Supplementary Materials and Methods

Preparation of feeder fibroblasts

The NIH/3T3 mouse embryonic fibroblast cell line was purchased from the American Type Culture Collection (ATCC CRL-1658). NIH/3T3-GFP was obtained by transducing NIH3T3 with pLenti PGK GFP Puro (Addgene). Both were maintained in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Darmstadt, Germany) supplemented with 10% fetal calf serum (Biochrom), 2 mM L-glutamine (Life Technologies) and 10 mM sodium pyruvate (Sigma-Aldrich, Taufkirchen, Germany). NIH/3T3 and NIH/3T3-GFP were gamma-irradiated with 30 Gy by Gammacell Exactor (Best Theratronics, Hertfordshire, UK) before seeding at a density of $1 \times 10^5/cm^2$ in tissue culture vessels, and kept in culture medium as above until use.

Passaging of primary distal lung cells

Cells were passaged when they reached 70-80% confluence. For human LECs, the feeder fibroblasts were first detached with phosphate buffered saline (PBS) containing 2 mM ethylenediaminetetraacetic acid (EDTA, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) before epithelial cells were harvested by trypsinization. Collected cells were passaged to new culture vessels at a ratio of 1:5, unless otherwise specified, or frozen stocked in liquid nitrogen until needed. Differential detachment of feeders with EDTA was skipped for chicken LECs, as they attach to the culture surface very weakly.

Population doubling (PD) calculation

The NIH3T3-GFP feeder cell lines was used to calculate population doublings. The cells were suspended in culture medium after harvesting, and the number of GFP-negative cells was counted under a fluorescent microscope. 2×10^5 cells were passaged onto a new 6-well tissue

culture plate previously seeded with irradiated NIH3T3-GFP. Population doubling level at each passage was calculated as follows:

$PDn=\log_2 Y-\log_2(2\times10^5)$, where Y indicates the number of GFP-negative cells harvested [55]. The culture was terminated when LECs did not reach 80% confluence within two weeks after splitting. The step of differential detachment with EDTA was skipped for chicken LECs, and they were harvested only with trypsin as described above. The other procedures were the same as that for human cells.

Flow cytometry

Human LECs grown with NIH/3T3 feeders were harvested by differential trypsinization and labeled with the following antibodies: fluorescein isothiocyanate (FITC)-conjugated mouse anti-human integrin α6, phycoerythrin (PE)-conjugated mouse anti-human integrin β4, allophycocyanin (APC)-conjugated CD324 (CDH-1). All antibodies for flow cytometry were purchased from Milteny Biotec (Bergisch Gladbach, Germany) and used according to the manufacturer's instructions. Propidium iodide (Life Technologies) was used to exclude dead cells from analysis.

Flow cytometry was also used to purify the epithelial cells from NIH/3T3-GFP cells. Briefly, cells were harvested by sequential treatment with 2 mM EDTA and trypsin when applicable to enrich the epithelial cells. The cells negative for both GFP and PI were collected. All analyses and sorting were performed on BD FACS Aria™ flow cytometers (Beckton Dickinson, Heidelberg, Germany). Both human and chicken LECs were seeded in DMEM supplemented with 10% FCS, 2 mM L-glutamine and 10 mM sodium pyruvate after sorting. Y27632 was supplemented to the medium at 9 μM for the first 24 h to prevent anoikis.

Fluorescent Immunocytochemistry

Cells cultured on coverslips were fixed with 3.7% paraformaldehyde at room temperature for 20 minutes. The cells were incubated in blocking/permeabilization solution containing 10%

FCS and 0.3% TritonX-100 (Carl Roth) followed by primary antibody solution at 4°C overnight. The primary antibodies used was as follows: anti-CDH-1 (BD Biosciences, Heidelberg, Germany), anti-ΔN-p63 (), anti-cytokeratin 5 (Abcam, Cambridge, United Kingdom), anti-prosurfactant protein C (EMD-Millipore, Darmstadt, Germany), anti-aquaporin 5 (Santa Cruz, Heidelberg, Germany) and anti-SCGB1A1 (Abcam). After washing with PBS/0.1% Tween-20 (Carl Roth), the coverslips were incubated with secondary antibodies conjugated with fluorochromes (Dianova, Hamburg, Germany). Hoechst 33258 (Life Technologies) was used for nuclear staining. Images were obtained with a Leica SP-8 confocal microscope (Leica Microsystems, Wetzler, Germany) and processed with FIJI [56].

Quantitative RT-PCR

Total RNA was extracted with the Gene Jet RNA Purification Kit (Thermo Scientific) according to the manufacturer's instructions. One-step qRT-PCR was performed using Power SYBR® Green RNA-to-CT™ (Life Technologies). Values were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene using the $2^{-\Delta\Delta C}_{T}$ method [61]. The following primer sequences were used: human scgb1a1: 5'-CAGAGGGGAAACGAGTGAAG-3' (forward) and 5'-GCTGTGTGGCTGAAAACAGA-3' (reverse), human aqp5 5'-CGTCAACGCGCTCAACAAC-3' (forward) and 5'-GTGACAGACAGGCCAATGGAC-3' (reverse), chicken sftpa 5'-ACTCGGCAATGTTTTACTGAATC-3' (forward) and 5'-TCCTCCAGTCTCTTCACAGG-3' (reverse),

chicken aqp5 5'-CTCCGGACGCTCTTCTATGT-3' (forward) and 5'-GTTGTTGAGCGAATTGATGGC-3' (reverse).

