify (due to Ctnnb1null mutation) or expand (due to Fgf21null mutation) early respiratory endodermal progenitors. Rescued neonates survived into adulthood and had lungs functionally indistinguishable from those of wild-type littermates. Efficient chimera formation and lung complementation required newly developed culture conditions that maintained the developmental potential of the donor PSCs and were associated with global DNA hypomethylation and increased H4 histone acetylation. These results pave the way for the development of new strategies for generating lungs in large animals to enable modeling of human lung disease as well as cell-based therapeutic interventions4–6.

Nearly 12 million adults in the United States, or 5% of the population, have been diagnosed with chronic obstructive pulmonary disease, and about 180,000 patients die annually with end-stage refractory lung diseases1. The only option currently available is lung transplantation, and patients die nearly every day without ever receiving this treatment because of the scarcity of donor organs. Bioengineering approaches for lung regeneration using endogenous progenitors or human PSCs in decellularized scaffolds emerged as promising options8–11. However, modeling the structural and functional complexities of the lung in vitro has been insurmountably challenging given its extraordinary cellular diversity, with more than 40 cell types from all embryonic layers, and its complex three-dimensional (3D) architecture that demands precise alignment of the epithelial and vascular components to form nearly 480 million alveoli for efficient gas exchange12,13. Lastly, maintaining tissue integrity and homeostasis in the presence of the continuous periodic changes in mechanical forces by breathing movement and by the high blood-flow output in the vascular compartment represents a major bioengineering problem to overcome12,13. We posited that the generation of a functional lung as a platform for regenerative purposes could be achieved if these challenges were bypassed by modeling lung organogenesis in vivo. Thus, we explored the possibility of generating lungs in chimeric animals from donor PSCs using a blastocyst complementation (BC)-based approach14–16.

Exogenous PSCs injected into an embryo at the blastocyst stage can join in the recipient’s developmental program and compete with the host’s cells for a niche. Donor cells can take over a particular progenitor defective niche made vacant by inactivation of a gene that is crucial for the initiation of the developmental program of that organ in the host. BC has been used successfully to generate a PSC-derived pancreas and to rescue the neonatal lethality of Pancreatic and Duodenal homeobox 1 (PDX1)-deficient embryos. Although innovative, BC remained challenging, as it often did not result in postnatal survival16,17. Gene deletion, when systemic, could lead to defects in multiple organs not rescued by the donor cells17,18. Moreover, successful BC requires the maintenance of donor PSC’s pluripotency and the ability to form chimeras1. The PSC must reach the targeted organ niche and faithfully respond to the specific cues of that organogenesis program. Thus, we sought to identify strategies to overcome these issues in the host and donor to allow lung formation. Lung organogenesis initiates when trachea and lung progenitors are collectively specified in the foregut endoderm by Wnt-β-catenin (Ctnnb1) activation19,20. Subsequent endodermal activation of fibroblast growth factor receptor 2 (Fgf2r2) by local fibroblast growth factor 10 (Fgf10) from the foregut mesoderm is required to selectively expand these lung progenitors to form the primordial lung21. Indeed, genetic disruption of Fgf2r2 in mice does

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not prevent tracheal formation but results in lung agenesis\textsuperscript{21–23}. Although it is an attractive option for lung BC modeling, systemic Fgfr2\textsuperscript{-} deletion was problematic because of lethality by impaired trophoblast formation or later due to multiple defects, including limb agenesis\textsuperscript{21–23}.

To prevent undesired non-lung phenotypes in the host, we devised a conditional gene-ablation strategy using a lineage-specific promoter to vacate a specific niche in the host endoderm for complementation, named conditional blastocyst complementation (CBC). The CBC then should target an endoderm-derived lineage for which the gene of choice has a crucial non-redundant function, independent of its expression in other organs. We tested this concept by generating mice in which Fgfr2 was conditionally deleted in the foregut endoderm immediately before the onset of lung organogenesis (Shh\textsuperscript{cre}/Fgfr2\textsuperscript{flm/flm}, hereafter, Fgfr2\textsuperscript{cmu})\textsuperscript{24,25}. Analysis of E13.5 Fgfr2\textsuperscript{cmu} embryos revealed the absence of lungs but preserved the trachea and esophagus, as in the wild type (WT) (Fig. 1a), confirming the essential function of Fgfr2 selectively in lung formation\textsuperscript{21–23}. The lack of placenta and limb defects in Fgfr2\textsuperscript{cmu} further suggested that the targeted gene-deletion strategy was well suited for the generation of hosts for lung CBC (Fig. 1b). In an initial attempt to complement the defective lung organ niche in Fgfr2\textsuperscript{cmu} mutants, we used donor PSCs derived from a Nkx2.1-GFP knock-in mouse\textsuperscript{e}. These cells not only carry both Fgfr2 alleles but also express green fluorescent protein (GFP) from the NK2 homeobox box 1 (Nkx2-1) locus, marking all lung epithelial progenitors and descendants from their earliest stages. Complemented PSCs Nkx2.1-GFP were expected to respond to Fgf10 from the host to form the lungs. PSCs Nkx2.1-GFP were cultured in medium containing selective GSK3\beta and Mek 1/2 inhibitors and Leukemia Inhibitory Factor (2i/LIF)\textsuperscript{f}, reported to maintain the ground-state pluripotency and the naive state of PSCs, and then were injected into Fgfr2-mutant host blastocysts and transferred to pseudopregnant mothers. The PSCs Nkx2.1-GFP indeed outcompeted the host Fgfr2\textsuperscript{-} cells and carried on lung morphogenesis. GFP labeling was detected in nearly 100% of the epithelium overlapping with Nkx2-1, and differentiation markers of airways (Scbg1a1, acetylated \(\alpha\)-tubulin) and alveolar (Sftpc, Pdpn) cell types (Extended Data Fig. 1). Although they did not have obvious abnormalities in lobation or branching morphogenesis, these lungs did not form the distal sacculles for gas exchange and remained immature, and pups died at birth (Extended Data Fig. 1b). Thus, 2i/LIF-treated PSCs Nkx2.1-GFP in Fgfr2\textsuperscript{cmu} hosts were likely to be unable to establish the epithelial–mesenchymal cross-talks that are required for the proper maturation of the lung at late gestation.

Then, we systematically tested different culture conditions for optimal maintenance of PSC pluripotency. This included medium with valproic acid (VPA)—a histone deacylase inhibitor that with valproic acid (VPA)—a histone deacylase inhibitor that optimally maintains PSC pluripotency. This included medium required for the proper maturation of the lung at late gestation. Flow cytometry analyses of the neonatal lungs from these animals showed that the proportion of GFP\textsuperscript{+} cells was consistently higher in the lung epithelial cells (Epcam\textsuperscript{+}CD45\textsuperscript{−}Pecam\textsuperscript{−}: 62.0±24.7%). Moreover, the proportion of GFP\textsuperscript{+} cells in Epcam\textsuperscript{−}Pecam\textsuperscript{−} and supplementary Table 1). Indeed, it was remarkable that the consistent (epithelial) versus variable (mesenchymal) complementation that resulted from emptying the host’s epithelial niche was already evident, even in the relatively small number of Fgfr2\textsuperscript{cmu} pups compared with the other genotypes. FACS-based assessment of chimerism in the liver showed only a low proportion of GFP\textsuperscript{+} cells, regardless of the host genotype (Extended Data Fig. 8a,b, Table 1 and Supplementary Table 1). This was further confirmed using a population of a2i/VPA/LIF-treated Sea1\textsuperscript{hsp} PSCs Nkx2.1-GFP isolated by fluorescence-activated cell sorting (FACS) (Sea1\textsuperscript{hsp}, 71.4%; Sea1\textsuperscript{dim}, 15.4%; Fig. 2a).

Injection of a2i/VPA/LIF-cultured Sea1\textsuperscript{hsp} PSCs Nkx2.1-GFP into blastocysts from Fgfr2\textsuperscript{cmu} hosts consistently rescued the lung agenesis phenotype, regardless of host genetic background (Table 1). Of note, the CBC-rescued Fgfr2\textsuperscript{cmu} neonates were viable and active at birth, and were indistinguishable from their WT littermates. Flow cytometry analyses of the neonatal lungs from these animals showed that the proportion of GFP\textsuperscript{+} cells was consistently higher in the lung epithelial cells (Epcam\textsuperscript{+}CD45\textsuperscript{−}Pecam\textsuperscript{−}: 62.0±21.5%) and to other lung mesenchymal cells (the CD45\textsuperscript{−}Epcam\textsuperscript{−}Pecam\textsuperscript{−}: 64.3±24.7%). Moreover, the proportion of GFP\textsuperscript{+} cells in Epcam\textsuperscript{−}Pecam\textsuperscript{−} and supplementary Table 1). This was further confirmed using a population of a2i/VPA/LIF-treated Sea1\textsuperscript{hsp} PSCs Nkx2.1-GFP isolated by fluorescence-activated cell sorting (FACS) (Sea1\textsuperscript{hsp}, 71.4%; Sea1\textsuperscript{dim}, 15.4%; Fig. 2a).