Replication assay

Forty-eight hours after siRNA transfection, cells were washed twice with PBS and challenged with A/33/WSN H1N1 influenza A virus at MOI 0.05 for 1 h at room temperature. Cells were then washed with DMEM containing 0.7% BSA and 1µg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (sigma). At 72 h post-infection supernatants containing newly produced viral particles were transferred onto freshly seeded MDCK cells.

MDCK cells were then incubated for 6 h and fixed with 3.7% formaldehyde for 30 minutes. Cells were stained with Hoechst and immunolabelled with anti-NP (BIO-RAD, Puchheim, Germany) followed by anti-mouse IgG secondary antibody (Dianova). Images were obtained by using automated microscopy and analyzed by ScanR Analysis Software (Olympus, Hamburg, Germany).

Microarray

Microarray experiments were performed as single-color hybridization. Quality control and quantification of total RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies) and a NanoDrop 1000 UV-Vis spectrophotometer (Kisker). Total RNA was isolated with TRIzol (Life Technologies) according the supplier's protocol using Glycogen as co-precipitant. RNA labeling was performed with the one-color Low Input Quick Amp Labeling Kit (Agilent Technologies). In brief, mRNA was reverse transcribed and amplified using an oligo-dT-T7 promoter primer, and resulting cRNA was labeled with Cyanine 3-CTP. After precipitation, purification, and quantification, 0.75 µg labeled cRNA was fragmented and hybridized to custom whole genome human 8 × 60k multipack microarrays (Agilent-048908) according to the supplier's protocol (Agilent Technologies). Scanning of microarrays was performed with 3 µm resolution using a G2565CA high-resolution laser microarray scanner (Agilent Technologies). Microarray image data were analyzed with the Image Analysis/Feature Extraction software G2567AA v. A.11.5.1.1 (Agilent Technologies) using default settings and the GE1_1105_Oct12 extraction protocol. The extracted single-color raw data txt files were further analyzed using R and the associated BioConductor package limma [57]. Microarray data have been deposited in the Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo/) of the National Center for Biotechnology Information and can be assessed with the GEO accession number GSE86449. Gene ontology analyses were performed with Genecodis online analysis tool [58-60].

Supplementary Figure Legends

Figure S1 Expression of mature pneumocyte markers in human LECs at early passage (Refers to Figure 2)

(A) Immunofluorescent micrograph of human LECs in co-culture after the first passage. Intensity of proSP-C (green) and the shape of the cells revealed heterogeneity among CDH-1 (red)-positive human LECs. These cells show compact round shape and express proSP-C (green) with its distribution suggesting localization in vesicles.

Figure S2 Higher concentration of DBZ increases *aqp5* expression and suppress *scgb1a1* after separation from feeders (Refers to Figure 4)

(A) Agarose gel electrophoresis of RT-PCR products. The white arrowhead shows the expected size of the product (122bp). Lanes: 1 and 6, 50 bp DNA ladder; 2 induced human LECs with 20 µM DBZ and CHIR99021, FGF7, IBMX, 8-Br-cAMP, dexamethasone and FGF10 (CKIADF), 72 h after passage; 3, induced with 100 nM DBZ and CKIADF; RNA extract from tissue, 5 No template control. 5 µL of each crude mix after reaction was loaded on the gel, except for lane 4 where the solution was diluted to 1:10 to avoid saturation.

(B) qRT-PCR shows more prominent up-regulation of *aqp5* when the concentration of DBZ was increased to 20 µM; * $p < 0.001$

Figure S3 Morphological change and expression of pneumocyte markers in human LECs after feeder removal (Refers to Figure 4)

(A) and (B) Immunofluorescent micrograph of human LECs after feeder removal. The cells are compact with strong staining of proSP-C (red) at 48 h (A), while widely spread cytoplasm with staining of AQP5 (green, arrowhead) is observed at 144 h (B).

Figure S4 A/Vietnam/1203/2004 (H5N1) replicates equally well in human and chicken LECs (Refers to Figure 6)

Human and chicken LECs were infected with A/Vietnam/1203/2004 (H5N1) at MOI 0.05. The supernatants were harvested 24, 48 and 72 h post-infection. Viral progeny in the supernatant was titrated by infecting Madin-Darby canine kidney cells and subsequently detecting viral nucleoprotein by immunofluorescent staining. Data are shown as mean ± SD.

Figure S5. Schematic illustration of primary lung cell expansion and differentiation

(A) Proliferating LECs and their fate after induction and feeder removal *in vitro*. Treating the cells with high doses of the Notch pathway inhibitor dibenzazepine (DBZ) and other factors during the expansion phase induces commitment to a pneumocyte phenotype. Differentiation is initiated by feeder withdrawal. (B) Lineage-negative epithelial progenitors (LNEPs) and distal airway stem cells (DASCs) are the specialized tissue stem cells that proliferate upon tissue damage (e.g. influenza infection) [18], [17]. Vaughan et al proposed that notch signaling regulates the fate of epithelial cells [17, 18].

Abbreviations: CHIR, CHIR99021; FGF, fibroblast growth factor; IBMX, 3-isobutyl-1-methylxanthine; 8-Br-cAMP, 8-bromoadenosine 3',5'-cyclic monophosphate.

Supplementary Tables

Table S1. List of small molecules used in the study

Suppl. Table 1

Name	Action	Tested conc.	Effect
RepSo	Inhibit ALK4, 5, 7	0.001-25 µM	++
SB202190	Inhibit p38	0.05 – 5 µM	-
CHIR99021	Inhibit GSK3b	0.1 – 10 µM	--
Nicotinamide	Activate P38 pathway	5 – 50 mM	-
Dibenzazepine	Inhibit g-secretase	0.001 – 10 µM	++ → -* ("-" at higher conc.)
FGF7	(Promote alveologenesis in mouse)	10, 20 ng/ml	-