VPA is known to improve chimera formation\textsuperscript{28,29}, so we tested its effect in each culture condition to identify the combination with the greatest capacity to induce pluripotency markers and sustain chimerism throughout development. We found that VPA and LIF (VPA/LIF) and a2i, VPA, and LIF (a2i/VPA/LIF) treatments were equally efficient in generating the highest proportion of Sea1\textsuperscript{hsp} PSCs Nkx2.1-GFP. However, the expression of the key pluripotent factor octamer-binding transcription factor 4 (Oct-4) was significantly higher in a2i/VPA/LIF cultures (Extended Data Fig. 7a,b). When injected into WT blastocysts, donor a2i/VPA/LIF-treated Sea1\textsuperscript{hsp} PSCs Nkx2.1-GFP nearly maximized the chimera-forming ability in all tested conditions (Extended Data Fig. 7c,d and Table 1). This was further confirmed using a population of a2i/VPA/LIF-treated Sea1\textsuperscript{hsp} PSCs Nkx2.1-GFP isolated by fluorescence-activated cell sorting (FACS) (Sea1\textsuperscript{hsp}, 71.4%; Sea1\textsuperscript{dim}, 15.4%; Fig. 2a).

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We had, however, no clear indication about why the PSCs cultured with a2i/VPA/LIF were more effective than the others. Given the key role of chromatin modifications and DNA methylation in PSC pluripotency and developmental potential, we investigated the epigenetic status of these PSCs to gain insights into the prospective mechanisms underlying these differences. Analysis of global DNA methylation showed significantly lower levels of 5-mC long interspersed nucleotide element 1 (LINE-1) repeats in a2i/VPA/LIF-treated PSCs compared with those treated with LIF alone. This overall lower methylation status favoring pluripotency was

**Fig. 1 | CBC rescues lung agenesis in Fgfr2-deficient mice.** a, Imaging of WT and homozygous Fgfr2^cnull (Shh^cre/+;Fgfr2^flox/flox) E13.5 mouse embryos. Top, whole-mount images of freshly isolated embryos. Dotted lines outline the lung (lu) and heart (Ht). An asterisk indicates the absence of lungs in Fgfr2^cnull mice. Middle, whole-mount immunofluorescence-confocal imaging of Sox2, Sox9 and Nkx2-1, depicting the esophagus (arrows) and trachea (arrowheads) in both WT (left) and Fgfr2^cnull (right) mice. Lungs are present in WT but not mutant mice (the asterisk indicates a blunt-ended trachea). Bottom, immunofluorescence imaging of Sox2 and Nkx2-1 depicts distal lung buds in WT mice, contrasting with a blunt-ended trachea in Fgfr2^cnull mice. b, Whole-mount images of freshly isolated E13.5 WT, Fgfr2^hetero (control heterozygous mutant Fgfr2^Shh^cre/+;Fgfr2^flox/+ ) and Fgfr2^cnull embryos. Limb (arrow) and placenta (pl) are present in mice of all genotypes (n = 5 per group). c, GFP imaging of freshly isolated lungs from newborn (P0) chimeric WT, Fgfr2^hetero and Fgfr2^cnull mice, in which PSC^CAG–GFP^ were used for CBC. The lung from a littermate Fgfr2^cnull^ mouse without PSC^CAG–GFP^ injection is shown as a negative control. d, Quantitative analysis of GFP distribution in the indicated lung cell types by immunofluorescence co-labeling with markers of differentiation in sections of P0 lungs from WT + PSC^CAG–GFP^ and Fgfr2^cnull + PSC^CAG–GFP^ mice. The percentage of GFP labeling is shown in alveolar type 1 (Pdpn), type 2 (Sftpc), club (Sgb1a1) and multiciliated (β-tubulin-4) cells, or in the airway compartment regardless of cell type (Sox2). Values are shown as mean ± s.e.m. in ten random fields per sample from a representative WT and mutant lung. Student's t-test, **P < 0.01. e, Representative confocal immunofluorescence images of GFP, Pdpn and DAPI in newborn lungs of WT, Fgfr2^hetero and Fgfr2^cnull mice complemented with VPA/LIF-treated PSC^CAG–GFP^. Complementation rescued the formation of distal saccules and alveolar type 1 differentiation. Fgfr2^hetero^ mice without complementation were used as a negative control. Boxed areas are shown enlarged in bottom panels and depict thin-walled distal saccules (arrows indicate GFP^+;Pdpn^+ cells; asterisk indicates GFP–Pdpn^+ cells). Scale bars, 150 μm (a), 2 mm (b), 1 mm (c) and 5 μm (e).
### Table 1 | Summary of PSC donor lines, culture conditions, host mouse strains and data for chimera formation, lung complementation and survival in mice subjected to CBC

| Cell line | Passage number | Donor PSC background | PSC treatment | Number of injected PSCs per blastocyst | % survival (number of pups or embryos/number of transferred blastocysts) | % chimera formation* | % complementation (number of complementation/number of chimeric animals) |
|-----------|----------------|----------------------|---------------|----------------------------------------|-------------------------------------------------|---------------------|-------------------------------------------------|
| cPSC\textsuperscript{1}GFP | 15 | W4/12956 | 2i/LIF | 25 | 129 x B6 x CD1 | 10 | 40% (10 neonates at P0) | 50% (5 chimeras) | 40% (2/5) at P0 (Extended Data Fig. 1) defective complementation (Fgfr2\textsuperscript{null}) |
| ESt\textsuperscript{2}CAG-tomato | 12 | C57BL/6N | LIF | 40 | 129 x B6 x CD1 | 10 | 40% (10 neonates at P0) | 10% (1 chimaera) | 0% (0/1) at P0 no complementation (Fgfr2\textsuperscript{null}) |
| ESt\textsuperscript{2}CAG-tomato | 14 | C57BL/6N | LIF | 25 | 129 x B6 x CD1 | 10 | 24% (6 neonates at P0) | 33% (2 chimeras) | 0% (0/2) at P0 no complementation (Fgfr2\textsuperscript{null}) |
| ESt\textsuperscript{2}CAG-tomato | 14 | C57BL/6N | a2i/LIF | 30 | 129 x B6 x CD1 | 10 | 23% (7 neonates at P0) | 71.4% (5 chimeras) | 0% (0/5) at P0 no complementation (Fgfr2 cnull) |
| cPSC\textsuperscript{2}GFP | 10 | F1 hybrid mouse (C57Bl/6 x 129) | LIF/VPA | 43 | 129 x B6 x CD1 | 20 | 53.5% (23 neonates at P0) | 66.6% (15 chimeras) | 13.3% (2/15) at P0 (Fig. 1) functional complementation (Fgfr2\textsuperscript{null}) |
| cPSC\textsuperscript{2}GFP | 10 | F1 hybrid mouse (C57Bl/6 x 129) | LIF/VPA | 12 | 129 x B6 x CD1 | 20 | 53.5% (6 embryos at E15.5) | 88% (5 chimeras) | 20% (1/5) at E15.5 (Extended Data Fig. 6) defective complementation (Fgfr2\textsuperscript{null}) |
| cPSC\textsuperscript{2}GFP | 10 | F1 hybrid mouse (C57Bl/6 x 129) | LIF/VPA | 40 | 129 x B6 x CD1 | 20 | 38% (15 neonates at P0) | 33.3% (5 chimeras) | 0% (0/5) at P0 no complementation (Fgfr2\textsuperscript{null}) |
| cPSC\textsuperscript{2}GFP | 11 | F1 hybrid mouse (C57Bl/6 x 129) | a2i/VPA/LIF | 43 | B6 | 20 | 47% (20 neonates at P0) | 85% (17 chimeras) | 11.8% (2/17) at P40 functional complementation (Ctnnb1\textsuperscript{null}) |
| cPSC\textsuperscript{2}GFP | 11 | F1 hybrid mouse (C57Bl/6 x 129) | a2i/VPA/LIF | 40 | 129 x B6 x CD1 | 20 | 50% (20 neonates at P0) | 64.2% (13 chimeras) | 30.8% (4/13) at P80 (Fig. 2) functional complementation (Fgfr2\textsuperscript{null}) |
| cPSC\textsuperscript{2}GFP | 11 | F1 hybrid mouse (C57Bl/6 x 129) | a2i/VPA/LIF | 87 | B6 | 20 | 35.6% (31 neonates at P0) | 65.2% (20 chimeras) | 15% (3/20) at P50 (Fig. 3) functional complementation (Ctnnb1\textsuperscript{null}) |
| cPSC\textsuperscript{2}GFP | 11 | F1 hybrid mouse (C57Bl/6 x 129) | a2i/VPA/LIF | 18 | 129 x B6 x CD1 | 20 | 50% (9 embryos at E12.5) | 100% (9 chimeras at E12.5) | 22.2% (2/9) at E12.5 Functional complementation (Fgfr2\textsuperscript{null}) |
| cPSC\textsuperscript{2}GFP | 13 | F1 hybrid mouse (C57Bl/6 x 129) | a2i/VPA/LIF | 80 | 129 x B6 x CD1 | 20 | 25% (20 neonates at P0) | 70% (14 chimeras) | 14.3% (2/14) at P1 (Extended Data Fig. 8) functional complementation (Fgfr2\textsuperscript{null}) |
| cPSC\textsuperscript{2}GFP | 11 | F1 hybrid mouse (C57Bl/6 x 129) | a2i/VPA/LIF | 36 | 129 x B6 x CD1 | 20 | 44% (16 embryos at E15.5) | 100% (16 chimeras at E15.5) | N.A. (Extended Data Fig. 7d) |
| cPSC\textsuperscript{2}GFP | 11 | F1 hybrid mouse (C57Bl/6 x 129) | a2i/VPA/LIF | 51 | 129 x B6 x CD1 | 20 | 50% (26 neonates at P0) | 88.9% (23 chimeras) | N.A. |
| cPSC\textsuperscript{2}GFP | 11 | F1 hybrid mouse (C57Bl/6 x 129) | a2i/VPA/LIF | 35 | 129 x B6 x CD1 | 20 | 43% (15 neonates at P0) | 100% (15 chimeras) | N.A. |
| CSL2J | 11 | C57Bl/6J C2J | 2i/VPA/LIF | 20 | C57BL/6J | 10 | 20% (4 neonates at P0) | 25% (1 chimaera) | N.A. |
| FL19 ARR3-1 | 11 | C57BL/6N | a2i/VPA/LIF | 20 | C57BL/6J | 10 | 60% (12 neonates at P0) | 100% (12 chimeras) | N.A. |
| FL19 ARR3-2 | 11 | C57BL/6N | a2i/VPA/LIF | 20 | C57BL/6J | 10 | 40% (8 neonates at P0) | 100% (8 chimeras) | N.A. |
| B7 | 15 | C57BL/6N | 2i/LIF | 40 | CD1 | 10 | 33% (13 neonates at P0) | 62% (8 chimeras) | N.A. |
| SUN107.4 | 16 | CD1 | 20 | CD1 | 8 | 30% (6 neonates at P0) | 50% (3 chimeras) | N.A. |

N.A., not applicable for complementation analyses, since host embryos were WT. *Percentage chimera formation was based on the presence of different skin color derived from donor cells (GFP, tdTomato fluorescence or pigmentation) of P0 host neonates or embryos, as indicated. Percentage complementation was determined as the number of chimeric animals with CBC-rescued lungs in homozygous Fgfr2\textsuperscript{null} or Ctnnb1\textsuperscript{null} mutants divided by the total number of chimeric animals in the litter. The genotype of the recipient mice and figure panels containing corresponding representative images are indicated. The degree of complementation is indicated as: (1) functional complementation: CBC rescue of lungs allowed survival postnatally when examined at P0, P1 or as adults (when examined prenatally, lungs had morphological and marker expression compatible with developmental stage); (2) defective complementation: rescued lungs with developmental abnormalities (no postnatal survival); and (3) no complementation: no rescue of lung agenesis. PSCs and conditions that resulted in lung complementation.
**Fig. 2 | Generation of fully functional adult lungs in Fgfr2-deficient mice via CBC.**

**a,** Schematic of the experimental design: a2i/VPA/LIF-treated PSC<sub>CAG-GFP</sub> were sorted into Ssea<sub>1high</sub> and Ssea<sub>1dim</sub> subpopulations and injected into WT blastocyst hosts, which were analyzed for chimera formation, as identified by GFP labeling in chimeric blastocysts (middle) or in the skin of chimeric P0 pups (right; arrows indicates GFP<sup>+</sup> chimeric neonates). **b,** Top, adult Fgfr<sub>2<sup>cnull</sup></sub> (asterisk) and control WT littermates complemented with Ssea<sub>1high</sub> PSC<sub>CAG-GFP</sub>. Middle, resistance (R<sub>rs</sub>), compliance (C<sub>rs</sub>) and elastance (E<sub>rs</sub>) of the respiratory system, as assessed by Flexivent analysis, in chimeric WT and Fgfr<sub>2<sup>cnull</sup></sub> mice. Bottom, response of lungs to increasing doses of methacholine (R<sub>n</sub>, resistance of conducting airways). Graphs represent mean ± s.e.m. of n = 6 (WT) and n = 4 (mutant) mice per group. Student’s t-test was used for statistical analysis. NS, statistically non-significant. **c,** Top, whole-mount GFP imaging of freshly isolated postnatal P80 adult lungs (lu), heart (ht) and esophagus (es) from Fgfr<sub>2<sup>cnull</sup></sub>+PSC<sub>CAG-GFP</sub> and littermate control WT+PSC<sub>CAG-GFP</sub> mice. Brackets indicate areas where GFP staining differed between complemented control and mutant lungs. Middle, immunofluorescence images of GFP and Pdpn (a marker of alveolar type 1 cells). Bottom, immunofluorescence images of GFP and Sftpc (a marker of alveolar type 2 cells) in lung sections. Boxed areas in double-labeled images are shown as single-labeled enlarged images below (arrows, GFP<sup>+</sup> marker cells; asterisk, GFP<sup>-</sup> marker cells). DAPI was used for nuclear staining. Scale bars, 10 μm (**a**), 1mm (**b**), and 10 μm (**c**).
similarly found in 2i/LIF-treated conditions, consistent with the global DNA hypomethylation reported in the naive state of mouse PSC pluripotency (Extended Data Fig. 9a). Moreover, gene-expression analysis showed that a2i/LIF and 2i/LIF treatment of PSCs led to a trend toward a decrease in levels or significantly lower levels of the de novo DNA methyltransferase Dnmt3b, compared to LIF or VPA/LIF treatments, respectively. Interestingly, the differences in Dnmt3b expression in cells treated with a2i/LIF/VPA compared with other groups and the somewhat decreased but variable changes in expression of Dnmt3a (Extended Data Fig. 9b) and cofactor Dnmt3l (not shown) suggested the presence of additional players, such as the Dnmt-interacting proteins.

We next investigated potential differences in patterns of histone modifications. Western blot analyses showed that among all groups, a2i/VPA/LIF PSCs expressed the lowest levels of the repressive chromatin mark H3K27me3 (Extended Data Fig. 9c). Also, a2i/LIF-treated PSCs, regardless of VPA addition, were markedly enriched in H4 pan acetylation compared with 2i-treated PSCs. Immunofluorescence staining of H4 acetylation (Ace-H4) and quantitative analysis of relative MFI showed the strongest signals most consistently in a2i/LIF (with or without VPA) compared to other conditions. Although some LIF-treated PSCs exhibited strong signals, overall, there was a broad range of intensities from very high to low, reflecting high variability and inconsistent effect (Extended Data Fig. 9c).
Data Fig. 9d). Notably, activation marks other than Ace-H4, such as H3K4me or H3K27ac (Extended Data Fig. 9c and data not shown) were not enriched by a2i/LIF treatment. Therefore, the a2i/VPA/LIF cocktail generates a unique epigenetic state that includes DNA hypomethylation, low levels of H3K27 methylation and increased abundance of H4 acetylation. We propose that the combination of these epigenetic changes, resulting in a more accessible (open) chromatin, underlie the a2i/VPA/LIF-mediated enhanced expression of pluripotency-associated proteins, such as Oct4, Pecam and Ssea1. A more detailed analysis of these mechanisms are currently under investigation.

We then used our CBC approach to test whether a2i/VPA/LIF enhanced the developmental potential of Ssea1high PSCCAG–GFP in which tracheal and lung progenitors are specified but the lung progenitors are selectively unable to expand, and the Ctnnb1null (Shhcre;Ctnnb1floxed/floxed), in which none of these progenitors are specified and thus neither lung nor trachea forms19,20. These enabled us to assess whether targeting different genes that are crucial for respiratory organogenesis under the same CBC strategy allowed these phenotypes to be rescued as expected. Remarkably, in both models, the chimeric animals with lung complementation developed normally to adulthood and reached full maturity (Figs. 2 and 3 and Extended Data Fig. 10). Pulmonary function tests (Flexivent) performed in adult mice showed values of airway resistance, lung elastance and compliance comparable with those of WT littermates. When challenged with a spasmogenic agent (methacholine), CBC-complemented lungs showed concentration-dependent bronchoconstriction indistinguishable from the response of controls (Figs. 2b,3c, Extended Data Fig. 10b and Table 1). Macросscopic analysis of Ctnnb1null, PSCCAG–GFP mice showed strong GFP signals in both trachea and lungs, suggesting efficient donor complementation in all respiratory progenitors (Fig. 3a, Extended Data Fig. 10e). In Fgffr2null PSCCAG–GFP complementation occurred preferentially in the lung, compared to the trachea (Fig. 2c, Extended Data Fig. 4c and data not shown). Immunofluorescence of lungs from Fgffr2null PSCCAG–GFP and Ctnnb1null, PSCCAG–GFP animals confirmed extensive GFP overlap with markers of the airway and alveolar epithelial cell types (Figs. 2c and 3a,b and Extended Data Fig. 10a,e).

In summary, we established a conditional gene-ablation strategy to vacate a specific niche for complementation (CBC) coupled with a new methodology for enhancing PSC chimerism and developmental potential, which uniquely maintains an open chromatin status and pluripotency in donor cells. The approach takes advantage of bona fide tissue interactions in vivo to overcome the substantial hurdles in generating lung and trachea in host embryos with a severe genetic defect that prevents these structures from forming. Complementation of defective respiratory progenitor niches resulted in the formation of fully functional lungs in vivo. The observations presented here open exciting perspectives for the use of CBC to generate epithelial and non-epithelial components of the lung employing tissue-specific Cre lines in hosts. Ongoing testing of various strategies of Cre-mediated recombination and our PSCs culture conditions suggest that CBC, coupled with efficient PSC maintenance, is versatile and may be used to complement multiple layers to generate a functional lung wholly from donor cells. Our work also lays the conceptual and technical platforms for investigating mechanisms of cell competition during mammalian organogenesis or as part of the surveillance mechanisms that maintain tissue integrity in adult homeostasis and regeneration–repair. Challenges for future use of this technology in translational and clinical practice include the need for a better understanding of the mechanisms of immune tolerance and interspecific histocompatibility barriers to overcome tissue rejection, and strategies to ablate residual host cells selectively in the targeted organs preserving tissue integrity. Further progress in these areas will facilitate engineering complex organs, such as the lung in large animals, to be ultimately used in cell-based interventions for regenerative purposes in human diseases.

Online content
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**Methods**

Mouse lines and genotyping. Shh<sup>cnull</sup> mice (cat. no. 05622) and Ctnnb1<sup>flox/flox</sup> (Ctnnb1<sup>mc37sc6</sup>; cat. no. 004152) were obtained from the Jackson Lab. For conditional deletion of Fgfr2 (Fgfr2<sup>Cre</sup> or Fgfr2<sup>floxed</sup>; cat. no. 005627), or Ctnnb1 (Ctnnb1<sup>Cre</sup>), we crossed Fgfr2<sup>floxed</sup> or Ctnnb1<sup>Cre</sup> males to Shh<sup>cnull</sup> or Shh<sup>cnull</sup>Ctnnb1<sup>Cre</sup> females, respectively. Genotyping of the Shh<sup>cnull</sup> allele was performed by PCR according to the protocol provided by the vendor (Jackson Lab, cat. no. 05622). Fgfr<sup>Cre</sup> mice were kindly gifted by X. Zhang. We further backcrossed these mice for more than three generations with CD-1 mice (Charles River, strain code: 022). For the lung complementation analyses, we injected the following PSCs into blastocysts of Fgfr2<sup>Cre</sup> and Ctnnb1<sup>Cre</sup> and littermate controls (WT, Fgfr2<sup>Cre</sup>/<sup>WT</sup> or Ctnnb1<sup>Cre</sup>/<sup>WT</sup>), respectively. For the statistical analyses, we performed PCR at a concentration of 2 x 10<sup>6</sup> per well in DMEM-based MEF medium that was supplemented with 5% FBS. For genotyping of PSCs<sup>cnull</sup> or PSCs<sup>Cre</sup>/<sup>WT</sup>, we used the protocol previously reported<sup>9,10</sup> with minor modification for the P1 respiratory tissue analyses. Briefly, we harvested the lungs and tracheas from those Em4-chimeric mice at P1 and placed the tissues into 50 ml tubes containing 10 ml of cold Ham's F12 medium. After transferring the tissues into 1.5 ml Eppendorf tubes, we finely minced the tissues by scissors. We then added 100 µl of pre-warmed dissociation buffer (1 mg ml<sup>−1</sup> DNase (Sigma), 5 mg ml<sup>−1</sup> collagen (BD Biosciences) and 0.2% BSA) according to the manufacturer's protocol: SSEA1+BV421 (BD Pharmingen, 125613: 1:200 dilution), PECAM-APC (BD eLegend, 102509: 1:200 dilution), Zombie Aqua Fixable Viability Kit (BioLegend, 423101). We performed flow cytometry analysis by BD LSR II Flow Software (Fig. 2a and Extended Data Fig. 2b,c). Statistical analyses were performed using a one-way analysis of variance (ANOVA) for the evaluation of the proportion of Ssea1<sup>+Pecam<sup>−</sup></sup>Psc<sup>cnull</sup> or Psc<sup>Cre</sup>/<sup>WT</sup> among the parental population (live cells) in three independent biological samples per each condition. For immunofluorescence analyses of Oct4 and Ssea1 expression, we fixed the cells with 4% PFA at 4°C at room temperature and stained with each antibody, as described above.

**Quantification of the pluripotency markers in the colonies of pluripotent stem cells.** Images of Oct4, Ssea1 or pan-acetyl Histone H4 staining were captured using the same parameters for each cell culture condition using a DMi8 (Leica Microsystems) inverted microscope. We performed quantitative analyses using ImageJ software. Briefly, we acquired Oct4- or pan-acetyl Histone H4 MFI of each colony cultured in the six different conditions (LIF, a2i/LIF, VPA/LIF; 2i/VPA/LIF, a2i/VPA/LIF; 2i/VPA/LIF) on the basis of the histogram of ImageJ program (Analyze > Histogram). The edge of each colony was manually selected using a freehand polygon tool of ImageJ. The average of MFI of the 20 colonies in LIF condition. The graphs are representative of three independent experiments. Results were analyzed by the statistical analyses of Oct4 and Ssea1 co-staining or pan-acetyl Histone H4 staining per experiment. To acquire relative MFI, we subtracted the background MFI of randomly selected non-colony area from 5 different fields, and then each subtracted MFI was normalized by dividing the average of MFI of the 20 colonies in LIF condition. The graphs are representative of three independent experiments. Results were analyzed by the statistical analyses of Oct4 and Ssea1 co-staining or pan-acetyl Histone H4 staining for the tested conditions. For the quantification of single Ssea1 staining by immunofluorescence, 5 random fields containing 11.5 colonies per field were captured using a DMi8 Leica microscope. Then, we classified those colonies into three categories based on the signal intensity of Ssea1 (Ssea<sub>1</sub><sup>low</sup>, Ssea<sub>1</sub><sup>mid</sup>, or Ssea<sub>1</sub><sup>high</sup>). As the graph to represent mean and standard errors (n = 5 fields) depicting the proportion of those categories in each condition. Each graph of Ssea1 analyses was representative of three independent experiments. Results from Ssea1<sup>low</sup> PSCs in each condition were subjected to statistical analyses (two-way ANOVA), expressed as mean ± s.e.m., and differences were considered statistically significant at P < 0.05.

**Analysis of GFP<sup>+</sup> chimerism by flow cytometry.** To obtain the percentage of GFP<sup>+</sup> cells in lung endothelial, mesenchymal and epithelial cells simultaneously in Ssea1<sup>low</sup>, Ssea1<sup>mid</sup>, or Ssea1<sup>high</sup>, we removed free-floating or WT, we used the protocol previously reported<sup>9,10</sup> with minor modification for the P1 respiratory tissue analyses. Briefly, we harvested the lungs and tracheas from those Em4-chimeric mice at P1 and placed the tissues into 50 ml tubes containing 10 ml of cold Ham's F12 medium. After transferring the tissues into 1.5 ml Eppendorf tubes, we finely minced the tissues by scissors. Then, we added 100 µl of pre-warmed dissociation buffer (1 mg ml<sup>−1</sup> DNase (Sigma), 5 mg ml<sup>−1</sup> collagen (Worthington Biochemical Corporation) and 15 µl DNP<sup>−</sup> Disperse (Corning) in HBSS). We then minced the tissues again to make them smaller than 3 mm in size. Then we added 900 µl of pre-warmed dissociation buffer (1 ml total dissociation buffer), and incubated them at 37°C on the roller with 135 r.p.m. speed. After 30 min of the incubation, we used a 1.0 mm filter (FALCON cat. no. 523825). The filtered tissues were centrifuged at 1400 r.p.m., 4°C, for 5 min, and we removed its supernatant. We resuspended the cell pellets with 1 ml of cold ACK lysis buffer to lyse remaining erythrocytes for 3 min at RT. We added 3 ml cold HBSS and centrifuged them down at 1,400 r.p.m., 4°C for 5 min to remove the lysed blood cells. Then, we resuspended the cell pellets with 1 ml cold HBSS+0.2% BSA and counted the cell number (about 3 million per P1 lung) with a hematocytometer. We transferred 1–1.5 million cells in 500 µl (final volume) of flow buffer (cold HBSS, 0.2% BSA and 3 µl Y27632), and then added 10 µl Fe Block (BD Pharmingen; cat. no. 535141) per sample and incubate for 10 min at 4°C. Then, we added the following antibodies: PE/CAM-521 (BD Pharmingen, cat. no. 562589), PE/CAM-711 (BioLegend, 118233, 1:200 dilution), ZenBrite Mouse IgG (BioLegend, 423101, 1:500), CD45-BV605 (BioLegend, 103155, 1/200) for 30 min on ice. After the staining, we washed the samples with flow buffer and resuspended them in 500 µl flow buffer for the subsequent analyses by Flow cytometry (FACS Aria gating
strategy in Extended Data Fig. 8). For setting up compensation and the voltage of each channel to avoid the background of autofluorescence and spill over each fluorophore signal to the other channels, we acquired 5 × 10^4 events from unstrained control group (WT C57BL/6N mice P1 lungs and trachea), each single staining sample (WT CD1 mice P1 lungs and trachea), PSCCAG–GFP (for GFP+ events) and ESI1green (for tdTomato+ events). For Zombie Aqua staining, we used WT CD1 mice P1 lungs and trachea incubated on ice to increase the population of dead cells (shown in Extended Data Fig. 8).

Blastocyst preparation and embryo transfer. Blastocysts were prepared by mating Shh−/−, Fgf21−/− males with superovulated Fgf21+/+ females. For testing the effect of different culture conditions, we used WT (C57BL/6N × 129S6/Cd1)1 males and females. Briefly, PMSG (pregnant mare serum gonadotropin, 5 IU in 0.1 ml PBS per mouse) was administered 48 h prior to mating, and hCG (3 IU in 0.1 ml PBS per mouse) was administered 12 h before its mating as an intraperitoneal injection. Blastocysts were harvested at E12.5, and 8–20 PSCs were injected into each blastocyst (Table 1). After the PSC injection, blastocysts were cultured in M2 medium (Cosmophio) for a few hours in a 37 °C, 5% CO2 incubator for its recovery. Then, blastocysts were transferred to the uterus of the pseudopregnant foster mother, according to a protocol approved by the Columbia University Institutional Animal Care and Use Committee.

Sorting liver cells for genotyping. Genotyping of the chimeric mice was performed in liver cells prior to fixation for histological analyses. Briefly, the dissected liver tissue from each pup (P0, P1) or embryos (E12.5, E13.5) was minced for 5 min using scissors. Tissues were trypsinized with 500 µl 0.25% trypsin (Invitrogen) for 15 min at 37 °C, and the enzyme was inactivated with an equal amount of cold 100% fetal bovine serum (FBS). These cells were centrifuged at 1,000 r.p.m., 4 °C, 5 min, and the supernatant was removed. Cells were resuspended in 1 ml flow buffer (PBS + 0.2% bovine serum albumin (BSA), pH 7.4) and then centrifuged. Finally, these cells were washed on nylon mesh filters and transferred into a flow tube. Cells were stained with DAPI (1/1,000) for 5 min to remove dead cells. More than 10,000 cells per liver were sorted by FACS Area with the GFP-negative gate used for genotyping. Sorting the purity was usually more than 90%. After removing the flow tube by centrifuging at 1,000 r.p.m., 4 °C, 5 min, cells were then stored at −20 °C. We performed PCR with Tim-adjusted primers and the GFP-negative population as a template. The chimerism (measured as % GFP+/live cells) in the liver cells was analyzed from the fcs files of sorted data by FlowJo.

Hematopoietic progenitor cells in colony-forming unit assays for genotyping of adult chimeric mice. To determine the genotyping before the pulmonary function test in the live chimeric adult mice, we performed a colony-formation assay in the MethoCult medium (StemCell, no. M3434). Briefly, we harvested the single-cell suspension with 300 µl MethoCult medium containing 100 µl 0.25% trypsin (Invitrogen) for 15 min at 37 °C. After counting these cells, 0.1× 10^5 cells were seeded in 0.2 ml of 1 ml Eppendorf tube containing 20 µl 0.5 mM EDTA blood from the submandibular vein of chimeric mice by using a 5 mm lancet. After the treatment with RBC lysis buffer (BioLegend, no. 420301) to each blood sample according to the manufacturer’s protocol, we removed lysed blood cells contained in the supernatant and, kept cells pelleted in 100 µl cold PBS. After counting these cell numbers, we plated each 0.4 × 10^5 cells per well resuspended in 300 µl MethoCult medium containing 100 µg/ml Primocine (IvovoGen, ant.-pm-1) in a 24-well plate. Since the MethoCult medium is highly viscous, we used blunt-end needles, 16 gauge (Becton Dickinson, no. 380010) to plate the cells contained in 24-well plates, we could obtain about 10 isolated colonies per 3 wells, and then we manually picked up at least 5 colonies from each mouse blood sample by using a 10-µl pipette for the subsequent genotyping analyses by using EZ Fast Blood/Cell PCR Genotyping Kit (EZ BioResearch cat. no. G1002).

Morphometric analyses. To determine the relative number of specific cell populations in P0 chimeric lungs, 10 non-overlapping random fields per mice were analyzed (×20 magnification) after capturing the images by confocal microscopy (Zeiss LSM710)11. For each field, we counted the number of GFP+ cells co-immunostained with specific antibodies. DAPI co-staining was used to determine the submandibular vein of chimeric mice by using a 5 mm lancet41. After the removal of the enzyme, cells were resuspended in 1 ml flow buffer (PBS + 0.25% trypsin (Invitrogen) for 15 min at 37 °C, and the enzyme was inactivated with an equal amount of cold 100% fetal bovine serum (FBS). These cells were centrifuged at 1,000 r.p.m., 4 °C, 5 min, and the supernatant was removed. Cells were resuspended in 1 ml flow buffer (PBS + 0.2% bovine serum albumin (BSA), pH 7.4) and then centrifuged. Finally, these cells were washed on nylon mesh filters and transferred into a flow tube. Cells were stained with DAPI (1/1,000) for 5 min to remove dead cells. More than 10,000 cells per liver were sorted by FACS Area with the GFP-negative gate used for genotyping. Sorting the purity was usually more than 90%. After removing the flow tube by centrifuging at 1,000 r.p.m., 4 °C, 5 min, cells were then stored at −20 °C. We performed PCR with Tim-adjusted primers and the GFP-negative population as a template. The chimerism (measured as % GFP+/live cells) in the liver cells was analyzed from the fcs files of sorted data by FlowJo.

Western blot analysis. PSCs were cultured for 5 days (LIF, LIF/21, LIF/21/21, VAP/ LIF, LIF/21/VAP and LIF/21/VAP/21) and Western blot analysis was performed as reported previously40. Briefly, the images were captured and quantified (Oncosoft, no. A25741). The relative abundance of the RNA for each gene to β-actin mRNA was determined using the 2−ΔΔCt method. The following primers were used: Dnmt3a forward 5′-CTTCTAGATCTCCATCTCT-3′; Dnmt3b reverse 5′-CAGGCTTGGTGAAAGGGG-3′; Fgfr2 forward 5′-TGGTGAATTGCGAAGGC-3′; Dnmt3b reverse 5′-AATGACGCTGTTCGCC-3′. Data were represented as mean ± s.e.m. of measurements in each culture condition and analyzed by Student’s t-test. Differences were considered statistically significant if P < 0.05; the number of animals per group is provided in the legends.

Quantitative real-time PCR. We treated the cells for 5 days in 6 different cell-condition combinations (LIF, LIF/21, LIF/21/21, VAP/LIF, LIF/21/VAP and LIF/21/VAP/21). Total RNA from each cell sample was extracted using the RNeasy Mini Kit (Qiagen, no. 74104) and reverse-transcribed using Superscript III (Invitrogen, no. 18080-051). Reactions (25 µl) were performed using ABI 7000 (Applied Biosystems) and SYBR Green Master Mix (Thermo Fisher, no. A25741). The relative abundance of the RNA for each gene to β-actin mRNA was determined using the 2−ΔΔCt method. The following primers were used: Dnmt3a forward 5′-CTTCTAGATCTCCATCTCT-3′; Dnmt3b reverse 5′-CAGGCTTGGTGAAAGGGG-3′; Fgfr2 forward 5′-TGGTGAATTGCGAAGGC-3′; Dnmt3b reverse 5′-AATGACGCTGTTCGCC-3′. Data were represented as mean ± s.e.m. of measurements in each culture condition and analyzed by Student’s t-test. Differences were considered statistically significant if P < 0.05; the number of animals per group is provided in the legends.

Pulmonary function assessment. Chimeric mutant mice (Fgf21+−/− + PSCCAG–GFP or Cimbl+/−/− + PSCCAG–GFP) and its littermate control WT mice were anesthetized with pentobarbital (intraperitoneally, 50 mg per kg (body weight)). Once surgical anesthesia was achieved, mice were tracheotomized with an 18G cannula and connected to a flexiVent (SciResq, Montreal, Quebec, Canada) with an FX1. Module and an in-line nebulizer were described previously42. Mice were mechanically ventilated at 150 breaths per min, with a tidal volume of 10 ml per kg and a positive end-expiratory pressure of 3 cm H2O. Muscle paralysis was achieved...
with succinylcholine (i.p., 10 mg per kg (body weight)) to prevent respiratory effort. By using the forced oscillation technique, baseline measurements of the resistance (Rrs), compliance (Crs) and elastance (Ers) of the respiratory system were measured. A baseline measure of central airway resistance (Rn) was performed, followed by subsequent Rn measurements during nebulized methacholine (0, 6.25, 12.5, 25 and 50 mg ml⁻¹) challenge (10-s nebulization, 50% duty cycle). Values for all measurements represent an average of three replicates. EKG and temperature were continuously monitored. Statistical analysis was performed by Student’s t-test, paired. Results were expressed as mean ± s.e.m. and differences considered statistically significant if P<0.05. The number of animals per group are provided in the legends.

Study approval. All experiments involving animals were performed according to the protocol approved by the Columbia University Institutional Animal Care and Use Committee and USAMRMC Animal Care and Use Review Office (ACURO).

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

Data availability
All files and processed data are also available from the corresponding author upon request. No database was generated. Source Data for Figs. 1–3 and Extended Data Figs. 2, 4, 5 and 7–10 are available online.

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Author contributions
M.M. designed and conducted all experiments; J.A.D. and C.W.E. performed pulmonary function assessment; C.-S.L. and Y.T. supported and performed microinjection and embryo transfer; M.M., P.R. and M.O. maintained mutant mice for the injection; K.F. supported imaging of whole-mount staining; K.F., Y.H., M.O. and M.K. supported and performed hematopoietic cell-colony-formation assay; X.X. and C.L. performed epigenetic experiments; M.M., C.L. and W.V.C. wrote the paper; H.N. gave crucial insights on the experiments and the manuscript.

Competing interests
The authors declare no competing interests.

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Peer review information Michael Basson was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.
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Extended Data Fig. 1 | PSC<sup>Nkx2-1-GFP</sup> rescues lung agenesis in Fgfr2-deficient mutants, but lungs are immature. **a**, Schematic of experimental procedure (left) and representative macroscopic view of the lungs and heart (ht) from newborn (P0) mice Fgfr2<sup>2null</sup> complemented by donor 2i/LIF-cultured PSC<sup>Nkx2-1-GFP</sup>. Right panel depicts GFP signals in the lungs not present in the heart (outlined). **b**, Representative GFP expression in lung section of P0 Fgfr2<sup>2null</sup>+PSC<sup>Nkx2-1-GFP</sup>; strong signals throughout all epithelial tubes, less prominent in the mesenchyme and its derivatives (Ex. large vessels, center). Asterisk (*) marks distal epithelial tubules unable to form distal saccules resulting in immature non-functional lungs. Immunofluorescence of Nkx2-1 and quantitative analysis confirming extensive double-labeling with GFP (single channels shown in small panels). Graph represents mean ± s.e.m. of % GFP+ lung epithelial cells in five random fields per sample (n = 2 animals). **c–f**, Representative immunofluorescence and confocal images of lungs double-labeled with GFP and markers of alveolar type 1 (Pdpn) and type 2 (Sftpc) or airway multiciliated (acetylated α-tub) or secretory (Scgb1a1) cells (n = 3–4 per group). Boxed area (f) enlarged in the right panels. **b–f**, images also displayed as single channels; DAPI in grey. Scale bars: **a, b, c–e, f**, 1 mm, 20 μm, 10 μm, and 20 μm, respectively.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Effect of PSC cell culture conditions in pluripotency markers. a, Representative morphology of PSCCAG–GFP colonies and immunofluorescence image of Ssea1 and Pecam expression under the culture conditions listed. b, Representative flow cytometry pseudocolor images (top) and respective histograms (bottom) showing the increased yield of the Ssea1highPecam+ PSCCAG–GFP population and high Ssea1 MFI in cultures treated with VPA/LIF and a2i/VPA/LIF. c, FACS analysis of PSCNkx2-1-GFP cultured on the conditions indicated. Graph shows differences in the yield of Ssea1Pecam+ (%). Note the effect of the addition of VPA to LIF or a2i/LIF treatment. Bars are mean ± standard error of n = 3 independent experiments in each culture condition. Data were analyzed by one-way ANOVA; differences were significant at **P < 0.01. Scale bars: a = 10 μm.
Extended Data Fig. 3 | Complemented distal lung of VPA/LIF-treated PSC^{CAG-GFP} Fgfr2 mutants undergo sacculation. **a** Representative immunofluorescence confocal imaging depicting the expression of GFP and markers for alveolar type 2 (Sftpc) in the walls of distal saccules in lung sections of P0 VPA/LIF-treated PSC^{CAG-GFP} Fgfr2\textsuperscript{cnull} mice (left panel, WT P0 littermate). **b**, the 3D-SIM image of PSC^{CAG-GFP} Fgfr2\textsuperscript{cnull} mice showing at high resolution the staining of alveolar type I surface double-labeled with GFP. Scale bars: **a** and **b**, 10 μm.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Airway epithelial complementation in Fgfr2 cnull PSC CAG–GFP newborn chimeric pups.  

**a, b.** Immunofluorescence and confocal imaging depicting GFP double-labeling (arrows) with cell differentiation markers Scgb1a1 (secretory), β-tubulin4 (multiciliated) and Cgrp (neuroendocrine) in lung sections of P0 mice from VPA/LIF-treated PSC CAG–GFP + Fgfr2 cnull and WT chimeric littermates; small panels in **b** depict single channels.  

**c.** Percentage of GFP+ cells in the lung and tracheal epithelium as determined by quantitative analysis of GFP signals in sections of newborn P0 PSC CAG–GFP Fgfr2 cnull animals.  

Graph, Mean ± s.e.m. of measurements in 5 random fields per section per sample. Student’s t-test; **P < 0.01. Right panel, Representative GFP and Sma immunostaining in a histological section of P0 complemented mutant depicting epithelial signals (arrows) consistently strong in intrapulmonary airways (bracket), but variable or low (asterisks) in the extrapulmonary airway (dashed box) and trachea (Tr). Boxed area enlarged on the right. Sma (alpha-smooth muscle actin) labeling airway smooth muscle. Scale bars: **a, b, c**: 10 μm, 10 μm, and 20 μm, respectively.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Chimerism in the lung alveolar vascular compartment (a–c) and extrapulmonary organs of animals complemented with VPA/LIF-treated PSCCAG–GFP (d–f). a, Immunofluorescence and confocal imaging of newborn (P0) WT + PSCCAG–GFP, Fgfr2hetero + PSCCAG–GFP and Fgfr2null + PSCCAG–GFP chimeric lungs. Representative image of blood vessels showing GFP–Isolectin B4 double-labeled endothelial cells (arrows). b, Percentage of GFP labeling in endothelial (top) and alveolar type I (bottom) cells in P0 WT + PSCCAG–GFP and Fgfr2null + PSCCAG–GFP chimeric lungs as determined by morphometric analysis of sections immunostained with Pecam (top) or Hopx and Pdpn (bottom). Graphs: mean ± s.e.m. of measurements in ten non-overlapping random fields per group (see also Supplementary Fig. 2 and Methods). c, GFP-Sma double-labeling of smooth muscle cells (arrow) in blood vessels (bv) and airways (aw) by immunofluorescence of P0 lungs from PSCCAG–GFP-complemented Fgfr2null mice. d, Proportion of GFP+ cells in the liver from E15.5 and P0 chimeric mice isolated by FACS. Graph represents mean ± s.e.m. of PSCCAG–GFP-complemented WT, Fgfr2null or Fgfr2null animals (n = 13, 8, 6 animals, respectively). e, Representative images of GFP expression in liver (left) and kidney (right) from P0 Fgfr2null + PSCCAG–GFP (whole-mount and histological sections) and quantitative analysis (bottom) of GFP labeling; graph: mean ± s.e.m. of % GFP+ cells per field in 5 random fields per group. f, Representative images of GFP expression in the intestine from WT or Fgfr2null injected with PSCCAG–GFP (histological sections) and graph showing % GFP+ (mean ± s.e.m., 5 random fields per 5 per group). Statistical analysis (b, d–f): Student’s t-test; **P < 0.01, NS: statistically non-significant. Scale bars: a, b = 10 μm, 5 μm, respectively; e: top panel: 20 μm, bottom panel: 1 mm; f: 10 μm.
Extended Data Fig. 6 | Low chimerism in VPA/LIF-treated PSCs$^{CAG-GFP}$ $Fgfr2^{cnull}$ embryos and defective lung organogenesis. GFP expression in whole-mount E15.5 embryos and tissues. a, Variable chimerism in E15.5 WT + PSCs$^{CAG-GFP}$ embryos with different levels of GFP signals in the skin, lung (lu) and heart (ht: outlined). b, Unilateral rescue of the lung (boxed) in E15.5 $Fgfr2^{cnull}$ embryos complemented with VPA/LIF PSCs$^{CAG-GFP}$; note low chimerism in the esophagus (es) and heart. Scale bars: 1 mm.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Comparative effects of a2i/VPA/LIF with other culture conditions in PSC pluripotency and chimerism. a, Immunofluorescence and quantitative analysis of Ssea1 expression in PSCCAG–GFP colonies in the various media tested. Top panel, representative images of Ssea1high, Ssea1dim and Ssea1negative colonies. Bottom panel, graphs showing the percent of Ssea1-expressing colonies high, dim or negative as above (left) and percent of Ssea1high colonies (right) in each culture condition. Data are mean ± s.e.m of the number of colonies per field in five non-overlapping random fields per condition. b, IF of Oct4 and SSEA1 in PSCCAG–GFP cultured in the conditions indicated. Left: representative image of Oct4 staining. Right: Graph showing Oct4 expression levels in SSEA1high (dots) or Ssea1 dim/negative pooled (empty dots) PSC colonies cultured as indicated. Mean fluorescent intensity (MFI) of Oct4 as assessed by imaging of 35 random colonies per culture condition (ImageJ). Relative MFI per colony shown as the Oct4 MFI of each colony normalized by the average fluorescent intensity of the PSCs cultured in LIF condition. Graphs in a and b depicting the VPA’s ability to enrich for Ssea1high PSCs and the a2i/VPA/LIF effect in enhancing Oct4 expression but no significant difference in Oct4 levels between Ssea1high and Ssea1dim a2i/VPA/LIF-treated PSCs. c, Percentage of chimera formation as determined by analysis of skin/coat color in pups at P0 from blastocysts injected with PSCs cultured in each condition and transferred to foster mothers. Graph represents mean ± s.e.m. of the percentage of chimeric pups generated from the PSCs indicated (number of experiments represented by each point in the graph, see Methods). d) Whole-mount brightfield and GFP images of E15.5 embryos showing the high frequency of GFP-expressing chimera formation from WT hosts injected with a2i/VPA/LIF-treated PSCCAG–GFP (see also Table 1). Statistical analysis: one-way ANOVA (a,b) and Student’s t-test (c); differences considered statistically significant if *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, NS: non-significant. Scale bars: a, b, d = 10 μm, 20 μm, 1 mm respectively.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | FACS-based assessment of cell type-specific chimerism in lung and liver from a2i/VPA/LIF -treated Ssea1<sup>high</sup> PSC<sup>CAG-GFP</sup> WT, Fgfr2<sup>hetero</sup>, or Fgfr2<sup>cnull</sup> hosts. a, Schematics of the CBC approach (Ssea1<sup>high</sup> a2i/VPA/LIF-treated PSC donor cells injected into blastocysts hosts, chimeric pups identified at birth), tissue isolation/dissociation and gating strategy for flow cytometry (FACS) analysis of different cell types in the lung. Lung cells (FSC/SSC panels) and singlets (FSC-H/FSC-A) were selected. Within the lung singlets, live cells were gated as an unstained negative control (NC) and hematopoietic (CD45<sup>+</sup> versus CD45<sup>−</sup>). In the CD45<sup>−</sup> gate, we separated epithelial (Epcam<sup>+</sup>), endothelial (Pecam<sup>+</sup>) and other non-endothelial lung mesenchymal cells (Pecam/Epcam double negative). The percentage of GFP<sup>+</sup> cells was calculated based on the unstained WT negative control gate.

b, FACS analysis of complemented lungs (left panels) and liver (right panel) from Ssea1<sup>high</sup> a2i/VPA/LIF -treated PSC<sup>CAG-GFP</sup> WT (<i>n</i> = 5) Fgfr2<sup>hetero</sup> (<i>n</i> = 7) or Fgfr2<sup>cnull</sup> (<i>n</i> = 2) animals showing the percentage of GFP labeling in lung (epithelial, endothelial or non-endothelial lung mesenchymal) cells and liver. Graphs (mean ± s.e.m) depicting GFP labeling in nearly all lung epithelial cells from Fgfr2<sup>cnull</sup> compared to heterozygous and WT, inconsistent variable labeling in lung endothelial/mesenchymal cells and low proportion of GFP<sup>+</sup> cells in liver regardless of the genotype.
Extended Data Fig. 9 | Effect of culture conditions in global DNA methylation, DNA methyltransferase gene expression and the patterns of histone modifications by PSC<sup>CAG-GFP</sup>. a, Analysis of 5-mC levels of Line-1 repeats in cell homogenates from PSC<sup>CAG-GFP</sup> cultures in the conditions indicated (n = 3 independent experiments); graph represents mean ± s.e.m. showing significantly lower levels of relative DNA methylation in all conditions compared to LIF. b, Relative levels expression of Dnmt3b and Dnmt3a in PSC<sup>CAG-GFP</sup> cultures by qPCR analysis; data were normalized by the LIF averaged values and represented as mean ± s.e.m (n = 3 independent experiments). c, Western blot of histone methyltransferases and acetylase from PSCs homogenates (n = 3 per condition); expression levels quantitated by densitometry analyses and normalized by LIF values. d, Immunofluorescence of histone H4ac in the PSC-treated cultures indicated (top: representative images). Mean fluorescent intensity (MFI) per colony as assessed by imaging of 20 random colonies per condition, 5 random fields (ImageJ). Graph (bottom) depicting mean ± s.e.m of MFI confirm the consistently high levels of H4-pan-acetyl expression in a2i/VPA/LIF also seen in Western blots. Statistical analyses: one-way ANOVA (a,b) and unpaired Student’s t-test (d), significance at *P < 0.05, **P < 0.01, ****P < 0.0001, NS: non-significant.
Extended Data Fig. 10 | Normal growth, differentiation and function of adult lungs from complemented a2i/VPA/LIF-treated PSCCAG–GFP mature animals (see also Figs. 2 and 3). a. Representative immunofluorescence confocal imaging of P80 a2i/VPA/LIF-treated PSCCAG–GFP Fgfr2\textsuperscript{cnull} and control WT + PSCCAG–GFP lungs double-labeled with GFP and airway differentiation markers multiciliated (β-tubulin4) and secretory (Scgb1a1) cells. Arrows: strong GFP overlapping signals in Fgfr2\textsuperscript{cnull} mutants, contrasting with the less prominent signals (* asterisks) in WT; lower panels: single-channel images. b, Flexivent analysis of pulmonary function in CBC-complemented day 80 WT (n = 6) and Fgfr2\textsuperscript{cnull} (n = 4); graph (mean ± s.e.m) showing non-significant (NS) difference in the resistance of conducting airways (Rn). c, d, Analysis of body weight in a2i/VPA/LIF PSCCAG–GFP complemented day 80 WT (n = 6), Fgfr2\textsuperscript{cnull} (n = 3), Fgfr2\textsuperscript{cnull} (n = 4) animals (c), and day 50 WT (n = 12), Ctnnb1\textsuperscript{hetero} (n = 5), Ctnnb1\textsuperscript{cnull} (n = 3) animals (d). Graph (mean ± s.e.m) indicating no significant difference in body weight between genotypes in both Fgfr2 and Ctnnb1 models. e, IF of day50 a2i/VPA/LIF-treated PSCCAG–GFP + Ctnnb1\textsuperscript{cnull} lungs (n = 3). Representative image of GFP double labeling with markers of secretory (Scgb1a1) or basal (p63) cells in adult airways (arrows: depicts strong GFP staining contrasting). Statistical analyses: Student’s t-test (b), one-way ANOVA (c,d); NS: statistically non-significant: P > 0.05. Scale bars: a,e = 10 μm, 5 μm, respectively.
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| For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings | ✔️        |
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### Software and code

**Policy information about availability of computer code**

**Data collection**

Prism8 SOFTWARE for statistical analyses, Zen 2.3 SP1 (Black edition from Zeiss company) equipped with Zeiss LSM710 confocal microscopy for the acquisition of the confocal imaging, Leica imaging software (LAS X 3.4.2.18368) equipped with DMi8 station for the acquisition of the PSC culture imaging, and Image J (1.52n from NIH) software for the analyses of tissue or PSC imaging.

**Data analysis**

Prism8 ([https://www.graphpad.com/scientific-software/prism/](https://www.graphpad.com/scientific-software/prism/))

Zen, LASX software will be available in Zeiss or Leica microscopy company, respectively.

Image J is available from NIH ([https://image.nih.gov/ij/](https://image.nih.gov/ij/)).

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

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The data that support the findings of this study are available from the corresponding author (M.M., W.C.) upon request.
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

Sample size for each of the experiments involving molecular or phenotypic characterization of the chimeric/BC animals or manipulation of PSCs culture conditions were estimated based on our previous reports (analysis of mouse genetic models) and studies from others (Stadfeld et al. Nature 2010; Kobayashi et al. Cell 2010). In all experiments a minimum of 3 replicates was tested per condition. The number of replicates (n) was provided for all parameters measured and sample sizes are listed in the figure legends. In the relatively few cases we found high variability in the results, sample size was increased to infer about statistical significance.

Data exclusions

none.

Replication

All data analyzed resulted from observations performed in 3 or more independent experiments per culture conditions and tested in at least 3 animals per group in the experiments involving generation of chimeric mice and BC. All attempts at replication of these experiments were successful.

Randomization

Methods of randomization were not required in this study. Samples and corresponding controls were always processed at the same time.

Blinding

The investigators were not blinded to group allocation during experiments and outcome assessment of the cultures and animal experiments. Blinding was not used because the genotyping was determined in advance and the presence of GFP was obvious in the analyses of the specimens.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a involved in the study

- [ ] Antibodies
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- [ ] Animals and other organisms
- [ ] Human research participants
- [ ] Clinical data

Methods

n/a involved in the study

- [ ] ChIP-seq
- [ ] Flow cytometry
- [ ] MRI-based neuroimaging

Antibodies

The following antibodies were used for Immunofluorescence analyses (Page#20-21): anti-acetylated β-tubulin IV (Abcam, ab11315, 1:500), anti-GFP chicken pAb (Thermo Scientific, 1:500), anti-Sgcb1a1 goat (Santa Cruz, #sc-9772, 1:500), anti-Pdpn (R&D, AF3244, 1:200), anti-Hop (Santa Cruz #sc-398703, 1:100), anti-Sftpc (Seven Hills #WRAB-9373, 1:1000), anti-Cgrp (Sigma, #C3198, 1:1000), anti-Sox2 rat (eBioscience, #14-9811-82), anti-SMA rabbit (Cell signaling, #19245), anti-Oct4 (Genetex, #GT486, 1:100), anti-SESA1 (Santa Cruz, sc-101462, 1:50), anti-Oct4 (R & D, #AF1759), and Histone H4ac (pan-acetyl) (Active Motif, #99925). Isolectin B4 (Life tech., I32450) was used at a dilution of 1:500 in PBS overnight at 4 degrees prior to the staining above to visualize vascular endothelial cells.

The following antibodies were used for flowcytometry analyses (Page#21, #22): For the PSC marker analyses (Page#21), we used SSEA1-BV421 (BioLegend, 125613: 1:200 dilution), PECAM-APC (BioLegend, 102509: 1:200 dilution), Zombie Aqua™ Fixable Viability Kit (BioLegend, 423101). For the chimerism analyses to postnatal chimeric animals (Page#22), we used Fc Block (BD Pharmingen, Cat# 553141), CD31-BV421 (BD Pharmingen, # 562939, 1/250 dilution), EPCAM-BV711 (BioLegend, 118233, 1/200), Aqua Zombie (BioLegend, 423101, 1/500), and CD45-BV605 (BioLegend, 103155, 1/200).

Validation

All antibodies used in the current study have been previously validated by the manufacturers (see below) and the previous studies in our lab (Mori et al., Development 2015; Mori et al., Nature Communications 2017; Yang et al. Dev Cell 2018) and used according to the manufacturers' instructions. Antibodies were also validated by titration studies and comparison with positive controls and negative controls.

- anti-acetylated β-tubulin IV (Abcam, ab11315, 1:500)
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  All of the cell lines used in this manuscript are described in Page#20-21 in Materials and Methods (See also below).

Authentication  The PSC CAG-GFP cell line was authenticated by MTI-GlobalStem at purchase. PSC CAG-GFP and MEFs are commercially available from Life tech (CF1 mouse embryonic fibroblasts, #A34181) or MTI-GlobalStem (C57BL/6N x 129S6 background: Cat# GSC-5003), respectively and maintained according to the manufacture’s protocol. ES1 CAG-tdTomato(C57BL/6N background), B7 (C57BL/6N background), SUN107.4 (CD1 background) were derived and authenticated by Dr. Hiromitsu Nakauchi’s lab at Stanford University; CSL2J2 (C57BL/6J C2J background), FL19 ARR3-1 (albino agouti C57BL/6N background), FL19 ARR3-2 (C57BL/6N background) derived and authenticated by Dr. Lin Chyuan-Sheng at the Columbia University Transgenic Core Facility. These PSC lines are available upon request. The authenticated PSCNkx2-1-GFP passage 15 (W4/129S6) were kindly gifted by Dr. Laertis Oikonomou and Dr. Darrell Kotton, CReM, Boston University.

Mycoplasma contamination  All of the cells were tested negative for Mycoplasma contamination.

Commonly misidentified lines (See ICCLAC register)  No misidentified cell line was used.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals  All lines used for generation of chimeric and CBC mice in this manuscript are described in Page#20 in the Materials and Methods section and Table1.

Wild animals  This study did not involve wild-animals.

Field-collected samples  This study did not involve field-collected samples at all.
Ethics oversight
All experiments involving animals were performed according to the protocol approved by the Columbia University Institutional Animal Care and Use Committee and USAMRMC Animal Care and Use Review Office (ACURO).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots
Confirm that:
☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
☒ All plots are contour plots with outliers or pseudocolor plots.
☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
The methodology for sample preparation is detailed in Page#22 in Materials and methods.

Instrument
FACS Aria flow cytometer (Becton Dickinson).

Software
Data were analyzed on FlowJo software.

Cell population abundance
More than 10,000 cells/liver or PSC-CAG-GFP were sorted using FACS Aria flow cytometer. GFP negative gate was used for genotyping. SSEA1 high or dim PSC-CAG-GFP populations were compared for checking efficacy of chimera formation (Fig. 2c). The sorting purity was checked by re-sorting the cells and purity was found to be more than 90% (Page #23).

Gating strategy
The gating strategy is detailed in Supp. Fig. 9 and its legend.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.